

Edited by  
Karl Esser

# THE MYCOTA

A Comprehensive Treatise on Fungi  
as Experimental Systems for Basic and Applied Research

---

## Environmental and Microbial Relationships IV

Second Edition

Christian P. Kubicek and Irina S. Druzhinina  
*Volume Editors*

---

# The Mycota

Edited by  
K. Esser

---

# The Mycota

- I     *Growth, Differentiation and Sexuality*  
1st edition ed. by J.G.H. Wessels and F. Meinhardt  
2nd edition ed. by U. Kües and R. Fischer
- II    *Genetics and Biotechnology*  
Ed. by U. Kück
- III   *Biochemistry and Molecular Biology*  
Ed. by R. Brambl and G. Marzluf
- IV    *Environmental and Microbial Relationships*  
1st edition ed. by D. Wicklow and B. Söderström  
2nd edition ed. by C.P. Kubicek and I.S. Druzhinina
- V     *Plant Relationships*  
1st edition ed. by G. Carroll and P. Tudzynski  
2nd edition ed. by H.B. Deising
- VI    *Human and Animal Relationships*  
1st edition ed. by D.H. Howard and J.D. Miller  
2nd edition ed. by A. Brakhage and P. Zipfel
- VII   *Systematics and Evolution*  
Ed. by D.J. McLaughlin, E.G. McLaughlin, and P.A. Lemke<sup>†</sup>
- VIII   *Biology of the Fungal Cell*  
Ed. by R.J. Howard and N.A.R. Gow
- IX     *Fungal Associations*  
Ed. by B. Hock
- X     *Industrial Applications*  
Ed. by H.D. Osiewacz
- XI    *Agricultural Applications*  
Ed. by F. Kempken
- XII   *Human Fungal Pathogens*  
Ed. by J.E. Domer and G.S. Kobayashi
- XIII   *Fungal Genomics*  
Ed. by A.J.P. Brown

---

# The Mycota

A Comprehensive Treatise  
on Fungi as Experimental Systems  
for Basic and Applied Research

Edited by K. Esser

---

## IV

*Environmental  
and Microbial Relationships*  
2nd Edition

Volume Editors:  
C.P. Kubicek · I.S. Druzhinina

---

With 55 Figures and 16 Tables

---

*Series Editor*

Professor Dr. Dr. h.c. mult. Karl Esser  
Allgemeine Botanik  
Ruhr-Universität  
44780 Bochum, Germany

Tel.: +49 (234)32-22211  
Fax.: +49 (234)32-14211  
e-mail: Karl.Esser@rub.de

*Volume Editors*

Professor Dr. Christian P. Kubicek  
Tel.: + 43 1 58801 17250  
Fax.: + 43 1 58801 17299  
e-mail: ckubicek@mail.zserv.tuwien.ac.at

Dr. Irina S. Druzhinina  
Tel.: + 43 1 58801 17202  
Fax.: + 43 1 58801 17299  
e-mail: druzhini@mail.zserv.tuwien.ac.at

TU Wien  
Institut für Verfahrenstechnik  
Umwelttechnik und Technische Biowissenschaften  
Getreidemarkt 9  
1060 Wien  
Austria

Library of Congress Control Number: 2007927885

ISBN 978-3-540-71839-0 Springer Berlin Heidelberg New York  
ISBN 3-540-58005-0 1st ed. Springer Berlin Heidelberg New York

This work is subject to copyright. All rights are reserved, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilm or in any other way, and storage in data banks. Duplication of this publication or parts thereof is permitted only under the provisions of the German Copyright Law of September 9, 1965, in its current version, and permissions for use must always be obtained from Springer-Verlag. Violations are liable for prosecution under the German Copyright Law.

Springer is a part of Springer Science+Business Media  
springer.com  
© Springer-Verlag Berlin Heidelberg 1997, 2007

The use of general descriptive names, registered names, trademarks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.

Editor: Dr. Dieter Czeschlik, Heidelberg, Germany  
Desk editor: Dr. Andrea Schlitzberger, Heidelberg, Germany  
Cover design: Erich Kirchner and WMXDesign GmbH, Heidelberg, Germany  
Production and typesetting: LE-T<sub>E</sub>X Jelonek, Schmidt & Vöckler GbR, Leipzig, Germany

Printed on acid-free paper SPIN 10987223 31/3180 5 4 3 2 1 0

**Karl Esser**

(born 1924) is retired Professor of General Botany and Director of the Botanical Garden at the Ruhr-Universität Bochum (Germany). His scientific work focused on basic research in classical and molecular genetics in relation to practical application. His studies were carried out mostly on fungi. Together with his collaborators he was the first to detect plasmids in higher fungi. This has led to the integration of fungal genetics in biotechnology. His scientific work was distinguished by many national and international honors, especially three honorary doctoral degrees.

**Christian P. Kubicek**

(born 1951) studied chemistry with specialization in Biotechnology and Food Chemistry. In 1977, he graduated at the University of Technology of Vienna with a thesis on citric acid production by *Aspergillus niger*. He later on habilitated at the Technical University in Microbial Biochemistry in 1983, at the Biocenter of the University of Vienna in Applied Microbiology in 1988, and received the honorary title of Professor for Microbial Biochemistry at the Vienna University of Technology in 1991. In 2003, he was appointed full Professor for Biotechnology and Microbiology at the same university. He is the author of about 250 papers in peer-reviewed journals, more than 30 book articles, several patents and three books, an editorial board member for several microbiological journals, and has been editor of *Applied and Environmental Microbiology* for 10 years. His scientific interests are various aspects of the biochemistry, molecular genetics, evolution and genomics of mitosporic fungi, particularly *Trichoderma*, *Aspergillus* and *Penicillium*.

**Irina S. Druzhinina**

(born 1974) graduated at the Department of Mycology and Algology of Lomonosov's Moscow State University (Russia). Her Master's thesis was dedicated to the species concept in fungi and population genetics of *Pleurotus ostreatus* (Fr.) Kumm. In 1998, she moved to Austria and switched her attention to radioecology and environmental risk assessment, by studying the physiology of radionuclide accumulation in fruit bodies of edible mushrooms. In 2001, she completed her thesis "Radioactive contamination of wild mushrooms: mycological approach and risk perception" at the University of Vienna (Austria), and received her doctoral degree with specialization in Botany. Soon thereafter, she was appointed Assistant Professor in the Department of Gene Technology and Applied Biochemistry at the Institute of Chemical Engineering of the Vienna University of Technology (Austria). Here, she returned to her main area of scientific interest and established a research group on Fungal Evolution and Speciation. Her work focuses on the phylogeny of Hypocreales fungi, and on the development of bioinformatics tools for the molecular identification of fungi. In a second line of research, she is interested in applying phenotype microarray techniques in investigating the metabolomics of filamentous fungi.

---

## Series Preface

Mycology, the study of fungi, originated as a subdiscipline of botany and was a descriptive discipline, largely neglected as an experimental science until the early years of this century. A seminal paper by Blakeslee in 1904 provided evidence for selfincompatibility, termed “heterothallism”, and stimulated interest in studies related to the control of sexual reproduction in fungi by mating-type specificities. Soon to follow was the demonstration that sexually reproducing fungi exhibit Mendelian inheritance and that it was possible to conduct formal genetic analysis with fungi. The names Burgeff, Kniep and Lindegren are all associated with this early period of fungal genetics research.

These studies and the discovery of penicillin by Fleming, who shared a Nobel Prize in 1945, provided further impetus for experimental research with fungi. Thus began a period of interest in mutation induction and analysis of mutants for biochemical traits. Such fundamental research, conducted largely with *Neurospora crassa*, led to the one gene: one enzyme hypothesis and to a second Nobel Prize for fungal research awarded to Beadle and Tatum in 1958. Fundamental research in biochemical genetics was extended to other fungi, especially to *Saccharomyces cerevisiae*, and by the mid-1960s fungal systems were much favored for studies in eukaryotic molecular biology and were soon able to compete with bacterial systems in the molecular arena.

The experimental achievements in research on the genetics and molecular biology of fungi have benefited more generally studies in the related fields of fungal biochemistry, plant pathology, medical mycology, and systematics. Today, there is much interest in the genetic manipulation of fungi for applied research. This current interest in biotechnical genetics has been augmented by the development of DNA-mediated transformation systems in fungi and by an understanding of gene expression and regulation at the molecular level. Applied research initiatives involving fungi extend broadly to areas of interest not only to industry but to agricultural and environmental sciences as well.

It is this burgeoning interest in fungi as experimental systems for applied as well as basic research that has prompted publication of this series of books under the title *The Mycota*. This title knowingly relegates fungi into a separate realm, distinct from that of either plants, animals, or protozoa. For consistency throughout this Series of Volumes the names adopted for major groups of fungi (representative genera in parentheses) are as follows:

### *Pseudomycota*

- Division: Oomycota (*Achlya, Phytophthora, Pythium*)  
Division: Hyphochytriomycota

### *Eumycota*

- Division: Chytridiomycota (*Allomyces*)  
Division: Zygomycota (*Mucor, Phycomyces, Blakeslea*)  
Division: Dikaryomycota  
Subdivision: Ascomycotina

Class: Saccharomycetes (*Saccharomyces, Schizosaccharomyces*)  
Class: Ascomycetes (*Neurospora, Podospora, Aspergillus*)

Subdivision: Basidiomycotina

Class: Heterobasidiomycetes (*Ustilago, Tremella*)

Class: Homobasidiomycetes (*Schizophyllum, Coprinus*)

We have made the decision to exclude from *The Mycota* the slime molds which, although they have traditional and strong ties to mycology, truly represent nonfungal forms insofar as they ingest nutrients by phagocytosis, lack a cell wall during the assimilative phase, and clearly show affinities with certain protozoan taxa.

The Series throughout will address three basic questions: what are the fungi, what do they do, and what is their relevance to human affairs? Such a focused and comprehensive treatment of the fungi is long overdue in the opinion of the editors.

A volume devoted to systematics would ordinarily have been the first to appear in this Series. However, the scope of such a volume, coupled with the need to give serious and sustained consideration to any reclassification of major fungal groups, has delayed early publication. We wish, however, to provide a preamble on the nature of fungi, to acquaint readers who are unfamiliar with fungi with certain characteristics that are representative of these organisms and which make them attractive subjects for experimentation.

The fungi represent a heterogeneous assemblage of eukaryotic microorganisms. Fungal metabolism is characteristically heterotrophic or assimilative for organic carbon and some nonelemental source of nitrogen. Fungal cells characteristically imbibe or absorb, rather than ingest, nutrients and they have rigid cell walls. The vast majority of fungi are haploid organisms reproducing either sexually or asexually through spores. The spore forms and details on their method of production have been used to delineate most fungal taxa. Although there is a multitude of spore forms, fungal spores are basically only of two types: (i) asexual spores are formed following mitosis (mitospores) and culminate vegetative growth, and (ii) sexual spores are formed following meiosis (meiospores) and are borne in or upon specialized generative structures, the latter frequently clustered in a fruit body. The vegetative forms of fungi are either unicellular, yeasts are an example, or hyphal; the latter may be branched to form an extensive mycelium.

Regardless of these details, it is the accessibility of spores, especially the direct recovery of meiospores coupled with extended vegetative haploidy, that have made fungi especially attractive as objects for experimental research.

The ability of fungi, especially the saprobic fungi, to absorb and grow on rather simple and defined substrates and to convert these substances, not only into essential metabolites but into important secondary metabolites, is also noteworthy. The metabolic capacities of fungi have attracted much interest in natural products chemistry and in the production of antibiotics and other bioactive compounds. Fungi, especially yeasts, are important in fermentation processes. Other fungi are important in the production of enzymes, citric acid and other organic compounds as well as in the fermentation of foods.

Fungi have invaded every conceivable ecological niche. Saprobic forms abound, especially in the decay of organic debris. Pathogenic forms exist with both plant and animal hosts. Fungi even grow on other fungi. They are found in aquatic as well as soil environments, and their spores may pollute the air. Some are edible; others are poisonous. Many are variously associated with plants as copartners in the formation of lichens and mycorrhizae, as symbiotic endophytes or as overt pathogens. Association with animal systems varies; examples include the predaceous fungi that trap nematodes, the microfungi that grow in the anaerobic environment of the rumen, the many insect-associated fungi and the medically important pathogens afflicting humans. Yes, fungi are ubiquitous and important.

There are many fungi, conservative estimates are in the order of 100,000 species, and there are many ways to study them, from descriptive accounts of organisms found in nature to laboratory experimentation at the cellular and molecular level. All such studies expand our knowledge of fungi and of fungal processes and improve our ability to utilize and to control fungi for the benefit of humankind.

We have invited leading research specialists in the field of mycology to contribute to this Series. We are especially indebted and grateful for the initiative and leadership shown by the Volume Editors in selecting topics and assembling the experts. We have all been a bit ambitious in producing these Volumes on a timely basis and therein lies the possibility of mistakes and oversights in this first edition. We encourage the readership to draw our attention to any error, omission or inconsistency in this Series in order that improvements can be made in any subsequent edition.

Finally, we wish to acknowledge the willingness of Springer-Verlag to host this project, which is envisioned to require more than 5 years of effort and the publication of at least nine Volumes.

Bochum, Germany  
Auburn, AL, USA  
April 1994

KARL ESSER  
PAUL A. LEMKE  
*Series Editors*

---

## Addendum to the Series Preface

In early 1989, encouraged by Dieter Czeschlik, Springer-Verlag, Paul A. Lemke and I began to plan *The Mycota*. The first volume was released in 1994, 12 volumes followed in the subsequent years. Unfortunately, after a long and serious illness, Paul A. Lemke died in November 1995. Thus, it was my responsibility to proceed with the continuation of this series, which was supported by Joan W. Bennett for Volumes X–XII.

The series was evidently accepted by the scientific community, because several volumes are out of print. Therefore, Springer-Verlag has decided to publish completely revised and updated new editions of Volumes I, II, III, IV, V, VI, and VIII. I am glad that most of the volume editors and authors have agreed to join our project again. I would like to take this opportunity to thank Dieter Czeschlik, his colleague, Andrea Schlitzberger, and Springer-Verlag for their help in realizing this enterprise and for their excellent cooperation for many years.

Bochum, Germany  
February 2007

KARL ESSER

---

## Volume Preface to the Second Edition

In the concept of *The Mycota* series, Karl Esser (Series Editor) felt a need for a volume which summarized existing knowledge on fungal communities, their interactions with each other and with biotic and abiotic factors of the environment, and which emphasized the role of fungi in ecosystem processes such as symbiotic relations and nutrient cycling. This was consequently published as Volume IV, *Environmental and Microbial Relationships*, in 1997. Now – 10 years later and after a revolution in molecular techniques and tools in this domain – there appeared an urgency for updating the volume, and we were invited to organize a second edition. Consequently, some reconstruction of content became necessary, particularly because some new chapters were deemed appropriate to document the progress in recently emerged areas of research. The original concept that authors should concentrate largely on overviews of main phenomena and most recent achievements was maintained.

The volume is partitioned into four sections: ‘Life History and Genetic Strategies’; ‘Determinants of Fungal Communities’; ‘Fungal Interactions and Biological Control Strategies’; and ‘Decomposition, Biomass and Industrial Applications’.

In the first, J.H. Andrews and R.F. Harris attempt to relate the evolution of fungi to their ecological and physiological properties, approaching this question by adding the dimension of trophic property to the conventional phylogenetic tree, and exploring biochemical aspects further by using bioenergetic electron flow diagrams. The chapter by K. Brunner, S. Zeilinger and R.L. Mach reviews various modern molecular biological methods applied for the correct identification of environmental isolates and communities, which have been essential in the progress in unravelling fungal ecology this past decade.

The second section deals with the role of main environmental factors and ecosystem disturbances in determining the species and population structure of fungal communities. S.J. Morris, C.F. Friese and M.F. Allen evaluate the diversity and biomass of microbial communities as direct indicators of the extent of the functional role played by these organisms in the dynamics of different ecosystems. They conclude that, if anthropogenic changes alter the structure and biodiversity of microbial communities, then critical functional roles in ecosystem- and global-level nutrient cycling are also likely impacted. R.M. Miller and D.J. Lodge elaborate on the impacts of various management practices associated with agriculture and forestry in influencing fungal structure and function, and discuss the hierarchical nature of soils and how this affects system response to disturbance by fungi. G.M. Gadd emphasizes how newly developed approaches using molecular biology and biomarkers are enabling a better understanding of community structure and responses to environmental factors, and pollutants. He concludes that – because of the complexity of the fungal growth form, multiplicity of biological responses and interactions with pollutants, coupled with the complexity of terrestrial (and other) environments – “a wealth of knowledge still awaits discovery”. In reviewing examples of fungi living in extreme environments, N. Magan stresses a critical need for experiments which better simulate fluctuating abiotic parameters and their impacts on the ecophysiology of these fungi. E.M.J. Arnolds illustrates how a knowledge of global, regional

and endemic patterns of fungal distribution can serve to enhance understanding of evolutionary processes and biodiversity patterns.

The use of fungi for the biological control of various agricultural pests has been a leading theme in applied mycology during the past 10 years. This is dealt with in the third section, on fungal interactions and biological control strategies. Mycoparasitism is considered to have an important influence on competitive interactions involving fungi in nature, and in the biological control of phytoparasitic fungi. A. Viterbo, J. Inbar, Y. Hadar and I. Chet use *Trichoderma* as a model system of a typical mycoparasite, and critically review how research on this phenomenon can be used to improve plant resistance to fungal pathogens. In the following chapter, S. Casas-Flores and A. Herrera-Estrella emphasize the application of fungi in the biocontrol of nematodes, highlighting the biochemical and molecular information available to date on this process. A.K. Charnley and S.A. Collins outline the current state of knowledge of insect fungal pathogens as it relates to their present use and future potential as mycoinsecticides. W.G.D. Fernando, R. Ramarathnam and T. de Kievit illustrate the use of bacteria in the biocontrol of *Sclerotinia* stem rot and blackleg diseases in canola. T.P. McGonigle shows how grazing can affect fungal communities through effects on species richness, community diversity, and replacement of some species by others. P. Bayman illustrates the recent progress made in the detection and understanding of endophytic fungi. M. Girlanda, S. Perotto and P. Bonfante emphasize the position of mycorrhizal fungi as a "tie that binds the host plant to the biotic and abiotic environment". J.B. Gloer offers numerous examples showing how observations in fungal ecology have generated hypotheses about fungal antagonism and defence which, in turn, have led to the discovery of novel bioactive fungal metabolites.

The fourth section highlights fungus-mediated decomposition and the nutrient-mobilizing potentials of fungi in both aquatic (marine and freshwater) and terrestrial ecosystems. J. Dighton illustrates how traditional concepts of nutrient cycling, which still today rely mainly on an understanding of leaf litter and wood decomposition, must be expanded when the role of fungal communities in driving nutrient cycling in other, more transiently changing ecosystems is investigated. M.O. Gessner, V. Gulis, K.A. Kuehn, E. Chauvet and K. Suberkropp examine the role of fungi in plant litter decomposition in two aquatic ecosystems in which fungal decomposers and plant litter decomposition have been studied to the greatest extent: standing-dead shoots of emergent vascular plants in salt and freshwater marshes. Finally, in order to stimulate the application of fungal degradation of key plant cell wall macromolecules, which accumulate in enormous amounts annually, C. Gamauf, B. Metz and B. Seibold illustrate the biochemical and molecular genetic properties of different genera of fungi involved in the turnover of these compounds.

We hope that this volume will prove useful both for scientists who wish to update themselves in any of these research areas as well as to graduate students interested in obtaining a first, multifaceted overview. We are grateful to all individual contributors who took the time and invested effort in collaborating with us on the updating of this volume, and especially that they all helped us to get this task finished within the expected time schedule.

Wien, Austria  
March 2007

CHRISTIAN P. KUBICEK  
IRINA S. DRUZHININA  
*Volume Editors*

---

## Volume Preface to the First Edition

In their concept of *The Mycota*, Karl Esser and Paul Lemke (Series Editors) determined that a volume was needed to examine research on fungal populations and communities. We were invited to organize Volume IV, *Environmental and Microbial Relationships*, and were instructed to concentrate on fungal responses to the physical environment, interactions with other fungi, microorganisms and invertebrates, and the role of fungi in ecosystem processes such as ecomposition and nutrient cycling. Individual chapter authors were asked to concentrate on ecological themes that could be supported by selected studies in depth and to judge fungal systems for their promise as research tools. We were advised not to solicit exhaustive reviews to cover all ecological groups of fungi.

Several authors were asked to emphasize the technology transfer of ecological information, showing how specific knowledge of the ecology and biology of fungi has application in biological control, in enzymatic conversions of plant biomass, biodegradation of toxic organic pollutants, and the discovery of natural products. Here, we asked chapter authors to take into account the basic ecological underpinnings of such technologies. Initially, we intended to place chapters emphasizing biological control strategies or industrial mycology in a section entitled "Technology transfer of ecological information." However, it was found more desirable to integrate these specific chapters within the ecological framework of the volume. We hope that this approach will better unite aspects of ecological research classified as fundamental vs. applied mycology. The immediate challenge for mycological ecology is to identify those examples where ecological studies of fungi in nature provide basic information leading to the development of a particular technology. The book begins with a section entitled "Fungal life history and genetic strategies." Here, J.H. Andrews and R.F. Harris examine the interconversion of different growth forms in a fungal life cycle, noting that the precise nature of the environmental signals which trigger these phases and how they are transduced by the organism are unknown. Fungal ecological genetics has become the most dynamic area of mycological research in the 1990s, largely due to the efforts of A.D.M. Rayner and colleagues at the University of Bath. In the present volume, M. Ramsdale and A.D.M. Rayner outline some of the highlights of progress made in recent years and also present their vision for the future of fungal ecological genetics.

The second section is concerned with the role of selected environmental factors and ecosystem disturbance in determining the species structure of fungal communities. J.C. Zak and S.C. Rabatin begin by examining various experimental designs, approaches and methodologies for analyzing and describing fungal communities. They emphasize the importance of scale in community ecology and argue that disturbance may be the single most important process regulating the structure and functioning of fungal communities in nature. C.F. Friese, S.J. Morris and M.F. Alien evaluate disturbance dynamics over a wide array of scales and interacting factors, highlighted by a case linking site disturbance by harvester ants and the renewal of plant communities every 100 to 1000 years. R.M. Miller and D.J. Lodge identify research needed to understand how fungi respond to disturbance created by management practices in agriculture and forestry. Disruption of mycorrhizal hyphal networks and the response of saprophytic hyphae have impacts on soil structure and nutrient pools associated with the fungal hyphae.

M. Wainwright and G.M. Gadd show how a more accurate assessment of the effects of pollutants on the growth and activity of fungi in the environment has been made possible by new methods for determining active fungal biomass and enlightened approaches to in vitro experimentation. In reviewing examples of fungi living in extreme environments, N. Magan argues a critical need for experiments which better simulate fluctuating abiotic parameters and their impact on the ecophysiology of these fungi. A knowledge of global, regional and endemic patterns of fungal distribution is important for the understanding of evolutionary processes and biodiversity patterns and E.J.M. Arnolds observes that such information may be applied to the control and spread of crop pests as well as in fungal conservation.

The interactions of fungi with one another, with other microbes, nematodes and arthropods have produced a wealth of ecological information with the potential for development of biological control strategies. This is considered in the third section, "Fungal interactions and biological control strategies." In reviewing fungal competition, P. Widden relates this knowledge to what is known about plant competition and the predictive value of competition theory. Because many decomposer fungi have to replace an existing microflora in order to colonize a substrate, interference competition can have an important impact on decomposition. P. Jeffries recognizes mycoparasitism as a widespread phenomenon and presents numerous examples of mycoparasitic interactions. Mycoparasitism is believed to have an important influence on competitive interactions involving fungi in nature, and in the biological control of phytoparasitic fungi. *Trichoderma harzianum* is presented as a model system of a typical mycoparasite. I. Chet, J. Inbar and Y. Hadar also examine research to improve plant resistance to fungal pathogens by integrating cloned fungal chitinase with antifungal polypeptides. Again, *Trichoderma* is shown to be a useful model system. While recognizing that mycoinsecticides have had little impact on insect pest control to date, A.K. Charnley suggests a promising future for these entomopathogenic fungi. This optimism is based on the current rate of progress in research on epizootiology, mass production, formulation, application and mechanisms of pathogenesis. Fungal agents for controlling plant pathogenic nematodes often perform poorly or inconsistently because they have been released prematurely, without sufficient basic knowledge of their biology and ecology. B. Kerry and B. Jaffee explain the importance relating basic information on the mode of action and epidemiology of selected fungal biocontrol agents to methods of mass production, formulation and application. In his examination of the potential of mycoherbicides, D.A. Shisler emphasizes the importance of understanding phylloplane microbial dynamics in order to obtain consistent field efficacy of a mycoherbicide, including strategies for bolstering the pathogen at this same weak point. T. McGonigle shows how the selective grazing of soil and litter fungi by arthropods can have an impact on community structure. Fungi have responded in different ways to reduce the negative effects of predation. J.B. Gloer offers numerous examples showing how observations in fungal ecology have generated hypotheses about fungal antagonism and defense which, in turn, have led to the discovery of novel bioactive fungal metabolites.

The final section recognizes fungus-mediated decomposition and the nutrient mobilizing potentials of fungi in both aquatic (marine and freshwater) and terrestrial ecosystems. J. Dighton considers aspects of metal ion accumulation and enzymatic competence of saprotrophic fungi. Such basic information can be applied to the degradation of toxic organic compounds and the uptake and accumulation of metal ions in contaminated soils. J.R. Leake and D.J. Read review evidence that mycorrhizae in decomposing litter have a direct role in recycling of organic nutrients. There is surprisingly little information on the extent to which processes of nutrient mobilization occur in nature. M.O. Gessner, K. Suberkropp and E. Chauvet examine the role of fungi in plant litter decomposition in aquatic environments (e.g. salt marshes, mangrove swamps and streams), and they observe that mechanisms controlling the allocation of resources

between mycelium and reproductive structures are not yet understood. In an effort to encourage lateral thinking, M.J.R. Nout and co-authors examine different approaches for estimating fungal biomass in food fermentations and consider the appropriateness of various approaches for fungal ecologists investigating litter decomposition. Likewise, the bioconversion of plant fibres to fuel, feed or precursors for chemical syntheses is examined by R. Sinsabaugh and M.A. Liptak, scientists whose principal research interest is in the biochemistry of plant litter decomposition and the ecology of the decomposers in nature.

We hope that not only professional biologists who wish to learn research directions and opportunities in mycological ecology will find this volume interesting, but also that graduate students in mycology and microbial ecology will find it useful. Thanks are especially due to those individual contributors who produced outstanding original figures and to all for promptly responding to our various editorial requests.

Peoria, Illinois, USA  
Lund, Sweden  
January 1997

DONALD WICKLOW  
BENGT SÖDERSTRÖM  
*Volume Editors*

---

## Contents

### Life History and Genetic Strategies

1 Evolutionary Ecology of the First Fungi J.H. ANDREWS, R.F. HARRIS .....	3
2 Molecular Approaches for Studying Fungi in the Environment K. BRUNNER, S. ZEILINGER, R.L. MACH .....	17

### Determinants of Fungal Communities

3 Disturbance in Natural Ecosystems: Scaling from Fungal Diversity to Ecosystem Functioning S.J. MORRIS, C.F. FRIESE, M.F. ALLEN .....	31
4 Fungal Responses to Disturbance: Agriculture and Forestry R.M. MILLER, D.J. LODGE .....	47
5 Fungi and Industrial Pollutants G.M. GADD .....	69
6 Fungi in Extreme Environments N. MAGAN .....	85
7 Biogeography and Conservation E.J.M. ARNOLDS .....	105

### Fungal Interactions and Biological Control Strategies

8 Plant Disease Biocontrol and Induced Resistance via Fungal Mycoparasites A. VITERBO, J. INBAR, Y. HADAR, I. CHET .....	127
9 Antagonism of Plant Parasitic Nematodes by Fungi S. CASAS-FLORES, A. HERRERA-ESTRELLA .....	147
10 Entomopathogenic Fungi and Their Role in Pest Control A.K. CHARNLLEY, S.A. COLLINS .....	159
11 Bacterial Weapons of Fungal Destruction: Phyllosphere-Targeted Biological Control of Plant Diseases, with Emphasis on Sclerotinia Stem Rot and Blackleg Diseases in Canola ( <i>Brassica napus</i> L.) W.G.D. FERNANDO, R. RAMARATHNAM, T. DE KIEVIT .....	189

12 Effects of Animals Grazing on Fungi T.P. McGONIGLE .....	201
13 Fungal Endophytes P. BAYMAN .....	213
14 Mycorrhizal Fungi: Their Habitats and Nutritional Strategies M. GIRLANDA, S. PEROTTO, P. BONFANTE .....	229
15 Applications of Fungal Ecology in the Search for New Bioactive Natural Products J.B. GLOER .....	257

**Decomposition, Biomass and Industrial Applications**

16 Nutrient Cycling by Saprotophobic Fungi in Terrestrial Habitats J. DIGHTON .....	287
17 Fungal Decomposers of Plant Litter in Aquatic Ecosystems M.O. GESSNER, V. GULIS, K.A. KUEHN, E. CHAUVET, K. SUBERKROPP .....	301
18 Degradation of Plant Cell Wall Polymers by Fungi C. GAMAUF, B. METZ, B. SEIBOTH .....	325
Biosystematic Index .....	341
Subject Index .....	345

---

## List of Contributors

**M.F. ALLEN**

(e-mail: michael.allen@ucr.edu)

Center for Conservation Biology, University of California,  
Riverside, CA 92521, USA

**J.H. ANDREWS**

(e-mail: jha@plantpath.wisc.edu)

Plant Pathology Department, University of Wisconsin,  
1630 Linden Drive, Madison, WI 53706, USA

**E.J.M. ARNOLDS**

(e-mail: arnolds21@hetnet.nl)

Holthe 21, 9411 Beilen, The Netherlands

**P. BAYMAN**

(e-mail: pbayman@uprrp.edu)

Departamento de Biología, Universidad de Puerto Rico–Río Piedras,  
P.O. Box 23360, San Juan, PR 00931, USA

**P. BONFANTE**

(e-mail: paola.bonfante@unito.it)

Department of Plant Biology, University of Torino,  
Viale PA Mattioli 25, Torino 10125, Italy

Extra Address

IPP-CNR, Viale PA Mattioli 25, Torino 10125, Italy

**K. BRUNNER**

(e-mail: brunner@mail.zserv.tuwien.ac.at)

FB Gentechnik und Angewandte Biochemie, Institut für Verfahrenstechnik,  
Umwelttechnik und Technische Biowissenschaften, TU Wien,  
Getreidemarkt 9/166/5/2, 1060 Vienna, Austria

**S. CASAS-FLORES**

(e-mail: scasas@ipicyt.edu.mx)

División de Biología Molecular,

Instituto Potosino de Investigación Científica y Tecnológica,  
Camino a la Presa de San José 2055, 78210 Tangamanga, San Luis Potosí SLP, México

**A.K. CHARNLEY**

(e-mail: bssakc@bath.ac.uk)

Department of Biology and Biochemistry, University of Bath,  
Claverton Down, Bath BA2 7AY, UK

**E. CHAUVET**

(e-mail: echauvet@cict.fr)

Laboratoire d'écologie fonctionnelle – EcoLab, UMR 5245 CNRS,  
University Toulouse 3, National Polytechnic Institute of Toulouse,  
29, rue Jeanne Marvig, 31055 Toulouse Cedex, France

**I. CHET**

(ilan.chet@weizmann.ac.il)

Department of Plant Sciences, Weizmann Institute of Science,  
Rehovot 76100, Israel

**S.A. COLLINS**

Department of Biology and Biochemistry, University of Bath,  
Claverton Down, Bath BA2 7AY, UK

**J. DIGHTON**

(e-mail: dighton@camden.rutgers.edu)

Rutgers University Pinelands Field Station,  
P.O. Box 206, 501 Four Mile Road, New Lisbon, NJ 08064, USA

**W.G.D. FERNANDO**

(e-mail: D\_Fernando@Umanitoba.ca)

University of Manitoba, Department of Plant Science,  
66 Dafoe Road, Winnipeg, Manitoba R3T 2N2, Canada

**C.F. FRIESE**

(e-mail: Carl.Friese@notes.udayton.edu)

Department of Biology, University of Dayton,  
300 College Park, Dayton, OH 45469, USA

**G.M. GADD**

(e-mail: g.m.gadd@dundee.ac.uk)

Division of Environmental and Applied Biology,  
College of Life Sciences, University of Dundee,  
Dundee, DD1 4HN, Scotland, UK

**C. GAMAUF**

(e-mail: ch\_gamauf@gmx.at)

FB Gentechnik und Angewandte Biochemie, Institut für Verfahrenstechnik,  
Umwelttechnik und Technische Biowissenschaften, TU Wien,  
Getreidemarkt 9/166/5/2, 1060 Vienna, Austria

**M.O. GESSNER**

(e-mail: gessner@eawag.ch)

Department of Aquatic Ecology,  
Eawag: Swiss Federal Institute of Aquatic Sciences and Technology,  
and Institute of Integrative Biology (IBZ), ETH Zurich,  
Überlandstrasse 133, 8600 Dübendorf, Switzerland

**M. GIRLANDA**

(e-mail: mariangela.girlanda@unito.it)

Department of Plant Biology, University of Torino,  
Viale PA Mattioli 25, Torino 10125, Italy

**J.B. GLOER**

(e-mail: james-gloer@uiowa.edu)

Department of Chemistry, University of Iowa,  
Iowa City, IA 52242, USA

**V. GULIS**

(e-mail: v.gulis@ua.edu)

Department of Biological Sciences, University of Alabama,  
Tuscaloosa, AL 35487-0206, USA

## Current address:

Department of Biology, Coastal Carolina University,  
Conway, SC 29528-6054, USA**Y. HADAR**

(e-mail: hadar@agri.huji.ac.il)

Department of Plant Pathology and Microbiology,  
The Hebrew University of Jerusalem, Faculty of Agriculture,  
Rehovot 76100, Israel**R.F. HARRIS**

(e-mail: rfharris@wisc.edu)

Soil Science Department, University of Wisconsin,  
1525 Observatory Drive, Madison, WI 53706, USA**A. HERRERA-ESTRELLA**

(e-mail: aherrera@ira.cinvestav.mx)

Laboratorio Nacional de Genómica para la Biodiversidad,  
Cinvestav Campus Guanajuato, Km 9.6 Libramiento Norte Carretera Irapuato-León,  
A.P. 629, Irapuato 36500, Guanajuato, México**J. INBAR**

(kobi.inbar@gmail.com)

Department of Plant Sciences, Weizmann Institute of Science,  
Rehovot 76100, Israel

## Present address:

P.O. Box 5592, Yavne 70600, Israel

**T. DE KIEVIT**

(e-mail: dekievit@ms.umanitoba.ca)

University of Manitoba, Department of Microbiology,  
Winnipeg, Manitoba R3T 2N2, Canada**K.A. KUEHN**

(e-mail: kevin.kuehn@usm.edu)

Department of Biological Sciences, The University of Southern Mississippi,  
Hattiesburg, MS 39406-0001, USA**D.J. LODGE**

(e-mail: djlodge@caribe.net)

International Institute of Tropical Forestry, USDA - Forest Service,  
P.O. Box 1377, Luquillo, PR 00773, USA**R.L. MACH**

(e-mail: rmach@mail.zserv.tuwien.ac.at)

FB Gentechnik und Angewandte Biochemie, Institut für Verfahrenstechnik,  
Umwelttechnik und Technische Biowissenschaften, TU Wien,  
Getreidemarkt 9/166/5/2, 1060 Vienna, Austria**N. MAGAN**

(e-mail: n.magan@cranfield.ac.uk)

Applied Mycology Group, Cranfield Health, Cranfield University,  
Barton Road, Silsoe, Bedford MK45 4DT, UK

**T.P. McGONIGLE**  
(e-mail: mcgoniglet@brandonu.ca)  
Department of Botany, Brandon University,  
270-18th Street, Brandon, Manitoba R7A 6A9, Canada

**B. METZ**  
(e-mail: bmetz@mail.zserv.tuwien.ac.at)  
FB Gentechnik und Angewandte Biochemie, Institut für Verfahrenstechnik,  
Umwelttechnik und Technische Biowissenschaften, TU Wien,  
Getreidemarkt 9/166/5/2, 1060 Vienna, Austria

**R.M. MILLER**  
(e-mail: rmmiller@anl.gov)  
Biosciences Division, Argonne National Laboratory,  
9700 S. Cass Avenue, Argonne, IL 60439, USA

**S.J. MORRIS**  
(e-mail: sjmorris@bradley.edu)  
Biology Department, Bradley University,  
1501 W. Bradley Avenue, Peoria, IL 61625, USA

**S. PEROTTO**  
(e-mail: silvia.perotto@unito.it)  
Department of Plant Biology, University of Torino,  
Viale PA Mattioli 25, Torino 10125, Italy

**R. RAMARATHNAM**  
(umramara@cc.umanitoba.ca)  
University of Manitoba, Department of Plant Science,  
66 Dafoe Road, Winnipeg, Manitoba R3T 2N2, Canada

**B. SEIBOTH**  
(e-mail: bseiboth@mail.zserv.tuwien.ac.at)  
FB Gentechnik und Angewandte Biochemie, Institut für Verfahrenstechnik,  
Umwelttechnik und Technische Biowissenschaften, TU Wien,  
Getreidemarkt 9/166/5/2, 1060 Vienna, Austria

**K. SUBERKROPP**  
(e-mail: ksuberkp@bama.ua.edu)  
Department of Biological Sciences, University of Alabama,  
Tuscaloosa, AL 35487-0206, USA

**A. VITERBO**  
(e-mail: ada.viterbo@weizmann.ac.il)  
Department of Plant Sciences, Weizmann Institute of Science,  
Rehovot 76100, Israel

**S. ZEILINGER**  
(e-mail: szeiling@mail.zserv.tuwien.ac.at)  
FB Molekulare Biochemie der Pilze, Institut für Verfahrenstechnik,  
Umwelttechnik und Technische Biowissenschaften, TU Wien,  
Getreidemarkt 9/166/5/2, 1060 Vienna, Austria

---

## **Life History and Genetic Strategies**

---

# 1 Evolutionary Ecology of the First Fungi

J.H. ANDREWS<sup>1</sup>, R.F. HARRIS<sup>2</sup>

## CONTENTS

I. Introduction .....	3
II. Biogeochemical Setting and Geological Timescale .....	3
III. Phylogenetic Evidence and its Interpretation .....	4
IV. Eukaryote Evolution and Fungal Phylogeny .....	5
V. Bioenergetic Analyses Applied to Evolutionary and Phylogenetic Relationships .....	11
VI. Conclusions.....	13
References .....	14

## I. Introduction

Other than the origin of life, arguably the other major evolutionary benchmarks are the symbiotic origin and phylogenetic radiation of eukaryotes, and the inception of aerobiosis. Intertwined with these developments, and of particular interest to mycologists, is a third event, the origin of the fungi.

Here, we review current thinking on the origin of eukaryotes, and the position of the fungi within the so-called tree of life. To provide a context for this discussion, we begin with a consensus geological timescale for dating the key geological and evolutionary events. This leads to an overview of the types and strength of evidence used to construct phylogenies, followed by the phylogenies themselves summarized as a phylogenetic tree starting with the origin of life, and focusing on eukaryotes, fungi, and alphaproteobacteria (the presumed ancient mitochondrial endosymbiont). The tree is expanded to include energy-generating and other ecophysiological trophic traits of the major taxa, which allows identification of the taxa

that are closest trophically to the mitochondrial and eukaryotic root. We conclude by depicting the energy-generating trophic relationships between fungi and alphaproteobacteria as a function of a simple, quantitative, bioenergetic electron flow framework (linked electron donors and acceptors) based on integrated thermodynamic and biochemical principles. The framework allows visual comparison of dissimilatory reactions and pathways that can, and cannot, proceed under ambient and standard environmental conditions. Further, it identifies energy connections between the mitochondrial endosymbiont and aerobic and anaerobic fungi, and allows quantitative assessment of hypotheses for the symbiotic origin and evolutionary ecology of eukaryotes.

## II. Biogeochemical Setting and Geological Timescale

Diverse evidence suggests that Earth originated about 4.5 Gyr (Gyr = Giga or billion years ago) from a collision between planetismals, followed by coalescence of the debris (Nisbet and Sleep 2001). After a period of cataclysmic chaos as Earth consolidated and cooled, a lithospheric crust was formed, and temperatures became conducive to the prolonged existence of liquid water as part of a functional hydrosphere. This set the primordial stage for chemical evolution toward systems supporting the organic precursors of life, followed by evolution of self-replicating, progenotic assemblages bridging into prokaryotic and eukaryotic organisms. The four geological timescales (eons) are the:

- (a) Hadean (from origin of planet, ~4.5 Gyr, to the origin of life, ~4.0 Gyr);
- (b) Archaean (4.0–2.5 Gyr);
- (c) Proterozoic (2.5–0.56 Gyr); and
- (d) Phanerozoic (0.56 Gyr–present).

<sup>1</sup> Plant Pathology Department, 1630 Linden Drive, University of Wisconsin, Madison, WI 53706, USA

<sup>2</sup> Soil Science Department, 1525 Observatory Drive, University of Wisconsin, Madison, WI 53706, USA

Our focus is on the late Archaean and Proterozoic (Nisbet and Sleep 2001).

Life probably originated by the early Archaean ( $\sim 3.8$  Gyr). Fossil evidence is strong (see section below on Phylogenetic Evidence) by the mid-Archaean (3.6–3.3 Gyr) for widespread bacterial biofilms and stromatolites (“organosedimentary structures produced by microbial trapping, binding, and precipitation, generally but not always photosynthetic”; Nisbet and Sleep 2001). Geochemical markers, including those typical of cyanobacteria, imply that oxygenic photosynthesis was occurring by 3.5 Gyr, and by 2.8–2.2 Gyr atmospheric oxygen levels were significant, as interpreted from oxidized rock (Nisbet and Sleep 2001; Bekker et al. 2004). This was sufficient to support aerobic respiration, a key event associated with the evolution of mitochondria, though high rates of oxygen utilization kept the levels at 1–2% of present-day concentrations until about 1.9 Gyr, when levels rose to at least 15% of present-day values (Knoll 1992; Bekker et al. 2004). Of course, anaerobic environments persist to this day in habitats such as the gut tract and anoxic sediments.

Unicellular eukaryotes (protists), arising more or less concurrently with mitochondria, were present by 1.5 Gyr, and possibly much earlier (Knoll 1992; Nisbet and Sleep 2001; Dyall et al. 2004). Fossils interpreted as presumptive eukaryotes are dated at 1.45 Gyr (Javaux et al. 2001), and a well-established fossil benchmark for a multicellular red alga protist (Bangiophyceae), i.e., implying the presence of both mitochondria and plastids, was recovered from 1.25–0.75 Gyr carbonate (sedimentary) rocks in arctic Canada (Butterfield et al. 1990). A “probable fungus” (*Tappania* sp.) dated at 1.43 Gyr (Butterfield 2005) has been recovered from Australian rock.

### III. Phylogenetic Evidence and its Interpretation

Primarily two forms of evidence have been used to reconstruct the history of life: paleontological (fossil or morphological), and molecular sequence (nucleotide or protein) information. The former is dated by biophysical or geochemical methods (Schopf et al. 1983; Knoll 1992); the latter, by molecular clock assumptions (Wilson et al. 1987). (This categorization excludes evidence of life inferred from geochemical profiles or signals; Knoll 1992;

Nisbet and Sleep 2001.) While morphological and molecular lines of data are complementary and have generally been in broad agreement (Baker and Gatesy 2002; Smith and Peterson 2002; Donoghue and Smith 2004), there are notable exceptions that have not been reconciled. Both approaches involve numerous inferences and assumptions, and have significant constraints, alluded to briefly as follows.

#### Fossils

Robust fossil evidence depends on a readily interpretable, accurately dated specimen. The fossil provides a *minimum* possible age for that taxon (and for sister taxa and preceding lineages; Berbee and Taylor 1999, 2001; Smith and Peterson 2002). Departure from the ideal specimen occurs because of both geological and biological limitations. Even among paleontologists, there is considerable controversy and subjectivity in interpreting retrieved specimens (Brasier et al. 2002). No rocks are yet known from the estimated origin of the planet (the oldest rocks reported to date are about 4 Gyr; Stern et al. 1998). For body fossilization to occur, generally the organism needs to be coated very quickly by sediment. Sedimentation associated with the early planet has been altered frequently, obscuring fossils, and under the best of conditions (shallow water) sediments are only sporadically distributed (Schopf et al. 1983). Thus, the early fossil records are more or less obliterated, with the first evidence for microbes appearing as stromatolites (3.5 Gyr, see above; Nisbet and Sleep 2001), and most of the macrofossils appearing over the past 0.6 Gyr (Schopf et al. 1983; Knoll 1992). Fossilization is facilitated by mineralized, or otherwise resistant materials not possessed by many organisms; those readily fossilized may not occur in geographic regions geologically suitable for preservation (Schopf et al. 1983). Because of their size and composition, fungi are not as well suited as dinosaurs for fossilization! Furthermore, much of microbial evolution is internal, reflecting changes in genetics, biochemistry, or ultrastructure, rather than in external body form (see Sect. IV., Eukaryote Evolution and Fungal Phylogeny). This results in what has imaginatively been called “the Volkswagen syndrome” (Schopf et al. 1983), alluding to the many generations of visually identical VW ‘beetles’. Finally, homoplasy (similarity in features among taxa for reasons such as convergence, i.e., other than common ancestry) may be a confounding factor, particularly in interpreting morphological evidence

(Givnish and Sytsma 1997; but see Baker and Gatesy 2002).

### Molecules

While it is often asserted confidently that “sequences don’t lie”, the data and analyses leading to inference of a particular sequence or phylogeny are not as objective as implied by this remark (Hillis et al. 1996; for fungi, see Berbee and Taylor 1999). The molecular approach dates essentially from the work by Zuckerkandl and Pauling, among others (reviewed by Wilson et al. 1987; Donoghue and Smith 2004), showing that the rates of amino acid replacement due to mutational change were about constant among lineages for the proteins cytochrome *c* and hemoglobin. This suggested that the times of divergence for pairs of species can be calculated. If fossil evidence is available for certain specimens to calibrate the clock, then it can be used in principle to estimate divergence times for species for which no fossil evidence exists (Wilson et al. 1987), including fungi (Berbee and Taylor 2001). Despite much research showing that the clock ticks irregularly (Wilkins 2002; Thomas et al. 2006), it is still used widely as a phylogenetic instrument. Among the early, retrospectively erroneous placements on the universal ‘tree of life’ were the amitochondriate eukaryotes, the hyperthermophilic bacteria, and probably, the root of the tree (Gribaldo and Philippe 2004).

Perhaps the largest assumption from molecular approaches, which is only rarely even mentioned, is that the gene- or protein-based trees portrayed in countless diagrams actually equate with organism trees, notwithstanding the fact that they are labeled as such. Beyond this, there are two basic sorts of deficiencies (Gribaldo and Philippe 2004) arising from either reconstruction models or basic biological processes. A single phylogenetic tree is usually specified by a model, yet trees are constructed by different rules (e.g., based on evolutionary distance, or maximum parsimony, or maximum likelihood) with different topologies and estimations of ancestral relationships (Lutzoni et al. 2001; Pace 2004). Early generation mathematical depictions made misleadingly simplistic assumptions that potentially caused the wrong tree to be specified with strong support (Embley and Martin 2006). Probably the most notorious artifact, termed long-branch attraction, refers to clustering of the longest branches due to variable evolution rates (i.e., the most rapidly evolving taxa, not necessarily

the most closely related, are grouped; Gribaldo and Philippe 2004). Other errors arise from loss of mutational signal in excessive noise (mutational saturation); clustering of taxa due to DNA base composition (e.g., biased by similar G+C content); and variable substitution rates within a protein across lineages (heterotachy; Gribaldo and Philippe 2004). When phylogenies from different proteins or genes conflict for the same group of species, this may be due to artifacts, or to lateral gene transfer (Gribaldo and Philippe 2004). At least partial solutions to most of these problems are now available, including more sophisticated grouping functions in newer mathematical models (Gribaldo and Philippe 2004; Embley and Martin 2006).

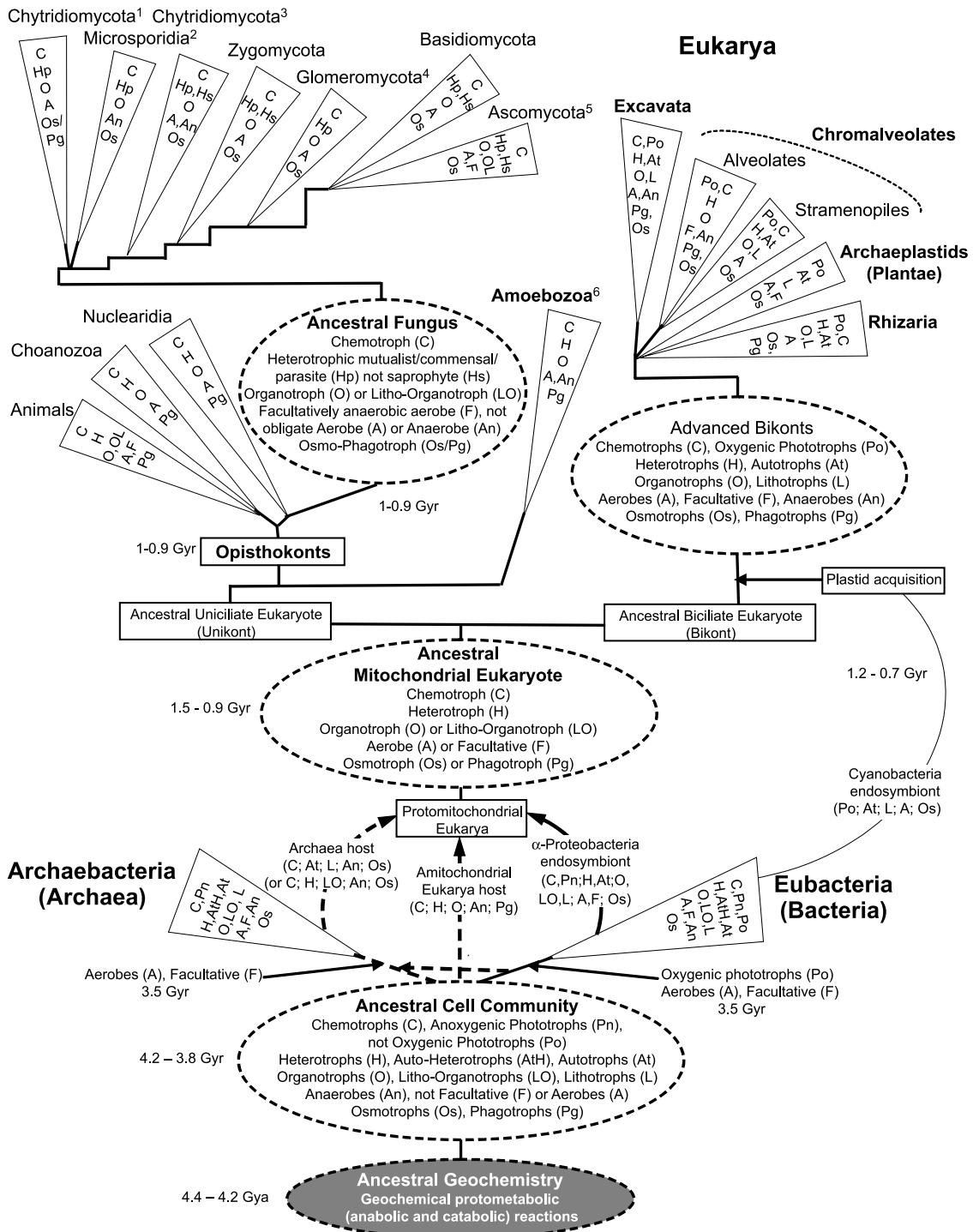
Further discussion of this important issue is beyond the scope of this chapter, but recognition that significant pitfalls exist for both morphological and molecular methods is important, so that they are used and interpreted cautiously, preferably in unison (Givnish and Sytsma 1997; Baker and Gatesy 2002).

## IV. Eukaryote Evolution and Fungal Phylogeny

### Eukaryotic Emergence

Figure 1.1 presents a stylized phylogenetic tree starting with the origin of life, and focusing on eukaryotes, fungi, and alphaproteobacteria (the hypothetical ancient mitochondrial endosymbiont). We have expanded the tree to include energy-generating and other ecophysiological trophic properties of the major taxa relative to those of alphaproteobacteria. The closest functional matches potentially identify the taxa closest to the mitochondrial eukaryotic root (either complementing or conflicting with the phylogenetic position) and/or the taxa exposed to environmental evolutionary pressures similar to those of the original alphaproteobacterial endosymbiont.

While it was once tacitly assumed that eukaryotes simply emerged from prokaryotes, the current interpretation is contentious and much more complex (Roger 1999; Martin and Russell 2003; Horner and Hirt 2004; Martin et al. 2003; Embley and Martin 2006; de Duve 2007). (For convenience and consistency with convention in virtually all the literature [e.g., Adl et al. 2005], we refer collectively to the bacteria and archaea as prokaryotes; we note that Pace [2006] has argued



**Fig. 1.1.** A view of eukaryote evolution from the origin of life, recognizing trophic properties of the major lineages and focusing on the fungi. This view integrates recent information and concepts (Roger 1999; Richards and Cavalier-Smith 2005; Embley and Martin 2006; Pace 2006; James et al. 2006; Kurland et al. 2006; Steenkamp et al. 2006; de Duve 2007), with the trophic ancestral origin of life (Kurland et al. 2006) amplified and expanded to include the

ancestral origin of eukaryotes and fungi, and the trophic properties of the major lineages. Broken lines identify controversy (e.g., existence or not of amitochondriate Eukarya, and sisterhood or descendant relationship of Eukarya to Archaea). All trophic categories assigned to the different taxa are based on extant properties. Unless specified otherwise (footnotes<sup>1–6</sup>), the unikont taxa are trophically simple mitochondrial organisms

against continued use of this term.) In terms of energy generation trophic properties, the alphaproteobacterial mitochondrial ancestor of eukaryotes is generally considered to have been chemotrophic (chemical energy), rather than phototrophic (primary energy light), organotrophic (organic electron donor) or litho-organotrophic (organic and supplementary inorganic sulfur  $e^-$  donor), rather than lithotrophic (inorganic electron donor), and aerobic ( $O_2$  electron acceptor for energy generation) or facultatively anaerobic ( $O_2$  and alternative electron acceptor for energy generation) (Cavalier-Smith 2004; Richards and Cavalier-Smith 2005; Embley and Martin 2006), although as recognized in Fig. 1.1, extant alphaproteobacteria are more diverse than this. The fact that eukaryotes split into either chemotrophs or oxygenic phototrophs argues against an anoxygenic phototrophic alphaproteobacterium as the ancestral mitochondrial endosymbiont.

The two most common scenarios for the emergence of eukaryotes are either (1) a nucleus-bearing, amitochondriate, proto-eukaryote cell acquiring an alphaproteobacterial endosymbiont, and giving rise to a mitochondrion-bearing eukaryote, or (2) a prokaryote (specifically archaeal) host acquiring an alphaproteobacterial endosymbiont that became a mitochondrion, with the recipient becoming progressively more eukaryote-like (acquiring or evolving a nucleus, cytoskeleton, etc.; Dyall et al. 2004; Embley and Martin 2006; Margulis et al. 2006). Neither hypothesis is entirely compelling, the former being deficient because no extant, primitively amitochondriate eukaryotic organisms have yet been found (Embley and Martin 2006), the latter because extant prokaryotes are not phagocytic, and with one exception, are not

<sup>1,3</sup> Chytridiomycota (para- or polyphyletic) include hydrogenosomal anaerobes such as *Neocallimastix* and *Piromyces* (Yarlett and Hackstein 2005). The initial lineage is represented by *Rozella*.

<sup>2</sup> Microsporidia are composed of mitosomal obligate anaerobes such as *Trichipleistophora* and *Encephalitozoon* (Van der Giezen et al. 2005).

<sup>4</sup> Glomeromycota (formerly Glomales of Zygomycota) are obligate endoparasites of photoautotrophs (Schüßler et al. 2001).

<sup>5</sup> Ascomycota include mitochondrial, facultatively anaerobic fermenters such as *Saccharomyces* (Tielens et al. 2002); denitrifiers and ammonia fermenters such as *Aspergillus* and *Fusarium* (Takasaki et al. 2004); methylotrophic yeasts such as *Pichia* and *Candida* (Nakagawa et al. 2005) and mycelial fungi such as *Trichoderma*, *Gliocladium*, and *Paecilomyces* (Tye and Willetts 1977).

<sup>6</sup> Amoebozoa include mitochondrial aerobes such as *Phalansterium*, the potential ancestral heterotrophic aerobic uniciliate eukaryote (Cavalier-Smith 2004), and mitosomal anaerobic Arachamoeba/Endamoeba such as *Entamoeba* (Van der Giezen et al. 2005).

known to harbor symbionts (Cavalier-Smith 2002, 2004; Embley and Martin 2006). While there is considerable evidence that eukaryotic informational genes have archaeal homologs, and that eukaryotic operational genes have bacterial homologs (Embley and Martin 2006; Kurland et al. 2006), the Eukarya have unique “signature proteins”, as do the other two domains (Hartman and Fedorov 2002). Such commonalities may suggest early, failed experiments in symbiosis, or gene transfer events among the three major domains Bacteria, Archaea, and Eukarya (see below, and Pace 2004). Pace states that “molecular trees based on rRNA and other reliable genes show unequivocally that the eukaryotic nuclear line of descent is as old as the archaeal line” (2005, p. 57). He further believes that “the modern kind of eukaryotic cell [sic], with organelles, probably also arose early, more than 3.5 Byr ago” ([sic; Byr = Gyr] Pace 2004, p. 84). Consequently, we show in Fig. 1.1 the origin of life and the universal ancestor as unspecified, with various evolutionary interconnections possible (dotted lines) among the three domains.

### Fungal Emergence

Proceeding up the tree, the most fundamental division among the eukaryotes is the split between the ancestrally uniciliate (unikont) and biciliate (bikont) lineages (Fig. 1.1; Cavalier-Smith 2002; Stechmann and Cavalier-Smith 2003; Richards and Cavalier-Smith 2005; Embley and Martin 2006). Generally speaking, the unikonts include the animals, fungi, and amoebozoans, while the bikonts comprise the plants, algae, and protozoans (Embley and Martin 2006). Among organisms examined to date, the genes for dihydrofolate reductase (DHFR) and thymidylate synthetase (TS) are fused in bikonts, but separate in unikonts; additionally, protein trees and other gene fusion/duplication data support this major eukaryotic division (Stechmann and Cavalier-Smith 2003; Cavalier-Smith 2004). From such evidence is drawn the presumptive conclusion that the root of the eukaryote tree is between the bikonts and unikonts (Stechmann and Cavalier-Smith 2003; Cavalier-Smith 2004; Embley and Martin 2006).

Fungi, animals, and various unicellular eukaryotes (the Choanozoa) together form the monophyletic Opisthokonta (Fig. 1.1), phenotypically diverse but linked mainly by a common molecular phylogeny (Steenkamp et al. 2006). This is an ancient lineage, perhaps at least 1 Gyr old (Knoll 1992;

Butterfield 2005). Depending on the classification system, the microsporidia (obligate intracellular parasites mainly of animals) either are (Bullerwell and Lang 2005; Adl et al. 2005; James et al. 2006) or are not (Steenkamp and Baldauf 2004; Embley and Martin 2006; Steenkamp et al. 2006) considered with the fungi, with which they are regarded to be at least very closely aligned (Hirt et al. 1999). A recent reconstruction of fungal evolution based on a six-gene phylogeny (James et al. 2006) shows the earliest divergence within the kingdom Fungi contains the microsporidia and the endoparasitic chytrid *Rozella allomycis*. The closest known relative to an ancestral fungus is an amoeboid, phagotrophic *Nuclearia* (Fig. 1.1, and James et al. 2006).

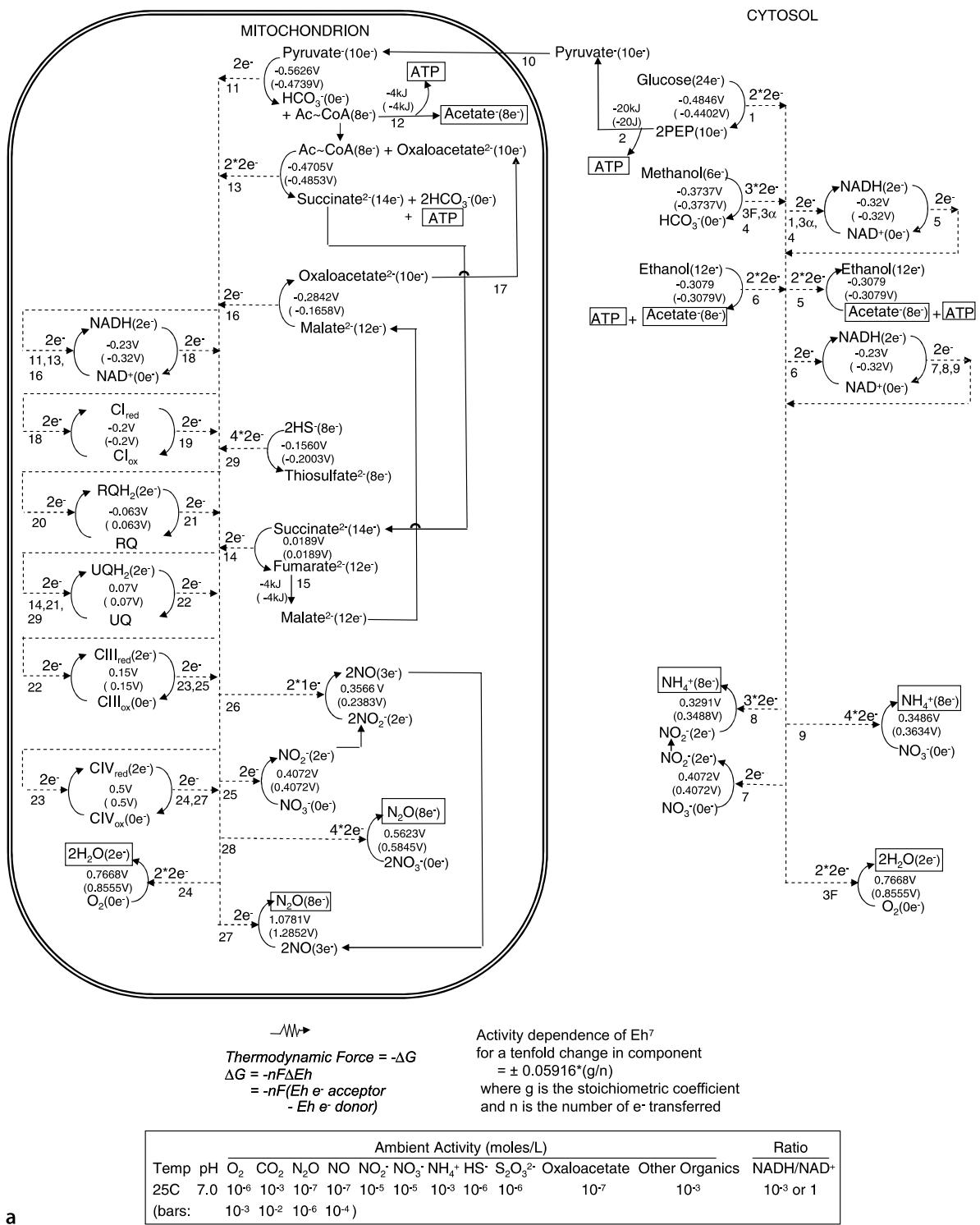
The ancestral fungus in Fig. 1.1 was likely a chemoheterotroph, and obtained its food as a parasite (or possibly as a commensal or mutualist), osmo/phagotrophically, with mitochondria producing energy by organotrophic or litho-organotrophic, aerobic, and anaerobic metabolism (e.g., integration of Fig. 1.2a and b). Depending on habitat selection pressures, the primordial osmo/phagotrophic fungus could early have evolved into *Rozella*-type aerobic chytrids, and into anaerobic microsporidia, by mitochondrial degeneration into anaerobic mitosomes, and loss of phagotrophism. The chytrid line later evolved into *Neocallimastix*-type anaerobic chytrids, by degeneration of mitochondria into hydrogenosomes and loss of phagotrophism, and then into aerobic chytrids. Still later, the more advanced extant fungi evolved in part by physiological losses, and lateral gene transfer (LGT) gains with respect to the ancestral fungus (Fig. 1.1).

The general consensus from evidence based on SSUrDNA sequences, protein trees, and limited information from cellular structure and physiology, is that the animal and fungal kingdoms are each monophyletic and the most closely related to one another (Steenkamp and Baldauf 2004; however, see comments below on geological age). The Choanozoa, consisting of four classes, constitute a paraphyletic group, and the order of branching of lineages within the opisthokonts is unclear (Steenkamp and Baldauf 2004). The choanoflagellates (aquatic uniflagellates) have both animal (sponges) and fungal similarities, suggesting that the Chytridiomycota, the oldest true fungal phylum, may have arisen from a chitinous thecate member (Steenkamp and Baldauf 2004; Steenkamp et al. 2006). Chytrids, which are aquatic (occasionally marine) and to some extent soil-borne, have

**Fig. 1.2.a** Bioenergetic electron flow diagram of mitochondrial and cytosolic dissimilatory reactions of extant aerobic and facultatively anaerobic fungi, and overlapping dissimilatory reactions of extant facultative alphaproteobacteria, under microaerophilic conditions. The  $e^-$  transfer half-reaction couples are located in non-scalar line with decreasingly less negative  $e^-$  transfer potential ( $Eh^7$ ); standard activity neutral pH potentials ( $Eh^{07}$ ) are in parentheses. The potentials are expressed to 4 decimals to allow  $\Delta G$  derivation identical to that using the  $\Delta G_f^0$  of the components in a completely mass-balanced equation. The total available  $e^-$  are in parentheses behind the compound name. Dotted lines identify  $e^-$  flow from  $e^-$  donor to  $e^-$  acceptor couples. In general, the number of  $e^-$  transferred from/to a couple is standardized on a single or multiple  $2e^-$  basis. Appropriate prorating of the half-reaction couples is needed to achieve transfer of the same number of  $e^-$ ,  $n$ , from the donor to the acceptor for a complete reaction. The energetics of a complete reaction is a function of  $n$ , the Faraday constant (F), and the difference in  $e^-$  transfer potential between the  $e^-$  acceptor and  $e^-$  donor couples, as shown in the equation, with  $e^-$  flowing downhill for an exergonic (energy generating) reaction. The non-redox reactions show the ambient ( $\Delta G^7$ ) and standard ( $\Delta G^{07}$ , in parentheses) energetics of the reactions. Compounds enclosed by boxes are terminal metabolites. The enzymes or processes for a linked  $e^-$  donor and  $e^-$  acceptor, or non-redox reaction, are numerically coded as follows. 1 NAD-linked glycolysis to phosphoenol pyruvate (PEP), 2 pyruvate kinase, 3F oxygen-linked alcohol oxidase (fungi), 3a methanol dehydrogenase ( $\alpha$ -proteobacteria), 4 NAD-linked formaldehyde dehydrogenase and formate dehydrogenase, 5 NAD-linked acetate reduction to ethanol via acetyl CoA (reverse of reaction 6), 6 NAD-linked alcohol dehydrogenase, CoA-acylating aldehyde dehydrogenase, acetyl CoA synthetase, 7 NAD-linked nitrate reductase, 8 NAD-linked nitrite reductase, 9 NAD-linked nitrate and nitrite reductase, 10 pyruvate transport into mitochondria (fungi), 11 NAD-linked pyruvate dehydrogenase, 12 ATP production via acetate:succinate CoA-transferase, 13 NAD-linked acetate oxidation via TCA cycle conversion of oxaloacetate to succinate, 14 UQ-linked succinate dehydrogenase, 15 fumarase, 16 NAD-linked malate dehydrogenase, 17 recycling of oxaloacetate back into the TCA cycle, 18–24 respiratory  $e^-$  carrier system, 25 nitrate reductase, 26 nitrite/nitric oxide reductase, 27 nitric oxide/nitrous oxide reductase, 28 denitrification to nitrous oxide, 29 UQ-linked sulfide oxidase

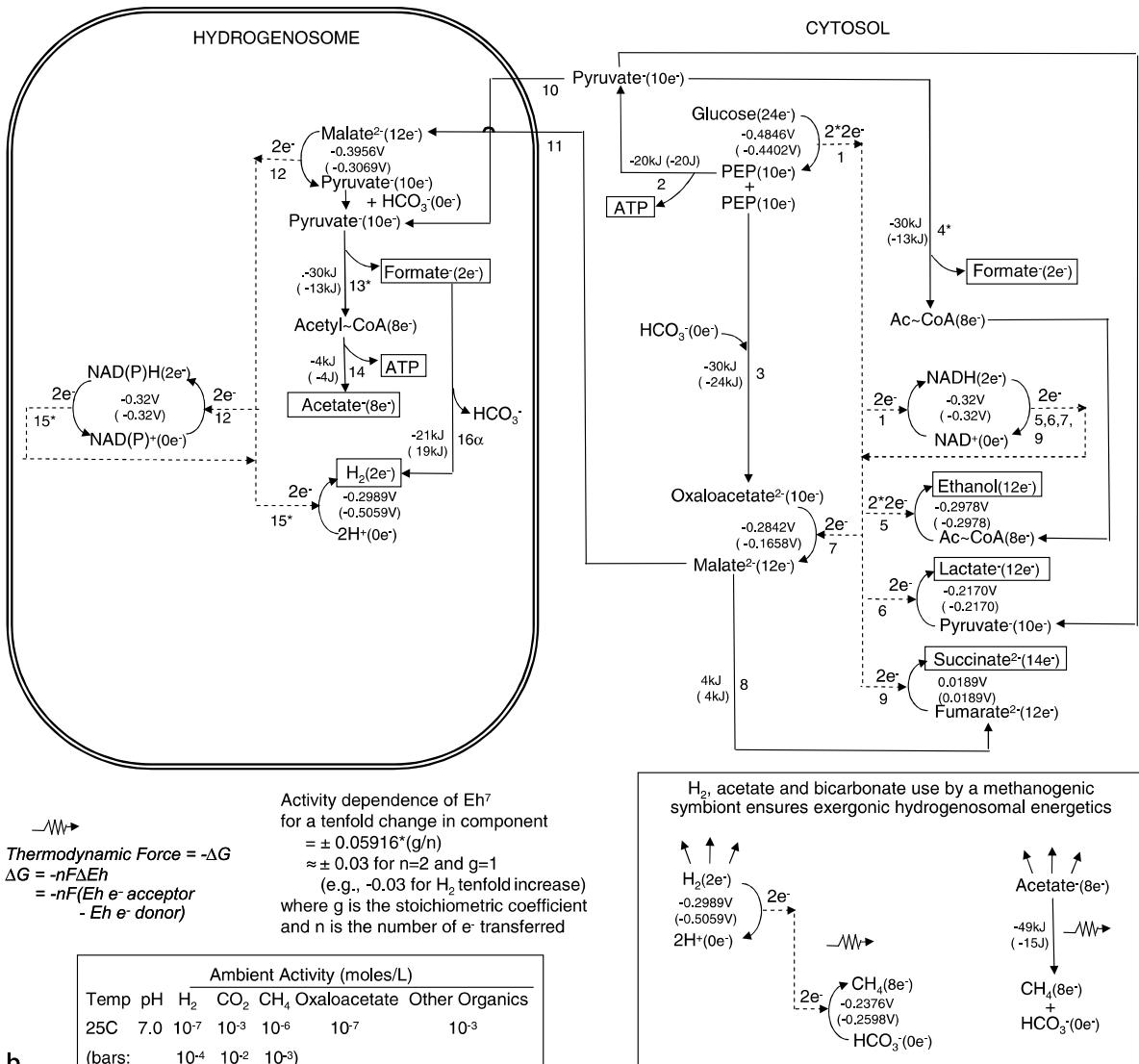
generally relatively sparse, coenocytic thalli, and are unique among the fungi in possessing uniflagellated (rarely polyflagellated) zoospores and gametes. Most extant species are saprobes, though several are parasites of plants, animals, or fungi.

The unifying characteristics of the monophyletic group Fungi (Eumycota) include a nutritive mode based on absorptive heterotrophy (osmotrophy, rather than phagotrophy), and possession of typically tube-like walls composed of  $\beta$ -glucan and chitin enclosing multi-nucleate



protoplasm, with mitochondria or derivatives such as hydrogenosomes or mitosomes present (Martin et al. 2003; Steenkamp and Baldauf 2004; Adl et al. 2005). The so-called higher fungi, comprising the

Ascomycota and Basidiomycota, each of which is monophyletic, form a sister clade with the monophyletic Glomeromycota (as endomycorrhizae, obligate symbionts of photoautotrophs; Schüßler



**Fig. 1.2.b** Bioenergetic electron flow diagram of hydrogenosomal dissimilatory reactions of extant anaerobic fungi, and overlapping dissimilatory reactions of extant facultatively anaerobic alphaproteobacteria (see Fig. 1.2a caption for explanation of figure structure). The codes for the enzymes or processes are as follows. 1 NAD-linked glycolysis to phosphoenol pyruvate (PEP), 2 pyruvate kinase, 3 phosphoenolpyruvate carboxykinase, 4 pyruvate:formate-lyase, 5 NAD-linked alcohol dehydro-

genase, 6 NAD-linked lactate dehydrogenase, 7 NAD-linked malate dehydrogenase, 8 fumarase, 9 NAD-linked fumarate reductase, 10 and 11 pyruvate and malate import into hydrogenosome (fungi), respectively, 12 NAD-linked malic enzyme, 13 pyruvate:formate-lyase, 14 ATP production via acetate:succinate CoA transferase, 15 NAD-linked hydrogenase, 16a formic hydrogen lyase (alphaproteobacteria). An asterisk means that the reaction is unique to fungi within the anaerobic eukaryotes

et al. 2001; Steenkamp and Baldauf 2004; James et al. 2006; Steenkamp et al. 2006). The basal branches are occupied by the Zygomycota and Chytridiomycota, both of which are paraphyletic or polyphyletic, and for which the relationships and evolutionary pathways are unclear and controversial (Steenkamp and Baldauf 2004; James

et al. 2006; Steenkamp et al. 2006). A general inference is that the primordial fungi were primarily aquatic, and lacked aerial dispersal (James et al. 2006). Numerous adaptations are apparent in the life cycle as fungi colonized land (see below) along with animals and plants. Among these are an increase in filamentous habit optimized for

absorptive nutrition based on saprotrophy or parasitism; loss of flagellated spores (on at least four separate occasions; James et al. 2006); and multiple innovations for spore production, aerial dispersal, and survival.

For reasons discussed above (see Phylogenetic Evidence and its Interpretation), estimation of the geological history of the fungi based on fossil evidence is often not in accord with inference from molecular clock assumptions. Based on their own calibrated molecular clock calculations and review of the literature, Berbee and Taylor (2001) estimated that the fungi diverged from the animal lineage at about 1.0–0.9 Gyr, prior to terrestrial flora, though Butterfield's (2005) claim of a “probable fungus” with characteristics similar to the zygomycetes dated at 1.43 Gyr from Australian rock, if correct, moves this back substantially. A major multi-gene analysis of the plant–animal–fungi divergence times estimated these to be approximately concurrent and relatively ancient at  $1.58 \pm 0.09$  Gyr (Wang et al. 1997). Subsequent radiation of the terrestrial fungi, roughly at 0.6 Gyr, likely followed closely the origin of land plants (Berbee and Taylor 2001; see also Selosse and Le Tacon 1998), but also involved affiliations with algae before the evolution of vascular plants (Heckman et al. 2001; Lutzoni et al. 2001; Yuan et al. 2005). The final major divergence, occurring perhaps at about 0.4–0.5 Gyr, was the splitting of the Ascomycota from the Basidiomycota (Berbee and Taylor 2001; Taylor et al. 2004).

Since much of microbial evolution is internal, rather than external, as alluded to at the outset in this chapter, internal changes beyond ultrastructure and physiology involve informational (replication, transcription, and translation) and operational (assimilatory biosynthesis, and energy-generating dissimilatory metabolism) genes (Embley and Martin 2006). Energy generation is the province of mitochondria, and remnant mitochondria such as hydrogenosomes and mitosomes. LGT from the mitochondrion to the nucleus has occurred extensively over time, depending on ecological role and environmental conditions (see below; Kurland and Andersson 2000; Bullerwell and Lang 2005). In addition, LGT from external sources to the nucleus also has occurred frequently (see below; Kurland and Andersson 2000; Gribaldo and Philippe 2004; Bullerwell and Lang 2005). Accordingly, any mitochondrial endosymbiont ancestry of the dissimilatory processes carried out under nuclear control in the cytosol is shrouded

in history. However, LGT from the nucleus or external sources to the mitochondrion is much less likely (Kurland and Andersson 2000; Bullerwell and Lang 2005). Thus, genes retained in extant or remnant mitochondria may well be ancestral to the original mitochondrial endosymbiont. The general consensus is that extant eukaryotic aerobes and facultative anaerobes have evolutionarily retained the inherited mitochondrial endosymbiont dissimilatory respiratory chain properties within their mitochondria, but have undergone extensive loss or intracellular transfers of other mitochondrial genes to the nucleus, and also may have gained nuclear genes via external lateral gene transfer. Similarly, many extant eukaryotic anaerobes have apparently lost much of their superfluous respiratory chain machinery, so that their mitochondria have evolved into ATP-generating, H<sub>2</sub>-producing hydrogenosomes or mitosomes (Embley and Martin 2006; Yarlett and Hackstein 2005). The only common denominator of mitochondria, hydrogenosomes, and mitosomes is iron-sulfur cluster assembly (Van der Giezen et al. 2005; Embley and Martin 2006).

From Fig. 1.1 it can be seen that, as a group, extant alphaproteobacteria have much more diverse metabolic properties than any of the eukaryotes. Standard logic in phylogenetics is that properties of ancestors can be inferred from commonalities of the descendants (Pace 1991). This leads to the further inference that more can be understood from modern organisms that are more closely related to the earliest organisms than from those that are evolutionarily more distant (Pace 1991). Applying that rationale to physiology, the extant eukaryotic lineage with the most metabolic overlap with extant alphaproteobacteria is evolutionarily closest to the endosymbiotic mitochondrial ancestor (Tielens et al. 2002) – fungi are that group (Fig. 1.1).

## V. Bioenergetic Analyses Applied to Evolutionary and Phylogenetic Relationships

Figure 1.2a and b provide a mechanistic expansion of the energy-generating trophic relationships between fungi and alphaproteobacteria identified in Fig. 1.1, as a function of an operationally simple, quantitative, bioenergetic electron flow (linked electron donors and acceptors) framework based on integrated thermodynamic and biochemical

principles. This framework allows direct comparative visualization of dissimilatory reactions and pathways that can and cannot proceed under ambient and standard environmental conditions, and identifies evolutionary energy connections between the mitochondrial endosymbiont and aerobic and anaerobic fungi. It also allows quantitative assessment of hypotheses for the symbiotic origin of eukaryotes.

Construction and interpretation of the electron flow diagrams are identified in the figure captions and footnotes, and described in more detail elsewhere (Harris and Arnold 1995; Andrews and Harris 2000; Zwolinski et al. 2000). The focus of Fig. 1.2a and b is on comparison of the dissimilatory reactions in fungal mitochondria and hydrogenosomes, respectively, as distinct from the cytosol, which overlap with the dissimilatory reactions of extant alphaproteobacteria. In brief, the electron transfer half-reactions shown in the figures are dissimilatory electron donors and electron acceptors linked in diverse combinations; specific reactions and pathways of interest are coded for the extant catalytic enzymes involved. The  $e^-$  transfer half-reactions are ranked according to decreasingly less negative  $e^-$  transfer potential (decreasing  $e^-$  donor/increasing  $e^-$  acceptor potential) under ambient conditions. Qualitative energetics are readily apparent by visual examination (downhill flow of  $e^-$  identifying an exergonic reaction). If desired, quantitative energetics are readily derived as  $\Delta G = -nF\Delta E$  (Fig. 1.2a and b), where  $-\Delta G$  refers to the Gibbs free energy change of the reaction (with a negative sign denoting an exergonic reaction),  $n$  is the number of electrons transferred,  $F$  is the Faraday constant, and  $\Delta E$  is the difference between the electron transfer potential of the electron acceptor and electron donor. This procedure is straightforward compared to the labor-intensive classical construction of completely stoichiometrically balanced equations, and calculation of  $\Delta G$  as a function of energy of formation and ambient activities of all reaction components (Amend and Shock 2001, cited by Martin and Russell 2003). The proton ionizable forms of the metabolites used in the figures are those dominant at the ambient pH (7) as defined by the pKa; the Eh<sup>07</sup> data were derived from  $\Delta G_f^0$  properties (Amend and Shock 2001; Thauer et al. 1977), or for the respiratory chain  $e^-$  carriers, obtained directly from the literature (Tielens et al. 2002; Voet and Voet 2005). Ambient activities used for the figures were generally in the upper Km range, with the following

exceptions: (1) O<sub>2</sub> activity was set at an order of magnitude lower than the Km to provide highly microaerophilic conditions under which O<sub>2</sub> activity would be marginal for enzyme accessibility; (2) oxaloacetate and H<sub>2</sub> activity, NAD(P)H/NAD(P)<sup>+</sup> ratios, were adjusted to levels needed for exergonic pathways. Operationally simple Eh adjustments for tenfold changes in ambient activities are given in the figures.

Overlapping with alphaproteobacteria (Gorrell and Uffen 1977; Garrity et al. 2005), the following noteworthy trophic properties of fungi can be summarized from Fig. 1.2a for aerobes and facultative anaerobes (properties 1 and 2a-d), and from Fig. 1.2b for obligate anaerobes (property 3):

- (1) All aerobic fungi, together with other aerobic eukaryotes, have a classical mitochondrial respiratory chain (Fig. 1.2a, reactions 18–24) for energy-conserving  $e^-$  flow to O<sub>2</sub> (Tielens et al. 2002).
- (2) Certain ascomycete fungi:
  - (a) in common with amoeba and ciliated protozoans, can use hydrogen sulfide as a mitochondrial  $e^-$  donor (Fig. 1.2a, reaction 29) for detoxification, if not energy generation (Theissen et al. 2003);
  - (b) in common with facultative protists, can use intracellular organics as cytosolic  $e^-$  acceptors for NAD<sup>+</sup> regeneration (e.g., acetate reduction to ethanol, Fig. 1.2a, reaction 5) under O<sub>2</sub>-limiting conditions;
  - (c) are the only eukaryotes capable of cytosolic, methylotrophic use of 1 C compounds as aerobic  $e^-$  donors (Fig. 1.2a, reactions 3F and 4; Tye and Willetts 1977; Nakagawa et al. 2005);
  - (d) are the only eukaryotes capable of anaerobic mitochondrial respiration using nitrate denitrification to nitrous oxide as an alternate  $e^-$  acceptor (Fig. 1.2a, reactions 25–28), and cytosolic nitrate reduction to ammonium (Fig. 1.2a, reactions 6–9) as a mechanism for substrate-level ATP production and NAD<sup>+</sup> regeneration (Tielens et al. 2002; Takasaki et al. 2004).
- (3) Anaerobic chytrids are unique within the eukaryotes in having hydrogenosomes that generate energy by substrate-level ATP production from malate oxidation to pyruvate, followed by pyruvate degradation to acetate and formate (pyruvate formate lyase pathway, PFL; Fig. 1.2b, reactions 12–14), accompanied

by NAD(P)H-linked hydrogenase reduction of protons to H<sub>2</sub> (Fig. 1.2b, reaction 15; Yarlett and Hackstein 2005). The PFL pathway compares to the pyruvate-ferridoxin oxidase (PFO) pathway for pyruvate oxidation and H<sub>2</sub> production by non-fungal hydrogenosomal eukaryotes (Yarlett and Hackstein 2005; Embley and Martin 2006), a pathway that is minor and likely obtained by lateral gene transfer over evolutionary time by alphaproteobacteria such as *Rhodospirillum* (Gorrell and Uffen 1977).

For anaerobic fungi, trophic categorization (property 3) of the Chytridiomycota as closest to alphaproteobacteria supports their phylogenetic placement as the oldest fungal phylum (Fig. 1.1). In contrast, within aerobic and facultative eukaryotes, fungal ascomycetes are the closest to alphaproteobacteria based on energy-generation trophic properties, and from this standpoint are the closest to the mitochondrial root. This affinity is decreased if the cytosolic properties (2b, 2c and part of 2d) are discounted, but not enough to affect the outcome. However, this trophic identification of ascomycetes as the oldest conflicts with the phylogenetic-based location of ascomycetes as the youngest member of the fungal group (Fig. 1.1). A possible explanation is that the closest match hypothesis is compromised by LGT and/or the possibility that the ascomytes involved were exposed over time to environmental evolutionary pressures similar to those of the original alphaproteobacteria endosymbiont, thereby retaining the related, ecologically advantageous properties from the original alphaproteobacterium.

Included in Fig. 1.2b (bottom right) is recognition that hydrogenosomal anaerobic eukaryotes often form symbiotic relationships with chemoaustrotrophic partners that make a living from the H<sub>2</sub>, acetate, and bicarbonate waste products of the eukaryote. This maintains a low pool size of these metabolites, and results in beneficial dissimilatory e<sup>-</sup> transfer potentials favorable for exergonic reactions by the eukaryotic partner. The quantitatively based electron flow framework facilitates interpretation and prediction of whether such symbiotic relationships are thermodynamically feasible, or not, as a function of operational e<sup>-</sup> transfer potentials dictated by standard potentials and metabolite pool sizes. Accordingly, in the example shown in Fig. 1.2b, the symbiotic relationship will fail for an H<sub>2</sub> pool size above about 10<sup>5</sup> mol/l

(for this pool size, the Eh<sup>7</sup> for 2H<sup>+</sup>/H<sub>2</sub> would become  $\sim -0.51 + 5 * 0.03 = -0.36$  V, which is too negative (uphill e<sup>-</sup> flow) for proton reduction by NADH/NAD<sup>+</sup> of Eh<sup>7</sup> = -0.32 V).

The symbiotic relationship in Fig. 1.2b is the basis of the hydrogen hypothesis for the force driving the ancient endosymbiotic formation of protomitochondria from an H<sub>2</sub>-producing alphaproteobacterium endosymbiont and an H<sub>2</sub>-consuming archaeal methanogenic host (Embley and Martin 2006). The viability of other current and new hypotheses for primordial symbiotic associations may be supported, or discounted by use of the bioenergetic electron flow framework.

## VI. Conclusions

Efforts to understand the two fundamental benchmarks in the history of life on earth – the origin of living systems, and the origin of eukaryotes – have provoked arguably more thought and debate than any other scientific question. With increasingly comprehensive paleontological information, and substantial advances in molecular systematics in the past two decades, more coherent scenarios are emerging and being subjected to critical test. However, like an exponential decline curve that infinitely approaches, but never reaches the axis, scientists can approach, but will never achieve full comprehension of the primordial world, which is literally lost in time.

The root of the universal tree and the nature of the universal ancestor are unclear, and likely to remain so, with relatively strong opinions supported by relatively weak data (Cavalier-Smith 2002, 2004; Embley and Martin 2006; Kurland et al. 2006). Regardless of their ultimate position in the tree of life (perhaps now more shrub-like in form!), fungi and allies display incipient forms of key biological trends, and hence are a particularly informative lineage. Fungi inhabit, as parasites, mutualists, commensals, or saprobes, most if not all terrestrial and aquatic habitats. They show, perhaps more so than any other lineage, extreme phenotypic and genotypic plasticity (Andrews 1991, 1992). This includes a system of balanced, coexisting genomes within one cell and one individual (the dikaryon), and life cycles that are variously either exclusively asexual, that bypass the sexual by unusual means (parasexuality), that alternate between sexual and asexual, or that can apparently run indefinitely as

one or the other. They show interesting and occasionally complex life cycles, especially among the parasites, most notably the rust fungi. The phylogenetic transition from a relatively undifferentiated, motile, aquatic lifestyle (chytrids) to a differentiated, sessile, terrestrial lifestyle sets in motion competing forms of natural selection, one operating at the cell lineage, the other at the level of the individual (Buss 1987). This in turn invokes isolating mechanisms during ontogeny, and of self/non-self recognition systems characteristic of the eukaryotes (Buss 1987). These attributes are clearly quite different from those of the bacteria or archaea.

There is strong evidence that eukaryote cells arose by symbiosis, and that the symbiont (protomitochondrion) was an alphaproteobacterium, though the nature of the recipient (archaeabacterium or proto-eukaryote) is unclear. The consensus now is that all eukaryotes contain mitochondria of one sort or another (Martin et al. 2003; Embley and Martin 2006), and that certain early eukaryotes subsequently acquired other kinds of symbionts (cyanobacterial origin of plastids in the Plantae; Cavalier-Smith 2002; Dyall et al. 2004). Despite some genetic homology and other common attributes, the domains Archaea, Bacteria, and Eukarya are quite different in ultrastructure, and in many cellular properties, including the cell wall (Cavalier-Smith 2002; de Duve 2007), not to mention sizes and geometries. So, whatever the nature of the initial symbiosis, the eukaryotic descendants have radiated into remarkably diverse extant forms unlike their ancestor. While numerical superiority and genotypic diversity on Earth may be reflected in the prokaryotes, the phenotypic diversity is represented overwhelmingly by the eukaryotes. This is testimony to the nature of the eukaryotic cell, environmental heterogeneity that set the stage for such radiation to occur, and energetic forces discussed further below. With the evolution of progressively more sophisticated systems to harvest energy, especially light energy, together with the use of oxygen as an electron acceptor, energy availability increased exponentially, and with it, biodiversity.

An important issue not addressed mechanistically by phylogenetics is what drove the evolutionary origins of the fungi and other early eukaryotes. We have approached this functional question by adding the dimension of trophic property to the conventional phylogenetic tree (Fig. 1.1), and exploring the biochemical aspects further by using bioenergetic electron flow dia-

grams (Fig. 1.2a, b). This shows that the Fungi are clearly closely related metabolically to the ancestral mitochondrial eukaryote and to the alphaproteobacterial endosymbiont. The Fungi are notable in being osmotrophs, chemotrophs, heterotrophs, organotrophs (some litho-organotrophs) with aerobic, facultatively anaerobic, or anaerobic capabilities. Based on their osmotrophy and biochemical versatility, in particular with respect to diverse energetic pathways, “a eukaryotic tree with fungi first would make sense”, according to Martin et al. (2003) who have updated a hypothesis of Cavalier-Smith from the early 1980s. Though the trophic relationships are not always in close accord with phylogenetic placement, they add an important biological dimension in efforts to understand evolutionary patterns. Inconsistencies may be explicable by relatively straightforward phenomena such as LGT, or reflect the retention of primordial properties by some advanced lines, or be indicative that the gene-based trees are incorrect at least in some places. The bioenergetic electron flow diagrams complement gross-level trophic classifications by providing a visual, conceptually powerful tool for understanding and predicting the biogeochemical forces driving organism growth and ecology, and are applicable not only to the origin of fungi and eukaryotes, but to all eras – from germination through continuing evolution of the tree of life.

## References

- Adl SM, Simpson AGB, Farmer MA, and 25 other authors (2005) The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J Eukaryot Microbiol* 52:399–451
- Amend JP, Shock EL (2001) Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and Bacteria. *FEMS Microbiol Rev* 25:175–243
- Andrews JH (1991) Comparative ecology of microorganisms and macroorganisms. Springer, Berlin Heidelberg New York
- Andrews JH (1992) Fungal life-history strategies. In: Carroll GC, Wicklow DT (eds) *The fungal community*, 2nd edn. Marcel Dekker, New York, pp 119–145
- Andrews JH, Harris RF (2000) The ecology and biogeography of microorganisms on plant surfaces. *Annu Rev Phytopathol* 38:145–180
- Baker RH, Gatesy J (2002) Is morphology still relevant? In: DeSalle R, Giribet G, Wheeler W (eds) *Molecular systematics and evolution: theory and practice*. Birkhäuser, Basel, pp 163–186
- Bekker A, Holland HD, Wang P-L, Rumble III D, Stein HJ, Hannah JL, Coetzee LL, Beukes NJ (2004) Dating the rise of atmospheric oxygen. *Nature* 427:117–120

- Berbee ML, Taylor JW (1999) Fungal phylogeny. In: Oliver R, Schweizer M (eds) Molecular fungal biology. Cambridge University Press, New York, pp 21–77
- Berbee ML, Taylor JW (2001) Fungal molecular evolution: gene trees and geologic time. In: McLaughlin DJ, McLaughlin EG, Lemke PA (eds) The Mycota VII Part B. Systematics and evolution. Springer, Berlin Heidelberg New York, pp 229–245
- Brasier MD, Green OR, Jephcott AP, Kleppe AK, Van Kranendonk MJ, Lindsay JF, Steele A, Grassineau NV (2002) Questioning the evidence for Earth's oldest fossils. *Nature* 416:76–81
- Bullerwell CE, Lang BF (2005) Fungal evolution: the case of the vanishing mitochondrion. *Curr Opinion Microbiol* 8:362–369
- Buss LW (1987) The evolution of individuality. Princeton University Press, Princeton, NJ
- Butterfield NJ (2005) Probable Proterozoic fungi. *Paleobiology* 31:165–182
- Butterfield NJ, Knoll AH, Swett K (1990) A bangiophyte red alga from the Proterozoic of Arctic Canada. *Science* 250:104–107
- Cavalier-Smith T (2002) The phagotrophic origin of eukaryotes and phylogenetic classification of protozoa. *Int J System Evol Microbiol* 52:297–354
- Cavalier-Smith T (2004) Chromalveolate diversity and cell megaevolution: interplay of membranes, genomes and cytoskeletons. In: Hirt RP, Horner DS (eds) Organelles, genomes and eukaryote phylogeny. CRC Press, New York, pp 75–108
- de Duve C (2007) The origin of eukaryotes: a reappraisal. *Nature Rev Genetics* 8:395–403
- Donoghue PCJ, Smith MP (2004) Telling the evolutionary time: molecular clocks and the fossil record. CRC Press, New York
- Dyall SD, Brown MT, Johnson PJ (2004) Ancient invasions: from endosymbionts to organelles. *Science* 304:253–257
- Embley TM, Martin W (2006) Eukaryotic evolution, changes and challenges. *Nature* 440:623–630
- Garrity GM, Bell JA, Lilburn T (2005) Class I Alphaproteobacteria. In: Brenner DJ, Krieg NR, Staley JT (vol eds), Garrity GM (Editor-in-Chief) Bergey's Manual of Systematic Bacteriology vol 2 C, 2nd edn. Springer, Berlin Heidelberg New York, pp 1–574
- Givnish TJ, Sytsma KJ (1997) Homoplasy in molecular versus morphological data: the likelihood of correct phylogenetic inference. In: Givnish TL, Sytsma KJ (eds) Molecular evolution and adaptive radiation. Cambridge University Press, New York, pp 55–101
- Gorrell TE, Uffen RL (1977) Fermentative metabolism of pyruvate by *Rhodospirillum rubrum* after anaerobic growth in the darkness. *J Bacteriol* 131:533–543
- Gribaldo S, Philippe H (2004) Pitfalls in tree reconstruction and the phylogeny of eukaryotes. In: Hirt RP, Horner DS (eds) Organelles, genomes and eukaryote phylogeny. CRC Press, New York, pp 133–152
- Harris RF, Arnold SM (1995) Redox and energy aspects of soil bioremediation. In: Skipper HD, Turco RF (eds) Bioremediation: science and applications. Soil Science Society of America, Madison, WI, pp 55–85
- Hartman H, Fedorov A (2002) The origin of the eukaryotic cell: a genomic investigation. *Proc Natl Acad Sci USA* 99:1420–1425
- Heckman DS, Geiser DM, Eidell BR, Stauffer RL, Kardos NL, Hedges SB (2001) Molecular evidence for the early colonization of land by fungi and plants. *Science* 293:1129–1133
- Hillis DM, Moritz C, Mable BK (eds) (1996) Molecular systematics, 2nd edn. Sinauer, Sunderland, MA
- Hirt RP, Logsdon JM, Healy B, Dorey MW, Doolittle WF, Embley MT (1999) Microsporidia are related to Fungi: evidence from the largest subunit of RNA polymerase II and other proteins. *Proc Natl Acad Sci USA* 96:580–585
- Horner DS, Hirt RP (2004) An overview of eukaryote origins and evolution: the beauty of the cell and fabulous gene phylogenies. In: Hirt RP, Horner DS (eds) Organelles, genomes and eukaryote phylogeny. CRC Press, New York, pp 1–23
- James TY, Kauff F, Schoch CL, Matheny PB, Hofstetter V, and 65 other authors (2006) Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature* 443:818–822
- Javaux EJ, Knoll AH, Walter MR (2001) Morphological complexity and ecological complexity in early eukaryotic ecosystems. *Nature* 412:66–69
- Knoll AH (1992) The early evolution of eukaryotes: a geological perspective. *Science* 256:622–627
- Kurland CG, Andersson SGE (2000) Origin and evolution of the mitochondrial proteome. *Microbiol Mol Biol Rev* 64:786–820
- Kurland CG, Collins LJ, Penny D (2006) Genomics and the irreducible nature of eukaryote cells. *Science* 312:1011–1014 and supporting online material DOI: 10.1126/science.1121674
- Lutzoni F, Pagel M, Reeb V (2001) Major fungal lineages are derived from lichen symbiotic ancestors. *Nature* 411:937–940
- Margulis L, Chapman M, Guerrero R, Hall J (2006) The last eukaryotic common ancestor (LECA): acquisition of cytoskeletal motility from aerotolerant spirochetes in the Proterozoic Eon. *Proc Natl Acad Sci USA* 103:13080–13085
- Martin W, Russell MJ (2003) On the origins of cells: a hypothesis for the evolutionary transitions from abiotic geochemistry to chemoautotrophic prokaryotes, and from prokaryotes to nucleated cells. *Philos Trans R Soc Lond B* 358:59–85
- Martin W, Rotte C, Hoffmeister M, Theissen U, Gelius-Dietrich G, Ahr S, Henze K (2003) Early cell evolution, eukaryotes, anoxia, sulfide, oxygen, fungi first(?), and a tree of genomes revisited. *IUBMB Life* 55:193–204
- Nakagawa T, Yamada K, Fujimura S, Ito T, Miyaji T, Tomizuka N (2005) Pectin utilization by the methylotrophic yeast *Pichia methanolica*. *Microbiology* 151:2047–2052
- Nisbet EG, Sleep NH (2001) The habitat and nature of early life. *Nature* 409:1083–1091
- Pace NR (1991) Origin of life – facing up to the physical setting. *Cell* 65:531–533
- Pace NR (2004) The early branches in the tree of life. In: Cracraft J, Donoghue MJ (eds) Assembling the tree of life. Oxford University Press, Oxford, pp 76–85
- Pace NR (2005) The large-scale structure of the tree of life. In: Sapp J (ed) Microbial phylogeny and evolution: concepts and controversies. Oxford University Press, Oxford, pp 53–69
- Pace NR (2006) Time for a change. *Nature* 441:289

- Richards TA, Cavalier-Smith T (2005) Myosin domain evolution and the primary divergence of eukaryotes. *Nature* 436:1113
- Roger AJ (1999) Reconstructing early events in eukaryotic evolution. *Am Nat* 154 suppl:S146–S163
- Schopf JW, Hayes JM, Walter MR (1983) Evolution of earth's earliest ecosystems: recent progress and unsolved problems. In: Schopf JW (ed) *Earth's earliest biosphere: its origin and evolution*. Princeton University Press, Princeton, NJ, pp 361–384
- Schüßler A, Schwarzott D, Walker C (2001) A new phylum, the *Glomeromycota*: phylogeny and evolution. *Mycol Res* 105:1413–1421
- Selosse M-A, Le Tacon F (1998) The land flora: a phototrophic-fungus partnership? *Trends Ecol Evol* 13:15–20
- Smith AB, Peterson KJ (2002) Dating the time of origin of major clades: molecular clocks and the fossil record. *Annu Rev Earth Planet Sci* 30:65–88
- Stechmann A, Cavalier-Smith T (2003) The root of the eukaryote tree pinpointed. *Curr Biol* 13:665–666
- Steenkamp ET, Baldauf SL (2004) Origin and evolution of animals, fungi and their unicellular allies (Opisthokonta). In: Hirt RP, Horner DS (eds) *Organelles, genomes and eukaryote phylogeny*. CRC Press, New York, pp 109–129
- Steenkamp ET, Wright J, Baldauf SL (2006) The protistan origins of animals and fungi. *Mol Biol Evol* 23:93–106
- Stern RA, Bleeker W (1998) Age of the world's oldest rocks refined using Canada's SHRIMP: the Acosta Gneiss Complex, Northwest Territories, Canada. *Geosci Can* 25:27–31
- Takasaki K, Shoun H, Yamaguchi M, Takeo, K, Nakamura A, Hoshino T, Takaya N (2004) Fungal ammonia fermentation, a novel metabolic mechanism that couples the dissimilatory and assimilatory pathways of both nitrate and ethanol. *J Biol Chem* 279:1214–1220
- Taylor JW, Spatafora J, O'Donnell K, Lutzoni F, James T, Hibbett DS, Geiser D, Bruns TD, Blackwell M (2004) The fungi. In: Cracraft J, Donoghue MJ (eds) *Assembling the tree of life*. Oxford University Press, Oxford, pp 171–194
- Thauer RK, Jungerman K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev* 41:100–180
- Theissen U, Hoffmeister M, Grieshaber M, Martin W (2003) Single eubacterial origin of eukaryotic sulfide: quinone oxidoreductase, a mitochondrial enzyme conserved from the early evolution of eukaryotes during anoxic and sulfidic times. *Mol Biol Evol* 20:1564–1574
- Thomas JA, Welch JJ, Woolfit M, Bromham L (2006) There is no universal molecular clock for invertebrates, but rate variation does not scale with body size. *Proc Natl Acad Sci USA* 103:7366–7371
- Tielens AGM, Rotte C, van Hellemond JJ, Martin W (2002) Mitochondria as we don't know them. *Trends Biochem Sci* 27:564–572
- Tye R, Willetts A (1977) Fungal growth on C1 compounds: quantitative aspects of growth of a methanol-utilizing strain of *Trichoderma lignorum* in batch culture. *Appl Environ Microbiol* 33:758–761
- Van der Giezen M, Tovar J, Graham Clark C (2005) Mitochondrion-derived organelles in protists and fungi. *Int Rev Cytol* 244:175–225
- Voet V, Voet JG (2005) *Biochemistry* vol 1, 3rd edn. Wiley, New York
- Wang DY-C, Kumar S, Hedges SB (1999) Divergence time estimates for the early history of animal phyla and the origin of plants, animals and fungi. *Proc R Soc Lond B* 266:163–171
- Wilkins AS (2002) The evolution of developmental pathways. Sinauer, Sunderland, MA
- Wilson AC, Ochman H, Prager EM (1987) Molecular time scale for evolution. *Trends Genetics* 3:241–247
- Yarlett N, Hackstein JH (2005) Hydrogenosomes: one organelle, multiple origins. *BioScience* 55:657–658
- Yuan X, Xiao S, Taylor TN (2005) Lichen-like symbiosis 600 million years ago. *Science* 308:1017–1020
- Zwolinski MD, Harris RF, Hickey WJ (2000) Microbial consortia involved in the anaerobic degradation of hydrocarbons. *Degradation* 11:141–158

---

## 2 Molecular Approaches for Studying Fungi in the Environment

K. BRUNNER<sup>1</sup>, S. ZEILINGER<sup>2</sup>, R.L. MACH<sup>1</sup>

### CONTENTS

I. Introduction .....	17
II. Immunological Detection of Fungi .....	18
III. PCR-Based Methods .....	19
A. Qualitative Diagnostic PCR .....	20
B. Quantitative PCR .....	21
C. Molecular Fingerprinting Technologies .	22
1. Denaturing Gradient Gel Electrophoresis (DGGE) .....	22
2. PCR-Restriction Fragment Length Polymorphism (PCR-RFLP), Amplified Fragment Polymorphism (AFLP) and Random Amplified Polymorphic DNA (RAPD) .....	23
IV. Probe Hybridization Technologies .....	24
V. Conclusions .....	24
References .....	25

### I. Introduction

The accurate identification of fungi from different ecosystems is essential in the fields of medical science, plant pathology, environmental studies and biological control. In phytopathology as well as animal and human pathology, the early identification of the disease-causing agent is crucial for the timely initiation of countermeasures.

Fungal community structure is rather difficult to be completely profiled in many environments, as individual ecosystems are generally complex, with fungi forming only one component of huge community assemblages. Most fungal biology has concentrated on that part of the Fungal Kingdom which is culturable, visible to the naked eye, or discernible morphologically under the microscope. The very nature of physiological and biochemical studies requires organisms which can be cul-

tured, and species identification relies on isolation, cultivation, biochemical tests and morphology. Although culture-based approaches are still a cornerstone in fungal diagnostics, and have told us most of what we presently know about fungal ecology, the methods used to isolate fungi tend to select for species able to grow only on particular media, and are therefore quite limited. Many fungal species are not culturable on given media, or fast-growing species often obscure the slower growing ones and, thus, the analysis might not reflect the true fungal community in a sample (MacNeil et al. 1995). Furthermore, conventional identification methods are very time consuming, have to be performed by skilled personnel, and can lead to problems in identification – incorrect interpretation, diagnosis and disease treatment can be the consequence. Furthermore, conventional methods used for fungal detection are predominantly non-quantitative.

To overcome the drawbacks of culture-based identification of organisms, throughout the last decade rapid screening technologies have been developed and are nowadays established for almost all aspects of fungal identification. In contrast to conventional detection methods, samples can be tested directly without any elaborate isolation and cultivation steps needed before proper classification. These novel detection methods include immunological tests, PCR-based technologies like restriction length polymorphism (PCR-RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and denaturing gel electrophoresis (DGGE), and an ever increasing number of diagnostic microarrays. These novel techniques are insensitive to microbial backgrounds and non-target organisms. The most recent technique enabling identification of whole microbial communities, without the need to cultivate any organisms, is metagenomics. Metagenomics is the whole-genome shotgun sequencing of pooled environmental DNA sam-

<sup>1</sup> FB Gentechnik und Angewandte Biochemie, Institut für Verfahrenstechnik, Umwelttechnik und Technische Biowissenschaften, TU Wien, Getreidemarkt 9/166/5/2, 1060 Vienna, Austria

<sup>2</sup> FB Molekulare Biochemie der Pilze, Institut für Verfahrenstechnik, Umwelttechnik und Technische Biowissenschaften, TU Wien, Getreidemarkt 9/166/5/2, 1060 Vienna, Austria

ples. Although this method has been applied to environmental samples of diverse origin, and has successfully revealed the composition of numerous prokaryotic communities, the application of this novel approach has not yet been established for fungal DNA.

Immunological diagnostics rely on recognition by antibodies, with specific antigens either presented on the surface or secreted by the fungus as targets. As most of this methods are ELISA (enzyme-linked immunosorbent assays) based, they are simple to use and can be applied by unskilled personnel. Additionally, ELISA assays allow quantification of the detected object and laboratory-independent on-site testing. However, it can be difficult and expensive to raise antibodies with the required specificity, and the application of this technique remained limited to a few organisms. Although numerous detection assays have been developed and commercially exploited during the 1990s, the increasing progress of a novel technique led to a replacement of ELISA tests. Polymerase chain reaction (PCR) became the most reliable method for diagnostics and, since the end of the last century, almost no new immunological tests for fungal identification have been presented.

The main advantage of PCR is that it is highly specific and sensitive enough to detect even minute amounts of fungal DNA in environmental samples (Lee and Ward 1990). This method allows to distinguish between different species of a genus and even within different populations (Lee and Ward 1990; Ward 1995, 1998). Besides the high specificity and sensitivity, quantitative application (real-time PCR) has contributed to the breakthrough of PCR for diagnostic applications. Related techniques like PCR-RFLP, RAPD, AFLP and DGGE, all based on PCR amplification of DNA, provide enough resolution to separate and identify strains from whole fungal communities. To further enhance the detection and discrimination capability of PCR, two-step approaches have been developed for the identification of large numbers of fungal strains in a single test cycle: for a start, DNA is amplified with universal primers and, thereafter, hybridization with highly specific probes distinguishes amplicons of different nucleic acid composition (Vanittanakom et al. 1998; Ingianni et al. 2001; Wu et al. 2002). Throughout the last decade, probe hybridization technologies have developed into the modern microarray applications which have taken place also in fungal diagnostics. Generally, target DNA con-

taining organism-specific sequences is amplified by PCR with universal primers, labelled and hybridized to the array. Microarrays allow the identification of numerous organisms in a single run. Since the late 1990s, the use of fluorescent probes has allowed the direct analysis of organisms *in situ*. This fluorescent *in situ* hybridization (FISH) has been adapted for fungal identification, and investigations even on uncultivable organisms can be performed.

Even though the abovementioned up-to-date techniques are commonly accepted as cultivation independent, initial isolation and cultivation steps cannot be omitted completely. For ELISA tests, the target antigen has to be isolated in advance to allow the production of suitable antibodies. To design specific primer pairs for non-quantitative diagnostic PCR and real-time PCR or probes for various hybridization techniques, diagnostic target DNA fragments from pure cultures must be sequenced. The essential knowledge about nucleic acid composition results in the inability to use these techniques for analyses of complex fungal communities comprising representatives of unknown genera or species. To overcome this bottleneck, methods like RFLP, RAPD, AFLP, or the separation of mixtures of PCR amplicons on a DGGE gel, have been adapted. These allow gaining an overview of the composition of fungal populations in an ecosystem.

## II. Immunological Detection of Fungi

Most immunological tests are based on enzyme-linked immunosorbent assay (ELISA), which was developed during the 1970s (Clark and Adams 1977). This technique is simple to use, specific, allows high throughput testing, and can be used quantitatively. One key factor is to develop antibodies with the required degree of specificity. The development of antibodies against viruses has generally been successful but this approach has worked less well for complex organisms such as fungi (McCartney et al. 2003). Another key factor for a successful detection is the ability to recover sufficient amounts of antigens from the environmental sample, and the knowledge about degradation or retention time of the target antigen in the sample. The dynamics of the antigen in the sample has to be well known to avoid misinterpretation due to its rapid breakdown or long retention time (Dewey et al. 1996).

The use of monoclonal antibodies to detect fungi and to study fungal interactions with hosts has been applied mainly in two different fields, namely plant pathology and biological control. Iyer and Cousin (2003) developed an indirect ELISA to detect *Fusarium* contamination in foods. Antibodies against *F. graminearum* and *F. moniliforme* proteins succeeded in identifying 15 different *Fusarium* isolates – 13 species different from *F. graminearum* and *F. moniliforme*. The assay was tested for specificity with 70 different moulds belonging to 23 genera, and only two other strains gave a false positive signal.

Otten et al. (1997) describe methods to improve the use of immunoassays for the detection and quantification of the fungal soil-borne plant pathogen, *Rhizoctonia solani*. The authors used a monoclonal antibody which recognises a catechol oxidase secreted by the hyphae of *Rhizoctonia*. The influence of different soil types on the retention of the antigen was tested, and a thousand-fold reduction in sensitivity of the assay was determined for clay soil, compared to sand and loam. This detection method has thereafter been used to monitor the influence of the soil-borne biocontrol agent *Trichoderma* on population dynamics of *Rhizoctonia* during saprophytic growth in compost-based soils (Thornton and Gilligan 1999). For the quantification of *Trichoderma* during antagonistic interaction, a monoclonal antibody-based ELISA has been developed. The antibody MF2 detects antigens of numerous species of the genera *Hypocreales/Trichoderma* and *Gliocladium* but does not cross-react with common soil organisms (Thornton et al. 2002). This antibody binds to the hyphae and septa of its target organisms, and was used to stain *Trichoderma* sp. while coiling around *Rhizoctonia* during the mycoparasitic interaction. To specifically detect only actively growing mycelia, two different antibodies were produced which selectively recognise glycoproteins secreted from the growing tips of either *Trichoderma* or *Rhizoctonia* (Thornton 2004). The authors demonstrated that *Trichoderma* successfully competes with the pathogen for nutrients and prevents the saprophytic growth of *Rhizoctonia*, and no cross-reactivity was found with fungi naturally occurring in the soil. Other ELISA tests based on monoclonal antibodies are commercially available for the detection of *Pythium*, *Phytophtora* and *Sclerotinia* (Ali-Shtayeh et al. 1991; Timmer et al. 1993; Miller 1996; Miller et al. 1997).

### III. PCR-Based Methods

PCR is the exponential amplification of DNA with two short oligonucleotide primers which are complementary to the 5' and 3' ends of the target sequence fragment. PCR is highly sensitive, and a few template strands can be amplified up to some micrograms of product DNA. This sensitivity, in combination with the specificity obtained by the two primers, makes PCR the most important technique in molecular diagnostics nowadays. To further increase sensitivity and specificity, PCR-associated techniques like nested PCR have been developed. After performing the first conventional amplification step, a second PCR is added with two new primers lying within the previously synthesized DNA. Nested PCR allows the detection of minute amounts of DNA, several folds lower than normal PCR.

The successful application of PCR-based diagnostic tools generally requires three crucial steps: (1) the selection of a suitable DNA marker sequence to accurately identify an organism, (2) extraction of total DNA from the sample and (3) a method to identify the presence of the target sequence in the amplified DNA.

**Selection of a Specific Target DNA Sequence**  
A proper selection of the target fragment to be amplified allows the detection of whole genera, in the case of primers designed across conserved DNA regions, or the identification of a single species even in a background of taxonomically related organisms. One of the main targets for the development of diagnostic PCR assays are the genes coding for the ribosomal RNA, which are present in all organisms at high copy numbers. The abundance of this type of DNA facilitates detection and, thereby, improves the sensitivity of the assay (White et al. 1990). The fungal nuclear ribosomal DNA consists of three genes encoding the 28S, 18S and 5.8S subunits. These genes are separated by internal transcribed spacer regions (ITS), and this unit is repeated many times. The ITS region is of particular importance for fungal diagnostics as it consists of conserved areas and highly variable sequences as well. Fungal ITS regions can be isolated by universal primers (White et al. 1990) and, after sequencing, strain- or genus-specific primers can be used for the identification of fungi from various samples. Although Atkins et al. (2004) discriminated between two varieties of the same fungus based on ITS sequences, in

general, the variability of this area might not be sufficient to distinguish between closely related strains (Atkins and Clark 2004). Besides the rRNA and the ITS region, the  $\beta$ -tubulin-encoding gene is among the most prominent diagnostic genes (Fraaije et al. 1999, 2001; Hirsch et al. 2000; Atkins and Clark 2004). The database of  $\beta$ -tubulin gene sequences is not as large as that for ribosomal DNA but, for particular applications, diagnostics based on this gene can be useful. The detection and real-time PCR quantification of *Fusarium* species rely mainly on amplification of  $\beta$ -tubulin gene fragments (Ali-Shtayeh et al. 1991; Mach et al. 2004; Reischer et al. 2004). If the sequence of the ITS regions or  $\beta$ -tubulin genes is not suitable, random parts of the genome can be searched for areas unique to taxa or species to be identified (Schesser et al. 1991; Mutasa et al. 1995). Primers designed within these arbitrary regions are often less discriminating because, for comparison, only little information is available in databases. These tailor-made applications can often lead to false positive results due to recognition of non-target organisms.

A very recent approach for species identification is DNA barcoding. Barcoding rests on the idea of a 'universal product code' – a few nucleotides only which can unambiguously be attributed to a particular species. However, prior to using these species-barcodes, comprehensive publicly available libraries of diagnostic sequences have to be assembled. Although this system may facilitate species identifications in the near future, until now only a few specialised web databases can be consulted. A *Fusarium* barcode database is available at <http://fusarium.cbio.psu.edu>, but the sequence information provided is still limited. Most advances in barcoding fungal genera have recently been made for *Hypocreales/Trichoderma*. Druzhinina et al. (2005) provide the TrichOKey barcode identification system with a web interface located at [www.isth.info](http://www.isth.info). This system is based on a combination of several diagnostic oligonucleotides allocated within ITS1 and ITS2, and was developed on the basis of 979 sequences of 88 species.

**DNA Extraction** Various protocols are available for extracting DNA from environmental samples such as soil or infected plant material. Variations in yield and purity of DNA can have severe influence on subsequent analytical techniques such as PCR. Usually, samples are suspended in buffer and most protocols rely on *in situ* lysis of the fungal

cellwalls by mechanical forces like bead-beating or grinding under liquid nitrogen, followed by purification steps like phenol/chloroform extraction or spin column centrifugation, and a final precipitation with ethanol or isopropanol. However, these general protocols need often to be adapted to the particular problems arising from the sample material. DNA isolation from particular plants can lead to the co-extraction of inhibitory substances (Malwick and Grunden 2005), and common soil compounds including humic acids are known as strong inhibitors of PCR-polymerases (Watson and Blackwell 2000). To remove potentially unfavourable substances prior to subsequent analyses, many commercial kits – tailored for particular applications – are available nowadays from Qiagen, Mo-Bio Laboratories (both UK), Epicentre (WI) and Zymo Research (CA).

#### A. Qualitative Diagnostic PCR

Conventional PCR is not quantitative but qualitative, with very high specificity, and can be used to detect, identify and monitor fungi from a broad spectrum of environmental samples. Most fungal diagnostic PCR applications have been developed to detect phytopathogens, mainly directly from infected plant material, food and feed samples, or from infested soil. Numerous species-specific primer pairs have been designed within recent years, for almost all economically relevant plant pathogens (Fraaije et al. 1999, 2001; Wang and Chang 2003; Weerasena et al. 2004; Jurado et al. 2005; Rubio et al. 2005; Sanchez-Rangel et al. 2005; Vetraino et al. 2005; Chadha and Gopalakrishna 2006). Moreover, PCR has successfully been applied to identify airborne fungi for various air sampling methods (Mukoda et al. 1994; Williams et al. 2001). To further improve PCR specificity and sensitivity, nested PCR approaches with two subsequent amplification steps have been developed for fungal diagnostics from different samples, and DNA target amounts of a few attograms can be detected reliably (Zhang et al. 2005; Klemsdal and Elen 2006; Lockhart et al. 2006). As the sample throughput of conventional PCR tests is not sufficient for particular applications, multiplex assays can be used as an alternative. Two or more primer pairs with the same optimal annealing temperature are used, and several amplicons are generated from a single run (Casimiro et al. 2004; Zhang et al. 2005; Klemsdal and Elen 2006;

Bezuidenhout et al. 2006; Lockhart et al. 2006). Although multiplex PCR allows the cost-efficient creation of several amplicons in a single reaction, this technique is difficult to optimize for custom applications as the determination of the optimal concentration for each primer is difficult and time consuming. The reaction conditions have to be optimized accurately to allow efficient generation of all amplicons. Another multiplex approach was tested by Brandfass and Karlovsky (2006). A pair of primers was designed to amplify a fragment from *F. graminearum* and *F. culmorum* simultaneously, and the mixture of amplicons was further resolved by melting curve analysis.

In addition to the simple species detection, PCR has been used to target biosynthetic pathway-specific genes. Sanchez-Rangel et al. (2005) demonstrated that the presence of the *F. verticillioides fum1* gene correlates with fumonisin production in most isolates. The presence of the *tri5* gene indicates trichothecene production capability of *Fusarium* spp., and the *tri7* gene can be used to discriminate different chemotypes, namely nivalenol and deoxynivalenol producers. The molecular detection of *Fusarium* mycotoxin biosynthesis-related genes has only recently been reviewed (Mule et al. 2005). To target ochratoxin-producing *Penicillium* spp. in food- and feed-related sources, specific primers have been designed to detect a non-ribosomal peptide synthetase gene which is essential for the respective pathway (Geisen 2004).

## B. Quantitative PCR

As the lack of quantification of PCR products was the main bottleneck of this new technology, competitive PCR (cPCR) was introduced to allow quantitative evaluation of the target signals. This method involves the addition of another target sequence to the assay. The sequence must be recognised by the same primers but the products must be of different length. Serial dilutions across a wide range are added to individual PCR reactions, and visually quantified on an agarose gel. If the bands derived from both target sequences are of the same intensity, then the unknown amplified template must match the quantity of the added target sequence. Competitive PCR has been applied successfully for the quantification of the nematophagous fungus *Verticillium clamydosporium* from soil samples. Increases in fungal growth were observed in the rhizosphere of potato

cyst nematode-infested plants after 14 weeks using cPCR (Mauchline et al. 2002). Providenti et al. (2004) tracked the environmental fate of *T. reesei* over a 6-month period in soil in a growth chamber. Survival was tested in three different soils, and the effect of plant rhizosphere was investigated using the cPCR technique (Nicholson et al. 1996, 1998).

Although the accuracy of competitive PCR can be very high (Chunming and Cantor 2004), nowadays quantitative real-time PCR has been widely accepted as the gold standard for accurate DNA quantification. During a real-time PCR run, the accumulation of the product is measured by an integrated fluorimeter. The threshold cycles (ct) determined for each sample correlate with the initial amount of DNA. Target DNA is quantified by the construction of a calibration curve which relates the amounts of calibration-DNA to a certain threshold cycle. Using SYBR Green as intercalation dye is the most economic way for real-time analysis. Although this dye allows quantification with high accuracy, the degree of specificity can sometimes not be sufficient to detect specific DNA in a background of similar targets. To further increase specificity of assays, fluorescent probe-based techniques have been established. Molecular beacons (Tyagi and Kramer 1996; Tyagi et al. 1998), scorpions (Thelwell et al. 2000) and TaqMan™ probes (Roche Molecular Systems, Pleasanton, CA) are nowadays state of the art, and even sequences distinguished by single nucleotide polymorphisms can be discriminated (Tyagi and Kramer 1996). For more detailed reviews concerning DNA quantification with real-time PCR, the reader is referred to Bustin (2002), Ginzinger (2002), Klein (2002), Ong and Irvine (2002), Filion et al. (2003), Mackay (2004), Valasek and Repa (2005), Wong and Medrano (2005) and Bustin et al. (2005). The main advantage of real-time PCR over competitive PCR is the detection range of six magnitudes, compared to only two for cPCR (McCartney et al. 2003). Most applications of quantitative real-time PCR have been implemented in the field of phytopathogen diagnostics, *Fusarium* being the most prominent representative. Most assays have focused on the detection of mycotoxin-producing isolates from soil samples or directly from infected plant tissues (Schnerr et al. 2001; Filion et al. 2003; Bluhm et al. 2004; Reischer et al. 2004; Dyer et al. 2006). Reischer et al. (2004) succeeded in the detection and quantification of only five copies of the *tub1* gene of *F. graminearum* from wheat ears. To monitor the early infection process of *Botrytis* and *Alternaria* before any macro-

scopic symptoms become visible, a reliable assay was established by Gachon and Saindrenan (2004). *Biscogniauxia mediterranea* is a fungal pathogen which causes severe damages to oaks after a long, symptomless endophytic period. A real-time assay based on a TaqMan probe was used to identify this pathogen before disease outbreak (Luchi et al. 2005). Boyle et al. (2005) investigated compatible, incompatible and non-host interactions of different species of the obligate biotroph *Melampsora* with plants. Until recently, light microscopy was the method of choice to detect interaction between arbuscular mycorrhizal fungi, or between mycorrhiza and potential host plants. To describe the symbiotic contribution of different mycorrhizal strains, a real-time PCR assay was developed. This detection method allows discrimination and quantification of different species of *Glomus* in various rhizospheres (Alkan et al. 2006). Like conventional PCR, real-time PCR can be carried out in a multiplex assay to allow quantification of more than one gene in a single run (Wittwer et al. 2001; Bluhm et al. 2004).

### C. Molecular Fingerprinting Technologies

In contrast to conventional PCR detection, which is a highly adapted and sensitive identification method for a single strain or for a few organisms, molecular fingerprinting techniques allow the fungal ecologist to rapidly profile whole populations in an ecosystem. As many of the genes used for diagnostic purposes have conserved and less-conserved regions, primers can be designed to amplify many or most species of the Kingdom Fungi. A further resolution of the amplified mixture of products can be performed in a subsequent step based on nucleic acid composition or variation of the amplicon length.

#### 1. Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis is a frequently used and established approach for the restriction enzyme-independent detection of DNA sequence variations, such as single-base substitutions (Eng and Vijg 1997). DGGE exploits the principle that sequence alterations cause changes in the melting temperature of double-stranded DNA, which can be analysed by a linearly increasing gradient of DNA denaturants established in polyacry-

lamide gels. Initially, the fragments move according to their molecular weight but, as they progress into higher denaturing conditions, each (depending on its sequence composition) reaches a point where the DNA begins to melt. In practice, nearly all single-base substitutions in amplicons up to 500 bp joined to a GC-clamp can be detected by PCR-DGGE-based analysis (Sheffield et al. 1989). DGGE provides the means to investigate fungal communities, in particular shifts and changes in community composition. This technique benefits from the ability to analyse a high number of samples on a single gel, and provides sufficient resolution to compare whole fungal communities, rather than single isolates, without the need of precultivation.

To develop tools for early and specific detection of *Fusarium langsethiae*, and for distinguishing it from related species of section *Sporotrichiella* and *Discolor* (*F. poae*, *F. sporotrichioides*, *F. kyushuense*, *F. robustum*, *F. sambucinum* and *F. tumidum*), sequence variations in their  $\beta$ -tubulin-encoding (*tub1*) gene were employed to design a PCR-based denaturing gradient gel electrophoresis assay. DGGE reliably separated all these strains, even from mixtures and in the presence of DNA from their natural hosts *Zea mays*, *Triticum aestivum* and *Avena sativa* (Mach et al. 2004). Van Elsas et al. (2000) developed a DGGE application based on nested PCR to assess the persistence of selected fungi in soil and to analyse the response of the natural fungal community to a spill of petrol. The primers for the first PCR were designed to amplify the rRNA genes of numerous members of Ascomycetes, Basidiomycetes, Zygomycetes and Chytridiomycetes whereas the primers used for the second nested PCR produced amplicons separable on denaturing gradient gels. DGGE allowed the resolution of mixtures of PCR products of different fungi into distinct band patterns. The fungi colonizing the rhizosphere of pines were monitored during regeneration of woods by a DGGE-based assay. Fungal diversity was investigated by a denaturing gradient gel following the PCR amplification of ITS sequences. This method successfully detected mycorrhizal and non-mycorrhizal fungi (Anderson et al. 2003). The fungal population dynamics in soil and in the rhizospheres of two maize cultivars grown in tropical soils were studied by a cultivation-independent analysis of directly extracted DNA. A fragment of the 18S rRNA amplified from the total community DNA was analysed by DGGE, and 39 different isolates could be identified (Gomes et al. 2003).

## 2. PCR-Restriction Fragment Length Polymorphism (PCR-RFLP), Amplified Fragment Polymorphism (AFLP) and Random Amplified Polymorphic DNA (RAPD)

### PCR-RFLP

Typically, PCR-RFLP markers are used to detect variation of DNA-restriction fragment patterns in PCR products. Based on diversity in the restriction pattern of a 1.3-kb *tub1* fragment, a method to discriminate species belonging to the plant parasite *Ceratocystis* has been developed. In all, 200 isolates comprising seven different species have been successfully identified (Loppnau and Breuil 2003). Another application for RFLP was the monitoring of populations of two *Tricholoma populinum* and *T. sculpturatum*, both associated to black poplar. The analyses were based on the restriction fragment pattern of the ITS region (Gryta et al. 2006), and the authors revealed differences in the population dynamics of the two species. On one hand, RFLP provides a rapid technique to screen microbial populations but, on the other hand, this method has to be optimized carefully to provide reliable results. The greatest variation among profiles generated from the same DNA sample was reported to be produced by using different *Taq* DNA polymerases (Osborn et al. 2000). Incomplete digestion by the restriction enzyme may, as a result of the generation of partially digested fragments, lead to an overestimation of the overall diversity within a community.

### RAPD

The most common version of PCR-based fingerprinting techniques is RAPD analysis, in which the random amplification products are separated on agarose gels in the presence of ethidiumbromide, and visualized under ultraviolet light (Williams et al. 1990). The enormous attractions of these arbitrary priming techniques are that there is no requirement for DNA probes or sequence information for the design of specific primers, since the procedure involves no blotting or hybridizing steps. It is quick, simple, can be automated, and very small amounts of initial DNA (10 ng per reaction) are sufficient for amplification (Williams et al. 1990). In many cases, RAPD techniques are applied to fungal communities to identify an amplified fragment highly specific for a single representative member or a subgroup. Based on the sequence of these characteristic fragments, sequence-characterised am-

plified region (SCAR) primers are designed for conventional PCR. These SCAR primers allow the detection of underrepresented species in a background of highly similar organisms. This technique has commonly been applied to monitor biocontrol fungi like *Pochonia*, *Paecilomyces* and *Trichoderma* (Hermosa et al. 2001; Rubio et al. 2005; Zhu et al. 2006), the plant pathogenic species of *Fusarium* (Moeller et al. 1999), and ochratoxin-producing species of *Aspergillus* (Taniwaki et al. 2003; Pelegrinelli-Fungaro et al. 2004).

Although RAPD has been used to identify and distinguish species in several studies, it is now ever more widely recognised and critically discussed that, to obtain reproducible band profiles on the gels, it is absolutely essential to maintain consistent reaction conditions. Numerous studies have reported the negative effects of altering different parameters like the ratio of template DNA, primers, concentration of *Taq* polymerase and Mg concentration.

### AFLP

More recently, a new PCR-based technique has been developed, amplified fragment length polymorphism (AFLP; Vos et al. 1995), which is essentially an intermediate between RFLP and PCR. The initial step involves restriction digestion of the genomic DNA, which is then followed by selective rounds of PCR amplification of the restricted fragments. The amplified products are radioactively or fluorescently labelled, and separated on sequencing gels. Two studies used this novel technique to identify ochratoxin-producing *Aspergillus* strains from various environmental samples (Vos et al. 1995; Castella et al. 2002) but their results did not always show a correlation between AFLP genotype determination and ochratoxin production. However, based on the sequence of marker fragments identified consistently in several studies, three highly specific primer pairs have been developed for *A. ochraceus* detection (Vos et al. 1995; Castella et al. 2002; Schmidt et al. 2003, 2004). Gadkar and Rillig (2005) developed an AFLP assay with precedent Phi29-polymerase chain reaction to monitor arbuscular mycorrhizal fungi, and succeeded to resolve genomic DNA obtained from single spores of several fungal species. Only recently, AFLP was used to identify characteristic amplified fragments of three industrially relevant fungi, *A. niger*, *A. oryzae* and *Chaetomium globosum*. The sequence of the strain-specific marker fragments

was used to design primers suitable to determine the fate and persistence of these fungi in natural soil microcosms (Gadkar and Rillig 2005).

#### IV. Probe Hybridization Technologies

This technology uses radioactively or fluorescently labelled probes with sequence homology to the target DNA. Short oligonucleotides, or large fragments of several hundred base pairs, can be used as DNA probes. Once bound to a DNA sample, which is immobilized on a membrane, the probe is detected by exposure to X-ray films. This technique, originally developed by Southern (1975), has been further improved and, today, oligonucleotide fingerprinting of rRNA genes (OFRG) is well established as a means of identifying and discriminating genera or species obtained from complex samples. Amplified rDNA fragments are arrayed on a membrane and are hybridized with numerous probes according to a particular algorithm, which allows sorting of the spotted samples into taxonomic clusters (Borneman et al. 2001). This method was previously developed to examine bacterial community composition but has now been adapted for fungal samples. Valinsky et al. (2002) analysed 1,536 fungal rDNA clones derived from soil samples. A large fraction of the clones could be identified as members of the genera *Fusarium* and *Raciborskimycetes*; the other clones showed sequence similarity to probes characteristic for *Alternaria*, *Ascobolus*, *Chaetomium*, *Cryptococcus* and *Rhizoctonia*. Similar assays were developed to examine the occurrence of common airborne fungi, based on the sequence variation of the 18S rRNA gene (Wu et al. 2003) or the mitochondrial rDNA (Zeng et al. 2003).

The next step in hybridization technology is to reverse the process: the use of immobilized oligonucleotide probes allow testing a sample simultaneously with a high number of probes. Generally, target DNA is PCR amplified, labelled and subsequently hybridized to the array. This method can be used for the detection of very high amounts of organisms in a single assay, provided that sufficient polymorphisms exist within the amplified fragment. For diagnostic DNA arrays, the rRNA genes and the ITS region are commonly selected as targets. The discriminatory potential of the probe-oligonucleotides is crucial for successful application, since many species may vary only in

a few nucleotides or even in a single nucleotide polymorphism (SNP). As a consequence, the accurate detection of SNPs is the aim of modern DNA-chip technology. Lievens et al. (2006) demonstrated the utility of array technology to detect single mismatches at different positions of the oligonucleotide, focusing on the accurate discrimination of ITS fragments of various plant pathogenic fungi. The hybridization is carried out with a high excess of amplified DNA, and the results can be imaged and analysed qualitatively and quantitatively. The first environmental application of this new method was the detection of bacteria but the method was soon adapted to identify a range of *Pythium* and *Phytophthora* species (Levesque et al. 1998). Within the scope of fungal detection, DNA arrays have mainly been developed to identify either plant pathogens from various environmental samples or mycotoxin-producing strains in food samples (Levesque 2001; Lievens et al. 2003, 2004, 2005; Pelegrinelli-Fungaro et al. 2004; Nicolaisen et al. 2005; Kostrzynska and Bachand 2006; Zezza et al. 2006). Nowadays, numerous arrays for the detection of fungi have been developed by private or public laboratories, and systems like OLISA™ (OLIGO Sorbent Assay, Apibio Biochips, Grenoble, France) are commercially available.

A very recent technology, also based on probe hybridization, is fluorescent probe hybridization. FISH is a cytogenetic technique which can be used to detect and localize DNA sequences on chromosomes, as it hybridizes with the sample DNA at the target site. The probe signal can then be seen through a fluorescent microscope, and the sample DNA scored for the presence or absence of sequences homologous to the fluorescent probe. Although this probing technique has often been used for detections of organisms in medical samples, environmental applications have been rare. Baker et al. (2004) used a FISH-based test to identify and monitor the fungal community of biofilms on acidic mine drainage water. Since the members of *Ascomycetes* were morphologically indistinguishable, rRNA-specific fluorescent probes were designed to target *Dothideomycetes* and *Eurotiomycetes*.

#### V. Conclusions

Within the last decade, many methods for the molecular detection of fungi in diverse ecosystems have evolved. In the mid-1990s, identification

based on immunological tests was state of the art but this expensive technique was soon replaced by PCR-based detection systems. Nowadays, there are many applications of PCR in diagnostics, most of these focusing on rapid detection directly from the source material, without any time-consuming cultivation steps. This technique has changed the view of molecular analysis, as now even minute amounts of organisms, some attograms of DNA, are detectable. PCR technology has opened up new ways of investigations, in particular the analysis of whole fungal communities from complex sources. In contrast to conventional culture-based methods, even unculturable strains can be detected. The combination with other techniques, like DGGE, RFLP, AFLP, or the use of random primers (RAPD) allow the identification of yet unknown isolates, although the information about their DNA sequence is limited or nonexistent.

Recent reverse probing technologies (microarrays) emerged from the hybridization technique developed by Southern in 1975. These DNA chips can be scaled up, so that hundreds of different organisms are detected simultaneously in the sample. Microarray technology has maybe advanced more than other molecular analysis methods within the last few years. In contrast to PCR-based technologies, arrays represent a closed system technology, and the oligonucleotide probes on the chip limit the detection capability. A rapid adaptation to screen for new organisms is hardly realizable. However, due to the high throughput of DNA arrays and at least raw-quantitative results, they might be the most promising tool for commercially detection kits in future.

## References

- Ali-Shtayeh MS, Macdonald JD, Kabashima J (1991) A method for using commercial ELISA tests to detect zoospores of *Phytophthora* and *Pythium* species in irrigation water. Plant Disease 75:305–311
- Alkan N, Gadkar V, Yarden O, Kapulnik Y (2006) Analysis of quantitative interactions between two species of arbuscular mycorrhizal fungi, *Glomus mosseae* and *G. intraradices*, by real-time PCR. Appl Environ Microbiol 72:4192–4199
- Anderson IC, Campbell CD, Prosser JI (2003) Diversity of fungi in organic soils under a moorland–Scots pine (*Pinus sylvestris* L.) gradient. Environ Microbiol 5:1121–1132
- Atkins SD, Clark IM (2004) Fungal molecular diagnostics: a mini review. J Appl Genet 45:3–15
- Atkins SD, Sosnowska D, Evans VJ, Clark IM, Hirsch PR, Kerry BR (2004) Investigation of three nematophagous fungi in two potato cyst nematode suppressive soils. Bull IOLB/SROP 27(1):1–8
- Baker BJ, Lutz MA, Dawson SC, Bond PL, Banfield JF (2004) Metabolically active eukaryotic communities in extremely acidic mine drainage. Appl Environ Microbiol 70:6264–6271
- Bezuidenhout CC, Prinsloo M, Van der Walt AM (2006) Multiplex PCR-based detection of potential fumonisin-producing *Fusarium* in traditional African vegetables. Environ Toxicol 21:360–366
- Bluhm BH, Cousin MA, Woloshuk CP (2004) Multiplex real-time PCR detection of fumonisin-producing and trichothecene-producing groups of *Fusarium* species. J Food Prot 67:536–543
- Borneman J, Chrobak M, Della Vedova G, Figueroa A, Jiang T (2001) Probe selection algorithms with applications in the analysis of microbial communities. Bioinformatics 17 suppl 1:S39–S48
- Boyle B, Hamelin RC, Seguin A (2005) *In vivo* monitoring of obligate biotrophic pathogen growth by kinetic PCR. Appl Environ Microbiol 71:1546–1552
- Brandfass C, Karlovsky P (2006) Simultaneous detection of *Fusarium culmorum* and *F. graminearum* in plant material by duplex PCR with melting curve analysis. BMC Microbiol 6:4
- Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29:23–39
- Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR – a perspective. J Mol Endocrinol 34:597–601
- Casimiro S, Moura M, Ze-Ze L, Tenreiro R, Monteiro AA (2004) Internal transcribed spacer 2 amplicon as a molecular marker for identification of *Peronospora parasitica* (crucifer downy mildew). J Appl Microbiol 96:579–587
- Castella G, Larsen TO, Cabanes J, Schmidt H, Alboresi A, Niessen L, Farber P, Geisen R (2002) Molecular characterization of ochratoxin A producing strains of the genus *Penicillium*. Syst Appl Microbiol 25:74–83
- Chadha S, Gopalakrishna T (2006) Detection of *Magnaporthe grisea* in infested rice seeds using polymerase chain reaction. J Appl Microbiol 100:1147–1153
- Chunming D, Cantor CR (2004) Quantitative analysis of nucleic acids – the last few years of progress. J Biochem Mol Biol 37:1–10
- Clark MF, Adams AN (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J Gen Virol 34:475–483
- Deweys FM, Thornton CR, Gilligan CA (1996) Use of monoclonal antibodies to detect, quantify and visualize fungi in soils. Adv Bot Res 24:275–308
- Druzhinina IS, Kopchinskiy AG, Komon M, Bissett J, Szakacs G, Kubicek CP (2005) An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocreales*. Fungal Genet Biol 42:813–828
- Dyer RB, Kendra DF, Brown DW (2006) Real-time PCR assay to quantify *Fusarium graminearum* wild-type and recombinant mutant DNA in plant material. J Microbiol Methods 67:534–542

- Eng C, Vijg J (1997) Genetic testing: the problems and the promise. *Nature Biotechnol* 15:422–426
- Filion M, St-Arnaud M, Jabaji-Hare SH (2003) Direct quantification of fungal DNA from soil substrate using real-time PCR. *J Microbiol Methods* 53:67–76
- Fraaije BA, Lovell DJ, Rohel EA, Hollomon DW (1999) Rapid detection and diagnosis of *Septoria tritici* epidemics in wheat using a polymerase chain reaction/Pico green assay. *J Appl Microbiol* 86:701–708
- Fraaije BA, Lovell DJ, Coelho JM, Baldwin S, Hollomon DW (2001) PCR-based assays to assess wheat varietal resistance to blotch and rust diseases. *Eur J Plant Pathol* 107:905–917
- Gachon C, Saindrenan P (2004) Real-time PCR monitoring of fungal development in *Arabidopsis thaliana* infected by *Alternaria brassicicola* and *Botrytis cinerea*. *Plant Physiol Biochem* 42:367–371
- Gadkar V, Rillig MC (2005) Suitability of genomic DNA synthesized by strand displacement amplification (SDA) for AFLP analysis: genotyping single spores of arbuscular mycorrhizal (AM) fungi. *J Microbiol Methods* 63:157–164
- Geisen R (2004) Molecular monitoring of environmental conditions influencing the induction of ochratoxin A biosynthesis genes in *Penicillium nordicum*. *Mol Nutr Food Res* 48:532–540
- Ginzinger DG (2002) Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol* 30:503–512
- Gomes NC, Fagbola O, Costa R, Rumjanek NG, Buchner A, Mendona-Hagler L, Smalla K (2003) Dynamics of fungal communities in bulk and maize rhizosphere soil in the tropics. *Appl Environ Microbiol* 69:3758–3766
- Gryta H, Carriconde F, Charcosset JY, Jargeat P, Gardes M (2006) Population dynamics of the ectomycorrhizal fungal species *Tricholoma populinum* and *Tricholoma sculpturatum* associated with black poplar under differing environmental conditions. *Environ Microbiol* 8:773–786
- Hermosa MR, Grondona I, Diaz-Minguez JM, Iturriaga EA, Monte E (2001) Development of a strain-specific SCAR marker for the detection of *Trichoderma atroviride* 11, a biological control agent against soilborne fungal plant pathogens. *Curr Genet* 38:343–350
- Hirsch PR, Mauchline TH, Mendum TA (2000) Detection of the nematophagous fungus *Verticillium chlamydosporium* in nematode infested plant roots using PCR. *Mycol Res* 104:435–439
- Ingianni A, Floris M, Palomba P, Madeddu MA, Quartuccio M, Pompei R (2001) Rapid detection of *Listeria monocytogenes* in foods, by a combination of PCR and DNA probe. *Mol Cell Probes* 15:275–280
- Iyer MS, Cousin MA (2003) Immunological detection of *Fusarium* species in cornmeal. *J Food Prot* 66:451–456
- Jurado M, Vazquez C, Patino B, Gonzalez-Jaen MT (2005) PCR detection assays for the trichotheccene-producing species *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium poae*, *Fusarium equiseti* and *Fusarium sporotrichioides*. *Syst Appl Microbiol* 28:562–568
- Klein D (2002) Quantification using real-time PCR technology: applications and limitations. *Trends Mol Med* 8:257–260
- Klemsdal SS, Elen O (2006) Development of a highly sensitive nested-PCR method using a single closed tube for detection of *Fusarium culmorum* in cereal samples. *Lett Appl Microbiol* 42:544–548
- Kostrzynska M, Bachand A (2006) Application of DNA microarray technology for detection, identification, and characterization of food-borne pathogens. *Can J Microbiol* 52:1–8
- Lee SB, Ward TJ (1990) Isolation of DNA from fungal mycelia and single spores. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) *PCR Protocols. A guide to methods and applications*. Academic Press, San Diego, CA, pp 282–287
- Levesque CA (2001) Molecular methods for detection of plant pathogens – What is the future? *Can J Plant Pathol* 24:333–336
- Levesque CA, Harlton CA, de Cock AW (1998) Identification of some oomycetes by reverse dot blot hybridization. *Phytopathology* 88:213–222
- Lievens B, Brouwer M, Vanachter AC, Levesque CA, Cammue BP, Thomma BP (2003) Design and development of a DNA array for rapid detection and identification of multiple tomato vascular wilt pathogens. *FEMS Microbiol Lett* 223:113–122
- Lievens B, Hanssen IRM, Vanachter AC, Cammune BPA, Thomma BP (2004) Root and foot rot on tomato caused by *Phytophthora infestans* detected in Belgium. *Plant Disease* 88:86
- Lievens B, Brouwer M, Vanachter AC, Levesque CA, Cammue BP, Thomma BP (2005) Quantitative assessment of phytopathogenic fungi in various substrates using a DNA macroarray. *Environ Microbiol* 7:1698–1710
- Lievens B, Claes L, Vanachter AC, Cammue BP, Thomma BP (2006) Detecting single nucleotide polymorphisms using DNA arrays for plant pathogen diagnosis. *FEMS Microbiol Lett* 255:129–139
- Lockhart RJ, Van Dyke MI, Beadle IR, Humphreys P, McCarthy AJ (2006) Molecular biological detection of anaerobic gut fungi (neocallimastigales) from landfill sites. *Appl Environ Microbiol* 72:5659–5661
- Loppnau PA, Breuil C (2003) Species level identification of conifer associated *Ceratocystis sapstain* fungi by PCR-RFLP on a beta-tubulin gene fragment. *FEMS Microbiol Lett* 222:143–147
- Luchi N, Capretti P, Pinzani P, Orlando C, Pazzagli M (2005) Real-time PCR detection of *Biscogniauxia mediterranea* in symptomless oak tissue. *Lett Appl Microbiol* 41:61–68
- Mach RL, Kullnig-Gradinger CM, Farnleitner AH, Reischer G, Adler A, Kubicek CP (2004) Specific detection of *Fusarium langsethiae* and related species by DGGE and ARMS-PCR of a beta-tubulin (tub1) gene fragment. *Int J Food Microbiol* 95:333–339
- Mackay IM (2004) Real-time PCR in the microbiology laboratory. *Clin Microbiol Infect* 10:190–212
- MacNeil L, Kauri T, Robertson W (1995) Molecular techniques and their potential application in monitoring the microbiological quality of indoor air. *Can J Microbiol* 41:657–665
- Malwick DK, Grunden E (2005) Isolation of fungal DNA from plant tissues and removal of DNA amplification inhibitors. *Mol Ecol Notes* 5:958–960
- Mauchline TH, Kerry BR, Hirsch PR (2002) Quantification in soil and the rhizosphere of the nematophagous fungus *Verticillium chlamydosporium* by competitive PCR

- and comparison with selective plating. *Appl Environ Microbiol* 68:1846–1853
- McCartney HA, Foster SJ, Fraaije BA, Ward E (2003) Molecular diagnostics for fungal plant pathogens. *Pest Manag Sci* 59:129–142
- Miller SA (1996) Detecting propagules of plant pathogenic fungi. *Adv Bot Res* 23:73–102
- Miller SA, Madden LV, Schmitthenner AF (1997) Distribution of *Phytophthora* spp. in field soils determined by immunoassay. *Phytopathology* 87:101–107
- Moeller EM, Chelkowski J, Geiger HH (1999) Species-specific PCR assays for the fungal pathogens *Fusarium moniliforme* and *Fusarium subglutinans* and their application to diagnose maize ear rot. *J Phytopathol* 147:497–508
- Mukoda T, Todd LA, Sobsey MD (1994) PCR and gene probes for detecting bioaerosols. *J Aerosol Sci* 25:1523–1532
- Mule G, Gonzalez-Jaen MT, Hornok L, Nicholson P, Waalwijk C (2005) Advances in molecular diagnosis of toxicigenic *Fusarium* species: a review. *Food Addit Contam* 22:316–323
- Mutasa ES, Chwarszczynska D, Adams MJ, Ward E, Asher MJC (1995) Development of PCR for the detection of *Polymyxa beae* in sugar beet roots and its application in field studies. *Physiol Mol Plant Pathol* 47:303–313
- Nicholson P, Lees A, Maurin N, Parry D, Rezanoor HN (1996) PCR assay to identify and quantify *Microdochium nivale* var *nivale* and *Microdochium nivale* var *majus* in wheat. *Physiol Mol Plant Pathol* 48:257–271
- Nicholson P, Simpson DR, Weston G, Rezanoor HN, Lees A, Parry D, Joyce D (1998) Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* using PCR assays. *Physiol Mol Plant Pathol* 53:17–37
- Nicolaisen M, Justesen AF, Thrane U, Skouboe P, Holmstrom K (2005) An oligonucleotide microarray for the identification and differentiation of trichothecene producing and non-producing *Fusarium* species occurring on cereal grain. *J Microbiol Methods* 62:57–69
- Ong YL, Irvine A (2002) Quantitative real-time PCR: a critique of method and practical considerations. *Hematology* 7:59–67
- Osborn AM, Moore ER, Timmis KN (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environ Microbiol* 2:39–50
- Otten CA, Gilligan CA, Thornton CR (1997) Quantification of fungal antigens in soil with a monoclonal antibody-based ELISA: analysis and reduction of soil-specific bias. *Phytopathology* 87:730–736
- Pelegrinelli-Fungaro MH, Vissoto PC, Sartori D, Vioas-Boas LA (2004) A molecular method for detection of *Aspergillus carbonarius* in coffee beans. *Curr Microbiol* 49:123–127
- Providenti MA, Mautner SI, Chaudhry O, Bombardier M, Scroggins R, Gregorich E, Smith ML (2004) Determining the environmental fate of a filamentous fungus, *Trichoderma reesei*, in laboratory-contained intact soil-core microcosms using competitive PCR and viability plating. *Can J Microbiol* 50(8):623–631
- Reischer GH, Lemmens M, Farnleitner A, Adler A, Mach RL (2004) Quantification of *Fusarium graminearum* in infected wheat by species specific real-time PCR applying a TaqMan Probe. *J Microbiol Methods* 59:141–146
- Rubio MB, Hermosa MR, Keck E, Monte E (2005) Specific PCR assays for the detection and quantification of DNA from the biocontrol strain *Trichoderma harzianum* 2413 in soil. *Microbial Ecol* 49:25–33
- Sanchez-Rangel D, SanJuan-Badillo A, Plasencia J (2005) Fumonisin production by *Fusarium verticillioides* strains isolated from maize in Mexico and development of a polymerase chain reaction to detect potential toxicigenic strains in grains. *J Agric Food Chem* 53:8565–8571
- Schessier K, Luder A, Henson JM (1991) Use of polymerase chain reaction to detect the take-all fungus, *Gaeumannomyces graminis*, in infected wheat plants. *Appl Environ Microbiol* 57:553–556
- Schmidt H, Ehrmann M, Vogel RF, Taniwaki MH, Niessen L (2003) Molecular typing of *Aspergillus ochraceus* and construction of species specific SCAR-primers based on AFLP. *Syst Appl Microbiol* 26:138–146
- Schmidt H, Taniwaki MH, Vogel RF, Niessen L (2004) Utilization of AFLP markers for PCR-based identification of *Aspergillus carbonarius* and indication of its presence in green coffee samples. *J Appl Microbiol* 97:899–909
- Schnerr H, Niessen L, Vogel RF (2001) Real time detection of the tri5 gene in *Fusarium* species by lightcycler-PCR using SYBR Green I for continuous fluorescence monitoring. *Int J Food Microbiol* 71:53–61
- Sheffield VC, Cox DR, Lerman LS, Myers RM (1989) Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc Natl Acad Sci USA* 86:232–236
- Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 98:503–517
- Taniwaki MH, Pitt JI, Teixeira AA, Iamanaka BT (2003) The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. *Int J Food Microbiol* 82:173–179
- Thelwell N, Millington S, Solinas A, Booth J, Brown T (2000) Mode of action and application of Scorpion primers to mutation detection. *Nucleic Acids Res* 28:3752–3761
- Thornton CR (2004) An immunological approach to quantifying the saprotrophic growth dynamics of *Trichoderma* species during antagonistic interactions with *Rhizoctonia solani* in a soil-less mix. *Environ Microbiol* 6:323–334
- Thornton CR, Gilligan CA (1999) Quantification of the effect of the hyperparasite *Trichoderma harzianum* on the saprotrophic growth dynamics of *Rhizoctonia solani* in compost using a monoclonal antibody-based ELISA. *Mycol Res* 103:443–448
- Thornton CR, Pitt D, Wakley GE, Talbot NJ (2002) Production of a monoclonal antibody specific to the genus *Trichoderma* and closely related fungi, and its use to detect *Trichoderma* spp. in naturally infested composts. *Microbiology* 148:1263–1279
- Timmer LW, Menge JA, Zitko SE, Pond E, Miller SA, Johnson EL V (1993) Comparison of ELISA techniques and standard isolation methods for *Phytophthora* detection in citrus orchards in Florida and California. *Plant Disease* 77:791–796

- Tyagi S, Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridization. *Nature Biotechnol* 14:303–308
- Tyagi S, Bratu DP, Kramer FR (1998) Multicolor molecular beacons for allele discrimination. *Nature Biotechnol* 16:49–53
- Valasek MA, Repa JJ (2005) The power of real-time PCR. *Adv Physiol Educ* 29:151–159
- Valinsky L, Della Vedova G, Jiang T, Borneman J (2002) Oligonucleotide fingerprinting of rRNA genes for analysis of fungal community composition. *Appl Environ Microbiol* 68:5999–6004
- van Elsas JD, Duarte GF, Keijzer-Wolters A, Smit E (2000) Analysis of the dynamics of fungal communities in soil via fungal-specific PCR of soil DNA followed by denaturing gradient gel electrophoresis. *J Microbiol Methods* 43:133–151
- Vanittanakom N, Merz WG, Sittisombut N, Khamwan C, Nelson KE, Sirisanthana T (1998) Specific identification of *Penicillium marneffei* by a polymerase chain reaction/hybridization technique. *Med Mycol* 36:169–175
- Vetraino AM, Paolacci A, Vannini A (2005) Endophytism of *Sclerotinia pseudotuberosa*: PCR assay for specific detection in chestnut tissues. *Mycol Res* 109:96–102
- Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Horres M, Frijters A, Pot J, Peleman J, Kuiper M et al. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* 23:4407–4414
- Wang PH, Chang CW (2003) Detection of the low-germination-rate resting oospores of *Pythium myriotylum* from soil by PCR. *Lett Appl Microbiol* 36:157–161
- Ward E (1995) Improved polymerase chain reaction (PCR) detection of *Gaeumannomyces graminis* including a safeguard against false negatives. *Eur J Plant Pathol* 101:561–566
- Ward E (1998) Analysis of ribosomal DNA sequences of *Polymyxa* species and related fungi and the development of genus- and species-specific PCR primers. *Mycol Res* 102:965–974
- Watson RJ, Blackwell B (2000) Purification and characterization of a common soil component which inhibits the polymerase chain reaction. *Can J Microbiol* 46:633–642
- Weerasena OV, Chandrasekharan NV, Wijesundera RL, Karunanayake EH (2004) Development of a DNA probe and a PCR based diagnostic assay for *Rhizoctonia solani* using a repetitive DNA sequence cloned from a Sri Lankan isolate. *Mycol Res* 108:649–653
- White TJ, Brun T, Lee SB, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols. A guide to methods and applications. Academic Press, San Diego, CA, pp 315–320
- Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 18:6531–6535
- Williams RH, Ward E, McCartney HA (2001) Methods for integrated air sampling and DNA analysis for detection of airborne fungal spores. *Appl Environ Microbiol* 67:2453–2459
- Wittwer CT, Herrmann MG, Gundry CN, Elenitoba-Johnson KS (2001) Real-time multiplex PCR assays. *Methods* 25:430–442
- Wong ML, Medrano JF (2005) Real-time PCR for mRNA quantitation. *Biotechniques* 39:75–85
- Wu Z, Blomquist G, Westermark SO, Wang XR (2002) Application of PCR and probe hybridization techniques in detection of airborne fungal spores in environmental samples. *J Environ Monit* 4:673–678
- Wu Z, Tsumura Y, Blomquist G, Wang XR (2003) 18S rRNA gene variation among common airborne fungi, and development of specific oligonucleotide probes for the detection of fungal isolates. *Appl Environ Microbiol* 69:5389–5397
- Zeng QY, Wang XR, Blomquist G (2003) Development of mitochondrial SSU rDNA-based oligonucleotide probes for specific detection of common airborne fungi. *Mol Cell Probes* 17:281–288
- Zezza F, Pascale M, Mule G, Visconti A (2006) Detection of *Fusarium culmorum* in wheat by a surface plasmon resonance-based DNA sensor. *J Microbiol Methods* 66:529–537
- Zhang Z, Zhang J, Wang Y, Zheng X (2005) Molecular detection of *Fusarium oxysporum* f. sp. *niveum* and *Mycosphaerella melonis* in infected plant tissues and soil. *FEMS Microbiol Lett* 249:39–47
- Zhu ML, Mo MH, Xia ZY, Li YH, Yang SJ, Li TF, Zhang KQ (2006) Detection of two fungal biocontrol agents against root-knot nematodes by RAPD markers. *Mycopathologia* 161:307–316

---

## **Determinants of Fungal Communities**

---

### 3 Disturbance in Natural Ecosystems: Scaling from Fungal Diversity to Ecosystem Functioning

S.J. MORRIS<sup>1</sup>, C.F. FRIESE<sup>2</sup>, M.F. ALLEN<sup>3</sup>

#### CONTENTS

I. Introduction .....	31
II. Ecosystem Disturbance:	
A Conceptual Framework .....	32
A. Conceptual Model Overview .....	32
B. Disturbance and Species Diversity .....	32
III. Fungal Community Dynamics:	
A Natural Disturbance Model .....	36
A. Disturbance Types and Characteristics ..	36
B. Biotic and Abiotic Characteristics of the Disturbance Model .....	37
IV. Scaling from Patch to Catastrophic Disturbance .....	38
A. Individuals .....	38
B. Community-Level Effects .....	39
C. Ecosystem Characteristics and Feedback Loops .....	41
V. Conclusions .....	42
A. The Impact of Disturbance on the Functional Role of Fungi .....	42
B. Future Research Directions .....	42
References .....	43

#### I. Introduction

The unique structure and physiology of fungi make them both sensitive and resilient in the face of natural disturbances. The type and scale of disturbance determine the degree to which fungi will survive a given disturbance. Disturbance, as defined by Pickett and White (1985), includes elements of both scale and process. Specifically, “*a disturbance* is any relatively discrete event in time that disrupts ecosystem, community or population structure and changes resources, substrate availability, or the physical environment.” The structure and functioning of fungal communities are influenced by disturbances through changes in vegetation, substrate, environment, and a number of other components characteristic of the ecosystem

in which the fungal community is located. Many of the descriptive studies of fungal communities come from the development of associations with particular vegetation types, which is dependent upon the successional sere, or with specific decomposing substrates. These, in turn, comprise continuously and gradually changing substrates that have demonstrable trajectories. This led to relatively deterministic descriptions of fungal communities and their changing compositions (*sensu* Clements 1916, 1936; Christensen 1981, 1989).

Fungi play a number of important roles in ecosystem dynamics. Fungi, as saprophytes, are among the primary decomposers of substrates, and are essential for recycling nutrients to plants. Fungi are also essential as mutualists. Mycorrhizal fungi are involved in nutrient capture, and in expanding the resources available to plants. Some fungi achieve this goal through increasing surface area explored in the soil, while others increase activities such as active decomposition (Cairney and Burke 1998; Dighton et al. 2001) or parasitism (Johnson et al. 1997; Klironomos and Hart 2001). Pathogenic fungi are also essential components of ecosystems, and play key roles such as maintaining biodiversity and mediating competition (Price et al. 1986; Winder and Shamoun 2006). As disturbance at any scale will alter the biomass and diversity of fungi in ecosystems, fungi involved in these activities will be altered to differing degrees, based on the physical location of organisms in the ecosystem, and the type of disturbance (Snyder et al. 2002). With the differences in characteristics of these groups in mind, we will attempt to discuss fungi in general with examples from each of these groups. Unfortunately, there is greater information available for some of these groups with regard to disturbances than for others. This has potential to limit our discussion, and more importantly, our general understanding of the impacts of disturbance on fungi. It certainly brings to the forefront the necessity to understand the response

<sup>1</sup> Biology Department, Bradley University, 1501 W. Bradley Avenue, Peoria, IL 61625, USA

<sup>2</sup> Department of Biology, University of Dayton, 300 College Park, Dayton, OH 45469, USA

<sup>3</sup> Center for Conservation Biology, University of California, Riverside, CA 92521, USA

of these groups of organisms to disturbance, and to understand their impacts on ecosystems.

Fungi have a unique physiology, morphology, and reproductive biology. This makes attempts to describe their dynamics difficult in the rather conventional terms used for higher plants and animals. Fungi, because of the size of individual hyphae or yeast cells, and because they are predominantly studied in the laboratory under a microscope, are viewed as microorganisms that primarily respond to minute quantities of substrates in their environment. In mass and spatial extent, however, many fungi are macroorganisms, often extending across large patches. These patches may be rather static in space, and exist over long time periods, such as the mat-forming ectomycorrhizal fungi, or the *Armillaria* mycelial networks (E.B. Allen et al. 1995). Nevertheless, because these large organisms are still made up of microscopic hyphae with the potential to function independently, detailing their life-history characteristics means understanding both the macroscopic and microscopic aspects of their existence. As a result of this dichotomy in the structure of fungal “individuals”, any disturbance – from an individual gopher mound to a volcano – has important ramifications to the types of organisms and roles they play in ecosystem dynamics. For example, a large disturbance such as a volcanic eruption can destroy an entire fungal community, requiring subsequent reinvasion and establishment subject to classical models of succession (Allen et al. 1992, 2005b). In contrast, a gopher mound can disrupt mycelial networks within multispecies patches and within “individual” fungal types. This scenario allows for some recolonization from existing species, should the mycelial network rebuild from the remnant hyphal fragments. Each of these disturbance extremes directly and indirectly regulates the composition of the plant community and, in turn, the animal community. It is this range in the activity that we will describe. We will then attempt to develop a set of conceptual models that will allow us to begin to link our understanding of fungal biodiversity and ecosystem functioning.

## II. Ecosystem Disturbance: A Conceptual Framework

### A. Conceptual Model Overview

Understanding the factors influencing the diversity, distribution, and abundance of organisms in

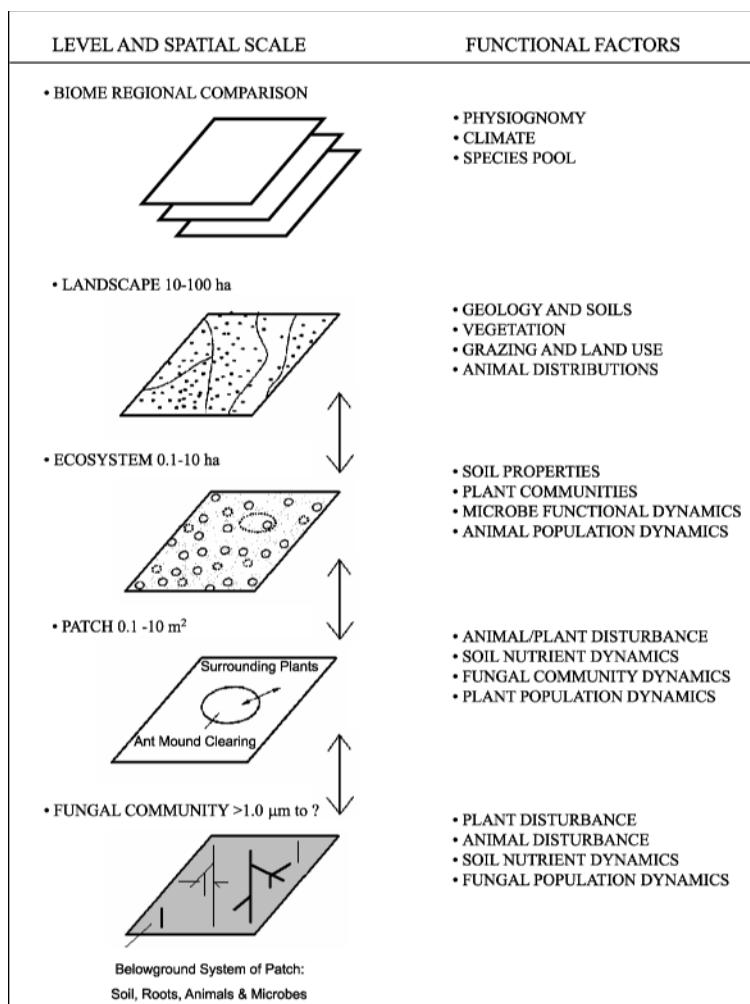
a community has long been a goal of ecologists and mycologists. Disturbance has been proposed to influence community diversity and structure through a variety of mechanisms. A single disturbance type, e.g., gopher mounds, can enhance (Allen et al. 1984, 1992), or retard (Koide and Mooney 1987) the rate of succession by altering soil resources, or species diversity. Disturbances may also affect communities by creating new sites for colonization (Platt 1975; Collins 1987), or creating new substrates (Cooke and Rayner 1984; Gams 1992). Ultimately, the ability of a disturbance to modify the substrate on which a fungal community exists will determine the extent to which the community will change. This modification can be to the substrate chemistry (e.g., differing litter types), or it can be the result of disturbances that affect the environmental factors under which those substrates can exist, such as changes in soil moisture content with removal of the litter layer. If disturbances differentially affect substrate and environment, then disturbances caused by a variety of agents will alter fungal species diversity within a community in different ways. We have developed a conceptual model of natural disturbance based on processes that scale from patch to ecosystem levels (Fig. 3.1). Our overview includes elements of these disturbance scales, and the factors that affect the various states of the disturbance cycle. The conceptual model integrates processes operating at the level of patch disturbance, such as soil enrichment, with those at the ecosystem level, such as spatial variations in natural disturbance density (e.g., fire, animal digging), soils, microbial and plant community structure (Fig. 3.1). We believe that emphasis on the feedback between patch dynamics and local ecosystem processes is an important key to increasing our understanding of the role of natural disturbance in fungal community structure and ecosystem function.

### B. Disturbance and Species Diversity

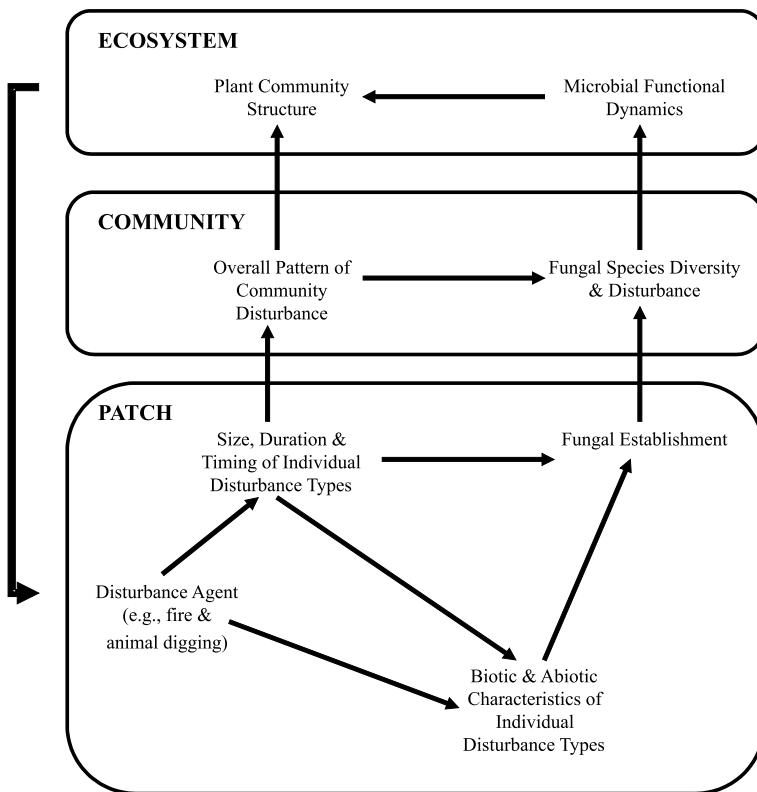
Disturbances influence the composition and species richness of communities through a variety of mechanisms. Some disturbances result in decreases in fungal biodiversity, while others are hypothesized to release ecosystems from unproductive states of “retrogression” (Wardle 2006). Within a short time scale, some disturbances affect the entire community simultaneously, such as volcanic eruptions (Andersen and MacMahon

1985; Allen et al. 1984, 2005b; Allen 1988) and catastrophic winds (e.g., Dunn et al. 1983), while other disturbances such as animal diggings (Koide and Mooney 1987; Allen et al. 1992; Friese and Allen 1993) or invasive species (Roberts and Anderson 2001; Evener and Chapin 2003; Bohlen 2006) influence only a relatively small proportion of the community at a time. Others initially start small, and expand over longer time scales, such as for invasive species (van Mantgem et al. 2004), N deposition (Egerton-Warburton and Allen 2000), and glacial expansions and retreats (Helm et al. 1999; Jumpponen et al. 2002). The scale and intensity of disturbances can, in turn, significantly affect the response of organisms and resulting successional patterns (e.g., Bazzaz 1983; Sousa 1984; Pickett and White 1985; Pickett et al. 1989; McClendon and Redente 1990; Egerton-Warburton and Allen 2000).

Large-scale disturbances affect fungal communities in different ways. Volcanoes, representing the most extreme large-scale disturbance, can completely destroy fungal communities, leaving topsoil buried under sterile tephra (Allen et al. 1984; Hendrix and Smith 1986) or lava (Gemma and Koske 1990), or even creating new lands (Henriksson and Henriksson 1974). Fungal communities in the most severely damaged areas can be destroyed completely. The recovery of these areas is driven by wind or small animal vectors capable of bringing new propagules from surviving areas, patches, or from source areas at differing distances. Materials deposited as a consequence of the eruption, such as tephra or lava, can have different characteristics, such as altered bulk density, chemistry or pH, which leads to the creation of new communities. On the most devastated area of Mt. St. Helens following



**Fig. 3.1.** Conceptual framework illustrating how ecological concepts of hierarchy and scale can be used to integrate and model the relationships of small-scale phenomena, such as the impact of disturbance on microbial functional groups, to larger-scale spatial patterns and processes (e.g., ecosystem dynamics) (based on Friese et al. 1997)



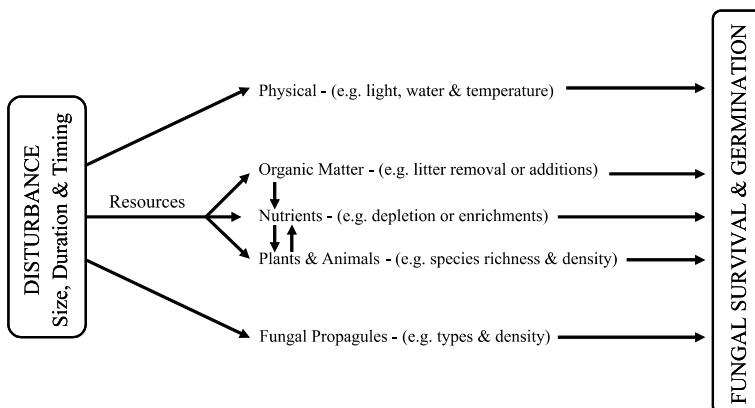
**Fig. 3.2.** Theoretical conceptual model depicting how patch-level disturbance processes can affect microbial functional dynamics, which, in turn, can shape larger-scale patterns and processes in community- and ecosystem-level dynamics (based on Friese et al. 1997)

the blast of 1980, Ascomycota were found to colonize the tephra within the first year, followed by Basidiomycota (Carpenter et al. 1987). After 10 years, AM and ectomycorrhizal fungi reestablished at the site, mediated by gophers and wind dispersal (Allen et al. 1992), although the ectomycorrhizal fungi were initially poorly developed.

The rate at which reinvasion progresses at the most severely disturbed sites depends on the availability of sources of fungal inoculum. With the existence of nearby or internal source patches of inoculum, fungal communities can begin invading and establishing. The rates of reinvasion of mycorrhizal fungi onto the pumice plain of Mt. St. Helens, to Krakatau following the 1883 eruption (Allen 1991), and from Hawaii (Gemma and Koske 1990) demonstrate that reinvasion of these fungi is dependent on the location and type of inocula, and upon the reinvasion of vegetation. Mt. St. Helens, by having mycorrhizae in the most damaged areas as soon as 1 year after the eruption, recovered more quickly than Krakatau, where facultatively mycorrhizal plant species were reported 3 years after the eruption. This is because the most severely damaged areas of Mt. St. Helens were surrounded by

remnant vegetation patches, rather than water, as on the island of Krakatau. On the Hawaiian Islands, there was a rapid invasion of mycorrhizal species from adjacent kipukas, or isolated patches of vegetation that remained untouched by disturbance (Gemma and Koske 1990). Volcanic eruptions such as the ones above occur relatively frequently, and are considered predictable and therefore subject to selective evolutionary pressure. Presumably, dispersal strategies and life histories of the plants on these islands are adapted to these disturbances, and it is probable that the same is true for the fungi.

Fire presents another example of large-scale disturbance that has the ability to affect fungal communities in different ways. Following a severe fire, there is an initial decrease in the number of propagules (Wright and Bollen 1961), and shift in the diversity of fungi present in an area (Wicklow 1973; E.B. Allen et al. 2003; Allen et al. 2005a; Bastias et al. 2006). Changes in soil pH and mineralization rates caused by fire regulate the fungi that can initially establish the area (Gochenaur 1981). Following the initial decrease in fungal propagules, a rapid increase in fungal biomass occurs, often to more than ten times the pre-fire value (Ahlgren and Ahlgren 1965; Wicklow 1973). These species,



**Fig. 3.3.** Detailed model of the biotic and abiotic characteristics of individual disturbance types. This model is a subset of the complete conceptual model depicted in Fig. 3.2. The disturbance agent determines aspects such as the size, duration, and timing of an individual disturbance event. All of these variables create a wide diversity of disturbance

patches, with unique biotic and abiotic characteristics. If each disturbance type creates a unique set of biotic and abiotic characteristics, then it is hypothesized that the fungal community will also be differentially affected within each of these disturbance types (based on Friese et al. 1997)

commonly referred to as pyrophilous fungi, are capable of taking advantage of the new resources made available by the fire.

Mycorrhizal species may decrease in number or diversity following fire (Vilarino and Arines 1991; E.B. Allen et al. 2003; Tuininga and Dighton 2004; Allen et al. 2005a), or remain unaffected within the plant root (Molina et al. 1992). However, some of these effects are relatively shallow, and do not affect diversity or density lower in the soil profile (Pattinson et al. 1999; Bastias et al. 2006). Fire can have a number of effects on mycorrhizal spores, depending on the maximum ground temperature reached while burning. Vilarino and Arines (1991) found that, following fire, the number and viability of AM spores decreased. They also determined that for at least one site, the dominant species of mycorrhizal fungus changed from *Acaulospora laevis* to *Acaulospora scrobiculata*. Percent root colonization by arbuscular mycorrhizae increased over the year following the burn, but did not reach the levels found before the fire. The depression in levels of soil colonization following fire at this site was detected for longer than in other similar research, such as that of Dhillon et al. (1988) on prairie soils. The authors suggested that the temperatures reached on these soils with a shrub and tree vegetation were greater than those reached with a herbaceous vegetation, the former producing longer-lasting effects. Treseder et al. (2004) found that fire in Alaskan boreal forests had little impact on AM fungi, but

recolonization by ECM appeared to be delayed up to 15 years following the disturbance. Alternatively, in secondary forests in the Yucatan Peninsula with a very shallow, highly organic soil, a “hot” fire virtually eliminated all inoculum (E.B. Allen et al. 2003), whereas following a “cooler” fire, where some organic matter persisted, the richness of fungi was much higher. The two types of fires can cause significantly different patterns of vegetation recovery (E.B. Allen et al. 2003; Allen et al. 2005a). Therefore, the frequency and intensity of a fire, which is determined largely by the structure of the plant community (e.g., forest vs. grassland), can determine both the spatial and temporal patterns of fungal community development.

Small-scale disturbance can also affect fungal communities in a variety of ways. These disturbances are often poorly characterized, because they create a mosaic of heterogeneous patches within the landscape that are often functionally and structurally different from those of the landscape that surrounds them. Patches are defined ecologically as discrete spatial patterns with easily identifiable boundaries (Pickett and White 1985). Disturbances such as mound building by animals create, or alter the patchiness of a landscape (Allen 1988; Friese and Allen 1993; Snyder et al. 2002). These patch disturbances disrupt existing external soil mycelial networks, such as those described by Finlay and Read (1986) for ectomycorrhizal fungi, and by Friese and Allen (1991) for arbuscular my-

corrhal fungi (e.g., absorptive hyphal networks, and hyphal bridges). Disruptions in the soil hyphal network will create openings for the colonization and spread of new fungi, thereby increasing fungal biodiversity, just as occurs for colonial animals (Connell 1961) and higher plants (Allen and Forman 1976). Gophers and ants are examples of animals that are capable of overturning soil, and moving mycorrhizal propagules within that soil to new patches in the soil matrix. Additionally, gophers trap spores within their fur, and can transport these fungi to new areas.

Ingestion and excretion of viable propagules at new locations by large mammals are also considered small-scale disturbances that have the capacity to change the community composition of a patch. The deposition of dung by elk, containing viable mycorrhizal spores from areas adjacent to the blast zone on the tephra at Mt. St. Helens, and this days following the blast, allowed the return of fungal propagules to a biotically sterile area (Allen 1987). Chronic small-scale disturbances can also be caused by large ungulates. Serengeti ecosystems are heavily grazed, resulting in elevated nitrogen and dung applications to the soil (Seagle et al. 1992). This increases nitrogen levels, and mineralizable carbon sources for microorganisms. Studies of mycorrhizal distribution in this ecosystem demonstrated an inverse relationship between soil fertility and the presence of mycorrhizal fungi (McNaughton and Oesterheld 1990). This relationship is also associated with a smaller gradient of nutritional status in the vegetation. The mycorrhizae allow the plants to maintain a high nutritional status across a broad range of soil nutritional ranges, which ultimately results in better forage for the animals, and a return of nutrients to the soil in the form of urine and dung.

### III. Fungal Community Dynamics: A Natural Disturbance Model

A natural disturbance model that evaluates fungi must examine fungal individuals and communities within the context of concomitant impacts of these organisms on ecosystem dynamics. Regardless of disturbance size, fungal biomass and diversity will be altered. The degree to which this alters specific ecosystem characteristics is dependent on the disturbance event itself, the substrates that exist following the disturbance, the subsequent

microclimate and its effects on the substrates, and the surrounding organisms. A disturbance event directly alters the fungal community by destroying hyphae and propagules of exposed species, although impacts of any disturbance will be modified by the intensity of the disturbance, and the seasonality of the event (Gochenaur 1981). A "hot" fire during a drought will devastate many litter fungi, whereas a "cool" one initiated during a wet season may affect only smoke-sensitive species, or those that exist exclusively on highly flammable materials. As the soil environment changes, e.g., decreased soil moisture in response to a lack of cover from direct radiation, the survival and growth of many hyphae are compromised (Boddy 1984). Loss of plant hosts, and changes in substrate quality and quantity will further alter the fungal community. Changes in diversity do not have to be large to impact a system. Change in the growth patterns of a single important fungal or non-fungal species as a consequence of disturbance can ramify through an entire community. These changes can alter, among other things, the competitive balance among species within a community, or ecosystem dynamics through changes to the types and activities of enzymes available.

An additional dilemma in determining the effects of disturbance at the patch or ecosystem scale is determining the extent of a fungal community (Cooke and Rayner 1984). These effects can be studied if the interpretation that communities are made of individuals with definable sets of species interactions (MacMahon et al. 1978) is used to define our concept of community. Large-scale disturbances can alter an entire landscape (Turner and Dale 1998), while small-scale disturbances may affect only a patch within the community. For this reason, the model developed here (Fig. 3.1) predicts the effects of disturbance within the patch, yet in some circumstances the patch may encompass an entire community. Following disturbance, the changes within the patch may affect the community of which it was a part, or may become a community of its own.

#### A. Disturbance Types and Characteristics

There are many types of disturbance that will affect a fungal community. In the fungal literature, disturbances are often subdivided into two main groups – enrichment, and destructive disturbances (Cooke and Rayner 1984). For understanding fungal com-

munity dynamics, these classifications are often too broad, because disturbances at the scale that relates to the fungal community can never be entirely enriching, or entirely destructive. For example, the effect of a forest fire on a fungal community is dependent on the type and size of fungal community being described, and the state that community is in when the fire occurs. The fire is destructive to the phylloplane fungal community, whereas soil fungi 5 to 10 cm deep may react to the fire as an enrichment disturbance. In areas prone to hurricane damage, high winds increase the amount of leaf and branch litter on the soil surface. However, the litter can decompose very rapidly, and overall, this decreases litter content, compared with the situation before a hurricane (Vargas and Allen, unpublished data). Further, the downed branch litter provides a large amount of fuel, often resulting in devastating wildfires that reduce, or even totally consume organic matter (see Gomez-Pompa et al. 2003).

Disturbances that alter plant and animal community dynamics will resonate through the fungal communities. Wind storms strip leaves and blow down canopy trees in a forest. These impacts are usually greater near the edge, which decreases habitat quality especially for small stands. These wind events provide substrate for saprophytic fungi, but they decrease host density for mycorrhizal fungi. In complicated food webs, such as those that involve truffle-eating flying squirrels, windthrow events can decrease food resources and canopy connectivity, which can diminish squirrel populations (Carey 2000; Ransome et al. 2004), and ultimately impair squirrel dispersal of mycorrhizal fungal spores contained in the truffles. As fungi participate in many different relationships in ecosystems, even a single disturbance, regardless of size or enrichment/destructive capacity, has the potential to impact fungi directly or indirectly through the organisms upon which fungi depend. Ultimately, this will feed back to alter fungal community dynamics.

## B. Biotic and Abiotic Characteristics of the Disturbance Model

The greatest effect of disturbance on the fungal community will be through influences on key factors that impact the growth of fungal structures, and the germination of spores. These factors, denoted as biotic and abiotic characteristics in Figs. 3.2 and 3.3, include physical characteristics, resources, soil flora and fauna, and fungal

propagules or hyphal fragments (Boddy 1984; Gams 1992). The physical components of the fungal community affected by disturbance include light, temperature, moisture, and pH (Gentry and Stiritz 1972; Rogers and Lavigne 1974; Mandel and Sorensen 1982). The resources include water, oxygen, organic matter, and a variety of mineral nutrients for growth and sporulation, of which some may be required in higher quantities than for vegetative growth (Moore-Landecker 1990). Another key resource included here is the physical environment within which organisms grow. For fungi in most terrestrial ecosystems, the complex system of decomposing wood and litter on the forest floor, or simply litter in grassland systems, is an essential part of their ecosystem. The litter layer can provide a fungal substrate, or it can modify the soil quality below it. As such, litter layer composition and characteristics, and the impact of disturbances on the litter layer can have severe consequences for fungi in terrestrial systems.

Plants and animals also regulate the composition of fungi in soils, but often in a nonlinear manner. Plants can provide both energy and carbon for fungal growth, but they can also provide many inhibitory substances. Animals can remove (graze), disperse, provide housing, or provide substrate (defecate or die) for different members of the fungal community, depending on fungal requirements. Additionally, changes in more than one of these parameters can act synergistically, or antagonistically, to further change the emergent fungal community (Snyder et al. 2002). In one case, the addition of CO<sub>2</sub> increased plant production, and C inputs to soil fungi. However, that input was matched by increased grazing by soil animals (Allen et al. 2005c). So, although the standing crop of fungal biomass remained the same under elevated CO<sub>2</sub>, the total throughput actually increased. Fire can also alter fungal-animal relationships. For example, the loss of the litter layer, a physical factor and/or substrate, by environmental factors such as fire, results not only in substrate loss, but also in increases in soil temperatures and decreases in water-holding capacity. As this happens, soil organisms such as mites and collembolans will migrate more deeply into the soil to escape drought, reducing grazing on fungi, but also reducing fungal dispersal near the surface (Klironomos and Kendrick 1995). Thus, changes in each of the above characteristics will be dependent on the type and intensity of the disturbance, and the interaction among the response variables.

The spatial structure of systems, as a consequence of environmental patterning or small-scale disturbance, has the potential to impact our ability to determine the effects of disturbance on organisms. For example, the existence of a pre-burn ant mound has the potential to alter the impact of a disturbance such as fire on an ecosystem. Alterations may be a consequence of different physical structures – for example, the movement of wind across the raised surface, or decreased burn heat as a consequence of increased mound moisture. The difficulty lies in separating the impacts of fire on the system from the impacts of pre-fire small-scale disturbance. Failure to detect changes in fire damage may be the result of sampling a mound without incorporating the specific characteristics of a mound. Boerner et al. (2000) found that enzyme dynamics related to bacterial and fungal activity in single-tree influence circles (see Zinke 1962) were altered by fire. Detecting changes in fungal dynamics following disturbance requires understanding of predisturbance spatial dynamics, and scale-appropriate postdisturbance sampling efforts. Failure to understand the impacts of patch-level disturbances, and of other agents capable of creating spatial structure in fungal communities will decrease the likelihood of detecting postdisturbance impacts.

#### IV. Scaling from Patch to Catastrophic Disturbance

Disturbances impact fungi at a number of levels. As discussed above, the smallest level reasonably available for description of disturbance impacts is the patch, and the largest is the ecosystem. While in theory large-scale disturbances can influence entire landscapes, and the last ice age certainly disturbed entire biomes, our discussion focuses on individuals, communities, and impacts at the ecosystem level, as these are the most common levels currently studied. Landscapes can be discussed as large mosaics of divergent ecosystems across a particular, extensive integrated distance, or they can be discussed as groupings of similar ecosystems at different successional stages recovering from similar disturbance regimes. This point is important, as adjacent ecosystems house communities that provide propagules for the reestablishment of similar communities. While not addressing this directly here, the availability of propagules similar to those of predisturbance communities is an essen-

tial component of reestablishment that, over evolutionary time, may have produced the mechanisms and community dynamics discussed below.

##### A. Individuals

Our understanding of impacts of disturbance on fungal individuals or species has been limited by a lack of appropriate techniques for identifying either. While culture techniques allowed us to examine some species, the availability of molecular techniques has greatly increased our knowledge and understanding of fungi as they exist in their natural environment. Studies prior to the common use of these techniques provide a great deal of the information on which our understanding of patch dynamics and the individual is based. So, while it is anticipated that many of the questions generated regarding individuals and the impacts of disturbance are only beginning to be addressed in greater detail, our discussion of individuals of necessity depends on data derived from a number of different approaches.

Patch-level disturbances often have greatest impact at the individual level. They alter fungal mycelial networks, and disturb the hyphal networks of individual fungi as they grow in soil. As such, individuals will change a great deal as a consequence of patch-level disturbances. Yet, this may, or may not impact fungal communities. The loss of a species from the ecosystem will occur only if the individual impacted is rare, has poor sporulation capacity or mechanisms, poor dispersal ability, is unable to germinate under new conditions, or is not represented in another, reasonably close resource patch. Differences in these characteristics among fungi, and consequences for recolonization following disturbance have been explored in the past. Recently, Drew et al. (2006) detected differences in the degree to which AM fungi can grow and colonize new hosts. In their study, *Glomus mosseae* demonstrated greater capacity for exploring a habitat to find new hosts than did *G. intraradices*, suggesting some fungi will be more successful in navigating patch disturbances than others. Each fungus exists in a system constrained by abiotic and biotic characteristics. Disturbances that alter these characteristics may result in the elimination of species from certain areas. This can occur across the disturbance intensity continuum; however, at the patch level, the fungus may be eliminated from the immediate vicinity of the disturbance, but not from

the fungal community at large. In one example, alternate year cropping and fallowing of a wheat-field in western Nebraska eliminated the dominant fungus of the native prairie, a small-spored *Glomus fasciculatum*. However, a larger-spored *Glomus mosseae* was able to persist through the fallow periods, and increased relative to *G. fasciculatum*. One outcome was a fungus that was less valuable, even detrimental, to wheat drought tolerance and production (Allen and Boosalis 1983).

Further research on the impacts of disturbance, such as tillage, on agricultural soils suggests that soil disturbance decreases fungal biomass, and alters soil structure (McGonigle and Miller 1996; Denef et al. 2001; Rillig and Mummey 2006). While these impacts often occur at the patch level in natural systems, the degree to which they impact individuals and species has been little studied. It is likely that the level of patch disturbance by digging animals and insects alters species composition at the patch level, while maintaining fungal community composition at the ecosystem level. Studies that have examined soil disturbances such as ant mounds have detected some of these patterns in arid systems. Friese and Allen (1993) found biotic enrichment of microorganisms in ant nests at study sites in Colorado and Wyoming. In this case, disturbance by harvester ant digging increased the total number of AM fungal propagules in the ant nests, compared to adjacent soil from blue grama grass (*Bouteloua gracilis*) at the site in Colorado, and under shrubs (*Artemisia tridentata*) at the site in Wyoming. The assemblages and dominant species of fungi also differed between mound and non-mound sites, with more fungi (characteristic of mesic sites) occurring in ant nests (C.F. Friese et al., unpublished data). Fungal species richness was higher in mound-associated material than in soil adjacent to mounds, or soil collected under shrubs at the Wyoming site, but not from soil adjacent to mounds at the Central Plains Experimental Range location (C.F. Friese et al., unpublished data). It appeared that for both locations, microenvironments selected a distinct assemblage of dominant fungi, with *Fusarium* spp. dominating the root material, and *Aspergillus* and *Penicillium* species predominating in seed cache soil (C.F. Friese et al., unpublished data). However, *Aspergillus fumigatus* had high densities in offmound soil from the Colorado site. Mucoraceous taxa, (i.e., *Cunninghamella*, *Rhizopus*, and *Syncephalastrum*) were isolated primarily from mound material, suggesting that the ant mounds may represent refugia for these

more mesic-adapted fungi (C.F. Friese et al., unpublished data). These results suggest the greatest impacts of these types of patch disturbances were on individual species through changes in biomass and organic matter, whereas overall fungal community diversity across the site was impacted to a much lesser degree.

## B. Community-Level Effects

Change in species composition within the patch may cause changes within the larger fungal community. The establishment of fungi within the patch can affect the diversity and distribution of fungi within the community, or it can cause the establishment of a new community that exists only within the patch. The exact outcome will be determined by the characteristics of the disturbance, especially the size of the disturbance, but also by the heterogeneity of the landscape prior to disturbance. A landscape matrix is established by a series of disturbances of increasing scale. The patches are nested within a habitat that can be relatively homogenous or heterogeneous, depending on the scale and intensity of other disturbances occurring across the landscape. From an area of no disturbance, smaller-scale disturbances are overlaid by the increasing intensity of larger-scale disturbance. The impact of smaller-scale disturbances may alleviate the effects of larger-scale disturbances, by acting as islands of inoculum, or dispersal agents. This was the case for the arbuscular mycorrhizal (AM) fungi at Mt. St. Helens. Disturbances by gopher digging and elk droppings resulted in AM fungal inoculum returning to a site completely decimated by the volcano blast (Allen et al. 1984, 1992, 2005b). Initially, EM fungi were predominantly dispersed back onto the site by wind (Allen 1987). Thus, across the pyroclastic flow zone, small AM fungal patches reformed along animal pathways, whereas the EM plants initially established at random locations. It was the reestablishment of these individuals, or of the small eclectic group of fungi that existed in the deposited inoculum, which led to recolonization following the most severe disturbance type. Similarly, Jumpponen (2003) found the fungal community in the youngest soils adjacent to a receding glacier to have dormant mycorrhizal fungi even before the arrival of the plant community. As these fungi are biotrophs dependent on plant hosts, airborne spore deposition preceded plant arrival providing symbionts for arriving seeds.

The composition of the postdisturbance community is also dependent on the competitive interactions among residuals and immigrants. A good deal of work has been undertaken on interactions of competitive (C), stress-tolerant (S), or ruderal (R) strategies in saprophytes following disturbance (Cooke and Rayner 1984). Immediately after a disturbance, the R-strategy is likely to predominate although, depending on the disturbance, many S-strategists may remain as residuals. Presumably, the C-, and more S-strategies will predominate later. Fungi such as *Mucor* and *Rhizopus* are presumed to be ruderals because they exploit simple carbohydrates rapidly (R-strategy). Alternatively, *Phanerochaete* grows slowly, but can degrade almost any type of substrate (S-strategy). *Cephalosporium* is an outstanding competitor because it expends a large amount of resources to produce antibiotics that restrict access to its own resource base. However, these subdivisions are highly artificial, and organisms exist rather along gradients between these extremes. For example, a common fungus of burned pine forests is *Morchella*. Is that because it tolerates the fires, and the harsh conditions following fires (S), competes well with other residuals and immigrants by growing hyphae rapidly and utilizing resources in the early spring, before other saprophytes become active (C), produces a massive sporulating fungus that disperses spores by wind and animals when released from competition (R), or (most probably) has some effective combination of all these strategies?

Taylor and Bruns (1999) examined the ECM community structure in a mature pine forest. They detected minimal overlap between the active mycorrhizal community and the community present as resistant propagules. This suggested that differences in colonization strategies, such as the C, S, and R described above, and resource preferences combined with resistant fungal structures allow diversity to be maintained in forest communities, so that organisms can respond to environmental cues and disturbances that have been historically part of the ecosystems. Other studies on mycorrhizal communities further suggest that community development is dependent on the type (enrichment vs. destructive), intensity, and frequency of disturbance. Lilleskov and Bruns (2003) found that differences in the timing of root colonization by two ECM fungi was altered by soil nutrient status. *Rhizopogon occidentalis*, an early successional species, colonized roots early, and then was replaced as a dominant species by *Tomentella sublilacina* as the forest ma-

tured. Under high nutrient conditions, however, this replacement was delayed, suggesting interspecific interactions between the two species were mediated by soil nutrient content. As disturbances also act as a stress upon a system, the return time of the stress can alter interspecific relationships. Puppi and Tartaglini (1991), evaluating the effects of fire on Mediterranean communities, found that although the communities were under similar environmental constraints, the vegetative and mycorrhizal community structure differed. These differences were attributed to the recurrence time of fire. In the more disturbed community, AM fungi were more common, whereas in the less disturbed community, EM were prevalent. EM were hypothesized to be more stress-tolerant than AM. Alternatively, in many grasslands, the plants forming AM were tolerant of fire, whereas those forming EM tended not to be. The fungi may simply be locked to the host strategy.

Fungal pathogens have unique roles in disturbances. Pathogen density and diversity can be impacted by disturbances. The role of pathogens in natural ecosystems, especially in relation to historical disturbance regimes, has not been addressed well in the literature. Increases in the loss of plants, especially forest trees, through pathogen attack has stimulated focus on fungi as pathogens. This may be a consequence of recent attention in the face of economic loss, or may be as a consequence of increased importance of pathogens as regulators of community dynamics. Allen et al. (2005a) suggested that altered weather patterns in the tropical dry forest they examined may have resulted in increased incidence of an indigenous fungal pathogen. Hence, altered historical disturbance regimes may change relative abundance and impact of pathogens on hosts. Parker et al. (2006) found that fire suppression resulted in increased levels of fungal pathogen activity in North American forests. This activity is now being altered by large-scale wildfires, and the reintroduction of more historically representative fire regimes using prescribed burns. However, these new disturbances are also causing increases in pathogen activities. Reintroduction of natural disturbance regimes are needed to reduce pathogen activities, yet the way to achieve these reductions may require greater understanding of the impacts of fire on the physiology and structure of these pathogens in their natural habitat. Changes in historical disturbance patterns and physical characteristics of the environment,

such as altered temperature and moisture, may be resulting in increases in pathogen activity.

Fungal pathogens also make interesting contributions as disturbances to plant communities. Fungi such as root rot fungi increase the likelihood of canopy gaps in forests (Bendel et al. 2006) directly through impacts on trees. These fungi increase substrate for saprophytes, and decrease habitat for mycorrhizal species. Other fungal pathogens have been found to aggravate the severity of natural disturbances. Papaik et al. (2005) found that beech bark disease does more damage to trees by decreasing the resistance of beech to disturbance events such as windthrow than it does by directly impacting the plant it infects.

### C. Ecosystem Characteristics and Feedback Loops

Changes in fungal community structure will have the greatest impact on the ecosystem if functioning changes with composition, or if the fungi affect plant diversity (e.g., Renker et al. 2004). Changes in primary production, quality of material produced, decomposition rates, or nutrient pool conversion can affect the stability, productivity, and ultimately the functioning of ecosystems (Chapin et al. 2002). Consequently, disturbances that modify soil processes or the vegetation will alter the corresponding ecosystem dynamics. Ecosystem-level feedback loops (Figs. 3.1 and 3.2) can influence patch structure through effects on disturbance types, characteristics, and the biotic and abiotic characteristics of the patch. Ecosystem dynamics will affect small-scale disturbance by influencing such things as animal types and densities, and large-scale disturbances by fuel loads and litter layer thickness. All scales of ecosystem disturbance, ranging from landslides to animal burrowing and to hyphal grazing by microarthropods, can disrupt critical points in the hyphal network that exist in the soil. Altering these hyphal network points changes nutrient availability and transfer to plant hosts, which, in turn, alters ecosystem productivity.

The most significant impacts of disturbance to fungal communities will be through changes in structure. It is predicted that a disturbed site tends to return to a community structure that does not entirely resemble the predisturbance state (Gochenaur 1981). The existence of a new assemblage of species, even of the same species of different age or density, may restrict the ability

of the community to return to a predisturbance state. This was observed in the simple experiment on cultured *Penicillium* and *Aspergillus* by Armstrong (1976), which demonstrated that although *Aspergillus* would exclude *Penicillium* in plated cultures of the same age, if *Aspergillus* spores were plated with *Penicillium* spores of a younger culture, then both would be maintained. While terrestrial ecosystems have a great deal more complexity than this two-species model, it is likely that changes induced by disturbance have differential effects on the species present, which will ultimately affect the composition of the postdisturbance community.

Mycorrhizal fungi may provide the links necessary to evaluate the impact of community structure, and of changes in community structure on ecosystem function (Read and Perez-Moreno 2003). The effects of individual fungal species on plant communities are expressed through their impacts on aboveground productivity and diversity. Fungal species can increase productivity through increased resource acquisition. They can alter plant diversity directly by presence or absence, through mechanisms of specificity and by altering the outcome of aboveground competition. They can cause changes to litter and tissue quality through differences in nutrient acquisition. The associations that link belowground community structure to aboveground dynamics are established, maintained, and disrupted by disturbances that alter fungal community dynamics.

Understanding the impacts of changes in communities of saprophytic fungi on ecosystem function is also essential for understanding the overall impacts of a given disturbance type. Modeling has been used recently to examine linkages between communities and ecosystem dynamics. While these models have been important for understanding global change scenarios, they have also allowed independent evaluation of key components of belowground communities in ecosystem dynamics. Hunt and Wall (2002) modeled effects of species loss on biodiversity. They found that deletion of saprophytic fungi and bacteria caused changes to net primary productivity. As they were modeling a large number of belowground groups, and detected little change with deletions of other groups, these results emphasize the importance of saprophytes as determinants of ecosystem characteristics. It also emphasizes that changes in this group as a result of disturbance has the potential to alter larger-scale functioning. There is currently a great deal of interest in tying community structure to

function at the ecosystem level. The response of fungal communities to disturbance events might provide good systems to evaluate these linkages.

## V. Conclusions

### A. The Impact of Disturbance on the Functional Role of Fungi

This chapter was designed to demonstrate that disturbance may be the single, most important process regulating the structure and functioning of fungal communities. This is due to the unique physiology, morphology, and reproductive biology of fungi. While microbial ecologists are beginning to describe the importance of individual disturbance events, we know far less about the interactions of small- and large-scale perturbations set within a larger landscape of single or multiple plant communities. This distinction becomes of even greater importance in the light of the global dimensions of anthropogenic influences on ecosystems, such as N fertilization, exotic species migrations, habitat fragmentation, and global climate change. We suggest that understanding the roles of disturbance in fungal communities, and the feedbacks from the fungal communities to ecosystem functioning, are crucial to understanding the results from these larger global concerns. Developing models for linking the range of scales that comprise disturbance dynamics depends on linking two distinct types of studies. First, we must begin to build an array of case studies from particular ecosystems in which we know the natural history of the fungi, and how these natural histories contribute to existing community composition and functioning. Second, we must develop a conceptual framework that integrates all of these various case studies into a comprehensive view of how communities work, and what factors regulate them. Finally, we must continuously reevaluate those conceptual models to develop a quantitative model of the complex roles of fungi within landscapes that are undergoing anthropogenic and natural change.

While it would be easiest to evaluate disturbance as a static entity, it is unfortunately a moving target. As anthropogenic impacts alter natural ecosystems, we are also altering disturbance regimes, and impacts of disturbance events. Ecosystems are comprised of organisms that have interacted with their biotic and abiotic environments over exceedingly long time scales.

The systems examined today are a consequence of organisms responding to the stresses experienced on predictable time scales. As anthropogenic disturbances have escalated only over the last 200 years, we cannot adequately predict the consequences of a given disturbance based on historical data, because we do not know if the system we are examining is similar to that which existed in the past. Ecologists use the terms “resistance” and “resilience” to evaluate the degree to which a system resists change in the face of disturbance, or the degree to which it returns to the predisturbance state. In theory, a system facing a predictable disturbance would respond in a resistant, or resilient fashion. However, with added unpredictable stresses such as chronic N deposition, herbicide use, global warming, and atmospheric pollution, systems may be less resistant or resilient to disturbances that they may have easily recovered from in the past. Loss of evolutionary history within ecosystems not only decreases our ability to understand the complex affects of disturbance on fungal communities, but it also has great potential to damage the ecosystems that currently exist within our biosphere.

### B. Future Research Directions

As a result of the widespread interest in anthropogenic changes to the earth’s atmosphere, and the effects that these changes may have on the biosphere, it is important to study and “tease out” a better understanding of how small-scale microbial processes (such as nutrient mobilization and immobilization) fit into the larger, global picture. The diversity and biomass of microbial communities is a direct indicator of the extent of the functional role that these organisms play in the dynamics of different ecosystems. If anthropogenic change alters the structure and biodiversity of microbial communities, then it is also likely that their critical functional roles in ecosystem and global-level nutrient cycling are also impacted. As is the case with other groups of organisms, it is as important to understand how the functional role of fungi is affected by various forms of human impact on the environment (e.g., Meyer 1993; Read 1993; M.F. Allen et al. 1995). One approach to evaluate this impact would be to examine the fungal communities present at ecological restoration sites that are at least 10–20 years old, and compare the microbial characteristics of these “established” restoration sites to those of their reference sites. Differences at these

sites would allow one to evaluate the impacts that human alteration of system characteristics has on fungal community dynamics. Future research on fungal community dynamics should also focus on linking the issues of fungal biodiversity and functionality with both natural disturbance and anthropogenic change. This research direction is critical for us to completely understand and explain the importance of fungi in ecosystem dynamics. Attempts to explore and integrate all of the above factors are crucial if we are ever to gain a comprehensive understanding of the functional role of fungi in diverse ecosystems, and the biosphere as a whole.

**Acknowledgements.** The authors wish to thank Shivcharn Dhillion and Tom Crist for technical assistance in the areas of field/laboratory work, and input on the development of conceptual models. Thanks also to Pua Borges and Anna Abts for help in editing and formatting.

## References

- Ahlgren IF, Ahlgren CE (1965) Effects of prescribed burning on soil microorganisms in a Minnesota jack pine forest. *Ecology* 46:304–310
- Allen MF (1987) Re-establishment of mycorrhizas on Mount St. Helens: migration vectors. *Trans Br Mycol Soc* 88:413–417
- Allen MF (1988) Re-establishment of VA mycorrhizae following severe disturbance: comparative patch dynamics of a shrub desert and a subalpine volcano. *Proc R Soc Edinb* 94:63–71
- Allen MF (1991) The ecology of Mycorrhizae. Cambridge University Press, Cambridge
- Allen MF, Boosalis MG (1983) Effects of two species of VA mycorrhizal fungi on drought tolerance of winter wheat. *New Phytol* 93:67–76
- Allen EB, Forman RTT (1976) Plant species removals and old-field community structure and stability. *Ecology* 57:1233–1243
- Allen MF, MacMahon JA, Andersen DC (1984) Reestablishment of Endogonaceae on Mount St. Helens: survival of residuals. *Mycologia* 76:1031–1038
- Allen MF, Crisafulli CM, Friese CF, Jeakins SJ (1992) Reformation of mycorrhizal symbioses on Mount St Helens, 1980–1990: interactions of rodents and mycorrhizal fungi. *Mycol Res* 96:447–453
- Allen EB, Allen MF, Helm DJ, Trappe JM, Molina R, Rincon E (1995) Patterns and regulation of mycorrhizal plant and fungal diversity. *Plant Soil* 170:47–62
- Allen MF, Morris SJ, Edwards F, Allen EB (1995) Microbe-plant interactions in Mediterranean-type habitats: shifts in fungal symbiotic and saprophytic functioning in response to global change. In: Moreno JM, Oechel WC (eds) Global change and Mediterranean-type ecosystems. Ecological Studies vol 117. Springer, Berlin Heidelberg New York, pp 287–305
- Allen EB, Allen MF, Egerton-Warburton L, Corkidi L, Gomez-Pompa A (2003) Impacts of early- and late-seral mycorrhizae during restoration in seasonal tropical forest, Mexico. *Ecol Appl* 13:1702–1717
- Allen MF, Allen EB, Gomez-Pompa A (2005a) Effects of mycorrhizae and nontarget organisms on restoration of a seasonal tropical forest in Quintana Roo, Mexico: factors limiting tree establishment. *Restor Ecol* 13:325–333
- Allen MF, Crisafulli CM, Morris SJ, Egerton-Warburton LM, MacMahon JA, Trappe JM (2005b) Mycorrhizae and Mount St. Helens: story of a symbiosis. In: Dale VH, Swanson FJ, Crisafulli CM. (eds) Ecological responses to the 1980 eruption of Mount St. Helens. Springer, Berlin Heidelberg New York, pp 221–231
- Allen MF, Klironomos JN, Treseder KK, Oechel WC (2005c) Responses of soil biota to elevated CO<sub>2</sub> in a chaparral ecosystem. *Ecol Appl* 15:1701–1711
- Andersen DC, MacMahon JA (1985) Plant succession following the Mount St. Helens volcanic eruption: facilitation by a burrowing rodent, *Thomomys talpoides*. *Am Midl Nat* 114:52–69
- Armstrong RA (1976) Fugitive species: experiments with fungi and some theoretical considerations. *Ecology* 57:953–963
- Bastias BA, Huang ZQ, Blumfield T, Xu Z, Cairney JWG (2006) Influence of repeated prescribed burning on the soil fungal community in an eastern Australian wet sclerophyll forest. *Soil Biol Biochem* 38:3492–3501
- Bazzaz FA (1983) Characteristics of populations in relation to disturbance in natural and man-modified ecosystems. In: Mooney HA, Godron M (eds) Disturbance and ecosystems. Ecological Studies vol 44. Springer, Berlin Heidelberg New York, pp 259–275
- Bendel M, Kienast F, Bugmann H, Rigling D (2006) Incidence and distribution of *Heterobasidion* and *Armillaria* and their influence on canopy gap formation in unmanaged mountain pine forests in the Swiss Alps. *Eur J Plant Pathol* 116:85–93
- Boddy L (1984) The micro-environment of basidiomycete mycelia in temperate deciduous woodlands. In: Jennings DH, Rayner ADM (eds) The ecology and physiology of the fungal mycelium. Cambridge University Press, Cambridge, pp 261–289
- Boerner REJ, Decker KLM, Sutherland EK (2000) Prescribed burning effects on soil enzyme activity in a southern Ohio hardwood forest: a landscape-scale analysis. *Soil Biol Biochem* 32:899–908
- Bohlen P (2006) Biological invasions: Linking the above-ground and belowground consequences. *Appl Soil Ecol* 32:1–5
- Cairney JWG, Burke RM (1998) Extracellular enzyme activities of the ericoid mycorrhizal endophyte *Hymenoscyphus ericae* (Read) Korf & Kernan: their likely roles in decomposition of dead plant tissue in soil. *Plant Soil* 205:181–192
- Carey AB (2000) Effects of new forest management strategies on squirrel populations. *Ecol Appl* 10:248–257
- Carpenter SE, Trappe JM, Ammirati Jr J (1987) Observations of fungal succession in the Mount St. Helens devasting zone, 1980–1983. *Can J Bot* 65:716–728

- Chapin FS, Matson PA, Mooney HA (2002) Principles of terrestrial ecosystem ecology. Springer, Berlin Heidelberg New York
- Christensen M (1981) Species diversity and dominance in fungal communities. In: Wicklow DT, Carroll GC (eds) The fungal community. Marcel Dekker, New York, pp 201–232
- Christensen M (1989) A view of fungal ecology. *Mycologia* 81:1–19
- Clements FE (1916) Plant succession: an analysis of the development of vegetation. Carnegie Institution of Washington Publication, Washington, DC
- Clements FE (1936) Nature and structure of the climax. *J Ecol* 24:252–284
- Collins SL (1987) Interactions of disturbances in tallgrass prairie: a field experiment. *Ecology* 68:1243–1250
- Connell JH (1961) The influence of interspecific competition and other factors on the distribution of the barnacle *Chthamalus stellatus*. *Ecology* 42:710–723
- Cooke RC, Rayner ADM (1984) Ecology of saprotrophic fungi. Longman, London
- Deneef K, Six J, Bossuyt H, Frey SD, Elliott ET, Merckx R, Paustian K (2001) Influence of dry-wet cycles on the interrelationship between aggregate, particulate organic matter, and microbial community dynamics. *Soil Biol Biochem* 33:1599–1611
- Dhillon SS, Anderson RC, Liberta AE (1988) Effect of fire on the mycorrhizal ecology of little bluestem (*Schizachyrium scoparium*). *Can J Bot* 66:706–713
- Dighton J, Mascarenhas M, Arbuckle-Keil GA (2001) Changing resources: assessment of leaf litter carbohydrate resource change at a microbial scale of resolution. *Soil Biol Biochem* 33:1429–1432
- Drew EA, Murray RS, Smith SE (2006) Functional diversity of external hyphae of AM fungi: ability to colonize new hosts is influenced by fungal species, distance and soil conditions. *Appl Soil Ecol* 32:350–365
- Dunn CP, Guntenspergen GR, Dorney JR (1983) Catastrophic wind disturbance in an old-growth hemlock-hardwood forest, Wisconsin. *Can J Bot* 61:211–217
- Egerton-Warburton LM, Allen EB (2000) Shifts in arbuscular mycorrhizal communities along an anthropogenic nitrogen deposition gradient. *Ecol Appl* 10:484–496
- Eviner VT, Chapin FS (2003) Gopher-plant-fungal interactions affect establishment of an invasive grass. *Ecology* 84:120–128
- Finlay RD, Read DJ (1986) The structure and function of the vegetative mycelium of ectomycorrhizal plants. I. Translocation of  $^{14}\text{C}$ -labelled carbon between plants interconnected by a common mycelium. *New Phytol* 103:143–156
- Friese CF, Allen MF (1991) The spread of VA mycorrhizal fungal hyphae in the soil: inoculum types and external hyphal architecture. *Mycologia* 83(4):409–418
- Friese CF, Allen MF (1993) The interaction of harvester ants and vesicular-arbuscular mycorrhizal fungi in a patchy semi-arid environment: the effects of mound structure on fungal dispersion and establishment. *Funct Ecol* 7:13–20
- Friese CF, Morris SJ, Allen MF (1997) Disturbance in natural ecosystems: Scaling from fungal diversity to ecosystem functioning. In: Wicklow DT, Söderström B (eds) The Mycota vol IV, 1st edn. Environmental and microbial relationships. Springer, Berlin Heidelberg New York, pp 47–63
- Gams W (1992) The analysis of communities of saprophytic microfungi with special reference to soil fungi. In: Winterhoff W (ed) Fungi in vegetation science. Kluwer Academic, Amsterdam, pp 183–223
- Gemma JN, Koske RE (1990) Mycorrhizae in recent volcanic substrates in Hawaii. *Am J Bot* 77:1193–1200
- Gentry JB, Stiritz KL (1972) The role of the Florida harvester ant, *Pogonomyrmex badius*, in old field mineral nutrient relationships. *Environ Entomol* 1:39–41
- Gochenaur SE (1981) Response of soil fungal communities to disturbance. In: Wicklow DT, Carroll GC (eds) The fungal community: its organization and role in the ecosystem. Marcel Dekker, New York, pp 459–479
- Gomez-Pompa A, Allen MF, Fedick SL, Jimenez-Osornio JJ (2003) The lowland Maya area: three millennia at the human-wildland interface. Haworth Press, New York
- Helm DJ, Allen EB, Trappe JM (1999) Plant growth and ectomycorrhiza formation by transplants on deglaciated land near Exit Glacier, Alaska. *Mycorrhiza* 8:297–304
- Hendrix LB, Smith SD (1986) Post-eruption revegetation of Isla Fernandina, Galapagos: II. *Natl Geogr Res* 2:6–16
- Henriksson LE, Henriksson E (1974) Occurrence of fungi on the volcanic island of Surtsey, Iceland. *Acta Bot Islandica* 3:82–88
- Hunt HW, Wall DH (2002) Modeling the effects of loss of soil biodiversity on ecosystem function. *Global Change Biol* 8:33–50
- Johnson NC, Graham JH, Smith FA (1997) Functioning of mycorrhizal associations along the mutualism-parasitism continuum. *New Phytol* 135:575–585
- Jumpponen A (2003) Soil fungal community assembly in a primary successional glacier forefront ecosystem as inferred from rDNA sequence analysis. *New Phytol* 158:569–578
- Jumpponen A, Trappe JM, Cazares E (2002) Occurrence of ectomycorrhizal fungi on the forefront of retreating Lyman Glacier (Washington, USA) in relation to time since deglaciation. *Mycorrhiza* 12:43–49
- Klironomos JN, Hart MM (2001) Food-web dynamics – animal nitrogen swap for plant carbon. *Nature* 410:651–652
- Klironomos JN, Kendrick B (1995) Relationships among microarthropods, fungi, and their environment. *Plant Soil* 170:183–197
- Koide RT, Mooney HA (1987) Spatial variation in inoculum potential of vesicular-arbuscular mycorrhizal fungi caused by formation of gopher mounds. *New Phytol* 107:173–182
- Lilleskov EA, Bruns TD (2003) Root colonization dynamics of two ectomycorrhizal fungi of contrasting life history strategies are mediated by addition of organic nutrient patches. *New Phytol* 159:141–151
- MacMahon JA, Phillips DL, Robinson JV, Schimpf DJ (1978) Levels of biological organization: an organism-centered approach. *BioScience* 28:700–704
- Mandel RD, Sorenson CJ (1982) The role of the western harvester ant (*Pogonomyrmex occidentalis*) in soil formation. *Soil Sci Soc Am J* 46:785–788
- McClendon T, Redente EF (1990) Succession patterns following soil disturbance in a sagebrush steppe community. *Oecologia* 85:293–300

- McGonigle TP, Miller MH (1996) Development of fungi below ground in association with plants growing in disturbed and undisturbed soils. *Soil Biol Biochem* 28:263–269
- McNaughton SJ, Oesterheld M (1990) Extramatrical mycorrhizal abundance and grass nutrition in a tropical grazing ecosystem, the Serengeti National Park, Tanzania. *Oikos* 59:92–96
- Meyer O (1993) Functional groups of microorganisms. In: Schulze ED, Mooney HA (eds) *Biodiversity and ecosystem function*. Springer, Berlin Heidelberg New York, pp 67–96
- Molina R, Massicotte H, Trappe J (1992) Specificity phenomena in mycorrhizal symbioses: community-ecological consequences and practical implications. In: Allen MF (ed) *Mycorrhizal functioning*. Chapman and Hall, New York, pp 357–423
- Moore-Landecker E (1990) *Fundamentals of the fungi*. Prentice Hall, New Jersey
- Papaik MJ, Canham CD, Latty EF, Woods KD (2005) Effects of an introduced pathogen on resistance to natural disturbance: beech bark disease and windthrow. *Can J Forest Res* 35:1832–1843
- Parker T, Clancy KM, Mathiasen RL (2006) Interactions among fire, insects and pathogens in coniferous forests of the interior western United States and Canada. *Agric Forest Entomol* 8:167–189
- Pattinson GS, Hammill KA, Sutton BG, McGee PA (1999) Simulated fire reduces the density of arbuscular mycorrhizal fungi at the soil surface. *Mycol Res* 103:491–496
- Pickett STA, White PS (1985) Patch Dynamics: a synthesis. In: Pickett STA, White PS (eds) *The ecology of natural disturbance and patch dynamics*. Academic Press, New York, pp 371–384
- Pickett STA, Kolasa J, Armesto JJ, Collins SL (1989) The ecological concept of disturbance and its expression at various hierarchical levels. *Oikos* 54:129–136
- Platt WJ (1975) The colonization and formation of equilibrium plant species associations on badger disturbances in a tall-grass prairie. *Ecol Monogr* 45:285–305
- Price PW, Westoby M, Rice B, Atsatt PR, Fritz RS, Thompson JN, Mobley K (1986) Parasite mediation in ecological interactions. *Annu Rev Ecol Systematics* 17:487–505
- Puppi G, Tartaglini N (1991) Mycorrhizal types in three Mediterranean communities affected by fire to different extents. *Acta Oecol* 12:295–304
- Ransome DB, Lindgren PMF, Sullivan DS, Sullivan TP (2004) Long-term responses of ecosystem components to stand thinning in young lodgepole pine forest I. Population dynamics of northern flying squirrels and red squirrels. *Forest Ecol Manage* 202:335–367
- Read DJ (1993) Plant-microbe mutualisms and community structure. In: Schulze ED, Mooney HA (eds) *Biodiversity and ecosystem function*. Springer, Berlin Heidelberg New York, pp 181–209
- Read DJ, Perez-Moreno J (2003) Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? *New Phytol* 157:475–492
- Renker C, Zobel M, Opik M, Allen MF, Allen EB, Vosatka M, Rydlova J, Buscot F (2004) Structure, dynamics and restoration of plant communities: do arbuscular mycorrhizae matter? In: Temperton VM, Hobbs RJ, Nuttle T, Halle S (eds) *Assembly rules and restoration ecology: bridging the gap between theory and practice*. Island Press, Washington, DC, pp 189–229
- Rillig MC, Mumme DL (2006) Mycorrhizas and soil structure. *New Phytol* 171:41–53
- Roberts KJ, Anderson RC (2001) Effect of Garlic Mustard *Alliaria petiolata* (Beib. Cavara & Grande) extracts on plants and arbuscular mycorrhizal (AM) fungi. *Am Midl Nat* 146:146–152
- Rogers LE, Lavigne RJ (1974) Environmental effects of western harvester ants on the shortgrass plains ecosystem. *Environ Entomol* 3:994–997
- Seagle SW, McNaughton SJ, Ruess RW (1992) Simulated effects of grazing on soil nitrogen and mineralization in contrasting Serengeti grasslands. *Ecology* 73:1105–1123
- Snyder SR, Crist TO, Friese CF (2002) Variability in soil chemistry and arbuscular mycorrhizal fungi in harvester ant nests: the influence of topography, grazing and region. *Biol Fertil Soils* 35:406–413
- Sousa WP (1984) The role of disturbance in natural communities. *Annu Rev Ecol Systematics* 15:353–391
- Taylor DL, Bruns TD (1999) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. *Mol Ecol* 8:1837–1850
- Treseder KK, Mack MC, Cross A (2004) Relationships among fires, fungi, and soil dynamics in Alaskan Boreal forests. *Ecol Appl* 14:1826–1838
- Tuinzing AR, Dighton J (2004) Changes in ectomycorrhizal communities and nutrient availability following prescribed burns in two upland pine-oak forests in the New Jersey pine barrens. *Can J Forest Res* 34:1755–1765
- Turner MG, Dale VH (1998) Comparing large, infrequent disturbances: what have we learned? *Ecosystems* 1:493–496
- van Mantgem PJ, Stephenson NL, Keifer M, Keeley J (2004) Effects of an introduced pathogen and fire exclusion on the demography of sugar pine. *Ecol Appl* 14:1590–1602
- Vilarino A, Arines J (1991) Numbers and viability of vesicular-arbuscular fungal propagules in field soil samples after wildfire. *Soil Biol Biochem* 23:1083–1087
- Wardle DA (2006) The influence of biotic interactions on soil biodiversity. *Ecol Lett* 9:870–886
- Wicklow DT (1973) Microfungal populations in surface soils of manipulated prairie stands. *Ecology* 54:1302–1310
- Winder RS, Shamoun SF (2006) Forest pathogens: friend or foe to biodiversity? *Can J Plant Pathol* 28:S221–S227
- Wright E, Bollen WB (1961) Microflora of Douglas-fir forest soil. *Ecology* 42:825–828
- Zinke PJ (1962) The pattern of influence of individual forest trees on soil properties. *Ecology* 43:130–133

---

## 4 Fungal Responses to Disturbance: Agriculture and Forestry<sup>1</sup>

R.M. MILLER<sup>2</sup>, D.J. LODGE<sup>3</sup>

### CONTENTS

I. Introduction .....	47
II. Disturbance as a General Phenomenon .....	48
III. Fungi as Control Points in Management Practices .....	49
A. The Habitats of the Fungus .....	49
B. Effects of Disturbance on Fungi .....	49
C. Contributions of Fungi to Nutrient Cycling .....	50
D. Contributions of Fungi to the Hierarchical View of Soil Aggregation .....	51
IV. Fungi and Agriculture .....	53
A. Tillage and Crop Rotation Effects on Fungi .....	53
B. Role of Fungi in Soil Restorations .....	55
V. Fungi and Forestry .....	56
A. Nutrient Additions in Forest Systems ..	56
B. Effects of Air Pollution and Fertilization on Fungi .....	58
C. Effects of Invasive Exotic Earthworms on Northern Forests and Forest Fungi ..	59
D. Forestry Practices for Pulpwood and Lumber Production .....	59
E. Effect of Site Preparation on Fungi .....	59
F. Effects of Woody Debris on Ecosystem Processes .....	61
G. Effect of Opening the Canopy and Moisture Fluctuations on Fungi .....	61
VI. Conclusions: Future Role of Fungi in Sustainable Practices .....	61
References .....	62

### I. Introduction

Soils are the most diverse and complex habitat on this planet, which have been formed by biological, chemical and physical processes, all persisting in parallel (Young and Crawford 2004). It has only

been in the last decade that an appreciation of the complexity of soils is being realized in the study of soil fungal communities and the processes they influence. Much of the past research on fungal responses to land management practices has been descriptive, being concerned with the composition and richness of fungal species. Such an approach may inform us on how a particular management practice or disturbance may affect fungal community structure; they contribute little to our understanding of the role of fungi in nutrient cycling and accumulation of organic matter. With the dawn of metagenomics, the opportunities for truly integrating fungal diversity with function should be soon realized.

Studies using trophic structure and food-web approaches to understanding the effects of disturbances of tillage, crop rotation, and silvicultural practices on the soil biota are identifying precisely how important fungi are for maintenance of a sustainable soil system (e.g., Wardle 1995; Wall and Moore 1999; Hedlund et al. 2004; Wardle et al. 2004; Moore et al. 2005); e.g., by integrating fungal responses with tillage practices, particularly informative linkages of fungi to processes associated with the dynamics of soil organic matter (SOM) have been identified (e.g., Hendrix et al. 1986; Hedlund et al. 2004; Moore et al. 2005). Also, investigations have corroborated the importance of fungi in the hierarchical model of soil structure (Tisdall and Oades 1982; Oades 1984) by demonstrating the structural role of hyphae, and the annealing properties of the polysaccharides and glycoproteins that they exude to form and maintain a stable aggregate structure (e.g., Miller and Jastrow 1990; Tisdall 1991; Degens 1997; Rillig and Mumme 2006).

Although there are many studies concerned with management practices in agriculture and forestry, a neglected area of research is the integration of fungal responses with these practices, especially as the responses relate to soil structure, nutrient cycling and organic matter

<sup>1</sup> This paper was written and prepared in part by a U.S. Government employee on official time, and is therefore in the public domain and not subject to copyright.

<sup>2</sup> Biosciences Division, Argonne National Laboratory, 9700 S. Cass Avenue, Argonne, IL 60439, USA

<sup>3</sup> International Institute of Tropical Forestry, USDA - Forest Service, P.O. Box 1377, Luquillo, PR 00773, USA

accumulation in soils (Miller and Jastrow 2000; Ritz and Young 2004; Six et al. 2004, 2006). One reason for this neglect is that plant and fungal responses and disturbance responses associated with land management practices are often studied at different spatial and temporal scales. Even so, from a conceptual viewpoint this research has demonstrated that fungi are a major contributor to system processes and functions that occur at various hierarchical organizational levels, indicating linkages and feedbacks between fungi and system responses (O'Neill et al. 1991; Miller and Jastrow 1994; Langley and Hungate 2003; Johnson et al. 2006; Six et al. 2006). The difficulty lies in our ability to focus questions and to measure responses or processes that function as control points.

Fungi are increasingly recognized as playing a critical role in mediating ecosystem responses to anthropogenic disturbances (Gadd 1993; Cairney and Meharg 1999; Staddon et al. 2002; Rillig 2004; Six et al. 2006). Anthropogenic disturbances include unintentional stresses, as well as stresses resulting from management practices associated with agriculture and silviculture. In addition, the responses of fungal-dominated ecosystems to chronic inputs of atmospheric nitrogen, and shifts in fungal-to-bacterial ratios and concomitant shifts in rates of nutrient cycling in response to introduced invasive earthworm species are major areas of concern (Lawrence et al. 2002; Lilleskov et al. 2002; Frey et al. 2004). Responses of soil microbial communities to alien earthworms in North American forests resemble responses to tillage in agriculture where invasive earthworms appear to be responsible for decreases in fungal-to-bacterial biomass ratios. While these are only a few examples from among many disturbances of anthropogenic origin, they help illustrate the hypothesized mechanisms of response and point to areas where more research is needed.

In this chapter, we will elaborate on the impacts of the various management practices associated with agriculture and forestry as they influence fungal structure and function. In addition, we will address examples of the impact of chronic stress in forest ecosystems from increasing nitrogen inputs and introduced soil fauna. Contributions of fungi to nutrient cycling, organic matter accumulation, and the formation of soil structure will be discussed. Furthermore, we will discuss the hierarchical nature of soils, and how this nature influences a systems response to disturbance. We will

use examples from agroecosystems, soil restorations, and forest systems.

## II. Disturbance as a General Phenomenon

Disturbance is a common feature of most systems, occurring at all levels of ecological organization and at numerous temporal and spatial scales (DeAngelis et al. 1985; Zak 1992). For our discussion, we define "disturbance" as the physical or chemical phenomena that disrupt communities and ecosystems. Disturbances may be either anthropogenic or natural, but it is the biota and the variation in terms of disturbance severity, frequency, and scale that result in different pathways of ecosystem response (rather than being the source of the disturbance; Waide and Lugo 1992). Some disturbances disrupt the physical structure of communities, such as soil tillage, bioturbation from introduced earthworms, clearcut harvesting of trees, and storm damage; other disturbances involve chemical additions, such as acid rain, fertilization, salinization, heavy metals, or biocides. Fungal communities may be directly affected by these physical or chemical disturbances. Fungi also respond to the indirect effects of disturbance, such as the mortality of litter decomposers that results from the increased drying frequency of the forest floor following disturbance to forest canopies, and repression of certain fungal enzyme systems by excess nitrogen (e.g., Sinsabaugh et al. 2002, 2005).

Substrata for decomposition can be viewed as discrete or continuous. Fungi of continuous substrata inhabit a niche that continually receives new resources, whereas fungi of discrete substrata inhabit a given substrate. In his classical study on ecological groupings of soil fungi, Garrett (1951) states that the succession of fungi on a substrate causes a progressive deterioration in the capacity of the substrate to support further growth. He further states that the substrate comes directly to the soil microorganism, i.e., roots grow through soil and die in it; dead leaves fall upon the soil. The former example views a substrate as discrete, whereas the latter views a substrate as being continuous. Accordingly, disturbance can be a perturbation to both substrate and fungus.

For a substrate, disturbance disrupts its delivery rate, its quality and its accessibility, whereas for the fungus disturbance represents the physical or

chemical disruption of the mycelial network. For example, tilling of soil breaks and damages the filamentous network of fungal hyphae inhabiting the soil. In turn, the loss of the hyphal network removes or damages the physical mechanism responsible for stabilizing soil macroaggregate structures. These macroaggregate structures physically protect particulate organic matter within them. The hyphal network disruption increases the potential for disruption of the macroaggregate structure, exposing the once-entrapped particulate organic matter to rendering by the microbial community (Angers et al. 1992; Jastrow and Miller 1998; Six et al. 2004).

### III. Fungi as Control Points in Management Practices

Along with Archaea and bacteria, fungi are the most numerically abundant organisms in the terrestrial ecosystem, and are the primary decomposers of organic residues in soil. Although fungi may be numerically less abundant than bacteria, fungi can account for as much as 78–90% by weight of the soil microbial biomass (Kjoller and Struwe 1982; Lynch 1983). These fungi may be free-living saprotrophs, pathogens, or in mutualistic associations with plant roots. Studies of grassland and agricultural soils indicate that fungal biomass typically outweighs bacterial biomass in undisturbed grassland communities and reduced tillage agroecosystems (Beare et al. 1992; Bardgett et al. 1996; Frey et al. 1999; Bailey et al. 2002), especially when low-quality residues are a primary input source (e.g., Bossuyt et al. 2001). It has been suggested that the size and composition of microbial communities in soils are primarily controlled by the quality, quantity, and distribution of substrata, all of which are influenced by land management practices (Anderson and Domsch 1980; Schnürer et al. 1985; Giller et al. 1997; Six et al. 2006). Differences in fungal-to-bacterial ratios likely reflect changes in microbial composition in response to management effects on the retention of litter and its quality, e.g., fungi typically dominate under reduced or no-till conditions (e.g., Hendrix et al. 1986; Beare et al. 1992; Six et al. 2006). Furthermore, many of the discrepancies reported in the literature may be explained by where bacteria and fungi tend to live in soil; e.g., a primary reason for fungal biomass being larger than bacterial biomass in surface litter is related to the ability of fungal hyphae to traverse

the gap between surface litter and soil more readily than do bacteria (Holland and Coleman 1987; Beare et al. 1992; Frey et al. 2000).

#### A. The Habitats of the Fungus

In an attempt to better conceptualize soil systems into biologically relevant regions on the basis of their spatial and temporal heterogeneity, the concept of “sphere of influence” has been proposed (Coleman et al. 1994; Beare et al. 1995). The five areas of concentrated activity in soils include:

- (1) The detritusphere, composed of the litter, fermentation, and humification layers above the soil surface that have considerable root, mycorrhizal and saprotrophic fungal biomass, and grazing of that biomass by the soil fauna;
- (2) The drilosphere, which is that portion of the soil that is influenced by the activities of earthworms and their casts;
- (3) The porosphere, which is a region of water films occupied by bacteria, protozoa, and nematodes, and of channels between aggregates occupied by microarthropods and the aerial hyphae of fungi;
- (4) The aggregatusphere, the region where the activity of microbes and fauna is concentrated in the voids between microaggregate and even macroaggregates; and
- (5) The rhizosphere, or the zone of soil influenced by roots, associated mycorrhizal hyphae, and their products.

The spheres are formed and maintained by biological influences that operate at different spatial and temporal scales. Moreover, each sphere has distinct properties that regulate interactions among organisms and the biogeochemical properties that they mediate (Coleman et al. 1994; Beare et al. 1995).

#### B. Effects of Disturbance on Fungi

Soils are spatially heterogeneous because of their biologically mediated properties (Beare et al. 1995; Young and Crawford 2004). A driving force for creating a spatially heterogeneous environment is the process of bioturbation (Hole 1982). These biologically mediated disturbances result in creation of a spatially heterogeneous environment, a very different outcome to system processes than is the case for disturbances associated with land management practices, such as tillage or clearcut harvesting that usually result in a loss of spatial heterogeneity.

For example, in the drilosphere, i.e., the region of soil influenced by the activities of earthworms and their casts, the grazing activities of microarthropods and millipedes change the size and distribution of litter on the soil surface, thereby increasing the surface area for fungal colonization and mixing the fragments with other debris (Beare et al. 1995). Macrofauna redistribute the litter in and upon the soil, creating a patchwork of both substrata and refugia for soil fungi, bacteria, and fauna (Lee and Pankhurst 1992). In the porosphere, the physical rearrangement of soil particles by growing roots and earthworm burrowing creates macropores that influence the preferential flow of water and nutrients. In the aggregatusphere and detritusphere, the relationship between soil organisms and bioturbation is considerable. Large amount of faunal feeding occurs on surface litter and associated fungal hyphae, resulting in the accumulation of particulate and fecal aggregates in surface soils. Earthworm casts also accumulate in the surface soils; however, the primary agent of aggregate stabilization is through the deposition of bacterial and fungal residues, and by hyphal entanglement of particles (Chenu 1989; Tisdall 1991; Degens 1997; Guggenberger et al. 1999). The continued inputs of microbial gums and glues, as well as the production of filamentous hyphae, are necessary for the long-term maintenance of the soil macroaggregate structure.

Conventional land management practices usually result in a loss of the spatial heterogeneity of soil. With more sustainable practices, the goal of management is to create a more spatially heterogeneous habitat; e.g., tillage results in the physical disruption of the more transient fungal hyphal network. Furthermore, tillage mixes surface residues vertically within the soil profile while usually doing little direct damage to the aggregate structure (Angers et al. 1992); however, without the physical continuity of the hyphal network, the rewetting of dried aggregates will cause their disruption or slaking. Another phenomenon associated with plowing is a temporary flush in C and N mineralization, which appears to be directly related to the exposure of organic residues to soil biota as a result of slaking (e.g., Elliott and Coleman 1988; Gupta and Germida 1988; Van Veen and Kuikman 1990; Kristensen et al. 2000). Some of the nutrient flush associated with tillage is attributed to disruption of the hyphal network. The amount of nutrient flush depends on (1) the overall amount of organic matter in the soil, (2) the quality of organic residues

sequestered within the aggregated portion of the soil, and (3) the amount of microbial biomass and its activity (Six et al. 2004).

### C. Contributions of Fungi to Nutrient Cycling

In agricultural and forest ecosystems, the primary role of saprotrophic fungi is as decomposers where their activities contribute to C and nutrient cycling (Kjoller and Struwe 1982). SOM dynamics is influenced by saprotrophic fungi through their regulation of the decomposition of plant and microbial residue, the production of polysaccharides, and the stabilization of soil aggregates (Six et al. 2004, 2006). Fungi are especially good decomposers of nutrient-poor plant polymers. Furthermore, saprotrophic fungi possess several growth habits that enable them to grow in nitrogen-deficient environments (Paustian and Schnürer 1987). These habits include:

- (1) Lysis and reassimilation of nitrogen from degenerated hyphae (Levi et al. 1968),
- (2) Directed growth to locally enriched nutrient sites (Levi and Cowling 1969; St. John et al. 1983; Boddy 1993), and
- (3) The translocation of cytoplasm to hyphal apices from mycelium in nitrogen-depleted regions (Cooke and Rayner 1984).

Fungal hyphae may also translocate mineral nitrogen to nitrogen-poor substrata where the absolute amount of nitrogen in a decomposing substrate increases during the early stages of decomposition (Aber and Melillo 1982; Holland and Coleman 1987; Frey et al. 2000). Also, the lateral and upward movement of <sup>15</sup>N-label inorganic nitrogen from mineral soil to decomposing litter has been demonstrated (Frey et al. 2000). Furthermore, the net immobilization of nitrogen in surface litter can be relieved by the application of fungicide (e.g., Beare et al. 1992; Frey et al. 2000). In addition to mineral N transfer to litter by saprotrophic fungi, a fungal-mediated litter-to-soil C transfer has been demonstrated where litter C enters the soil via fungal mycelia and becomes stabilized within the macroaggregate soil structure (Frey et al. 2003). Similar bidirectional translocation of nutrients by rhizomorphs of wood-decay fungi have also been observed (Lindahl et al. 2001).

Fungal biomass represents a significant pool of available nutrients in soils, and its turnover has important consequences for carbon and nutrient cycling (Langley and Hungate 2003; Zhu

and Miller 2003; Six et al. 2006). Soil microbial biomass, e.g., of a temperate grassland, contains 1–2 and 2–5 t ha<sup>-1</sup> for bacteria and fungi, respectively (Killham 1994). The availability of hyphal components and cell products in the form of cell walls, cytoplasm, and extracellular polysaccharides represents a relatively labile organic pool in soils. It has been estimated from a survey of arable soils that the biomass of the microflora in agricultural soils has about 108 and 83 kg ha<sup>-1</sup> of N and P, respectively (Anderson and Domsch 1980). In a forest stand composed of spruce and pine, the amount of ectomycorrhizal biomass found associated with mantles and mycelium is estimated to be 700–900 kg ha<sup>-1</sup> (Wallander et al. 2001). The amount of N in ectomycorrhizal fungal biomass is 187 kg N ha<sup>-1</sup> (Wallander et al. 2004). Hence, fungal mycelia represent a major soil sink for nutrients.

As reported above, the mycorrhizal fungus can represent a considerable portion of the fungal biomass, with reports of 20–30% of the microbial biomass carbon being composed of mycorrhizal fungal biomass in grassland and agroecosystems (Miller et al. 1995; Olsson et al. 1999; Leake et al. 2004). The importance of mycorrhizal fungi in nutrient cycling is that, as symbionts, they provide a direct physical link between primary producers and decomposers. Plant processes influenced by mycorrhizal fungi include host functions such as photosynthesis, nutrient uptake, and water usage. Mycorrhizal fungal influences on host functions can affect nutrient accumulation and alter nutrient ratios in plant tissues. In addition, mycorrhizae can affect plant nutrient uptake by affecting a host's growth rate and by influencing mineral ion uptake (Smith and Read 1997).

The contributions of externally produced arbuscular mycorrhizal (AM) fungal hyphae to nutrient cycling are considerable (Zhu and Miller 2003; Rillig 2004; Leake et al. 2004; Staddon 2005). Although studies of carbon allocation to below-ground structures are few, they indicate that external AM hyphae represent approximately 20% of the labeled extraradical organic carbon pool (Jakobsen and Rosendahl 1990). Annual production of external hyphae in prairie soils is estimated to be 28 m cm<sup>-3</sup> of soil, with a calculated annual hyphal turnover of 26% (Miller et al. 1995). Recently, accelerator mass spectrometry microanalysis of <sup>14</sup>C was used to quantify the turnover rate of extraradical hyphae in plants grown in a controlled environment (Staddon et al. 2003). A surprising outcome

of this study is that the turnover rate of hyphae attached to plant roots averaged only 5–6 days, suggesting that carbon flow from host plants to hyphae in soil might quickly be respiration back to the atmosphere. More importantly, the findings suggest a rapid pathway for atmospheric carbon to enter the soil carbon cycle (however, see Olsson and Johnson 2005). Since AM hyphal cell walls are composed primarily of chitin, a carbohydrate that is rather recalcitrant to decomposition, the rapid turnover of live hyphae would still allow for the accumulation of hyphal residues that could remain within the soil matrix for a considerable period of time (Zhu and Miller 2003).

Turnover rates appear to be faster for extraradical hyphae of mycorrhizal fungi than for intraradical hyphae of roots (Staddon et al. 2003). This may be true for the relatively thin-walled, small-diameter hyphae, but a substantial portion of the AM hyphal network is composed of thick-walled runner or arterial hyphae (Friese and Allen 1991; Read 1992). These runner hyphae are likely to be longer lived and more recalcitrant than the thinner-walled hyphae that are probably directly involved in nutrient acquisition. Moreover, a considerable proportion of the hyphae extracted from soil is either nonviable or highly vacuolated (Schubert et al. 1987; Sylvia 1988; Hamel et al. 1990), suggesting substantial persistence of these hyphae. Because fungal cell walls also contain chitin, they may be a relatively passive source of nitrogen, too (Langley and Hungate 2003).

#### D. Contributions of Fungi to the Hierarchical View of Soil Aggregation

An important component of a successful soil management strategy is the creation and maintenance of the soil aggregate structure (Jastrow and Miller 1998; Miller and Jastrow 2000; Six et al. 2004). The degree to which a soil has been degraded will determine the extent of formation versus maintenance of soil aggregates. The importance of aggregated soils in soil management comes not only from the role of aggregates in controlling soil erosion, but also because aggregates facilitate the maintenance of nutrient cycles. In arable systems, the nutrient reserve of a soil is typically maintained by inputs from crop residues and from fertilizers. In grassland and forest systems, the nutrient reserve is typically maintained by inputs from litter. In temperate

forest systems, litter accumulates mainly from leaf fall, while root inputs are more important in grasslands and the few tropical forests that have been studied. Unless organic inputs are protected within the soil macroaggregates, the accumulation of organic matter and the concomitant buildup of soil nutrients is usually minimal (Elliott and Coleman 1988; Six et al. 2004). Without the physical protection afforded within macroaggregates, organic matter and associated nutrients may be rapidly lost via both mineralization and erosion (Elliott 1986; Jastrow and Miller 1998; Six et al. 2004).

The hierarchical view of soil aggregation is based on the spatial and temporal actions of various organic and mineral binding agents (e.g. Tisdall and Oades 1982; Oades 1984; Jastrow and Miller 1998; Six et al. 2004). These binding agents can be grouped into three classes based on age and the degree of rendering of organic inputs. The first class is composed of microbial and plant-derived polysaccharides that can decompose quite rapidly and are referred to as transient binding agents. The second class, referred to as temporary agents, is composed mainly of living or dead fibrous roots and hyphae, and can normally persist through a growing season or even longer in perennial systems. The third class, referred to as persistent binding agents, is composed of decayed or more rendered materials having humic acid moieties in association with clays and/or amorphous mineral complexes. In the hierarchical model of soil aggregation, fungal hyphae play an essential role as temporary stabilizing agents of soil macroaggregates.

The characteristics that allow fungal hyphae to contribute to the formation and stabilization of soil macroaggregates are related to a wide range of factors including soil characteristics, vegetation type, management practices, and characteristics of the fungus itself (Degens 1997; Miller and Jastrow 2000; Six et al. 2004). For example, because of their physical dimensions, fungal hyphae can grow and ramify through and within pores the size of those found between soil macroaggregates. In addition, hyphae are believed to persist for longer periods of time in soil because of their filamentous nature, coarse branching habit, and rather large diameters. Tisdall and Oades (1979) reported that AM hyphae could stabilize aggregates up to 22 weeks after their host plants had died. By comparison, saprotrophic hyphae, with their more regular branching habit and typically thin walls, appear to have a more transient effect on aggregation, often lasting no more than a few weeks. Another factor enabling AM fun-

gal hyphae to contribute to soil aggregate stabilization is their obligate association with plant roots. Because AM hyphae have direct access to photosynthetic carbon, they represent a conduit for host carbon into the soil, bypassing the decomposition process. Additionally, AM hyphae have been found to produce a very stable glycoprotein, called glomalin, which may act as a longer-term binding agent (Wright and Upadhyaya 1996; Rillig and Mummy 2006).

Many of the early investigations of AM hyphae and aggregation were conducted in sandy soils or sand dune systems (Nicolson 1959; Miller and Jastrow 2000). These studies indicate hyphae are the primary mechanism for binding sand particles into aggregated units (Degens et al. 1996). However, for soils where organomineral binding agents are important to stabilizing soil aggregates, the contributions of AM hyphae go beyond the entanglement mechanism described for sandy soils. Rather, in loamy textured soils of alfisols, mollisols, and vertisols, AM hyphae have been found to contribute to the maintenance of a hierarchically arranged aggregated soil structure composed of both macroaggregate and microaggregate structures (Miller and Jastrow 2000). In oxisols, where oxides are the dominant binding agent of soil particles, aggregates do not appear to be hierarchically organized. This attribute does not preclude the entanglement or enmeshment of primary and secondary particles by saprotrophic and AM hyphae, but the role of the fungus may be secondary to the oxide-binding mechanism. For soils with relatively high clay content, the shrink-swell capacity of the soil may override or minimize the contributions of hyphae to soil aggregation. Hence, when viewed over a wide range of soil types, the contributions of fungi to soil stabilization depend largely on broad textural characteristics, and whether the structure of a soil is hierarchical in construction, among other factors (Table 4.1).

To date, little information exists for ectomycorrhizal fungi contributing to soil aggregate stabilization, although saprotrophic basidiomycetes have been demonstrated to contribute to aggregate stabilization (Caesar-TonThat and Cochran 2000). It has been suggested that ectomycorrhizal hyphae may create a very stable soil structure by producing stronger bonds with clays than do other hyphal types (Emerson et al. 1986). What is known is that ectomycorrhizal hyphae have the ability to extend considerable distances into the soil; exude polysaccharides and organic acids; and enmesh soil

**Table 4.1.** Fungal hyphal and abiotic influences on soil structure for contrasting soil textures (modified from Miller and Jastrow 2000)

Abiotic and biotic properties	Influence in soil type		
	Sand	Loam	Clay
Shrink-swell capacity	Minimal	Important	Maximum
Abiotic aggregation	Minimal	Important	Maximum
Fungal hyphal effects	Important	Important	Minimal

particles between them (Skinner and Bowen 1974; Foster 1981). Furthermore, the release of organic acids by ectomycorrhizal hyphae may be a factor in the dissolution of clays (Leyval and Berthelin 1991).

#### IV. Fungi and Agriculture

Because of the various management practices used in agroecosystems, fungi experience vastly different disturbance regimes. The impacts of these disturbances are expressed directly, and by their effects on food-web structure and function (Hendrix et al. 1986; Paustian et al. 1990; Hedlund et al. 2004; Moore et al. 2005). Under conventional management practices, the activities of the soil biota, including saprotrophic and mycorrhizal fungi, have been largely marginalized by the use of agrochemicals such as fungicides, herbicides, pesticides, and fertilizers, which suppress pests or bypass nutrient cycles. With stronger societal pressures to reduce the use of agrochemicals and fertilizers, however, an ever increasing reliance on processes influenced by soil biota, and especially fungi, will emerge.

Several areas of research with fungi offer promise for reducing the use of agrochemicals and intensive tillage regimes. These areas include the contributions of mycorrhizal fungi in plant production and soil aggregation, the use of fungi as biological agents for control of plant pathogens as well as insect and nematode pests (not discussed in this chapter), and the management of nutrient cycling through better use of saprotrophic and symbiotic processes (Elliott and Coleman 1988; Bethlenfalvay and Linderman 1992; de Leij et al. 1995; Cavagnaro et al. 2006; Six et al. 2006).

##### A. Tillage and Crop Rotation Effects on Fungi

Agricultural practices such as tillage, crop rotation, crop residue retention, and fertilizer use all affect the ecological niches available for occupancy by

the soil biota. Simply put, an agricultural field is basically an experiment in natural selection where those organisms best adapted to those habitats and niches gradually replace those individuals not so well adapted (Rovira 1994). Hence, soils managed by conventional, reduced or no-till practices have distinctly different soil biotic communities. Of these practices, tillage most disrupts the soil fungal community (Table 4.2), resulting in a reduction of the soil's macroaggregate structure (e.g., Gupta and Germida 1988; Beare et al. 1997; Kabir et al. 1997; Wright et al. 1999) and a reduction in fungal biomass (Gupta and Germida 1988; Frey et al. 1999). The loss or reduction of this basic structural component of soil results in the destruction of many of the ecological niches suitable for soil fungi and bacteria (Young and Ritz 2000). The practice of tillage serves many purposes, including preparation of seed bed, mechanical weed control, accelerated mineralization of nutrients from organic matter, and improved water capture and storage in the soil profile (Cook 1992). Unfortunately, tillage also sets the stage for soil erosion and loss of organic matter.

A major control point in conservation tillage is the management of crop residues. In conventional tillage practices, plowing results in the mixing of soil profiles and the burial of crop residues; whereas in no-tillage systems the soil is not plowed and residues are placed on the soil surface as mulch. These differences in soil disturbance and residue placement can influence the composition and activity of the fungal community (Hendrix et al. 1986; Beare et al. 1993; Six et al. 2006). Using a detritus food-web approach, it has been demonstrated that no-tillage systems favored the fungal component of the soil microflora, resulting in the buildup of fungivorous microarthropods, nematodes, and earthworms (e.g., Hendrix et al. 1986; Beare et al. 1992; Wardle et al. 2004). Alternatively, conventional tillage practices favored the bacterial component, resulting in the buildup of a completely different group of organisms by

**Table 4.2.** Fungal and microbial properties of soil aggregate size classes from a native grassland soil and adjacent soil

subjected to cultivation for 69 years (data from Gupta and Germida 1988)

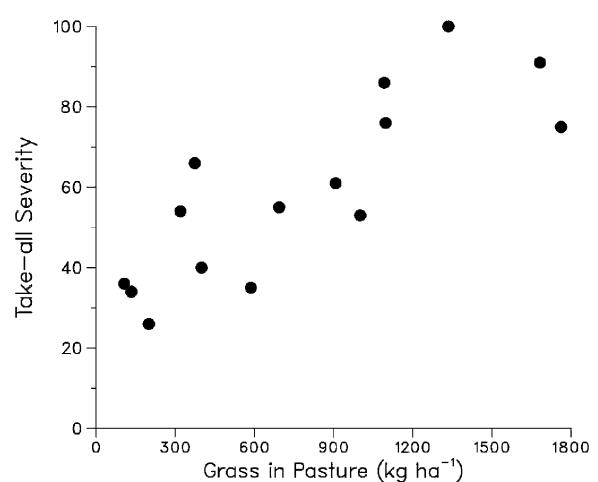
Aggregate size class (mm)	Fungal biomass Length (mg g <sup>-1</sup> )	Biovolume (mm <sup>3</sup> g <sup>-1</sup> )	Microbial biomass C (µg g <sup>-1</sup> )	N (µg g <sup>-1</sup> )
Native soil				
>1.00	874	3.74	1538	139
0.50–1.00	1276	12.89	1862	155
0.25–0.50	2163	10.85	1463	133
0.10–0.25	508	2.06	1161	124
<0.10	511	3.59	1106	124
Cultivated soil				
>1.00	180	0.98	886	86
0.50–1.00	166	1.12	946	92
0.25–0.50	543	3.63	859	90
0.10–0.25	144	1.70	655	82
<0.10	66	0.75	645	80

promoting protozoa, bacterivorous nematodes, and enchytraeids. The direct effects of the various tillage practices on fungi are related to physical disruption of the hyphal network, and to the mixing of surface residues within the soil profile. Crop residues placed upon the soil surface are colonized predominately by saprotrophic and facultatively pathogenic fungi, whereas residues mixed within the soil also have a significant bacterial component (Six et al. 2006).

Tillage and crop rotation practices affect the growth of facultatively pathogenic fungi; e.g., the adoption of conservation tillage practice can increase the incidence of several foliar and root diseases (Bockus and Shroyer 1998). This increase in disease incidence associated with reduced tillage would be expected because the survival of foliar pathogens is often dependent on their association with host residues, and the rate of residue decomposition is slower if the material is retained on the soil surface (Rothrock 1992). Conservation tillage and cropping practices also influence disease incidence by indirect means; e.g., many grass species can be the primary host of the take-all fungus *Gaeumannomyces graminis*, a major pathogen of many cereal crops. Hence, when soils are placed into a pasture rotation to improve soil organic matter content and soil structure, a potential unanticipated effect caused by the density of the pasture's grass content occurs with resumption of cropping, where a relationship exists between prior grass content and the amount of the take-all pathogen *G. graminis* in the subsequent cereal crop (Fig. 4.1). A reduction in tillage intensity also increases the colonization of roots by non-mycorrhizal fungi,

many of them possessing non-filamentous growth habits (Mozafar et al. 2000).

An association has also been demonstrated between the fungal pathogen *Rhizoctonia solani* and conservation tillage. In this case, tillage practices that conserve crop residues on or near the soil's surface create conditions ideal for the growth of *R. solani*. The relationship is caused by the strong competitive growth capability of *Rhizoctonia* on particulate organic matter (Rovira 1986, 1990). The practice of direct drilling allows for optimal conditions for the proliferation of the fungus. A primary mechanism for control of *Rhizoctonia* in conventionally tilled soils is that the plowing breaks up



**Fig. 4.1.** A significant positive association exists between grass content of pastures and the density of the take-all fungus *Gaeumannomyces graminis* var. *tritici* ( $r^2 = 0.71$ ,  $p < 0.001$ ; figure redrawn from MacNish and Nicholas 1987)

the hyphal network in the soil, and either kills portions of the hyphae or breaks the hyphae into less-infective units (Neate 1994). More recent studies indicate a soil's structure mediates the growth of *Rhizoctonia*, where hyphal growth is preferential in gaps found between soil aggregates (Otten et al. 2004). It appears that invasion of the fungus into host populations operates at two scales – at the microscopic scale, the fungus preferentially explores certain pathways, i.e., gaps in the soil that influence the mode of growth, and at a larger scale a critical density of host is necessary, allowing the fungus to switch from noninvasive to invasive growth (Otten and Gilligan 2006).

Tillage and crop rotation practices can also influence the AM fungus (Abbott and Robson 1994; Kabir 2005). Many studies have demonstrated that cropping and tillage practices influence the mycorrhizal fungi (e.g., Dodd et al. 1990; Johnson et al. 1991; Kabir et al. 1998; Jansa et al. 2003). Tillage results in maximum disturbance to the fungal propagula and the extraradical hyphal network, reducing crop nutrient uptake (O'Halloran et al. 1986; Kabir et al. 1998; Jansa et al. 2003). The use of fallow rotations can also result in the expression of phosphorus deficiency, even though there may be no decrease in available phosphorus (Thompson 1987). The mechanism for the deficiency response appears to be a reduction in AM fungal propagula associated with the fallow practice.

Although most crops are dependent upon mycorrhizal fungi, roots of crops belonging to the chenopod and crucifer families usually do not possess mycorrhizal fungi. These two families include crops such as spinach, sugar beet, canola, rapeseed, and mustards. When such crops are used in rotations, they tend to lead to a reduction in mycorrhizal propagula (Gavito and Miller 1998; Thompson 1991). Hence, the yield of mycorrhiza-dependent crops after a nondependent crop may decline, if the needs of that crop for symbiont-supplied nutrients cannot be fulfilled.

Cropping and tillage practices have been demonstrated to result in a loss of specific groups of AM fungal species (Sieverding 1991; Douds et al. 1995; Boddington and Dodd 2000; Jansa et al. 2003). Cropping and tillage can also select for less-effective mycorrhizal fungi. Rotations with sod crops are necessary to eliminate decline symptoms associated with the proliferation of a certain mycorrhizal fungi when grown with tobacco as the host crop (Hendrix et al. 1995). Furthermore, those AM fungi that proliferate

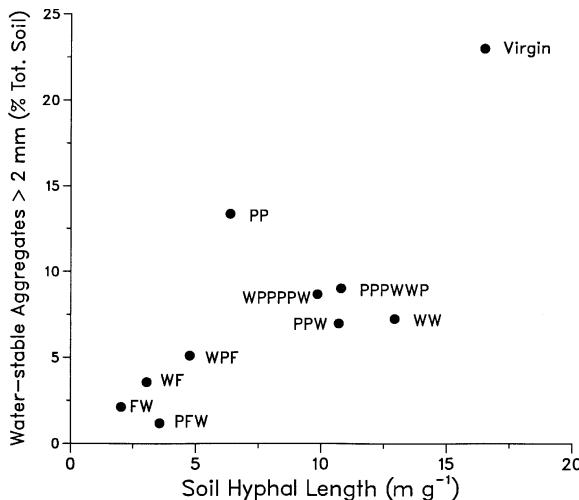
under tillage conditions may be less beneficial, or even detrimental to those crops in which they proliferate (Johnson et al. 1992).

## B. Role of Fungi in Soil Restorations

Depending on the degree of degradation of a soil, an important component of a successful restoration is the reestablishment of a nutrient reserve (Bradshaw et al. 1982). This reserve is initiated by the combination of atmospheric deposition, weathering, and detrital inputs from vegetation, or by additions of organic amendments and fertilizers. Unless these organic inputs are stabilized, accrual of organic matter and microbial biomass, and the concomitant buildup of nutrient reserves in soils are usually minimal. As described above, organic residues are generally protected or stabilized within soils through the formation of soil aggregates. Hence, a major goal of any soil restoration should be to establish conditions that favor formation of stable soil macroaggregates, thereby facilitating an important step in the creation of a nutrient reserve (Miller and Jastrow 1992a, b; Six et al. 2004).

Mycorrhizal fungal dynamics appears to be a good indicator for determining the consequences of different crop rotations on soil stability. Studies in Australia indicated that 50 years of crop rotation decreased the amount of stable macroaggregates, and simultaneously decreased the lengths of roots and AM fungal hyphae in the soil, compared with soils from long-term pasture and natural sites (Tisdall and Oades 1980). The study found that the positive association between the amount of macroaggregates and the length of external mycorrhizal hyphae was related to the type of crop rotation and the frequency of fallow rotations (Fig. 4.2). A similar relationship was also found between macroaggregates and root length. This study indicated that frequent use of fallow in crop rotations can significantly decrease the amount of soil held as macroaggregates. Conversely, the longer a soil has a cover crop, the greater the amount of soil held as macroaggregates. The study also suggested that the loss of stable soil macroaggregates associated with fallow rotations may be caused, at least in part, by a reduction in the mycorrhizal fungus population that is brought on by both tillage and fallow disturbances.

Using a series of prairie reconstructions in the central United States as a means of investigating the aggradative phase of a soil indicates that a stable



**Fig. 4.2.** The relationship between extraradical hyphae and percentage of water-stable aggregates for a soil under different crop rotations (PP=old pasture, PPW=pasture-pasture-wheat, WW=wheat every year, WPF=wheat-pasture-fallow, PFW=pasture-fallow-wheat, WF=wheat-fallow, FW=fallow-wheat, PPPWWP and WPPPWP=2 years wheat and 4 years pasture, respectively; redrawn from Tisdall and Oades 1980)

soil aggregate structure can develop rapidly under prairie and pasture vegetation (Jastrow 1987; Miller and Jastrow 1990; Jastrow et al. 1998). The soils of the study area had been under cultivation for over 150 years; however, within 8 years after being planted to prairie species, the proportions of stable macroaggregates had approached that of a nearby prairie remnant (Jastrow 1987). The rapid recovery of soil aggregates was most likely due to a well-developed microaggregate structure that remained relatively intact during cropping, in combination with a rapid reestablishment of a relatively dense root and hyphal network after the cessation of tillage (Miller and Jastrow 1992b; Jastrow and Miller 1998). Both total root length and the length of roots colonized by AM fungi increased with time from disturbance (Cook et al. 1988). Furthermore, root and soil hyphal lengths were associated with increases in the proportion of soil held as water-stable macroaggregates (Miller and Jastrow 1990; Jastrow et al. 1998).

The conversion of these soils from tillage-based agriculture to prairie also enhanced microbial biomass, with fungal biomass increasing at a proportionally great rate than bacterial biomass (Allison et al. 2005). Moreover, extramatrical AM fungal biomass responded more strongly to the prairie conversion than did saprotrophic fungal

biomass. Finally, in addition to modifications in hyphal length and biomass, the composition of the AM fungal community changed with the cessation of tillage and reconstruction of the prairie (Miller and Jastrow 1992b). Using spore biovolume as a measure of AM species contributions revealed that soils under conventional tillage practices were dominated by *Glomus constrictum* and *G. etunicatum*. However, with the cessation of cultivation, *Gigaspora gigantea* replaced *Glomus* as the dominant fungal group by the fifth growing season. The spore biovolume of *Glomus* species was negatively associated with recovery time since disturbance, external hyphal length, and percent of soil held as macroaggregates; in contrast, *Gigaspora* was positively associated with extraradical hyphal length and macroaggregation. Other investigators have also found *Gigaspora* to be more effective than *Glomus* in producing aggregates (Schreiner and Bethlenfalvay 1995). These trends suggest that AM fungi may differ in their sensitivities to disturbance and in their abilities to produce extraradical hyphae, both of which can influence the development of soil aggregates.

## V. Fungi and Forestry

Forests that are managed for recreation, timber, pulpwood, or other forest products differ widely in tree diversity, their dominant mycorrhizal fungal associates, the seral stage that is being managed, climate, seasonality, and decomposability of litter fall. For example, tree plantations and many native temperate and boreal forests are dominated by one or a few tree species, whereas many tropical forests are speciose; a single hectare of lowland Amazonian forest in Ecuador was reported to have over 470 tree species greater than 10 cm in diameter at breast height (Valencia et al. 1994). Such underlying differences among forest ecosystems will greatly influence how fungi respond to disturbances related to forestry practices.

### A. Nutrient Additions in Forest Systems

Fertilization and atmospheric inputs of nitrogen can influence the rate at which fungal decomposers recycle nutrients from organic matter, but such effects may differ among forest types. Rates of leaf decomposition differ among tree species and forest ecosystems, primarily because of limitations

on the growth and activity of fungal decomposers imposed by differences in concentrations of mineral nutrients in the fallen leaves, climate, and abundance of lignin and other recalcitrant or toxic secondary plant compounds (e.g., Meentemeyer 1978; Swift et al. 1979; Aber and Melillo 1982; Vogt et al. 1986). Low nitrogen concentrations in fallen litter are often more limiting to nutrient cycling in temperate forests, whereas the availability of phosphorus is often more important in lowland tropical forests (Vitousek and Sanford 1986), although phosphorus concentrations can also be important in temperate coniferous systems (Dyer et al. 1990). Many successional forest trees produce highly decomposable leaf litterfall containing higher concentrations of nutrients and labile carbon and lower concentrations of secondary chemicals than do species from later successional stages (Marks 1974; Bazzaz 1979; Grime 1979). Therefore, the effects of episodic fertilization or chronic additions of nitrogen via air pollutants on litter decomposer fungi and the rate of decomposition may depend on the forest species composition, and may be correlated with climate.

In a late successional subtropical wet forest in Puerto Rico, complete fertilization accelerated decomposition of fine roots that had very low nutrient concentrations, thereby lowering the dead root standing stocks after 9 months (Parrotta and Lodge 1991). Fertilization apparently also accelerated decomposition of leaf litter in the same experiment, as indicated by a higher turnover rate. Production of leaf litter was significantly increased by fertilization, while standing stocks of litter did not differ among treatments at low elevation; litterfall was greater, and litter standing stocks lower in fertilized than in control plots at high elevation (Zimmerman et al. 1995).

Despite higher rates of leaf decomposition, certain decomposer fungi were apparently negatively affected by fertilization in the above study. Superficial and interstitial mycelia of decomposer basidiomycetes completely disappeared from the litter layer in fertilized plots, and the diversity and abundance of their fruitifications were also reduced, compared with control plots (D.J. Lodge, S. Cantrell, and O. Oscar, unpublished data), suggesting that the fungal decomposer community may have changed in response to repeated fertilization. Similarly, Heinrich and Wojewoda (1976) found that fertilization of forest plots in Poland significantly decreased the number of fruiting bodies of basidiomycetes that decompose SOM and wood.

Shifts in the composition of the fungal decomposer community in response to fertilization may reflect differential sensitivity among species to salt stress (Castillo Cabello et al. 1994), or reduced competitive advantage of fungi that use hyphal cords and rhizomorphs to translocate nutrients into nutrient-depauperate food bases (Boddy 1993; Lodge 1993). The disappearance of cord-forming basidiomycetes may have a negative impact on the ecosystem in terms of loss of SOM and nutrients. Absence of cords formed by basidiomycetes in the litter layer had previously been found to significantly increase the rate of litter export from steep slopes during storms, and subsequent soil erosion from the exposed surfaces (Lodge and Asbury 1988). Thus, there may be contrasting responses to fertilization in different ecosystem-level processes that are mediated or influenced by fungi. In standard forestry practices, fertilization generally occurs only at planting or post-thinning at 10 to 15 years (Barrett 1962; Oliver 1986), so negative long-term impacts on litter fungi are probably less than in the experiments cited above.

Addition of mineral nutrients that are limiting to the growth of fungal decomposers might reasonably be expected to consistently increase the rate of decomposition, but divergent responses occur in different forest ecosystems (Sinsabaugh et al. 2005). Although higher nitrogen and phosphorus concentrations were correlated with higher initial rates of decomposition of coniferous litter in Sweden, later decomposition and N-mineralization were inhibited (Berg et al. 1982, 1987). As noted in a review by Berg (1986), later decomposition may be slowed by ammonium and amino acid repression of fungal ligninolytic enzymes, and by the complexing of nitrogen with phenolic compounds to form particularly recalcitrant products (Stevenson 1982; Nommik and Vahtras 1982). In nitrogen addition experiments used to simulate atmospheric inputs of N from air pollutants in northern USA hardwood forests, activity of enzymes that are important in delignification (phenol oxidase and peroxidase) decreased along with decomposition rates in low-quality (high lignin) litter, while oxidative enzyme activity (e.g., cellobiohydrolase) and rates of decomposition increased in high-quality (low lignin) litter (Carriero et al. 2000; DeForest et al. 2004; Waldrop et al. 2004; Sinsabaugh et al. 2005). Suppression of white-rot basidiomycete fungi or their enzyme activity has been implicated in altering forest floor biogeochemical processes that influence ecosystem level responses to increases in

nitrogen (Fog 1988; Berg and Matzer 1997; Carriero et al. 2000).

Sinsabaugh et al. (2005) have suggested that not all ecosystem-specific responses in forests to nitrogen additions can be attributed to suppression of white-rot basidiomycete fungi and their enzymes. Although white-rot basidiomycetes may be the organisms primarily responsible for delignification of leaf litter in some ecosystems, some soft-rot fungi, such as *Aspergillus wentii*, degrade lignin faster when nitrogen is more abundant (Fog 1988). Furthermore, Sinsabaugh et al. (2005) noted that not all white-rot fungi exhibit N-dependent expression, and that changes in specific enzyme activities occur in systems that vary widely in microbial community composition and fungal abundance (DeForest et al. 2004; Gallo et al. 2004; Waldrop et al. 2004). Nitrogen effects on litter decomposition may result from an uncoupling of polysaccharide and polyphenol degradation (Sinsabaugh et al. 2002), and hydrolytic enzymes such as cellulases and phosphatases may also play a role (Sinsabaugh et al. 2005). Berg and Ekbohm (1991) hypothesized that nitrogen and phosphorus initially stimulate microbial decomposition and nitrogen mineralization, thereby increasing the rate of release of nitrogen compounds that react with aromatic compounds to form resistant residues. Söderström et al. (1983) found that annual fertilization of Scots pine plantations caused slowing of the long-term decomposition rate, and a 50% reduction in microbial respiration and abundance in humus. The addition of nitrogen through fertilization or air pollutants may therefore be expected to increase the rate of total decomposition in forests where litter contains low concentrations of both nitrogen and polyphenolic compounds, but may slow complete decomposition in forests where litter contains high concentrations of polyphenolics and low concentrations of nitrogen (Berg and Ekbohm 1991). Palm and Sanchez (1990) found that leguminous leaves that naturally contained high concentrations of both nitrogen and polyphenolic compounds decayed more slowly in tropical agroecosystems than predicted from their nitrogen concentrations alone, indicating that the negative interaction between nitrogen and polyphenolics is a widespread phenomenon.

Some studies using experimentally elevated levels of nitrogen input have found decreased fungal-to-bacterial ratios (Waldrop et al. 2004), while others have not (DeForest et al. 2004). The key processes that initiate the chain of events that determine carbon flow and fate in forest ecosys-

tems occur in the litter layer (Zak et al. 2006). Methods such as PLFA that have been used to date to determine changes in overall microbial community structure in forest litter (e.g., ratios of bacteria, fungi and protozoans; DeForest et al. 2004) are not able to detect changes in basidiomycete versus soft-rot fungi, or changes in fungal species dominance that may be more relevant to ecosystem-level responses to elevated nitrogen inputs. Other methods, such as real-time PCR, will be needed to determine how fungal community composition shifts contribute to the radical changes in carbon cycling observed in some forest ecosystems.

## B. Effects of Air Pollution and Fertilization on Fungi

Atmospheric nitrate deposition, primarily from automobile exhaust and power plants, has increased 5- to 20-fold in northeastern USA and parts of Europe (Galloway 1995). In addition to the ecosystem process-level effects on fungal activity of increased nitrogen inputs noted above in Section V.A, there have been significant changes in ectomycorrhizal fungal communities in European forests. The decline in fruiting bodies of ectomycorrhizal fungi in Europe (Derbsch and Schmitt 1987; Termorshuizen and Schaffers 1987; Jakucs 1988; Arnolds 1989; Fellner 1989, 1993; Nauta and Vellinga 1993) has been attributed to direct and indirect effects of air pollution (Arnolds 1991). While ectomycorrhizal fungi have decreased, fruiting by wood-decay fungi has increased in central Europe (Fellner 1993). Application of nitrogen fertilizer was found to cause similar declines in the diversity or abundance (or both) of ectomycorrhizal fungal species (Fiedler and Hunger 1963; Heinrich and Wojewoda 1976; Schlechte 1991). Ectomycorrhizal and ericoid mycorrhizal fungi are thought to confer special advantages to plants in the uptake of nitrogen from organic residues in environments in which the availability or uptake of nitrogen is limited (Read 1991), which may help explain why chronic additions of nitrogen from air pollutants are associated with declines in ectomycorrhizal fungi and their associated host trees.

Some fungal responses to air pollution may be related to soil pH. Bååth et al. (1979) found that active hyphae of soil fungi were decreased by application of artificial acidified rain. Not all ectomycorrhizal fungi have been negatively affected by air pollution. For instance, Fellner (1993) found that

certain *Cortinarius* species in the subgenus *Dermocybe* that are favored by acidic conditions were increasing in highly acidified forests. Schlechte (1991) reported that moderate amelioration of soil pH through application of dolomitic limestone had a stabilizing effect on ectomycorrhizal fungal communities in chronically acidified acid forests. However, addition of some forms of lime has been found to adversely affect the abundance or diversity of ectomycorrhizal fungal fruiting bodies (Hora 1959; Fiedler and Hunger 1963; Heinrich and Wojewoda 1976).

### C. Effects of Invasive Exotic Earthworms on Northern Forests and Forest Fungi

The effects of invasions of exotic earthworms into previously undisturbed North American forests is of considerable concern for the maintenance of forest fungi and the nutrient cycling process they mediate (Bohlen et al. 2004a). Invasions by non-native earthworms, such as *Lumbricus terrestris* and *Dendrobaena octaedra*, have resulted in radical losses of forest floor (Hazelhoff et al. 1981; Alban and Berry 1994; Bohlen et al. 2004b), resulting in altered nutrient availability to trees (Paré and Bernier 1989; Lawrence et al. 2002) through changes in nutrient cycling (Walbridge et al. 1991; Alban and Berry 1994; Scheu and Parkinson 1994a, b; Bohlen et al. 2004a, b). In addition, earthworm invasions in North America have increased susceptibility of forests to erosion through the loss of soil cover (Hazelhoff et al. 1981; Alban and Berry 1994; Bohlen et al. 2004a). Forest floor is a key component that is critical for maintaining the stability of many forest ecosystems, for example, through protection against soil erosion, and for facilitating forest regeneration after disturbance (Bormann and Likens 1979). The presence and extent of negative effects of exotic earthworm invasions vary with the characteristics of forest stands, including forest type, soil characteristics, and history of previous disturbance (Bohlen et al. 2004a). Suarez et al. (2004) found that broadleaf forest plots invaded by *L. terrestris* had more total P at 0–12 cm depth than did reference plots, but more of the P was fixed by Al and Fe hydroxides, and was thus unavailable. Walbridge et al. (1991) also noted the potential for negative effects of mineral soil from worm casts on phosphorus availability in the organic horizon. In forests with typically higher development on strongly weathered soils that have

elevated phosphorus-fixing capacity, most of the available phosphorus, and thus most of the P uptake by trees via mycorrhizal fungi, occurs in the organic forest floor, rather than the mineral soil (Wood et al. 1984; Yanai 1992). Lawrence et al. (2002) found that mycorrhizal fungal colonization rates and percentage of colonized root length declined significantly in organic forest soil horizons invaded by earthworms, indicating they can affect the primary means of nutrient uptake by trees. Paré and Bernier (1989) found that stands of sugar maple in southern Canada had impaired phosphorus nutrition where earthworms were abundant. The activities of exotic earthworms that remove forest floor litter and process it below ground will undoubtedly be detrimental to litter decomposer fungi, especially basidiomycete fungi that form litter mats and are thus critical for preventing erosion losses on steep forest slopes, but this aspect has not been studied.

### D. Forestry Practices for Pulpwood and Lumber Production

In sustainable pulpwood forestry, monospecific stands are often planted and harvested on short rotations, using tree species from an early seral stage such as pines or other conifers, certain eucalyptus, white birch, aspen, and poplars. Such even-aged management practices have been predominant in the United States for pulpwood and lumber since 1950 (Oliver 1986). In such cases, clearcut harvesting sometimes followed by burning of slash is often used to regenerate the species. Though such forestry practices are often viewed as severe by environmentalists, these management practices often mimic natural disturbances to which those early seral stage tree species and their associated fungi are adapted (Oliver 1986). For example, some forest types, especially certain pines, are dependent on fire for their establishment and maintenance of their integrity (Oliver 1986).

### E. Effect of Site Preparation on Fungi

The effects of different intensities of site preparation and silvicultural practices on microbial biomass and microbial nutrient stores before replanting of pine were studied in the southeastern United States by Vitousek and Matson (1984). They compared (1) whole-tree versus stem-only harvest; (2) chopping the harvest debris versus

shearing and piling of all debris and forest floor into wind rows, followed by disk ing of the mineral soil; and (3) application of herbicide. Vitousek and Matson (1984, 1985) found that microbial nutrient immobilization was effective in preventing leaching losses of nitrogen when the forest floor was left intact (chopping) but not when the forest floor was removed (shearing/piling/disking). Losses of nitrate to stream water and groundwater via leaching (Borman and Likens 1979; Vitousek and Melillo 1979; Robertson and Tiedje 1984), and atmospheric losses through denitrification occur in forests that have been cleared, but microorganisms in the forest floor and soil immobilize and conserve most of the nitrogen that is mineralized in response to the disturbance (Marks and Borman 1972; Vitousek and Matson 1984, 1985). Large woody debris (slash) and whole-tree harvesting had little effect on microbial immobilization of nitrogen, at least during short-term study (2 years), but herbicides may have been toxic to microorganisms, thereby increasing leaching losses of nitrogen (Vitousek and Matson 1985).

Although burning of coarse woody debris (slash) is considered an appropriate management practice for regeneration of Douglas fir in the Pacific Northwest (Oliver 1986), broadcast burning following clearcuts (Harvey et al. 1980) and partial cuts (Harvey et al. 1997) has been found to greatly decrease the abundance of active ectomycorrhizae in the forest floor, and levels of ectomycorrhizal fungal inoculum became insufficient for regeneration if replanting was delayed until the next dry season. Broadcast burning has been misapplied to old-growth true fir forests, resulting in mortality of young trees, predisposition of the firs to fungi that decay stems (Oliver 1986), and destruction of the litter fungal community. Highly decayed coarse woody debris is important for maintenance of ectomycorrhizal fungi in the northern Rocky Mountains during the hot dry summers (Harvey et al. 1980, 1997). In addition to affording a moist refugium to mycorrhizae during drought, ectomycorrhizal fungi apparently obtain part of their nitrogen from organic sources including rotting wood (Read et al. 1989). Thus, removal of coarse woody debris from forests by whole-tree harvesting or burning of woody debris following harvest may have negative effects on ectomycorrhizal fungi and tree nutrition, especially in areas with seasonal drought (Harvey et al. 1980, 1997).

Others have also reported that the severity of a particular management activity can affect

the persistence of ectomycorrhizal fungi in the northwestern United States (e.g., Schoenberger and Perry 1982; Perry et al. 1982; Amaranthus and Perry 1987). Moreover, studies of younger clearcut stands indicate that prompt regeneration may be important to secure adequate formation of indigenous fungi, suggesting mycorrhizal nursery stock may be more useful on older and more severely burned sites (Pilz and Perry 1984).

Studies of site preparation effects on mycorrhizal fungi from tropical forest systems are practically nonexistent. The few studies that have been conducted demonstrate disturbances due to site preparation reduce AM fungal propagule levels and species composition, compared to undisturbed forest levels (Alexander et al. 1992; Mason et al. 1992; Wilson et al. 1992). In a Cameroon study, mycorrhizal spore loss appeared to be related to the severity of disturbance, with decreases being greatest in stands with vegetation completely removed. Reduced spore numbers in manually cleared stands were not as severe as in those cleared by mechanical methods, and planting of the stands with *Terminalia* seedlings allowed for their recovery, although mycorrhizal species composition remained altered (Mason et al. 1992). Similar findings were found in a *Terminalia* plantation in the Côte d'Ivoire, where even though site preparation effects on spore numbers were less easily differentiated, manual clearing of vegetation resulted in weaker spore reduction than did mechanical means (Wilson et al. 1992). Also, spore numbers recovered more rapidly in the manually cleared stand; AM fungal richness increased greatly under both methods of clearcutting, although species balance under manual clearcutting was closer to that of the undisturbed forest. These studies suggest selective harvest practices do minimal damage to the mycorrhizal community, in that they support extensive mycelial networks, which for natural systems are the primary source of infection for tree seedlings (Alexander et al. 1992). The reduced levels for mycorrhizal roots, hyphae, and spores in heavily logged systems are due to the mortality of root fragments containing mycorrhizal hyphae. Hence, clearing practices that allow for the continued existence of a mycelial network may be preferable ecologically and silviculturally (Table 4.3).

Stumps of felled trees, roots in the soil, and other woody residues left from forestry operations often provide essential resources for pathogenic fungi and those that cause root or stem decay

**Table 4.3.** The most probable number (MPN propagula per 100 g fresh soil $\pm$ SE) of AM fungi in the top 15 cm of soil for Malaysian forest sites under different logging intensities ( $n = 5$ ; data from Alexander et al. 1992)

Logging intensity	MPN <sup>a</sup>
Undisturbed	307.5 $\pm$ 13.8a
Selectively logged	291.2 $\pm$ 7.8a
Heavily logged	75.3 $\pm$ 2.6b
Heavily logged (eroded)	6.0 $\pm$ 1.5c

<sup>a</sup> Values followed by the same letter are not significantly different ( $p < 0.05$ , Duncan's Multiple Range Test)

(Boyce 1961); e.g., stumps and woody debris have been found to increase mortality from root rots by certain *Armillaria* species in tropical and temperate plantations (Rishbeth 1985; Wargo and Harrington 1991), although raised water tables and shock following thinning and partial cuts may be predisposing factors (Wargo and Harrington 1991). Increased availability of nutrients, especially nitrogen, after logging and fire is known to improve the growth and survival of many root pathogens and to increase the disease incidence (Matson and Boone 1984). In contrast, increased nitrogen mineralization following logging apparently increases the resistance of *Tsuga mertensiana* to laminated root rot caused by *Phellinus weiri* (Matson and Boone 1984). Feeder-root rots caused by *Fusarium* and *Phytophthora* species are favored by soil conditions following fire, as well as by increased soil moisture following harvesting.

#### F. Effects of Woody Debris on Ecosystem Processes

The removal of woody storm debris dramatically affected forest recovery and productivity in Puerto Rico following damage from hurricane Hugo, by altering fungal competition for nutrients (Zimmerman et al. 1995). The results of this study are relevant to management of woody debris (slash) following a partial or selective harvest. Plots from which woody debris was removed and those that received complete fertilization recovered their canopies and productivity more rapidly than the control plots (Zimmerman et al. 1995). The massive deposition of organic matter carbon on the forest floor apparently stimulated microbial production and nutrient immobilization, reducing the availability of nutrients to trees in the control plots, whereas fertilization released the trees from

nutrient competition with decomposers, predominantly fungi (Lodge et al. 1994; Zimmerman et al. 1995). However, bole increment growth was greater only in the fertilization treatment, and removal of woody debris is expected to have a longer-term negative impact on forest productivity through its effects on reduced SOM inputs from decomposing wood and consequent lower phosphorus availability (Sanford et al. 1991). The removal of woody debris following a forest disturbance may therefore have opposing short- and long-term consequences for forest recovery and productivity, so it is the balance of the short- and long-term goals that will determine the appropriate management strategy.

#### G. Effect of Opening the Canopy and Moisture Fluctuations on Fungi

Opening of a forest canopy by storms or from logging causes dramatic environmental changes on the forest floor. Canopy removal reduces transpiration and is often accompanied by higher soil moisture or raised water tables; however, the litter and humus layers may experience more rapid drying as a result of greater exposure to solar irradiation and wind. Moisture is often cited as a primary factor controlling fungal biomass (Parkinson et al. 1968; Söderström 1979; Lodge 1993; Lodge et al. 1994), but temperature can also be important (Flanagan and Van Cleve 1977). Fluctuations in fungal and microbial biomass in response to normal wetting and drying cycles are thought to be important in determining the fate of limiting nutrients and maintaining forest productivity (Lodge et al. 1994). Following a major disturbance, stress tolerance in some fungal species allows them to replace other species that are less tolerant, such as the replacement of *Collybia johnstonii* by marasmoid species of basidiomycete litter decomposers following hurricane Hugo in Puerto Rico (Lodge and Cantrell 1995). Thus, the overall response of fungal biomass to factors such as moisture fluctuations may be more obscure than the responses of individual species, and may depend on the severity and scale of the disturbance.

#### VI. Conclusions: Future Role of Fungi in Sustainable Practices

Unfortunately, few examples exist for fungi and their response to management practices used in agriculture and forestry. Nevertheless, this chap-

ter represents the gleanings from a wide variety of sources ranging from decomposition studies, to detrital food-web investigations, and to research on soil aggregation, all of which suggest an important role for fungi in managed systems. Moreover, obstacles to identifying the contribution of fungi in managed systems have been just as much methodological as conceptual.

Although our ability to quantify the various fungal components (especially their activities) is still limited, major conceptual breakthroughs have occurred on how we view soils. Two important conceptual advances are the hierarchical theory of soil structure (Tisdall and Oades 1982), and the “spheres of influence” view of soil systems (Coleman et al. 1994; Beare et al. 1995). In both of these conceptual views of soils, the contributions of fungi are considerable. Furthermore, the perturbations imposed by management practices have played an important role in the development of these views. Fungal responses to disturbance offer an opportunity to test these two compelling theories of soil structure and function.

A necessary step in developing sustainable management practices in agriculture and forestry will require identifying practices that allow for controlled manipulations of the fungal community. Although our ability to manipulate fungi is rather limited, such manipulations are not impossible. Research is needed to better understand the response of both the saprotrophic hyphae and the mycorrhizal hyphal network to disturbances associated with different management practices. Of crucial importance is the development of management practices that maximize nutrient uptake but are not detrimental to the litter layer and the soil aggregation process. Specifically, research is needed on how disruption of the mycorrhizal hyphal network via tillage and rotation (e.g., fallow or non-mycorrhizal host crops) affects in agriculture, and harvesting and site preparation (e.g., clearcut or slash removal) affect in forestry in terms of the soil aggregate structure. Research also needs to be directed at developing management practices that take advantage of the nutrient pools associated with the fungal hyphae. These kinds of studies would represent important steps in controlling the soil's labile nutrient pools.

**Acknowledgements.** The support of RMM for the preparation of this chapter was by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, Climate Change Research Division, under contract no. W-31-109-ENG-38.

## References

- Abbott LK, Robson AD (1994) The impact of agricultural practices on mycorrhizal fungi. In: Pankhurst CE, Doubt BM, Gupta VVSR, Grace PR (eds) *Soil biota management in sustainable farming systems*. CSIRO Melbourne, Australia, pp 88–95
- Aber JD, Melillo JM (1982) Nitrogen immobilization in decaying hardwood leaf litter as a function of initial nitrogen and lignin content. *Can J Bot* 60:2263–2269
- Alban DH, Berry E (1994) Effects of earthworm invasion on morphology, carbon, and nitrogen of a forest soil. *Appl Soil Ecol* 1:246–149
- Alexander I, Ahmad N, See LS (1992) The role of mycorrhizas in the regeneration of some Malaysian forest trees. *Philos Trans R Soc Lond B* 335:379–388
- Allison VJ, Miller RM, Jastrow JD, Matamala R, Zak DR (2005) Changes in soil microbial community structure in a tallgrass prairie chronosequence. *Soil Sci Soc Am J* 69:1412–1421
- Amaranthus MP, Perry DA (1987) Effect of soil transfer on ectomycorrhiza formation and survival and growth of conifer seedlings in disturbed forest sites. *Can J Forest Res* 17:944–950
- Anderson JPE, Domsch KH (1980) Quantities of plant nutrients in the microbial biomass of selected soils. *Soil Sci* 130:211–216
- Angers DA, Pesant A, Vigneux J (1992) Early cropping-induced changes in soil aggregation, organic matter, and microbial biomass. *Soil Sci Soc Am J* 56:115–119
- Arnolds E (1989) The changing macromycete flora in the Netherlands. *Trans Br Mycol Soc* 90:391–406
- Arnolds E (1991) Decline of ectomycorrhizal fungi in Europe. *Agric Ecosyst Environ* 35:209–244
- Bååth E, Lundgren B, Söderström B (1979) Effects of artificial acid rain on microbial activity and biomass. *Bull Environ Contam Toxicol* 23:737–740
- Bailey VL, Smith JL, Bolton H (2002) Fungal-to-bacterial ratios in soils investigated for enhanced C sequestration. *Soil Biol Biochem* 34:997–1007
- Bardgett RD, Hobbs PJ, Frostegård Å (1996) Changes in soil fungal-to-bacterial biomass ratios following reductions in the intensity of management of an upland grassland. *Biol Fert Soils* 22:261–264
- Barrett JW (1962) *Regional silviculture of the United States*. Ronald Press, New York
- Bazzaz FA (1979) The physiological ecology of plant succession. *Annu Rev Ecol Syst* 10:351–372
- Beare MH, Parmelee RW, Hendrix PF, Cheng W, Coleman DC, Crossley DA (1992) Microbial and faunal interactions and effects on litter nitrogen and decomposition in agroecosystems. *Ecol Monogr* 62:569–591
- Beare MH, Pohlad BR, Wright DH, Coleman DC (1993) Residue placement and fungicide effects on fungal communities in conventional and no-tillage soils. *Soil Sci Soc Am J* 57:392–399
- Beare MH, Coleman DC, Crossley Jr DA, Hendrix PF, Odum EP (1995) A hierarchical approach to evaluating the significance of soil biodiversity to biogeochemical cycling. In: Collins HP, Robertson GP, Klug MJ (eds) *The significance and regulation of soil biodiversity*. Kluwer, Dordrecht, pp 5–22

- Beare MH, Hu S, Coleman DC, Hendrix PF (1997) Influences of mycelial fungi on soil aggregation and organic matter storage in conventional and no-tillage soils. *Appl Soil Ecol* 5:211–219
- Berg B (1986) Nutrient release from litter and humus in coniferous forest soils – a mini review. *Scand J Forest Res* 1:359–369
- Berg B, Ekbohm G (1991) Litter mass-loss rates and decomposition patterns in some needle and leaf litter types. Long-term decomposition in a scots pine forest VII. *Can J Bot* 69:1449–1456
- Berg B, Matzner E (1997) Effect of N deposition on decomposition of plant litter and soil organic matter in forest systems. *Environ Rev* 5:1–25
- Berg B, Wessen B, Ekbohm G (1982) Nitrogen level and lignin decomposition in Scots pine needle litter. *Oikos* 38:291–296
- Berg B, Staaf H, Wessen B (1987) Decomposition and nutrient release in needle litter from nitrogen-fertilized Scots pine (*Pinus sylvestris*) stands. *Scand J Forest Res* 2:399–415
- Bethlenfalvay GL, Linderman RG (eds) (1992) Mycorrhizae in sustainable agriculture. In: ASA Special Publ 54. Agronomy Society of America, Crop Science Society of America, and Soil Science Society of America, Madison, WI, pp 71–99
- Bockus WW, Shroyer JP (1998) The impact of reduced tillage on soilborne plant pathogens. *Annu Rev Phytopathol* 36:485–500
- Boddington CL, Dodd JC (2000) The effects of agricultural practices on the development of indigenous arbuscular mycorrhizal fungi. I. Field studies in an Indonesian ultisol. *Plant Soil* 218:137–144
- Boddy L (1993) Cord-forming fungi: warfare strategies and other ecological aspects. *Mycol Res* 97:641–655
- Bohlen PJ, Groffman PM, Fahey TJ, Fisk MC, Suárez E, Pelletier DM, Fahey RT (2004a) Ecosystem consequences of exotic earthworm invasion of north temperate forests. *Ecosystems* 7:1–12
- Bohlen PJ, Groffman PM, Fahey TJ, Fisk MC (2004b) Influence of earthworm invasion on redistribution and retention of soil carbon and nitrogen in northern temperate forests. *Ecosystems* 7:13–27
- Bormann FH, Likens GE (1979) Pattern and process in a forested ecosystem. Springer, Berlin Heidelberg New York
- Bossuyt H, Denef K, Six J, Frey SD, Merckx R, Paustian K (2001) Influence of microbial populations and residue quality on aggregate stability. *Appl Soil Ecol* 16:195–208
- Boyce JS (1961) Forest pathology, 3rd edn. McGraw Hill, New York
- Bradshaw AD, Marrs RH, Roberts RD, Skeffington RA (1982) The creation of nitrogen cycles in derelict land. *Philos Trans R Soc Lond B* 296:557–561
- Cesar-TonThat TC, Cochran VL (2000) Soil aggregate stabilization by a saprophytic lignin-decomposing basidiomycete fungus. I. Microbial aspects. *Biol Fert Soils* 32:374–380
- Cairney JWG, Meharg AA (1999) Influences of anthropogenic pollution on mycorrhizal fungal communities. *Environ Pollut* 106:169–182
- Carreiro MM, Sinsabaugh RL, Report DA, Parkhurst DF (2000) Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. *Ecology* 81:2359–2365
- Castillo Cabello G, Georis P, Demoulin V (1994) Salinity and temperature effects on growth of three fungi from Laing Island (Papua New Guinea). In: Abstr Vol 5th Int Mycological Congr, Vancouver, BC, 14–21 August 1994, p 31
- Cavagnaro TR, Jackson LE, Six J, Ferris H, Goyal S, Asami D, Scow KM (2006) Arbuscular mycorrhizas, microbial communities, nutrient availability, and soil aggregates in organic tomato production. *Plant Soil* 282:209–225
- Chenu C (1989) Influence of a fungal polysaccharide, scleroglucan on clay microstructure. *Soil Biol Biochem* 21:299–305
- Coleman DC, Hendrix PF, Beare MH, Crossley DA, Hu S, van Vliet PCJ (1994) The impacts of management and biota on nutrient dynamics and soil structure in sub-tropical agroecosystems: impacts on detritus food webs. In: Pankhurst CE, Doube BM, Gupta VVSR, Grace PR (eds) Soil biota management in sustainable farming systems. CSIRO Melbourne, Australia, pp 133–143
- Cook RJ (1992) Wheat root health management and environmental concern. *Can J Plant Pathol* 14:76–85
- Cook BD, Jastrow JD, Miller RM (1988) Root and mycorrhizal endophyte development in a chronosequence of restored tallgrass prairie. *New Phytol* 110:355–362
- Cooke RC, Rayner ADM (1984) Ecology of saprophytic fungi. Longman, London
- DeAngelis DL, Waterhouse JC, Post WM, O'Neill RV (1985) Ecological modeling and disturbance evaluation. *Ecol Model* 29:399–419
- DeForest JL, Zak DR, Pregitzer KS, Burton AJ (2004) Anthropogenic  $\text{NO}_3^-$ -deposition alters microbial community function in northern hardwood forests. *Soil Sci Soc Am J* 68:132–138
- Degens BP (1997) Macro-aggregation of soils by biological bonding and binding mechanisms and factors affecting these: a review. *Aust J Soil Res* 35:431–459
- Degens BP, Sparling GP, Abbott LK (1996) Increasing the length of hyphae in a sandy soil increases the amount of water-stable aggregates. *Appl Soil Ecol* 3:149–159
- de Leij FAAM, Whipps JM, Lynch JM (1995) Traditional methods of detecting and selecting functionally important microorganisms from soil and the rhizosphere. In: Allsopp D, Colwell RR, Hawksworth DL (eds) Microbial diversity and ecosystem function. CABI, Wallingford, pp 321–336
- Derbsch H, Schmitt JA (1987) Atlas der Pilze des Saarlandes, Teil 2. Nachweise, Ökologie, Vorkommen und Beschreibungen. Minister für Umwelt des Saarlandes, Saarbrücken (aus Natur und Landschaft im Saarland 3)
- Dodd JC, Arias I, Kooman I, Hayman DS (1990) The management of populations of vesicular-arbuscular mycorrhizal fungi in acid-infertile soils of a savanna ecosystem. II. The effects of pre-crops on the spore populations of native and introduced VAM-fungi. *Plant Soil* 122:241–247
- Douds DD, Glaves L, Janke RR (1995) Effects of tillage and farming system upon populations and distribution of vesicular-arbuscular mycorrhizal fungi. *Agric Ecosyst Environ* 52:111–118
- Dyer ML, Meentemeyer V, Berg B (1990) Apparent controls of mass loss rate of leaf litter on a regional scale. Litter quality vs. climate. *Scand J Forest Res* 5:311–323

- Elliott ET (1986) Aggregate structure and carbon, nitrogen, and phosphorus in native and cultivated soils. *Soil Sci Soc Am J* 50:627–633
- Elliott ET, Coleman DC (1988) Let the soil work for us. *Ecol Bull* 39:23–32
- Emerson WW, Foster RC, Oades JM (1986) Organo-mineral complexes in relation to soil aggregation and structure. In: Huang PM, Schnitzer M (eds) *Interactions of soil minerals with natural organics and microbes*. Soil Science Society of America, Madison, WI, pp 521–548
- Fellner R (1989) Mycorrhizae-forming fungi as bioindicators of air pollution. *Agric Ecosyst Environ* 28:115–120
- Fellner R (1993) Air pollution and mycorrhizal fungi in Central Europe. In: Pegler DN, Boddy L, Ing B, Kirk PM (eds) *Fungi of Europe: investigation, recording and conservation*, Royal Botanic Gardens, Kew, pp 239–250
- Fiedler HJ, Hunger W (1963) Über den Einfluss einer Kalkdüngung auf Vorkommen, Wachstum und Nährlementgehalt höherer Pilze im Fichtenbestand. *Arch Forstw* 12:936–962
- Flanagan PW, Van Cleve K (1977) Microbial biomass, respiration and nutrient cycling in a black spruce taiga ecosystem. In: Lohm U, Persson T (eds) *Soil organisms as components of ecosystems*. *Ecol Bull* 25:261–273
- Fog K (1988) The effect of added nitrogen on the rate of decomposition of organic matter. *Biol Rev* 63:433–462
- Foster RC (1981) Polysaccharides in soil fabrics. *Science* 214:665–667
- Frey SD, Elliott ET, Paustian K (1999) Bacterial and fungal abundance and biomass in conventional and no-tillage agroecosystems along two climatic gradients. *Soil Biol Biochem* 31:573–585
- Frey SD, Elliott ET, Paustian K, Peterson GA (2000) Fungal translocation as a mechanism for soil nitrogen inputs to surface residue decomposition in a no-tillage agroecosystem. *Soil Biol Biochem* 32:689–698
- Frey SD, Six J, Elliott ET (2003) Reciprocal transfer of carbon and nitrogen by decomposer fungi at the soil-litter interface. *Soil Biol Biochem* 35:1001–1004
- Frey SD, Knorr M, Parrent JL, Simpson RT (2004) Chronic nitrogen enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests. *Forest Ecol Manage* 196:159–171
- Friese CF, Allen MF (1991) The spread of VA mycorrhizal fungal hyphae in the soil: inoculum types and external hyphal architecture. *Mycologia* 83:409–418
- Gadd GM (1993) Interactions of fungi with toxic metals. *New Phytol* 124:25–60
- Gallo ME, Amonette R, Lauber C, Sinsabaugh RL, Zak DR (2004) Short-term changes in oxidative enzyme activity and microbial community structure in nitrogen-amended north temperate forest soils. *Microbial Ecol* 48:218–229
- Galloway JN (1995) Acid deposition: perspectives in time and space. *Water Air Soil Pollut* 85:15–24
- Garrett SD (1951) Ecological groups of soil fungi: a survey of substrate relationships. *New Phytol* 50:149–166
- Gavito ME, Miller MH (1998) Changes in mycorrhizal development in maize induced by crop management practices. *Plant Soil* 198:185–192
- Giller KE, Beare MH, Lavelle P, Izac A-MN, Swift MJ (1997) Agricultural intensification, soil biodiversity and agroecosystem function. *Appl Soil Ecol* 6:3–16
- Grime JP (1979) *Plant strategies and vegetation processes*. Wiley, New York
- Guggenberger G, Frey SD, Six J, Paustian K, Elliott ET (1999) Bacterial and fungal cell-wall residues in conventional and no-tillage agroecosystems. *Soil Sci Soc Am J* 63:1188–1198
- Gupta VVSR, Germida JJ (1988) Distribution of microbial biomass and its activity in different soil aggregate size classes as affected by cultivation. *Soil Biol Biochem* 20:777–786
- Hamel C, Fyles H, and Smith DL (1990) Measurement of development of endomycorrhizal mycelium using three different vital stains. *New Phytol* 115:297–302
- Harvey AE, Jurgensen MF, Larsen MJ (1980) Clearcut harvesting and ectomycorrhizae: survival of activity on residual roots and influence on bordering forest stances in western Montana. *Can J Forest Res* 10:300–303
- Harvey AE, Page-Dumroese DS, Jurgensen MF, Graham RT, Tonn JR (1997) Site preparation alters soil distribution of roots and ectomycorrhizae on outplanted western white pine and Douglas-fir. *Plant Soil* 188:107–117
- Hazelhoff LP, van Hoof P, Imeson AC, Kwaad FJPM (1981) The exposure of forest soil to erosion by earthworms. *Earth Surf Processes Landforms* 6:235–250
- Hedlund K, Griffiths B, Christensen S, Scheu S, Setälä H, Tscharntke T, Verhoef H (2004) Trophic interactions in changing landscapes: responses of soil food webs. *Basic Appl Ecol* 5:495–503
- Heinrich Z, Wojewoda W (1976) The effect of fertilization on a pine forest ecosystem in an industrial region. IV. Macromycetes. *Ecol Polska* 24:319–330
- Hendrix PF, Parmelee RV, Crossley DA Jr, Coleman DC, Odum EP, Groffman P (1986) Detritus food webs in conventional and no-tillage agroecosystems. *Bioscience* 36:374–380
- Hendrix JW, Guo BZ, An Z-Q (1995) Divergence of mycorrhizal fungal communities in crop production systems. In: Collins HP, Robertson GP, Klug MJ (eds) *The significance and regulation of soil biodiversity*. Kluwer, Dordrecht, pp 131–140
- Hole F (1982) Effects of animals in soil. *Geoderma* 25:75–112
- Holland EA, Coleman DC (1987) Litter placement effects on microbial and organic matter dynamics in an agroecosystem. *Ecology* 68:425–433
- Hora FB (1959) Presidential address: Quantitative experiments on toadstool production in woods. *Trans Br Mycol Soc* 42:1–14
- Jakobsen I, Rosendahl L (1990) Carbon flow into soil and external hyphae from roots of mycorrhizal cucumber plants. *New Phytol* 115:77–83
- Jakucs P (1988) Ecological approach to forest decay in Hungary. *Ambio* 17:267–274
- Jansa J, Mozafer A, Kuhn G, Anken T, Ruh R, Sanders IR, Frossard E (2003) Soil tillage affects the community structure of mycorrhizal fungi in maize roots. *Ecol Appl* 13:1164–1176
- Jastrow JD (1987) Changes in soil aggregation associated with tallgrass prairie restoration. *Am J Bot* 74:1656–1664
- Jastrow JD, Miller RM (1998) Soil aggregate stabilization and carbon sequestration: Feedbacks through organomineral associations. In: Lal R, Kimble J, Follett R, Stewart B (eds) *Soil processes and the carbon cycle*. CRC Press, Boca Raton, FL, pp 207–223

- Jastrow JD, Miller RM, Lussenhop J (1998) Contributions of interacting biological mechanisms to soil aggregate stabilization in restored prairie. *Soil Biol Biochem* 30:905–916
- Johnson NC, Pfleger FL, Crookston RK, Simmons SR, Copeland PJ (1991) Vesicular-arbuscular mycorrhizas respond to corn and soybean cropping history. *New Phytol* 117:657–663
- Johnson NC, Copeland PJ, Crookston RK, Pfleger FL (1992) Mycorrhizae: possible explanation for yield decline with continuous corn and soybean. *Agronomy J* 84:387–390
- Johnson NC, Hoeksema JD, Bever J, Chaudhary VB, Gehring C, Klironomos J, Koide R, Miller RM, Moore J, Moutoglis P, Schwartz M, Simard S, Swenson W, Umbanhowar J, Wilson G, Zabinski C (2006) From Lilliput to Brobdingnag: extending models of mycorrhizal function across scales. *Bioscience* 56:889–900
- Kabir Z (2005) Tillage or no-tillage: impact on mycorrhizae. *Can J Plant Sci* 85:23–29
- Kabir Z, O'Halloran IP, Fyles JW, Hamel C (1997) Seasonal changes of arbuscular mycorrhizal fungi as affected by tillage practices and fertilization. I. Hyphal density and mycorrhizal root colonization. *Plant Soil* 192:285–293
- Kabir Z, O'Halloran IP, Fyles JW, Hamel C (1998) Dynamics of the mycorrhizal symbiosis of corn: effect of host physiology, tillage practice and fertilization on spatial distribution of extraradical hyphae in the field. *Agric Ecosyst Environ* 68:151–163
- Killham K (1994) *Soil ecology*. Cambridge University Press, Cambridge
- Kjoller A, Struwe S (1982) Microfungi in ecosystems: fungal occurrence and activity in litter and soil. *Oikos* 39:389–422
- Kristensen HL, McCarty GW, Meisinger JJ (2000) Effects of soil structure disturbance on mineralization of organic soil N. *Soil Sci Soc Am J* 64:371–378
- Langley JA, Hungate BA (2003) Mycorrhizal controls on belowground litter quality. *Ecology* 84:2302–2312
- Lawrence B, Fisk MC, Fahey TJ, Suarez ER (2002) Influence of non-native earthworms on mycorrhizal colonization of sugar maple (*Acer saccharum* Marsh). *New Phytol* 157:145–153
- Leake J, Johnson D, Donnelly D, Muckie G, Boddy L, Read D (2004) Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. *Can J Bot* 82:1016–1045
- Lee KE, Pankhurst CE (1992) Soil organisms and sustainable productivity. *Aust J Soil Res* 30:855–892
- Levi MP, Cowling EB (1969) Role of nitrogen in wood deterioration. VII. Physiological adaptation of wood-destroying and other fungi to substrates deficient in nitrogen. *Phytopathology* 59:460–468
- Levi MP, Merrill W, Cowling EB (1968) Role of nitrogen in wood deterioration. VI. Mycelial fractions and model nitrogen compounds as substrates for growth of *Polyporus versicolor* and other wood-destroying and wood-inhabiting fungi. *Phytopathology* 58:626–634
- Leyval C, Berthelin J (1991) Weathering of mica by roots and rhizospheric microorganisms of pine. *Soil Sci Soc Am J* 55:1009–1016
- Lilleskov EA, Fahey TJ, Horton TR, Lovett GM (2002) Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient in Alaska. *Ecology* 83:104–115
- Lindahl B, Finlay RI, Olsson S (2001) Simultaneous bidirectional translocation of  $^{32}\text{P}$  and  $^{33}\text{P}$  between wood blocks connected by mycelial cords of *Hypholoma fasciculare*. *New Phytol* 150:189–194
- Lodge DJ (1993) Nutrient cycling by fungi in wet tropical forests. In: Isaac S, Frankland JC, Watling R, Whalley AJS (eds) *Aspects of tropical mycology*. British Mycological Society Symposium Series 19. Cambridge University Press, Cambridge, pp 37–57
- Lodge DJ, Asbury CE (1988) Basidiomycetes reduce export of organic matter from forest slopes. *Mycologia* 80:888–890
- Lodge DJ, Cantrell S (1995) Fungal communities in wet tropical forests: variation in time and space. *Can J Bot* 73 suppl:S1391–S1398
- Lodge DJ, McDowell WH, McSwiney CP (1994) The importance of nutrient pulses in tropical forests. *Trends Ecol Evol* 9:384–387
- Lynch JM (1983) *Soil biotechnology*. Blackwell, London
- MacNish GC, Nicholas DA (1987) Some effects of field history on the relationship between grass production in subterranean clover pasture, grain yield and take-all (*Gaeumannomyces graminis* var. *tritici*) in a subsequent crop of wheat at Bannister, Western Australia. *Aust J Agric Res* 38:1011–1018
- Marks PL (1974) The role of cherry (*Prunus pensylvanica* L.) in the maintenance of stability in northern hardwood ecosystems. *Ecol Monogr* 44:73–88
- Marks PL, Borman FH (1972) Revegetation following forest cutting: mechanisms for return to steady state nutrient cycling. *Science* 176:914–915
- Mason PA, Musoko MO, Last FT (1992) Short-term changes in vesicular-arbuscular mycorrhizal spore populations in *Terminalia* plantations in Cameroon. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) *Mycorrhizas in ecosystems*. CABI, Oxon, pp 261–267
- Matson PA, Boone RD (1984) Natural disturbance and nitrogen mineralization: wave-form dieback of mountain hemlock in the Oregon Cascades. *Ecology* 65:1511–1516
- Meentemeyer V (1978) Macroclimate and lignin control of litter decomposition rates. *Ecology* 59:465–472
- Miller RM, Jastrow JD (1990) Hierarchy of root and mycorrhizal fungal interactions with soil aggregation. *Soil Biol Biochem* 22:579–584
- Miller RM, Jastrow JD (1992a) The application of VA mycorrhizae to ecosystem restoration and reclamation. In: Allen MF (ed) *Mycorrhizal functioning*. Chapman and Hall, New York, pp 438–467
- Miller RM, Jastrow JD (1992b) The role of mycorrhizal fungi in soil conservation. In: Bethlenfalvay GL, Linderman RG (eds) *Mycorrhizae in sustainable agriculture*. ASA Special Publ 54. Agronomy Society of America, Crop Science Society of America, and Soil Science Society of America, Madison, WI, pp 29–44
- Miller RM, Jastrow JD (1994) VA mycorrhizae and biogeochemical cycling. In: Pfleger FL, Linderman RG (eds) *Mycorrhizae and plant health*. American Phytopathology Society, St. Paul, MN, pp 189–212

- Miller RM, Jastrow JD (2000) Mycorrhizal fungi influence soil structure. In: Kapulnik Y, Douds D (eds) Arbuscular mycorrhizas: physiology and function. Kluwer, Dordrecht, pp 4–18
- Miller RM, Reinhardt DR, Jastrow JD (1995) External hyphal production of vesicular-arbuscular mycorrhizal fungi in pasture and tallgrass prairie communities. *Oecologia* 103:17–23
- Moore JC, McCann K, de Ruiter PC (2005) Modeling trophic pathways, nutrient cycling, and dynamic stability in soils. *Pedobiology* 49:499–510
- Mozafar A, Anken T, Ruh R, Frossard E (2000) Tillage intensity, mycorrhizal and nonmycorrhizal fungi and nutrient concentrations in maize, wheat, and canola. *Agron J* 92:1117–1124
- Nauta M, Vellinga EC (1993) Distribution and decline of macrofungi in the Netherlands. In: Pegler DN, Boddy L, Ing B, Kirk PM (eds) Fungi of Europe: investigation, recording and conservation. Royal Botanic Gardens, Kew, pp 21–46
- Neate SM (1994) Soil and crop management practices that affect root diseases of crop plants. In: Pankhurst CE, Doube BM, Gupta VVSR, Grace PR (eds) Soil biota management in sustainable farming systems. CSIRO Melbourne, Australia, pp 96–106
- Nicolson TH (1959) Mycorrhiza in the Gramineae. I. Vesicular-arbuscular endophytes, with special reference to the external phase. *Trans Br Mycol Soc* 42:421–438
- Nommik H, Vahtras K (1982) Retention and fixation of ammonium and ammonia in soils. In: Stevenson FJ (ed) Nitrogen in agricultural soils. Agronomy Monographs 22. Agronomy Society of America, Madison, WI, pp 123–171
- Oades JM (1984) Soil organic matter and structural stability: mechanisms and implications for management. *Plant Soil* 76:319–337
- O'Halloran IP, Miller MH, Arnold G (1986) Absorption of P by corn (*Zea mays* L.) as influenced by soil disturbance. *Can J Soil Sci* 66:287–302
- Oliver CD (1986) Silviculture, the next 30 years, the past 30 years. Part I. Overview. *J Forestry* 84:32–42
- Olsson PA, Johnson NC (2005) Tracking carbon from the atmosphere to the rhizosphere. *Ecol Lett* 8:1264–1270
- Olsson PA, Thingstrup I, Jakobsen I, Bååth E (1999) Estimation of the biomass of arbuscular mycorrhizal fungi in a linseed field. *Soil Biol Biochem* 31:1879–1887
- O'Neill EG, O'Neill RV, Norby RJ (1991) Hierarchy theory as a guide to mycorrhizal research on large-scale problems. *Environ Pollution* 73:271–284
- Otten W, Gilligan CA (2006) Soil structure and soil-borne diseases: using epidemiological concepts to scale from fungal spread to plant epidemics. *Eur J Soil Sci* 57:26–37
- Otten W, Harris K, Young IM, Ritz K, Gilligan CA (2004) Preferential spread of the pathogenic fungus *Rhizoctonia solani* through structured soil. *Soil Biol Biochem* 36:203–210
- Palm CA, Sanchez PA (1990) Decomposition and nutrient release patterns of leaves of 3 tropical legumes. *Biotropica* 22:330–338
- Paré D, Bernier B (1989) Changes in phosphorus nutrition of sugar maple along a topographic gradient in the Quebec Appalachians. *Can J Forest Res* 19:135–137
- Parkinson D, Balasooriya I, Winterhalder K (1968) Studies on fungi in a pinewood soil. III. Application of the soil sectioning technique to the study of amounts of fungal mycelium in the soil. *J Soil Sci* 16:258–269
- Parrotta JA, Lodge DJ (1991) Fine root dynamics in a subtropical wet forest following hurricane disturbance in Puerto Rico. *Biotropica* 23:343–347
- Paustian K, Schnürer J (1987) Fungal growth response to carbon and nitrogen limitation: application of a model to laboratory and field data. *Soil Biol Biochem* 19:621–629
- Paustian K, Andrén O, Clarholm M, Hansson AC, Johansson G, Lagerlöf J, Lindberg T, Pettersson R, Sohlenius B (1990) Carbon and nitrogen budgets of four agro-ecosystems with annual and perennial crops, with and without N fertilization. *J Appl Ecol* 27:60–84
- Perry DA, Meyer MM, Egeland D, Rose SL, Pilz D (1982) Seedling growth and mycorrhizal formation in clearcut and adjacent, undisturbed soils in Montana: a greenhouse bioassay. *Forest Ecol Manage* 4:261–273
- Pilz DP, Perry DA (1984) Impact of clearcutting and slash burning on ectomycorrhizal associations of Douglas-fir seedlings. *Can J Forest Res* 14:94–100
- Read DJ (1991) Mycorrhizas in Ecosystems – nature's response to the "law of the minimum". In: Hawksworth DL (ed) Frontiers in mycology. Proc 4th Int Mycological Congr, Regensburg, 1990. CABI, Kew, pp 101–130
- Read DJ (1992) The mycorrhizal mycelium. In: Allen MF (ed) Mycorrhizal functioning. Chapman and Hall, New York, pp 102–133
- Read DJ, Leake JR, Langdale AR (1989) The nitrogen nutrition of mycorrhizal fungi and their host plants. In: Boddy L, Marchant R, Read DJ (eds) Nitrogen, phosphorus and sulphur utilization by fungi. Proc Symp British Mycological Society, Birmingham, 1988. Cambridge University Press, Cambridge, pp 181–204
- Rillig MC (2004) Arbuscular mycorrhizae and terrestrial ecosystem processes. *Ecology Letters* 7:740–754
- Rillig MC, Mumme DL (2006) Mycorrhizas and soil structure. *New Phytol* 171:41–53
- Rishbeth J (1985) *Armillaria*: resources and hosts. In: Moore D, Casselton LA, Wood DA, and others (eds) Developmental biology of higher fungi. Cambridge University Press, Cambridge, pp 87–101
- Ritz K, Young IM (2004) Interactions between soil structure and fungi. *Mycologist* 18:52–59
- Robertson GP, Tiedje JM (1984) Denitrification and nitrous oxide production in successional and old-growth Michigan forests. *Soil Sci Soc Am J* 48:383–389
- Rothrock CS (1992) Tillage systems and plant disease. *Soil Sci* 154:308–315
- Rovira AD (1986) Influence of crop rotation and tillage on *Rhizoctonia* bare patch of wheat. *Phytopath* 76:669–673
- Rovira AD (1990) The impact of soil and crop management practices on soil-borne root diseases and wheat yields. *Soil Use Manage* 6:195–200
- Rovira AD (1994) The effect of farming practices on the soil biota. In: Pankhurst CE, Doube BM, Gupta VVSR, Grace PR (eds) Soil biota management in sustainable farming systems. CSIRO Melbourne, Australia, pp 81–87
- Sanford RL, Parton WJ, Ojima DS, Lodge DJ (1991) Hurricane effects on soil organic matter dynamics and forest production in the Luquillo Experimental Forest, Puerto Rico. *Soil Biol Biochem* 23:343–347

- Puerto Rico: results of simulation modeling. *Biotropica* 23:364–372
- Scheu S, Parkinson D (1994a) Effects of earthworms on nutrient dynamics, carbon turnover and microorganisms in soils from cool temperate forests of the Canadian Rocky Mountains – laboratory studies. *Appl Soil Ecol* 1:113–125
- Scheu S, Parkinson D (1994b) Effects of invasion on an aspen forest (Canada) by *Dendrobaena octaedra* (Lumbricidae) on plant growth. *Ecology* 75:2348–2361
- Schlechte G (1991) Zur Struktur der Basidiomyzeten-flora von unterschiedlich immissionsbelasteten Waldstandorten in Südniedersachsen unter besonderer Berücksichtigung der Mycorrhizabbildung. Jahn u Ernst, Hamburg
- Schnürer J, Clarholm M, Rosswall T (1985) Microbial activity in an agricultural soil with different organic matter contents. *Soil Biol Biochem* 17:6121–618
- Schoenberger MM, Perry DA (1982) The effect of soil disturbance on growth and ectomycorrhizae of Douglas-fir and western hemlock seedlings: a greenhouse bioassay. *Can J Forest Res* 12:343–353
- Schreiner RP, Bethlenfalvay GJ (1995) Mycorrhizal interactions in sustainable agriculture. *Crit Rev Biotechnol* 15:271–285
- Schubert A, Marzachi C, Mazzitelli M, Cravero MC, Bonfante-Fasolo P (1987) Development of total and viable extraradical mycelium in the vesicular-arbuscular mycorrhizal fungus *Glomus clarum* Nicol. and Schenck. *New Phytol* 107:183–190
- Sieverding E (1991) Vesicular-arbuscular mycorrhiza management in tropical agrosystems. Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ), Eschborn
- Sinsabaugh RL, Carreiro MM, Repert DA (2002) Allocation of extracellular enzymatic activity in relation to litter composition, N deposition, and mass loss. *Biogeochemistry* 60:1–24
- Sinsabaugh RL, Gallo ME, Lauber C, Waldrop MP, Zak DR (2005) Extracellular enzyme activities and soil organic matter dynamics for northern hardwood forests receiving simulated nitrogen deposition. *Biogeochemistry* 75:201–215
- Six J, Bossuyt H, Degryze S, Denef K (2004) A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics. *Soil Tillage Res* 79:7–31
- Six J, Frey SD, Thiet RK, Batten KM (2006) Bacterial and fungal contributions to carbon sequestration in agroecosystems. *Soil Sci Soc Am J* 70:555–569
- Skinner MF, Bowen GD (1974) The uptake and translocation of phosphate by mycelial strands of pine mycorrhizas. *Soil Biol Biochem* 6:53–56
- Smith SE, Read DJ (1997) Mycorrhizal symbioses 2nd edn. Academic Press, London
- Söderström B (1979) Some problems in assessing the fluorescein diacetate-active fungal biomass in soil. *Soil Biol Biochem* 11:147–148
- Söderström B, Bååth E, Lundgren B (1983) Decrease in soil microbial activity and biomasses owing to nitrogen amendments. *Can J Microbiol* 29:1500–1506
- Staddon PL (2005) Mycorrhizal fungi and environmental change: the need for a myco-centric approach. *New Phytol* 167:635–637
- Staddon PL, Heinemyer A, Fitter AH (2002) Mycorrhizas and global environmental change: research at different scales. *Plant Soil* 244:253–261
- Staddon PL, Ramsey CB, Ostie N, Ineson P, Fitter AH (2003) Rapid turnover of hyphae of mycorrhizal fungi determined by AMS microanalysis of  $^{14}\text{C}$ . *Science* 300:1138–1140
- Stevenson FJ (1982) Humus chemistry. Genesis, composition, reactions. Wiley, New York
- St. John TV, Coleman DC, Reid CPP (1983) Growth and spatial distribution of nutrient-absorbing organs: selective exploitation of soil heterogeneity. *Plant Soil* 71:487–493
- Suarez ER, Fahey TJ, Groffman PM, Bohlen PJ, Fisk MC (2004) Effects of exotic earthworms on soil phosphorus cycling in two broadleaf temperate forests. *Ecosystems* 7:28–44
- Swift MJ, Heal OW, Anderson JM (1979) Decomposition in terrestrial ecosystems. Studies in Ecology vol 5. Blackwell, Oxford
- Sylvia DM (1988) Activity of external hyphae of vesicular-arbuscular mycorrhizal fungi. *Soil Biol Biochem* 20:39–43
- Termorshuizen AJ, Schaffers AP (1987) Occurrence of carpopores of ectomycorrhizal fungi in selected stands of *Pinus sylvestris* in the Netherlands in relation to stand vitality and air pollution. *Plant Soil* 104:209–217
- Thompson JP (1987) Decline of vesicular-arbuscular mycorrhizae in long fallow disorder of field crops and its expression in phosphorus deficiency of sunflower. *Aust J Agric Res* 38:847–867
- Thompson JP (1991) Improving the mycorrhizal condition of the soil through cultural practices and effects on growth and phosphorus uptake in plants. In: Johansen C, Lee KK, Sahrawat KL (eds) Phosphorus nutrition of grain legumes in the semi-arid tropics. International Crops Research Institute for the Semi-arid Tropics (ICRISAT), Patancheru, India, pp 117–137
- Tisdall JM (1991) Fungal hyphae and structural stability of soil. *Aust J Soil Res* 29:729–743
- Tisdall JM, Oades JM (1979) Stabilization of soil aggregates by the root systems of ryegrass. *Aust J Soil Res* 17:429–441
- Tisdall JM, Oades JM (1980) The effect of crop rotation on aggregation in a red-brown earth. *Aust J Soil Res* 18:423–433
- Tisdall JM, Oades JM (1982) Organic matter and water-stable aggregates in soils. *J Soil Sci* 33:141–163
- Valencia R, Balslev H, Pax Y, Miño G (1994) High tree alpha-diversity in Amazonian Ecuador. *Biodivers Conserv* 3:21–28
- Van Veen JA, Kuikman PJ (1990) Soil structural aspects of decomposition of organic matter by micro-organisms. *Biogeochemistry* 11:213–233
- Vitousek PM, Matson PA (1984) Mechanisms of nitrogen retention in forest ecosystems: a field experiment. *Science* 225:51–52
- Vitousek PM, Matson PA (1985) Disturbance, nitrogen availability, and nitrogen losses in an intensively managed loblolly pine plantation. *Ecology* 66:1360–1376
- Vitousek PM, Melillo JM (1979) Nitrate losses from disturbed ecosystems: patterns and mechanisms. *Forest Sci* 25:605–619

- Vitousek PM, Sanford R (1986) Nutrient cycling in moist tropical forest. *Annu Rev Ecol Syst* 17:137–167
- Vogt KA, Grier CC, Vogt DJ (1986) Production, turnover, and nutrient dynamics of above- and below-ground detritus of world forests. *Adv Ecol Res* 15:303–377
- Waide RB, Lugo AE (1992) A research perspective on disturbance and recovery of a tropical montane forest. In: Goldammer JG (ed) Tropical forests in transition: ecology of natural and anthropogenic disturbance processes. Birkhäuser, Basel, pp 173–190
- Walbridge M, Richardson CJ, Swank WT (1991) Vertical distribution of biological and geochemical phosphorus sub-cycles in two southern Appalachian forest soils. *Biogeochemistry* 13:61–85
- Waldrop MP, Zak DR, Sinsabaugh RL (2004) Microbial community response to nitrogen deposition in northern forest ecosystems. *Soil Biol Biochem* 36:1443–1451
- Wall DH, Moore JC (1999) Interactions underground – soil biodiversity, mutualism, and ecosystem process. *BioScience* 49:109–117
- Wållander H, Nilsson LO, Hagerberg D, Bååth E (2001) Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field. *New Phytol* 151:753–760
- Wållander H, Göransson H, Rosengren U (2004) Production, standing biomass and natural abundance of  $^{15}\text{N}$  and  $^{13}\text{C}$  in ectomycorrhizal mycelia collected at different soil depths in two forest types. *Oecologia* 139:89–97
- Wardle DA (1995) Impacts of disturbance on detritus food webs in agro-ecosystems of contrasting tillage and weed management practices. *Adv Ecol Res* 26:105–185
- Wardle DA, Bardgett R, Klironomos J, Setälä H, van der Putten WH, Wall D (2004) Ecological linkages between aboveground and belowground biota. *Science* 304:1629–1633
- Wargo PM, Harrington TC (1991) Host stress and susceptibility. In: Shaw II GS, Kile GA (eds) *Armillaria* root disease. USDA-Forest Service Agriculture Handbook No. 691, Washington, DC, pp 88–101
- Wilson J, Ingleby K, Mason PA, Ibrahim K, Lawson GJ (1992) Long-term changes in vesicular-arbuscular mycorrhizal spore populations in *Terminalia* plantations in Côte d'Ivoire. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhizas in ecosystems. CABI, Oxon, pp 268–275
- Wood T, Bormann FH, Voigt GK (1984) Phosphorus cycling in a northern hardwood forest: biological and chemical controls. *Science* 27:391–393
- Wright SF, Upadhyaya A (1996) Extraction of an abundant and unusual protein from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi. *Soil Sci* 161:575–586
- Wright SF, Starr JL, Paltineanu IC (1999) Changes in aggregate stability and concentration of glomalin during tillage management transition. *Soil Sci Soc Am J* 63:1825–1829
- Yanai RD (1992) Phosphorus budget of 70-yr-old northern hardwood forest. *Biogeochemistry* 17:1–22
- Young IM, Crawford JW (2004) Interactions and self-organization in the soil-microbe complex. *Science* 304:1634–1637
- Young IM, Ritz K (2000) Tillage, habitat space and function of soil microbes. *Soil Tillage Res* 53:201–213
- Zak JC (1992) Response of soil fungal communities to disturbance. In: Carroll G, Wicklow DT (eds) The fungal community, 2nd edn. Marcel Dekker, New York, pp 403–425
- Zak DR, Holmes WE, Tomlinson MJ, Pregitzer KS, Burton AJ (2006) Microbial cycling of C and N in northern hardwood forests receiving chronic atmospheric  $\text{NO}_3^-$  deposition. *Ecosystems* 9:242–253
- Zhu YG, Miller RM (2003) Carbon cycling by arbuscular mycorrhizal fungi in soil-plant systems. *Trends Plant Sci* 8:407–409
- Zimmerman JK, Pulliam WM, Lodge DJ, Quiñones-Orfila V, Fetcher N, Guzman-Grajales S, Parrotta JA, Asbury CE, Walker LR, Waide RB (1995) Nitrogen immobilization by decomposing woody debris and the recovery of tropical wet forest from hurricane damage. *Oikos* 72:314–322

---

## 5 Fungi and Industrial Pollutants

G.M. GADD<sup>1</sup>

### CONTENTS

I. Introduction .....	69
II. Predicted Effects of Pollutants on Fungal Populations .....	70
III. Fungi and Xenobiotics .....	70
IV. Effects of Acid Rain and Airborne Pollutants on Fungal Populations .....	72
V. Effects of Toxic Metals on Fungi .....	73
A. Effects of Metals on Fungal Populations .....	74
B. Mycorrhizal Responses Towards Toxic Metals .....	75
C. Metal and Metalloid Transformations by Fungi .....	75
1. Metal Mobilization .....	76
2. Metal Immobilization .....	76
3. Organometal(loid)s .....	77
D. Accumulation of Metals and Radionuclides by Macrofungi .....	77
E. Accumulation of Radiocaesium by Macrofungi .....	77
F. Fungi as Bioindicators of Metal and Radionuclide Contamination .....	78
VI. Conclusions .....	79
References .....	79

### I. Introduction

Fungi may be exposed to a wide variety of organic and inorganic pollutants in the environment. Since fungi play a major role in carbon, nitrogen, phosphorus and other biogeochemical cycles (Wainwright 1988a,b; Gadd 2006), the impairment of fungal activity could have important consequences for ecosystem function. It is obviously desirable that more is known about the impact of pollutants on these organisms. Unfortunately, while it is easy to speculate on the likely effects of pollutants on fungi, it is often far more difficult to demonstrate such effects. Studies on pollutant effects on fungal populations are difficult, largely because of the inadequacy

of many of the techniques which are available to study fungi, and the complexity of microbial communities. However, an appreciation of the effects which pollutants can have on fungi can be obtained by a combination of the following measurements: (1) pollutant concentration, composition and distribution (2) pollutant bioavailability (3) pollutant concentrations which cause a toxic or physiological response *in vitro*, (4) effects of the pollutant on fungal population/community size and composition and (5) secondary changes resulting from pollution effects on fungal populations, e.g. impact on leaf litter decomposition. While pollutant concentration and composition may be determined using standard analytical techniques, with varying degrees of difficulty depending on the pollutant and the environmental matrix, analyses of pollutant bioavailability and speciation remain challenging problems.

The effect of pollutants on fungal population/community size and composition is particularly difficult to assess. Many earlier studies used the dilution plate count method to assess changes in fungal community composition. The shortcomings of this technique have been criticised at length and are now well known. To overcome problems relating to the use of plate counts, biomarkers such as phospholipid fatty acid (PLFA) composition, and extraction and analysis of DNA are now routinely used, though no method(s) are exempt from problems. Another problem is that it is unlikely that a meaningful picture of how fungi respond to pollutants in the environment can be gained from determining responses to pollutants added to solid or liquid growth media in laboratory experiments. The effects of toxic metals on soil fungi growing *in vitro*, for example, is markedly influenced by the composition of the medium used: metals are likely to be more toxic to fungi in low-carbon media than in carbon-rich media where the production of large amounts of extracellular polysaccharides and chemical interactions with

<sup>1</sup> Division of Molecular and Environmental Microbiology, College of Life Sciences, University of Dundee, Dundee, DD1 5EH, Scotland, UK

the medium will tend to reduce metal availability. Medium components may also complex metals out of solution, making them unavailable (Gadd and Griffiths 1978). Finally, interactions between different pollutants and their breakdown products may have a major influence on the toxicity of a pollutant in the natural environment.

This chapter will outline some of the main effects of organic and inorganic pollutants on fungi, and will include discussion of effects at the cellular and community levels as well as their applied and environmental significance.

## II. Predicted Effects of Pollutants on Fungal Populations

Environmental pollution might be expected to lead to both toxic (destructive) and enrichment disturbance on fungal populations (Wainwright 1988b). Although toxic disturbance is likely to predominate, instances will occur where both types of disturbance are found together. Toxic disturbance of fungal populations is likely to be particularly damaging to ecosystem function, while the rarer enrichment disturbance may occasionally produce beneficial effects on soil processes.

Toxic disturbance is likely to lead to a reduction in fungal numbers and species diversity, as well as biomass and activity changes which may detrimentally influence fundamentally important processes such as litter decomposition. The resultant degree of toxic disturbance will depend upon both toxicant concentration and its availability to the fungal population, as well as to the susceptibility of the individuals involved. Toxicants may be selective and affect only a few species, or they may have a more generalised effect. Selective inhibition may have less of an impact on overall soil fungal activity than might be imagined, since susceptible species can be replaced by more resistant fungi, some of which may be more active in a given physiological process than the original population. While concentration effects are generally emphasised, it is surprising how often the question of toxicant bioavailability is avoided in studies on the effects of pollutants on microorganisms. In soils, for example, bioavailability of a pollutant will generally depend upon factors such as (1) adsorption to organic and inorganic matter, (2) chemical speciation, (3) microbial transformation and/or degradation and (4) leaching. Another factor of importance in relation to the

effects of toxicants on soil fungi concerns nutrient availability. Fungi are generally thought to be already stressed by the low levels of available carbon present in most soils and other environments (Wainwright 1992). They will grow slowly, if at all, under these conditions, and may be more susceptible to pollutants than when growing under high nutrient conditions.

Fungal populations are unlikely to remain static when confronted with a toxic agent, and resistant populations are likely to develop which will be a major factor in determining population responses to the pollutant. On the other hand, a number of studies have shown that fungi isolated from metal-contaminated soils show less adaptation to toxic metals, such as copper, than might be expected (Yamamoto et al. 1985; Arnebrant et al. 1987). Mowll and Gadd (1985) also found no differences in the sensitivity of *Aureobasidium pullulans* to lead when isolates from contaminated and uncontaminated phylloplanes were compared.

Enrichment disturbances may also be either selective or non-selective. Non-selective enrichment disturbance might theoretically result from the input into the ecosystem of a pollutant which is widely used as a nutrient source. Since such enrichment is rare, most examples of this form of disturbance will be selective. Reduced forms of sulphur are, for example, likely to enrich the soil for S-oxidizing fungi, while phenolics and hydrocarbons may favour species capable of utilizing these compounds.

## III. Fungi and Xenobiotics

Some fungi have remarkable degradative properties, and lignin-degrading white-rot fungi, such as *Phanerochaete chrysosporium*, can degrade several xenobiotics including aromatic hydrocarbons, chlorinated organics, polychlorinated biphenyls, nitrogen-containing aromatics and many other pesticides, dyes and xenobiotics. Such activities are of bioremedial potential where ligninolytic fungi have been used to treat soil contaminated with pentachlorophenol (PCP) and polycyclic aromatic hydrocarbons (PAHs) (Singleton 2001). In general, treatment involves inoculation of the contaminated soil followed by nutrient addition, irrigation, and aeration and maintenance by general land farming procedures. Correct preparation of the fungal inoculum can be crucial: fungi may be grown on

lignocellulosic substrates prior to introduction into the soil (Singleton 2001). Treatment can take weeks to months or longer, depending on the level of contamination and environmental factors. In many cases, xenobiotic-transforming fungi need additional utilizable carbon sources because, although capable of degradation, they cannot utilize these substrates as an energy source for growth. Therefore, inexpensive utilizable lignocellulosic wastes such as corn cobs, straw and sawdust can be used as nutrients for enhanced pollutant degradation (Reddy and Mathew 2001). Wood-rotting and other fungi are also receiving attention for the decolorization of dyes and industrial effluents, various agricultural wastes such as forestry, pulp and paper by-products, sugar cane bagasse, coffee pulp, sugar beet pulp, apple and tomato pulp, and cyanide (Barclay and Knowles 2001; Cohen and Hadar 2001; Knapp et al. 2001).

Polycyclic aromatic hydrocarbons (PAHs) enter the environment via many routes, including fossil-fuel combustion, vehicle exhaust emissions, gas and coal tar manufacture, wood-preservation processes, and waste incineration (Harvey 1997; Pozzoli et al. 2004). Many PAHs are toxic towards microorganisms, plants and animals; PAHs of low molecular weight and high water solubility are the most toxic (Cerniglia and Sutherland 2006). PAHs disappear relatively slowly in the environment through physical, chemical and biological processes, some of which are mediated by bacteria and fungi. PAH recalcitrance of soils and sediments increases with molecular weight, but several other physico-chemical and biological factors can contribute to this, e.g. lack of PAH-degrading microorganisms, nutrient deficiency, low bioavailability, preferential utilization of more easily degradable substrates, and the presence of other toxic pollutants or breakdown products (Cerniglia and Sutherland 2006). Other related factors which affect PAH biodegradation in soil include soil type, pH, temperature, oxygen concentration, irradiation, as well as the solubility, volatility and sorption properties of the PAHs (Huesemann et al. 2003; Lehto et al. 2003; Rasmussen and Olsen 2004). Bioremediation by mixed communities may be enhanced by bacteria which produce degradative enzymes as well as biosurfactants (Straube et al. 1999; Cameotra and Bollag 2003). Aerobic biodegradation of PAHs by soil microorganisms uses monooxygenase, peroxidase and dioxygenase pathways; the first and third of these pathways are

utilized by bacteria while the first and second are found in fungi.

Many fungi can metabolize PAHs (Cerniglia and Sutherland 2001, 2006; Sutherland 2004; Verdin et al. 2004). Since fungi cannot generally use PAHs as the sole carbon and energy source (Cerniglia and Sutherland 2001), they must be supplied with nutrients to allow co-metabolism. A small number of yeasts and filamentous fungi have been reported to use some PAHs, including anthracene, phenanthrene, pyrene and benzo[a]pyrene, as carbon and energy sources (Romero et al. 2002; Lahav et al. 2002; Saraswathy and Hallberg 2002; Veignie et al. 2004). Some fungi co-metabolize PAHs to *trans*-dihydrodiols, phenols, quinones, dihydrodiol epoxides, and tetraols but seldom degrade them completely to CO<sub>2</sub> (Casillas et al. 1996; Cajthaml et al. 2002; da Silva et al. 2003).

The transformation of PAHs by ligninolytic, wood-decaying fungi involves several different enzymes. The enzymes produced by white-rot fungi which are involved in PAH degradation include lignin peroxidase, manganese peroxidase, laccase, cytochrome P450, and epoxide hydrolase (Haemmerli et al. 1986; Bezalel et al. 1996; Cerniglia and Sutherland 2006). Ligninolytic fungi metabolize PAHs via reactions involving reactive oxygen species to phenols and quinones (Pickard et al. 1999; Steffen et al. 2003), and these may be further degraded by ring-fission enzymes (Cerniglia and Sutherland 2006).

Several wood-decaying fungi, e.g. *Bjerkandera*, *Coriolopsis*, *Irpex*, *Phanerochaete*, *Pleurotus* and *Trametes* spp., have been investigated for bioremediation of PAH-contaminated soils (Baldrian et al. 2000; Novotný et al. 2000; Cerniglia and Sutherland 2006). Laboratory trials have demonstrated their ability to degrade complex mixtures of PAHs, such as those in creosote and coal tar, but actual bioremediation of contaminated soils using these fungi has met with varying success (Pointing 2001; Cerniglia and Sutherland 2001; Canet et al. 2001; Hestbjerg et al. 2003). Non-ligninolytic fungi, including *Cunninghamella*, *Mucor*, *Fusarium* and *Penicillium* spp., have also been considered for PAH bioremediation (Colombo et al. 1996; Pinto and Moore 2000; Ravelet et al. 2001; Saraswathy and Hallberg 2002).

Biodegradation may require the presence of mixed bacterial and fungal communities, although less is known about the pathways of PAH degradation by co-cultures (Juhasz and Naidu 2000). The evolution of <sup>14</sup>CO<sub>2</sub> from

<sup>14</sup>C-phenanthrene in soil was enhanced almost two-fold (from 19.5 to 37.7%) when *P. chrysosporium* was added to the indigenous soil microflora (Brodkorb and Legge 1992). Boonchan et al. (2000) combined *Penicillium janthinellum* with either *Stenotrophomonas maltophilia* or an unidentified bacterial consortium. The fungus could partially degrade pyrene and benzo[a]pyrene but could not use either as a carbon source; *S. maltophilia* could use pyrene as a carbon source and co-metabolize benzo[a]pyrene. The fungal-bacterial combinations grew on pyrene, chrysene, benz[a]anthracene, benzo[a]pyrene and dibenz[ah]anthracene, converting 25% of the benzo[a]pyrene to CO<sub>2</sub> in 49 days. The white-rot fungus *P. ostreatus* and the brown-rot fungus *Antrodia vaillantii* enhanced the degradation of fluorene, phenanthrene, pyrene and benz[a]anthracene in artificially contaminated soils (Andersson et al. 2003). Unlike *P. ostreatus*, which inhibited the growth of indigenous soil microorganisms, *A. vaillantii* stimulated soil microbial activity.

Ligninolytic fungi partially oxidize PAHs by reactions involving extracellular free radicals (Majcherczyk and Johannes 2000), making the PAHs more water-soluble so that they are able to serve as substrates for bacterial degradation (Meulenberg et al. 1997). Partial oxidation increases PAH bioavailability at most contaminated sites (Mueller et al. 1996; Meulenberg et al. 1997), and PAH-contaminated soils may contain large populations of PAH-transforming bacteria (Johnsen et al. 2002) and fungi (April et al. 2000; Saraswathy and Hallberg 2002). Combinations of several microorganisms are usually better able to degrade benzo[a]pyrene and other high-molecular-weight PAHs than pure cultures (Kanaly et al. 2000).

#### IV. Effects of Acid Rain and Airborne Pollutants on Fungal Populations

Although acid rain is generally regarded as a long-range pollution phenomenon, high concentrations of mineral acids will pollute ecosystems close to point source emissions (Tabatabai 1985; Francis 1986; Helander et al. 1993). Acid rain effects will also impinge on the availability and effects of other pollutants such as toxic metals, which may accompany atmospheric dispersal and/or be released from soil components as a result of increased acid-

ity (Wainwright et al. 1982). Baath et al. (1984) showed that soil biological activity, as determined by respiration rate, was significantly reduced following treatment with simulated acid rain. Mycelial lengths (FDA active) were also reduced by the treatment, while plate counts showed no response. Fritze (1987), on the other hand, showed that urban air pollution had no effect on the total length of fungal hyphae in the surface horizons of soils supporting Norway spruce (*Picea abies*). Bewley and Parkinson (1985) showed that the contribution which fungi make to the total respiration of a soil was reduced by acid rain. In contrast, Roberts et al. (1980) concluded that the addition of acid rain to forest soils did not affect the normal 9:1 balance of fungal to bacterial respirations. These studies clearly illustrate how difficult it is to generalise about the effects of atmospheric pollutants on soil microorganisms. Among higher fungi, simulated acid rain has been shown to increase the dominance of some ectomycorrhizal fungi, while decreasing species diversity among saprophytic species (Sastad and Jenssen 1993). Shaw et al. (1992) also showed that fumigation with sulphur dioxide or ozone had no effect on mycorrhizal populations. Acid treatments have been shown to impair the decomposition of both deciduous leaves and conifer needles (Baath et al. 1984; Prescott and Parkinson 1985). Small-scale inhibitory effects were common, although stimulatory effects were also observed. Pollution in the form of alkaline dust from iron and steel works was shown to lead to a doubling of the total length of fungal hyphae (Fritze 1991; Fritz and Baath 1993). The addition of lime has been shown to variously decrease soil fungal populations (Nodar et al. 1992) or to have no measurable effect (Persson et al. 1989).

The measurement of leaf litter and cellulose decomposition provides a means of assessing the impact of atmospheric pollutants on soils. However, in the absence of a means of partitioning the relative impact of the toxicants on fungi, bacteria and soil animals, such methods provide a measure of the effects of the pollutants only on the total soil community. Atmospheric pollutants from coking works can, for example, reduce populations of soil microarthropods, a response which retards the rate of litter decomposition in deciduous woodland soils (Killham and Wainwright 1981).

Few examples of the effects of enrichment disturbance by air pollutants on fungal populations can be found in the literature. However, some fungi have been reported to utilize atmospheric pollution deposits from coking works as a nutrient source,

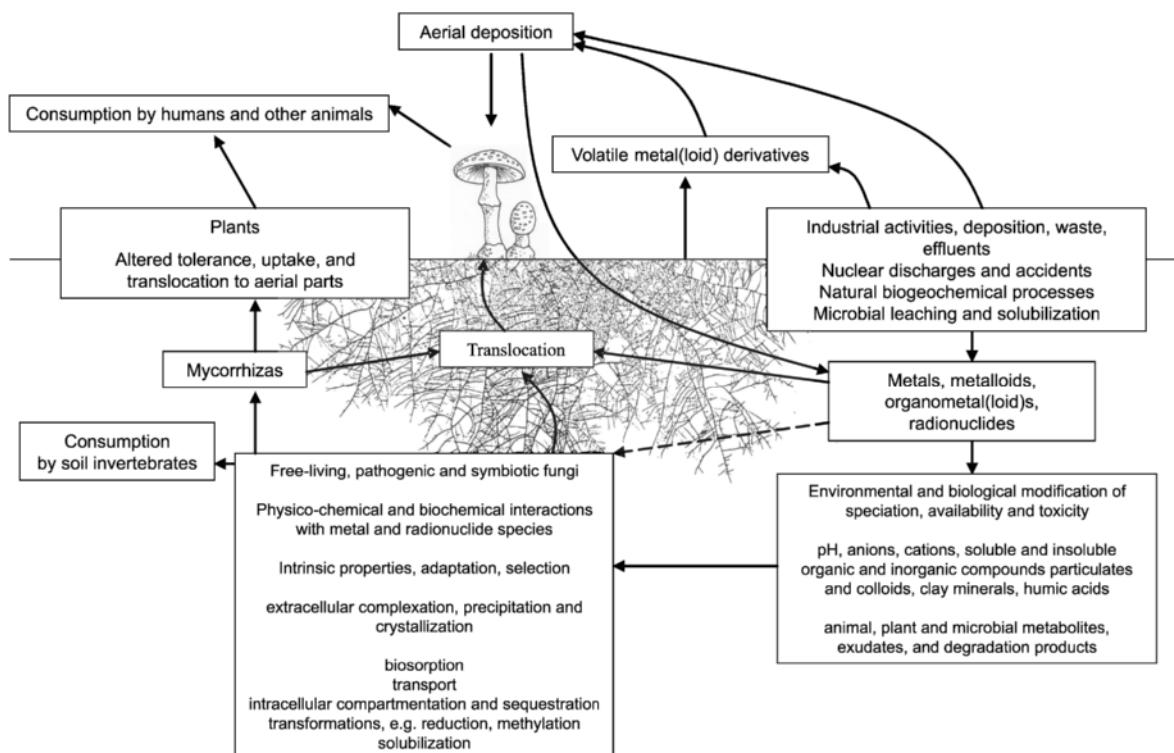
as well as being able to oxidize the reduced sulphur which these particles contain (Killham and Wainwright 1982, 1984).

## V. Effects of Toxic Metals on Fungi

The ability of fungi to survive in the presence of potentially toxic metals depends on a number of biochemical and structural properties, including physiological and/or genetical adaptation, morphological changes and, finally, environmental modification of the metal in relation to speciation, availability and toxicity (Fig. 5.1; Gadd and Griffiths 1978; Gadd et al. 1984; Gadd 1992, 2007).

Terms such as "resistance" and "tolerance" are often used interchangeably in the literature, and may be arbitrarily based on the ability to grow on a certain metal concentration in laboratory me-

dia (Baath 1991; Gadd 1992). "Resistance" is probably more appropriately defined as the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to the metal species concerned; the synthesis of metallothionein and  $\gamma$ -glutamyl peptides in response to Cu and Cd respectively providing perhaps the best examples (Mehra and Winge 1991). Metal tolerance may be defined as the ability of an organism to survive metal toxicity by means of intrinsic properties and/or environmental modification of toxicity (Gadd 1992). Intrinsic properties which can determine survival include possession of impermeable pigmented cell walls and extracellular polysaccharide, and metabolite excretion, especially where this leads to detoxification of the metal species by binding or precipitation (Gadd 1993a). However, such distinctions are often difficult to recognise because of the involvement, in fungal survival in response to metal toxicity, of several direct and in-



**Fig. 5.1.** Diagrammatic representation of the interactions of toxic metals and radionuclides with fungi in the terrestrial environment. The *dotted line* shows direct effects of metal species on fungi; this may sometimes occur and is more likely for metal species, such as Cs<sup>+</sup>, which are highly mobile. Release of metal/radionuclide species from dead and decomposing animal, plant and microbial biomass is

not shown but will be an important part of metal cycling. Fungal roles in metal solubilization from naturally occurring substrates and/or industrial materials are indicated (see Burgstaller and Schinner 1993). For more detailed information regarding physiological and cellular interactions, see Mehra and Winge (1991) and Gadd (1993a); for organometal(loid) transformations, see Gadd (1993b)

direct physicochemical and biological mechanisms (Gadd and White 1985).

Biological mechanisms implicated in fungal survival (as distinct from environmental modification of toxicity) include extracellular precipitation; complexation and crystallization; the transformation of metal species by, e.g. oxidation, reduction, methylation and dealkylation; biosorption to cell walls, pigments and extracellular polysaccharide; decreased transport or impermeability, efflux; intracellular compartmentation; and finally, precipitation and/or sequestration (Gadd and Griffiths 1978; Gadd et al. 1984; Gadd 1990, 1992; Mehra and Winge 1991).

#### A. Effects of Metals on Fungal Populations

A range of fungi from all the major groups may be found in metal-polluted habitats (Gadd 1993a). In general terms, toxic metals may affect fungal populations by reducing abundance and species diversity, and selecting for a resistant/tolerant population (Jordan and Lechevalier 1975; Babich and Stotzky 1985; Arnebrant et al. 1987). However, the effect of toxic metals on microbial abundance in natural habitats varies with the metal species and the organism present, and also depends on a variety of environmental factors, making generalisations difficult (Gadd and Griffiths 1978).

General reductions in fungal "numbers" (as assessed by the dilution plate count in many earlier studies) have often been noted in soils polluted with Cu, Cd, Pb, As and Zn (Bewley and Stotzky 1983; Babich and Stotzky 1985). However, numerical estimates alone may provide little meaningful information unless possible changes in fungal groups and species are considered, and the problems associated with plate counting are in any case well known. Frostegard et al. (1993) analysed the phospholipid fatty acid (PLFA) composition of soil in order to detect changes in the overall composition of the microbial community and provide more reliable information on fungal populations than can be produced using plate counts. Two soils were amended with Cd, Cu, Ni, Pb and Zn and analysed after 6 months. PLFA 18:2 $\omega$ 6 is regarded as an indicator of fungal biomass, and this increased with increasing metal contamination for all metals except Cu, possibly reflecting the well-known mycotoxicity of Cu. However, in forest soils, such an increase in PLFA 18:2 $\omega$ 6 was not observed because of masking by identical PLFAs derived from plant material (Frostegard et al. 1993).

Several studies have shown that microbial population responses to toxic metals are characterised by a shift from bacteria, including streptomycetes, to fungi (Mineev et al. 1999; Kostov and Van Cleemput 2001; Olayinka and Babalola 2001; Chander et al. 2001a, b; Khan and Scullion 2002). However, other studies have shown a higher metal sensitivity of the fungal component of the microbial biomass (Pennanen et al. 1996). What seems clear is that all nutritional groups of fungi (saprotrophs, biotrophs and necrotrophs) can be affected by toxic metals. Ruhling et al. (1984) found that the soil respiration rate, fluorescein diacetate active mycelium (FDA) and mycelial standing crop were all reduced with increasing copper concentration in soils proximal to a brass mill. Nordgren et al. (1983, 1985) also showed that fungal biomass and soil respiration decreased by ~75% along an increasing concentration gradient of metal pollution. A relative decrease in an indicator fatty acid for arbuscular mycorrhizal fungi and an increase for other fungi have been reported for zinc-polluted soil (Kelly et al. 1999). Toxic metals (Cd, Cr, Cu, Ni, Pb and Zn) led to a decrease in the number of arbuscular mycorrhizal fungi and low colonization of plant roots and, as a result, changes in mycorrhizal species diversity (Del Val et al. 1999; Moynahan et al. 2002; Mozafar et al. 2002). Toxic metals also reduce plant root colonization by ectomycorrhizal fungi and ectomycorrhizal species composition (Hartley et al. 1999; Markkola et al. 2002). The most frequent soil saprotrophic microfungi isolated from heavily metal-polluted habitats in Argentina, Czech Republic and Ukraine were reported to be species of *Penicillium*, *Aspergillus*, *Trichoderma*, *Fusarium*, *Rhizopus*, *Mucor* as well as *Paecilomyces lilacinus*, *Nectria invertum*, *Cladosporium cladosporioides*, *Alternaria alternata* and *Phoma fimi* (Kubatova et al. 2002; Massaccesi et al. 2002; Fomina, Manichev, Kadoshnikov and Nakonechnaya, unpublished data). Melanized fungi, such as *Cladosporium* sp., *Alternaria alternata* and *Aureobasidium pullulans*, were often isolated from soil containing high concentrations of copper and mercury (Zhdanova et al. 1986), and can be dominant members of the mycoflora of metal-contaminated phylloplanes (Mowll and Gadd 1985). Dark septate endophytes were found to be dominant fungi among isolates from roots of *Erica herbacea* L. in Pb-, Cd- and Zn-polluted soil (Cevnik et al. 2000).

Metal pollution of plant surfaces is widespread but many filamentous and polymorphic fungi appear to be little affected (Bewley 1979, 1980; Be-

wley and Campbell 1980; Mowll and Gadd 1985). On polluted oak leaves, *Aureobasidium pullulans* and *Cladosporium* species were the most numerous organisms (Bewley 1980). In fact, numbers of *A. pullulans* showed a good positive correlation with lead, whether derived from industrial or vehicular sources, and this fungus was frequently the dominant microorganism present (Bewley and Campbell 1980; Mowll and Gadd 1985).

In conclusion, elevated concentrations of toxic metals can affect both the qualitative and quantitative composition of fungal populations, although it is often extremely difficult to separate their effects from those of other environmental pollutants. It is apparent that certain fungi can exhibit considerable tolerance towards toxic metals and can become dominant microorganisms in some polluted habitats. However, while species diversity may be reduced in certain cases, resistance/tolerance can be exhibited by fungi from both polluted and non-polluted habitats. Physico-chemical properties of the environment, including changes associated with the metal pollution, may also influence metal toxicity and thereby affect species composition (Gadd 1984, 1992, 1993a; Baath 1989).

### B. Mycorrhizal Responses Towards Toxic Metals

Plant symbiotic mycorrhizal fungi can accumulate metals from soil components, and this may have consequences for metal nutrition of the symbiosis as well as increased or decreased toxicity. Since plants growing on metalliferous soils are generally mycorrhizal, an important ecological role for the fungus has frequently been postulated, although such a role, e.g. phytoprotection, is often difficult to confirm (Meharg and Cairney 2000).

Ericaceous plants appear to be entirely dependent on the presence of ericoid mycorrhizas for protection against copper, the fungus preventing metal translocation to plant shoots (Bradley et al. 1981, 1982). Arbuscular mycorrhizas (AM) from metal-contaminated sites are often more metal tolerant to, e.g. Cd and Zn, than other isolates, suggesting a benefit to the plant via increased metal resistance, nutrient uptake, etc.; in some cases, however, AM plants do not necessarily require fungal colonization for survival (Griffioen 1994). It is often postulated that mycorrhizas provide a barrier to the uptake of potentially toxic metals (Wilkins 1991; Hetrick et al. 1994; Wilkinson and Dickin-

son 1995; Leyval et al. 1997; Meharg and Cairney 2000), though this has not been confirmed in every case. Further, in some instances, AM may mediate enhanced accumulation of essential metals which, unless regulated, may lead to phytotoxicity (Killham and Firestone 1983). It is generally concluded that local conditions at metal-contaminated sites may determine the nature of the relationship between the plant and the AM fungus, since detrimental, neutral or beneficial interactions have all been documented (Meharg and Cairney 2000).

For ericaceous mycorrhizas, clear host protection is observed for host plants, e.g. *Calluna* sp., *Erica* sp. and *Vaccinium* sp. growing on polluted and/or naturally metalliferous soils (Bradley et al. 1981, 1982). Further, ericaceous plants are generally found on nutrient-deficient soils, and it is likely the mycorrhiza additionally benefit the plants by enhanced nutrient uptake (Smith and Read 1997). A protective metal-binding effect of ectomycorrhizal fungi (EcM) has been postulated frequently (e.g. Leyval et al. 1997), though other workers point out the lack of clear evidence (Denny and Wilkins 1987; Colpaert and Van Assche 1987, 1993; Dixon and Buschena 1988).

### C. Metal and Metalloid Transformations by Fungi

Fungi can transform metals, metalloids (elements with properties intermediate between those of metals and non-metals: the group includes arsenic, selenium and tellurium) and organometallic compounds by reduction, methylation and dealkylation. These are all processes of environmental importance, since transformation of a metal or metalloid may modify its mobility and toxicity. For example, methylated selenium derivatives are volatile and less toxic than inorganic forms while reduction of metalloid oxyanions, such as selenite or tellurite to amorphous elemental selenium or tellurium respectively, results in immobilization and detoxification (Thompson-Eagle and Frankenberger 1992; Morley et al. 1996). The mechanisms by which fungi (and other microorganisms) effect changes in metal speciation and mobility are important survival determinants as well as components of biogeochemical cycles for metals and many other elements, including carbon, nitrogen, sulphur and phosphorus (Fig. 5.1; Gadd 1999, 2007).

Metals and their compounds interact with fungi in various ways depending on the metal

species, organism and environment, while fungal metabolism also influences metal speciation and mobility. Many metals are essential, e.g. Na, K, Cu, Zn, Co, Ca, Mg, Mn and Fe, but all can exert toxicity when present above certain threshold concentrations (Gadd 1993b). Other metals, e.g. Cs, Al, Cd, Hg and Pb, have no known biological function but all can be accumulated by fungi (Gadd 1993b, 2001a, b). Metal toxicity is greatly affected by environmental conditions and the chemical behaviour of the particular metal species in question. Despite apparent toxicity, many fungi survive, grow and flourish in apparently metal-polluted locations and a variety of mechanisms, both active and incidental, contribute to tolerance. Fungi have many properties which influence metal toxicity, including the production of metal-binding peptides, organic and inorganic precipitation, active transport and intracellular compartmentalization, while fungal cell walls have significant metal-binding abilities (Gadd and Griffiths 1978; Gadd 1993b; Fomina and Gadd 2002).

## 1. Metal Mobilization

Metal mobilization from solids, e.g. rocks, minerals, soil, ash, mine spoil and other substrates, can be achieved by chelation by excreted metabolites and siderophores, and methylation which can result in volatilization. Fungi can solubilize minerals by means of proton efflux, the production of Fe(III)-binding siderophores, and as a result of respiratory carbon dioxide accumulation. In addition, other excreted metabolites with metal-complexing properties, e.g. amino acids, phenolic compounds and organic acids, may also be involved (Fig. 5.1). Fungal-derived carboxylic acids provide a source of protons for solubilization and metal-complexing anions (Gadd 1999, 2001a; Burgstaller and Schinner 1993; Gadd and Sayer 2000). Many metal citrates are highly mobile and not readily degraded. Oxalic acid can act as a leaching agent for those metals which form soluble oxalate complexes, including Al and Fe (Strasser et al. 1994). Solubilization phenomena can have consequences for mobilization of metals from toxic metal-containing minerals, e.g. pyromorphite ( $Pb_5(PO_4)_3Cl$ ), contaminated soil and other solid wastes (Sayer et al. 1999). Fungi can also mobilize metals and attack mineral surfaces by redox processes. Fe(III) and Mn(IV) solubility is increased by reduction to Fe(II) and Mn(II) respectively. Reduction of Hg(II) to volatile elemental

Hg(0) can also be mediated by fungi (Gadd 1993a, b).

The removal of metals from industrial wastes and by-products, contaminated soil, low-grade ores and metal-bearing minerals by fungal "heterotrophic leaching" is relevant to metal recovery and recycling and/or bioremediation of contaminated solid wastes, and perhaps the removal of unwanted phosphates. The ability of fungi, along with bacteria, to transform metalloids has also been utilized successfully in the bioremediation of contaminated land and water. Selenium methylation results in volatilization, a process which has been used to remove selenium from the San Joaquin Valley and Kesterson Reservoir, California, involving evaporation pond management and primary pond operation (Thompson-Eagle et al. 1989; Thompson-Eagle and Frankenberger 1992).

## 2. Metal Immobilization

Fungal biomass provides a metal sink, either by sorption to biomass (cell walls, pigments and extracellular polysaccharides), intracellular accumulation and sequestration, or precipitation of metal compounds onto and/or around hyphae (Fig. 5.1). Fungi are effective biosorbents for a variety of metals including Ni, Zn, Ag, Cu, Cd and Pb (Gadd 1990, 1993b), and this can be an important passive process in both living and dead biomass (Gadd 1990, 1993b; White et al. 1995; Sterflinger 2000). The presence of chitin, and pigments such as melanin, strongly influences the ability of fungi to act as biosorbents (Mowll and Gadd 1985; Manoli et al. 1997; Fomina and Gadd 2002). In a biotechnological context, fungi and their by-products have received considerable attention as biosorbents for metals and radionuclides (Gadd and White 1992; Gadd 2002). However, attempts to commercialize biosorption have been limited, primarily due to competition with commercially produced ion exchange media of high specificity.

Fungi can precipitate a number of inorganic and organic compounds, e.g. oxalates, oxides and carbonates (Grote and Krumbein 1992; Arnott 1995; Gadd 1999; Gharieb and Gadd 1999; Verrecchia 2000), and this can lead to formation of biogenic minerals (mycogenic precipitates). Precipitation, including crystallization, will immobilize metals but also leads to release of nutrients like sulphate and phosphate (Gadd 1999). Fungi can produce a variety of metal oxalates with a variety of different metals and metal-bearing

minerals, e.g. Cd, Co, Cu, Mn, Sr, Zn and Ni (Gadd 1999), which may provide a mechanism whereby fungi can tolerate toxic metal-containing environments. A similar mechanism occurs in lichens growing on copper sulphide-bearing rocks, where precipitation of copper oxalate occurs within the thallus (Purvis 1996).

Many fungi precipitate reduced forms of metals and metalloids in and around fungal hyphae, e.g. Ag(I) can be reduced to elemental silver Ag(0); selenate [Se(VI)] and selenite [Se(IV)] to elemental selenium; tellurite [Te(IV)] to elemental tellurium [Te(0)] (Gharieb et al. 1995, 1999).

### 3. Organometal(loid)s

Organometals (compounds with at least one metal–carbon bond) can be attacked by fungi, with the organic moieties being degraded and the metal compound undergoing changes in speciation. Degradation of organometallic compounds can be carried out by fungi either by direct biotic action (enzymes) or by facilitating abiotic degradation – for instance, by alteration of pH and excretion of metabolites. Organotins, such as tributyltin oxide and tributyltin naphthenate, may be degraded to mono- and dibutyltins by fungal action, inorganic Sn(II) being the final degradation product. Organomercury compounds may be detoxified by conversion to Hg(II) by fungal organomercury lyase, the Hg(II) being subsequently reduced to Hg(0) by mercuric reductase, a system analogous to that found in mercury-resistant bacteria (Gadd 1993a).

### D. Accumulation of Metals and Radionuclides by Macrofungi

Elevated concentrations of toxic metals and radionuclides can occur in the fruiting bodies of higher fungi sampled from polluted environments (cf. Fig. 5.1). This phenomenon is of significance in relation to the use of macrofungi as bioindicators of metal pollution, and because of human toxicity resulting from the consumption of wild fungi. In general, levels of Pb, Cd, Zn and Hg found in macrofungi from urban or industrial areas are higher than from corresponding rural areas, although there are wide differences in uptake abilities between different species and different metals (Tyler 1980; Bressa et al. 1988; Lepsova and Mejstrik 1989). Cadmium is accumulated

to quite high levels in macrofungi, averaging around 5 mg kg dry wt<sup>-1</sup>, although levels of up to 40 mg kg dry wt<sup>-1</sup> have also been recorded (Byrne et al. 1976). *Laccaria amethystina* caps exhibited total As concentrations of 100–200 mg kg dry wt<sup>-1</sup> (Stijve and Porette 1990; Byrne et al. 1991). Accumulation of <sup>110</sup>Ag and <sup>203</sup>Hg was studied in *Agaricus bisporus* and concentration factors (metal concentration in mushroom:metal concentration in substrate) were found to be up to 40 and 3.7 respectively, with the highest Ag and Hg contents recorded being 167 and 75 mg kg dry wt<sup>-1</sup> respectively (Byrne and Tusek-Znidaric 1990). As well as fruiting bodies, rhizomorphs (e.g. of *Armillaria* species) can concentrate metals up to 100 times the level found in soil. Concentrations of Al, Zn, Cu and Pb in rhizomorphs were 3,440, 1,930, 15 and 680 mg kg dry wt<sup>-1</sup> respectively, with the metals primarily located in extracellular portions (Rizzo et al. 1992).

### E. Accumulation of Radiocaesium by Macrofungi

Following the Chernobyl accident in 1986, there were several studies on radiocaesium (mainly <sup>137</sup>Cs) accumulation by fungi. Free-living and mycorrhizal basidiomycetes can accumulate radiocaesium (Haselwandter 1978; Elstner et al. 1987; Byrne 1988; Dighton and Horrill 1988; Haselwandter et al. 1988; Clint et al. 1991; Dighton et al. 1991; Heinrich 1992); these organisms appear to have a slow turnover rate for Cs, and comprise a major pool of radiocaesium in soil (Clint et al. 1991). Mean activities of 25 Ukrainian, six Swedish and ten North American collections were 4660, 9750 and 205 Bq kg dry wt<sup>-1</sup> respectively (Smith et al. 1993). Deviations in the <sup>137</sup>Cs:<sup>134</sup>Cs ratios attributable to Chernobyl have revealed considerable accumulation of pre-Chernobyl Cs in macrofungi, probably as the result of weapons testing (Byrne 1988; Dighton and Horrill 1988). It appeared that about 20% of the <sup>137</sup>Cs in Eastern Europe (Moscow area, Belarus, Ukraine) was of non-Chernobyl origin (Smith et al. 1993). Radiocaesium accumulation in basidiomycetes appears to be species-dependent, with influences exerted by soil properties. Significantly higher activities may be found in mycorrhizal species compared to saprotrophic and parasitic fungi (Smith et al. 1993). Smith et al. (1993) found that many prized edible mycorrhizal fungi may

contain unacceptably high levels of  $^{137}\text{Cs}$ , i.e. levels exceeding 1000 Bq kg dry wt $^{-1}$ . It has also been demonstrated that the fungal component of soil can immobilize the total Chernobyl radiocaesium fallout received in upland grasslands (Dighton et al. 1991), although grazing of fruiting bodies by animals may lead to radiocaesium transfer along the food chain (Bakken and Olsen 1990).

#### F. Fungi as Bioindicators of Metal and Radionuclide Contamination

As mentioned above, higher fungi growing at contaminated sites can show significantly elevated concentrations of metals in their fruiting bodies, and some experiments have demonstrated a correlation between the quantities of metals in a growth substrate and the amounts subsequently found in the fruiting bodies (Wondratschek and Roder 1993). The concept of bioindicators has been usually discussed in terms of reaction indicators and accumulation indicators. Reaction indicators may comprise individual organisms and/or communities which may decline or disappear (sensitive species) or show increases (tolerant species). For accumulation indicators, the indicator organism is analysed for the pollutant. Some organisms, in theory, can therefore serve as both reaction and accumulation indicators.

As described above, alteration of macrofungal communities by metal pollution has frequently been recorded. Ruhling et al. (1984) noted a decline from about 40 species per 100 m $^2$  to about 15 species near the source of metal contamination (smelter emissions), with only *Laccaria lacccata* increasing in frequency at more polluted locations. Other higher fungi which are apparently tolerant of high metal pollution include *Amanita muscaria* and several species of *Boletus*; some *Russula* species, on the other hand, appear metal sensitive (Wondratschek and Roder 1993).

Fungi possess several advantages over plants as metal accumulation indicators. The fruiting bodies may accumulate greater amounts of metals than is the case for plants, while the large area of mycelium ensures contact with and translocation from a large area of soil. Furthermore, fruiting bodies may project above the ground for only a short period, thereby minimising contamination from aerial or wet deposition of metal pollutants. Sporophores are also easily harvested, and amenable to rapid chemical analysis (Mejstrik and Lepsova 1993).

However, it is debatable whether a sufficiently clear relationship exists between indicator species and the metal pollution under consideration. For mercury, wide variations in metal content of fruiting bodies occur in different species sampled at the same site, ranging over as much as three orders of magnitude, with some species showing extremely high Hg accumulation values. Mercury concentrations in fungi generally occur in the range 0.03–21.6 mg kg dry wt $^{-1}$ , although concentrations greater than 100 mg kg dry wt $^{-1}$  have been recorded from polluted sites. Despite this, several macrofungi have been suggested as being suitable bioindicators of mercury pollution (see Mejstrik and Lepsova 1993; Wondratschek and Roder 1993; Table 5.1).

A wide variation in Cd content has also been recorded in macrofungi, with ranges of reported values from <0.1–229 mg kg dry wt $^{-1}$  (Tyler 1980). However, there is frequently a lack of correlation between the fungal Cd content and the Cd content of the soil (Wondratschek and Roder 1993). Compared to other common metal pollutants, lower concentrations of Pb tend to be found in macrofungi, with much of the Pb content being derived from aerial sources. Levels of Pb around 0.4–36 mg kg dry wt $^{-1}$  have been reported in sporophores, with higher levels occurring in urban areas (Tyler 1980). Zinc, an essential metal for fungal growth and metabolism, occurs at high concentrations within fungi, 50–300 mg kg dry wt $^{-1}$  (Tyler 1980), with a few genera apparently showing high affinities for the metal (Table 5.1). Copper may also be found at high levels (20–450 mg kg dry wt $^{-1}$ ) in higher fungi

**Table 5.1.** Higher fungi proposed as bioindicators for metal pollution, based on metal analyses of fruiting bodies (see Mejstrik and Lepsova 1993; Wondratschek and Roder 1993)

Species	Metal(s)
<i>Agaricus arvensis</i>	Hg, Cd
<i>Agaricus campestris</i>	Hg, Cd
<i>Agaricus edulis</i>	Hg, Cd
<i>Agaricus haemorrhoidearius</i>	Hg
<i>Agaricus xanthodermus</i>	Hg
<i>Agaricus</i> sp.	Pb, Zn, Cu
<i>Amanita rubescens</i>	Hg
<i>Amanita strobiliformis</i>	Hg
<i>Coprinus comatus</i>	Hg
<i>Lycoperdon perlatum</i>	Hg
<i>Lycoperdon</i> sp.	Pb, Zn, Cu
<i>Marasmius oreadus</i>	Hg
<i>Mycena pura</i>	Hg, Cd

(Tyler 1980). However, with both Cu and Zn, there is a tendency for metal concentrations in fruiting bodies to be independent of soil concentrations, which reduces their value as bioindicators (Gast et al. 1988).

It is clear that many factors contribute to the wide variations in recorded metal contents of macrofungal fruiting bodies, even in the same species sampled at the same site. Despite numerous studies, most investigations tend to be contradictory and provide little useful information (Wondratschek and Roder 1993). Apart from organism-related factors, environmental factors are of paramount importance in relation to metal accumulation by higher fungi, and include physico-chemical soil properties such as moisture and temperature, all of which influence metal availability as well as the physiological activity of the fungus. It can be concluded, therefore, that a perfect fungal bioindicator does not exist, although macrofungi may be useful in determining the extent of a polluted or unpolluted area.

## VI. Conclusions

It is clear from the above that fungi are of importance in the transformation of both organic and inorganic pollutants in the natural environment. While pollutants may exhibit toxicity, and cause changes in fungal community composition, fungi possess a range of mechanisms which confer resistance or tolerance, many of these resulting in pollutant transformation to less-toxic forms. Such activities are part of natural biogeochemical cycles for major elements such as C, N, O, P and S but also metals, metalloids and radionuclides, as well as having applications in the bioremediation and natural attenuation of polluted habitats. However, pollutant interactions are complex and greatly influenced by environmental factors. While the theoretical response of fungi to pollutants can readily be speculated upon, some effects are difficult to demonstrate and quantify because of the inadequacy of several common techniques used to study fungal populations and their activities. Despite this, newly developed approaches using molecular biology and biomarkers are allowing a better understanding of community structure and responses to environmental factors, including pollutants. Growth media containing low and, therefore, more realistic concentrations of avail-

able carbon should also be used if *in vitro* techniques are employed to help determine the effects of pollutants on fungal growth. However, it is clear that because of the complexity of the fungal growth form, their multiplicity of biological responses and interactions with pollutants, coupled with the complexity of the terrestrial (and other) environments, a wealth of knowledge still awaits discovery.

**Acknowledgements.** The author gratefully acknowledges research support from the Biotechnology and Biological Sciences Research Council, the Natural Environment Research Council, and British Nuclear Fuels plc.

## References

- Andersson BE, Lundstedt S, Tornberg K, Schnürer Y, Öberg LG, Mattiasson B (2003) Incomplete degradation of polycyclic aromatic hydrocarbons in soil inoculated with wood-rotting fungi and their effect on the indigenous soil bacteria. *Environ Toxicol Chem* 22:1238–1243
- April TM, Foght JM, Currah RS (2000) Hydrocarbon-degrading filamentous fungi isolated from flare pit soils in northern and western Canada. *Can J Microbiol* 46:38–49
- Arnebrant K, Baath E, Nordgren A (1987) Copper tolerance of microfungi isolated from polluted and unpolluted forest soil. *Mycologia* 79:890–895
- Arnott HJ (1995) Calcium oxalate in fungi. In: Khan SR (ed) Calcium oxalate in biological systems. CRC Press, Boca Raton, FL, pp 73–111
- Baath E (1989) Effects of heavy metals in soil on microbial processes and populations (a review). *Water Air Soil Pollut* 47:335–379
- Baath E (1991) Tolerance of copper by entomogenous fungi and the use of copper-amended media for isolation of entomogenous fungi from soil. *Mycol Res* 95:1140–1152
- Baath E, Lundgren B, Soderstrom B (1984) Fungal populations in podzolic soil experimentally acidified to simulate acid rain. *Microbial Ecol* 10:197–203
- Babich H, Stotzky G (1985) Heavy metal toxicity to microbe-mediated ecological processes: a review and potential application to regulatory policies. *Environ Res* 36:11–137
- Bakken LR, Olson RA (1990) Accumulation of radiocaesium in fungi. *Can J Microbiol* 36:704–710
- Baldrian P, in der Wiesche C, Gabriel J, Nerud F, Zadraúil F (2000) Influence of cadmium and mercury on activities of ligninolytic enzymes and degradation of polycyclic aromatic hydrocarbons by *Pleurotus ostreatus* in soil. *Appl Environ Microbiol* 66:2471–2478
- Barclay M, Knowles CJ (2001) Cyanide biodegradation by fungi. In: Gadd GM (ed) Fungi in bioremediation. Cambridge University Press, Cambridge, pp 335–358
- Bewley RJF (1979) The effects of zinc, lead and cadmium pollution on the leaf surface microflora of *Lolium perenne* L. *J Gen Microbiol* 110:247–254

- Bewley RJF (1980) Effects of heavy metal pollution of oak leaf microorganisms. *Appl Environ Microbiol* 40:1053–1059
- Bewley RJF, Campbell R (1980) Influence of zinc, lead and cadmium pollutants on microflora of hawthorn leaves. *Microbial Ecol* 6:227–240
- Bewley RJF, Parkinson D (1985) Bacterial and fungal activity in sulphur dioxide polluted soils. *Can J Microbiol* 31:13–15
- Bewley RJF, Stotzky G (1983) Effects of cadmium and zinc on microbial activity in soils: influence of clay minerals. Part 1. Metals added individually. *Sci Total Environ* 31:41–45
- Bezalel L, Hadar Y, Fu PP, Freeman JP, Cerniglia CE (1996) Metabolism of phenanthrene by the white rot fungus *Pleurotus ostreatus*. *Appl Environ Microbiol* 62:2547–2553
- Boonchan S, Britz ML, Stanley GA (2000) Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. *Appl Environ Microbiol* 66:1007–1019
- Bradley R, Burt AJ, Read DJ (1981) Mycorrhizal infection and resistance to heavy metals. *Nature* 292:335–337
- Bradley R, Burt AJ, Read DJ (1982) The biology of mycorrhiza in the Ericaceae. VIII. The role of mycorrhizal infection in heavy metal resistance. *New Phytol* 91:197–209
- Bressa G, Cima L, Costa P (1988) Bioaccumulation of Hg in the mushroom *Pleurotus ostreatus*. *Ecotoxicol Environ Safety* 16:85–89
- Brodkorb TS, Legge RL (1992) Enhanced biodegradation of phenanthrene in oil tar-contaminated soils supplemented with *Phanerochaete chrysosporium*. *Appl Environ Microbiol* 58:3117–3121
- Burgstaller W, Schinner F (1993) Leaching of metals with fungi. *J Biotechnol* 27:91–116
- Byrne AR (1988) Radioactivity in fungi in Slovenia, Yugoslavia, following the Chernobyl accident. *J Environ Radioact* 6:177–183
- Byrne AR, Tusek-Znidaric M (1990) Studies of the uptake and binding of trace metals in fungi. Part I. Accumulation and characterisation of mercury and silver in the cultivated mushroom, *Agaricus bisporus*. *Appl Organometal Chem* 4:43–48
- Byrne AR, Ravnik V, Kosta L (1976) Trace element concentrations in higher fungi. *Sci Total Environ* 6:65–78
- Byrne AR, Tusek-Znidaric M, Puri BK, Irgolic KJ (1991) Studies of the uptake and binding of trace metals in fungi. Part II. Arsenic compounds in *Laccaria amethystina*. *Appl Organometal Chem* 5:25–32
- Cajthaml T, Möder M, Kacer P, Šásek V, Popp P (2002) Study of fungal degradation products of polycyclic aromatic hydrocarbons using gas chromatography with ion trap mass spectrometry detection. *J Chromatogr A* 974:213–222
- Cameotra SS, Bollag J-M (2003) Biosurfactant-enhanced bioremediation of polycyclic aromatic hydrocarbons. *Crit Rev Environ Sci Technol* 30:111–126
- Canet R, Birnstingl JG, Malcolm DG, Lopez-Real JM, Beck AJ (2001) Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by native microflora and combinations of white-rot fungi in a coal-tar contaminated soil. *Bioresource Technol* 76:113–117
- Casillas RP, Crow SA, Heinze TM, Deck J, Cerniglia CE (1996) Initial oxidative and subsequent conjugative metabolites produced during the metabolism of phenanthrene by fungi. *J Indust Microbiol* 16:205–215
- Cerniglia CE, Sutherland JB (2001) Bioremediation of polycyclic aromatic hydrocarbons by ligninolytic and non-ligninolytic fungi. In: Gadd GM (ed) *Fungi in bioremediation*. Cambridge University Press, Cambridge, pp 136–187
- Cerniglia CE, Sutherland JB (2006) Relative roles of bacteria and fungi in polycyclic aromatic hydrocarbon biodegradation and bioremediation of contaminated soils. In: Gadd GM (ed) *Fungi in biogeochemical cycles*. Cambridge University Press, Cambridge, pp 182–211
- Cevnik M, Jurc M, Vodnik D (2000) Filamentous fungi associated with the fine roots of *Erica herbacea* L. from the area influenced by the Zerjav lead smelter (Slovenia). *Phyton – Annales Bot* 40:61–64
- Chander K, Dyckmans J, Hooper H, Joergensen, RG, Raubuch M (2001a) Long-term effects on soil microbial properties of heavy metals from industrial exhaust deposition. *J Plant Nutr Soil Sci* 164:657–663
- Chander K, Dyckmans J, Joergensen RG, Meyer B, Raubuch M (2001b) Different sources of heavy metals and their long-term effects on soil microbial properties. *Biol Fert Soil* 34:241–247
- Clint GM, Dighton J, Rees S (1991) Influx of <sup>137</sup>Cs into hyphae of basidiomycete fungi. *Mycol Res* 95:1047–1051
- Cohen R, Hadar Y (2001) The roles of fungi in agricultural waste conversion. In: Gadd GM (ed) *Fungi in bioremediation*. Cambridge University Press, Cambridge, pp 305–334
- Colombo JC, Cabello M, Aramburre AM (1996) Biodegradation of aliphatic and aromatic hydrocarbons by natural soil microflora and pure cultures of imperfect and lignolitic fungi. *Environ Pollut* 94:355–362
- Colpaert JV, Van Assche JA (1987) Heavy metal tolerance in some ectomycorrhizal fungi. *Funct Ecol* 1:415–421
- Colpaert JV, Van Assche JA (1993) The effect of cadmium on ectomycorrhizal *Pinus sylvestris* L. *New Phytol* 123:325–333
- da Silva M, Cerniglia CE, Pothuluri JV, Canhos VP, Espósito E (2003) Screening filamentous fungi isolated from estuarine sediments for the ability to oxidize polycyclic aromatic hydrocarbons. *World J Microbiol Biotechnol* 19:399–405
- Del Val C, Barea JM, Azcon-Aguilar C (1999) Diversity of arbuscular mycorrhizal fungus populations in heavy-metal-contaminated soils. *Appl Environ Microbiol* 65:718–723
- Denny HJ, Wilkins DA (1987) Zinc tolerance in *Betula* spp. IV. The mechanism of ectomycorrhizal amelioration of zinc toxicity. *New Phytol* 106:545–553
- Dighton J, Horrill Ad (1988) Radiocaesium accumulation in the mycorrhizal fungi *Lactarius rufus* and *Inocybe longicystis* in upland Britain following the Chernobyl accident. *Trans Br Mycol Soc* 91:335–337
- Dighton J, Clint GM, Poskitt J (1991) Uptake and accumulation of <sup>137</sup>Cs by upland grassland soil fungi: a potential pool of Cs immobilization. *Mycol Res* 95:1052–1056
- Dixon RK, Buschena CA (1988) Response of ectomycorrhizal *Pinus banksia* and *Picea glauca* to heavy metals in soil. *Plant Soil* 105:265–271

- Elstener EF, Fink R, Holl W, Lengfelder E, Ziegler H (1987) Natural and Chernobyl-caused radioactivity in mushrooms, mosses and soil samples of defined biotopes in S.W. Bavaria. *Oecologia* 73:553–558
- Fomina M, Gadd GM (2002) Metal sorption by biomass of melanin-producing fungi grown in clay-containing medium. *J Chem Technol Biotechnol* 78:23–34
- Francis AJ (1986) Acid rain effects on soil and aquatic processes. *Experientia* 42:455–465
- Fritze H (1987) The influence of urban air pollution on soil respiration and fungal hyphal length. *Ann Bot Finn* 24:251–256
- Fritze H (1991) Forest soil microbial responses to emissions from an iron and steel smelter. *Soil Biol Biochem* 23:151–155
- Fritze H, Baath E (1993) Microfungal species composition and fungal biomass in a coniferous forest soil polluted by alkaline deposition. *Microbial Ecol* 25:83–92
- Frostegard A, Tunlid A, Baath E (1993) Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. *Appl Environ Microbiol* 59:3605–2617
- Gadd GM (1984) Effect of copper on *Aureobasidium pullulans* in solid medium: adaptation not necessary for tolerant behaviour. *Trans Br Mycol Soc* 82:546–549
- Gadd GM, White C (1985) Copper uptake by *Penicillium ochro-chloron*: influence of pH on toxicity and demonstration of energy-dependent copper influx using protoplasts. *J Gen Microbiol* 131:1875–1879
- Gadd GM (1990) Fungi and yeasts for metal accumulation. In: Ehrlich HL, Brierley C (eds) *Microbial mineral recovery*. McGraw-Hill, New York, pp 249–275
- Gadd GM (1992) Metals and microorganisms: a problem of definition. *FEMS Microbiol Lett* 100:197–204
- Gadd GM (1993a) Interactions of fungi with toxic metals. *New Phytol* 124:25–60
- Gadd GM (1993b) Microbial formation and transformation of organometallic and organometalloid compounds. *FEMS Microbiol Rev* 11:297–316
- Gadd GM (1999) Fungal production of citric and oxalic acid: importance in metal speciation, physiology and biogeochemical processes. *Adv Microbial Physiol* 41:47–92
- Gadd GM (2001a) Accumulation and transformation of metals by microorganisms. In: Rehm H-J, Reed G, Puhler A, Stadler P (eds) *Biotechnology, a Multi-volume Comprehensive Treatise* vol 10. Special processes. Wiley, Weinheim, pp 225–264
- Gadd GM (2001b) Metal transformations. In: Gadd GM (ed) *Fungi in bioremediation*. Cambridge University Press, Cambridge, pp 359–382
- Gadd GM (2002) Interactions between microorganisms and metals/radionuclides: the basis of bioremediation. In: Keith-Roach MJ, Livens FR (eds) *Interactions of microorganisms with radionuclides*. Elsevier, Amsterdam, pp 179–203
- Gadd GM (ed) (2006) *Fungi in biogeochemical cycles*. Cambridge University Press, Cambridge
- Gadd GM, Griffiths AJ (1978) Microorganisms and heavy metal toxicity. *Microbial Ecol* 4:303–317
- Gadd GM (2007) Geomycology: biogeochemical transformation of rocks, minerals, metals and radionuclides by fungi, biowathering and bioremediation. *Mycol Res* 111:3–49
- Gadd GM, Sayer JA (2000) Fungal transformations of metals and metalloids. In: Lovley DR (ed) *Environmental microbe-metal interactions*. American Society of Microbiology, Washington, DC, pp 237–256
- Gadd GM, White C (1992) Removal of thorium from simulated acid process streams by fungal biomass: potential for thorium desorption and reuse of biomass and desorbent. *J Chem Technol Biotechnol* 55:39–44
- Gadd GM, Chudek JA, Foster R, Reed RH (1984) The osmotic responses of *Penicillium ochro-chloron*: changes in internal solute levels in response to copper and salt stress. *J Gen Microbiol* 130:1969–1975
- Gast CH, Jansen E, Bierling J, Haanstra L (1988) Heavy metals in mushrooms and their relationship with soil characteristics. *Chemosphere* 17:789–799
- Gharieb MM, Gadd GM (1999) Influence of nitrogen source on the solubilization of natural gypsum. *Mycol Res* 103:473–481
- Gharieb MM, Wilkinson SC, Gadd GM (1995) Reduction of selenium oxyanions by unicellular, polymorphic and filamentous fungi: cellular location of reduced selenium and implications for tolerance. *J Indust Microbiol* 14:300–311
- Gharieb MM, Kierans M, Gadd GM (1999) Transformation and tolerance of tellurite by filamentous fungi: accumulation, reduction and volatilization. *Mycol Res* 103:299–305
- Griffioen WAJ (1994) Characterization of a heavy metal-tolerant endomycorrhizal fungus from the surroundings of a zinc refinery. *Mycorrhiza* 4:197–200
- Grote G, Krumbach WE (1992) Microbial precipitation of manganese by bacteria and fungi from desert rock and rock varnish. *Geomicrobiol J* 10:49–57
- Haemmerli SD, Leisola MSA, Sanglard D, Fiechter A (1986) Oxidation of benzo[a]pyrene by extracellular ligninases of *Phanerochaete chrysosporium*: veratryl alcohol and stability of ligninase. *J Biol Chem* 261:6900–6903
- Hartley J, Cairney JWG, Freestone P, Woods C, Meharg AA (1999) The effects of multiple metal contamination on ectomycorrhizal Scots pine (*Pinus sylvestris*) seedlings. *Environ Pollut* 106:413–424
- Harvey RG (1997) *Polycyclic aromatic hydrocarbons*. Wiley, Hoboken, NJ
- Haselwandter K (1978) Accumulation of the radioactive nuclide <sup>137</sup>Cs in fruitbodies of basidiomycetes. *Health Phys* 34:713–715
- Haselwandter K, Berreck M, Brunner P (1988) Fungi as bioindicators of radiocaesium contamination: pre- and post-Chernobyl activities. *Trans Br Mycol Soc* 90:171–174
- Heinrich G (1992) Uptake and transfer factors of <sup>137</sup>Cs by mushrooms. *Radiat Environ Phys* 31:39–49
- Helander ML, Ranta H, Neuvonen S (1993) Responses of phyllosphere microfungi to simulated sulphuric and nitric acid deposition. *Mycol Res* 97:533–537
- Hestbjerg H, Willumsen PA, Christensen M, Andersen O, Jacobsen CS (2003) Bioaugmentation of tar-contaminated soils under field conditions using *Pleurotus ostreatus* refuse from commercial mushroom production. *Environ Toxicol Chem* 22:692–698

- Hetrick BAD, Wilson GWT, Figge DAH (1994) The influence of mycorrhizal symbiosis and fertilizer amendments on establishment of vegetation in heavy metal mine spoil. *Environ Pollut* 86:171–179
- Huesemann MH, Hausmann TS, Fortman TJ (2003) Assessment of bioavailability limitations during slurry biodegradation of petroleum hydrocarbons in aged soils. *Environ Toxicol Chem* 22:2853–2860
- Johnsen AR, Winding A, Karlson U, Roslev P (2002) Linking of microorganisms to phenanthrene metabolism in soil by analysis of  $^{13}\text{C}$ -labeled cell lipids. *Appl Environ Microbiol* 68:6106–6113
- Jordan MJ, Lechevalier MP (1975) Effects of zinc-smelter emissions on forest soil microflora. *Can J Microbiol* 21:1855–1865
- Juhasz AL, Naidu R (2000) Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. *Int Biodet Biodegrad* 45:57–88
- Kanaly RA, Bartha R, Watanabe K, Harayama S (2000) Rapid mineralization of benzo[a]pyrene by a microbial consortium growing on diesel fuel. *Appl Environ Microbiol* 66:4205–4211
- Kelly JJ, Haggblom M, Tate RL (1999) Changes in soil microbial communities over time resulting from one time application of zinc: a laboratory microcosm study. *Soil Biol Biochem* 31:1455–1465
- Khan M, Scullion J (2002) Effects of metal (Cd, Cu, Ni, Pb or Zn) enrichment of sewage-sludge on soil microorganisms and their activities. *Appl Soil Ecol* 20:145–155
- Killham K, Firestone MK (1983) Vesicular-arbuscular mycorrhizal mediation of grass responses to acidic and heavy metal depositions. *Plant Soil* 72:39–48
- Killham K, Wainwright M (1981) Deciduous leaf litter and cellulose decomposition in soil exposed to heavy atmospheric pollution. *Environ Pollut* A26:69–78
- Killham K, Wainwright M (1982) Microbial release of sulphur ions from atmospheric pollution deposits. *J Appl Ecol* 18:889–896
- Killham K, Wainwright M (1984) Chemical and microbiological changes in soil following exposure to heavy atmospheric pollution. *Environ Pollut* A33:122–131
- Knapp JS, Vantoch-Wood EJ, Zhang F (2001) Use of wood-rotting fungi for the decolorization of dyes and industrial effluents. In: Gadd GM (ed) *Fungi in bioremediation*. Cambridge University Press, Cambridge, pp 242–304
- Kostov O, Van Cleemput O (2001) Microbial activity of Cu contaminated soils and effect of lime and compost on soil resiliency. *Compost Sci Utilization* 9:336–351
- Kubatova A, Prasil K, Vanova M (2002) Diversity of soil microscopic fungi on abandoned industrial deposits. *Cryptog Mycol* 23:205–219
- Lahav R, Fareleira P, Nejidat A, Abielovich A (2002) The identification and characterization of osmotolerant yeast isolates from chemical wastewater evaporation ponds. *Microbial Ecol* 43:388–396
- Lehto K-M, Puhakka JA, Lemmettyinen H (2003) Biodegradation of selected UV-irradiated and non-irradiated polycyclic aromatic hydrocarbons (PAHs). *Biodegradation* 14:249–263
- Lepsova A, Mejstrik V (1989) Trace elements in fruit bodies of fungi under different pollution stress. *Agric Ecosyst Environ* 28:305–312
- Leyval C, Turnau K, Haselwandter K (1997) Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. *Mycorrhiza* 7:139–153
- Majcherczyk A, Johannes C (2000) Radical mediated indirect oxidation of a PEG-coupled polycyclic aromatic hydrocarbon (PAH) model compound by fungal laccase. *Biochim Biophys Acta* 1474:157–162
- Manoli F, Koutsopoulos E, Dalas E (1997) Crystallization of calcite on chitin. *J Cryst Growth* 182:116–124
- Markkola AM, Ahonen-Jonnarth U, Roitto M, Strommer R, Hyvarinen M (2002) Shift in ectomycorrhizal community composition in Scots pine (*Pinus sylvestris* L.) seedling roots as a response to nickel deposition and removal of lichen cover. *Environ Poll* 120:797–803
- Massaccesi G, Romero MC, Cazau MC, Bucsinszky AM (2002) Cadmium removal capacities of filamentous soil fungi isolated from industrially polluted sediments, in La Plata (Argentina). *World J Microbiol Biotechnol* 18:817–820
- Meharg AA, Cairney JWG (2000) Co-evolution of mycorrhizal symbionts and their hosts to metal-contaminated environments. *Adv Ecol Res* 30:69–112
- Mehra RK, Winge DR (1991) Metal ion resistance in fungi: molecular mechanisms and their related expression. *J Cell Biochem* 45:30–40
- Mejstrik V, Lepsova A (1993) Applicability of fungi to the monitoring of environmental pollution by heavy metals. In: Market B (ed) *Plants as biomonitor*s. VCH Verlagsgesellschaft, Weinheim, pp 365–378
- Meulenbergh R, Rijnaarts HHM, Doddema HJ, Field JA (1997) Partially oxidized polycyclic aromatic hydrocarbons show an increased bioavailability and biodegradability. *FEMS Microbiol Lett* 152:45–49
- Mineev VG, Gomonova NF, Zenova GM, Skvortsova IN (1999) Changes in the properties of soddy-podzolic soil and its microbocenosis under intensive anthropogenic impact. *Eurasian Soil Sci* 32:413–417
- Morley GF, Sayer JA, Wilkinson SC, Gharieb MM, Gadd GM (1996) Fungal sequestration, solubilization and transformation of toxic metals. In: Frankland JC, Magan N, Gadd GM (eds) *Fungi and environmental change*. Cambridge University Press, Cambridge, pp 235–256
- Mowll JL, Gadd GM (1985) The effect of vehicular lead pollution on phylloplane mycoflora. *Trans Br Mycol Soc* 84:685–689
- Moynahan OS, Zabinski CA, Gannon JE (2002) Microbial community structure and carbon-utilization diversity in a mine tailings revegetation study. *Restoration Ecol* 10:77–87
- Mozafar A, Ruh R, Klingel P, Gamper H, Egli S, Frossard E (2002) Effect of heavy metal contaminated shooting range soils on mycorrhizal colonization of roots and metal uptake by leek. *Environ Monitoring Assess* 79:177–191
- Mueller JG, Cerniglia CE, Pritchard PH (1996) In: Crawford RL, Crawford DL (eds) *Bioremediation: principles and applications*. Cambridge University Press, Cambridge, pp 125–194

- Nodar R, Acea MJ, Carballas T (1992) Microbiological response to  $\text{Ca}(\text{OH})_2$  treatments in a forest soil. *FEMS Microbiol Ecol* 86:213–219
- Nordgren A, Baath E, Soderstrom B (1983) Microfungi and microbial activity along a heavy metal gradient. *Appl Environ Microbiol* 45:1837–1839
- Nordgren A, Baath E, Soderstrom B (1985) Soil microfungi in an area polluted by heavy metals. *Can J Bot* 63:448–455
- Novotný C, Erbanová P, Cajthaml T, Rothschild N, Dosoretz C, Šašek V (2000) *Irpex lacteus*, a white rot fungus applicable to water and soil bioremediation. *Appl Microbiol Biotechnol* 54:850–853
- Olayinka A, Babalola GO (2001) Effects of copper sulphate application on microbial numbers and respiration, nitrifier and urease activities, and nitrogen and phosphorus mineralization in an alfisol. *Biol Agric Hort* 19:1–8
- Pennanen T, Frostegard A, Fritze H, Baath E (1996) Phospholipid fatty acid composition and heavy metal tolerance of soil microbial communities along two heavy metal-polluted gradients in coniferous forests. *Appl Environ Microbiol* 62:420–428
- Persson T, Lundkvist H, Wiren A, Hyvonen R, Wessen B (1989) Effect of acidification and liming on carbon and nitrogen mineralization and soil organisms in moor humus. *Water Air Soil Pollut* 45:77–70
- Pickard MA, Roman R, Tinoco R, Vázquez-Duhalt R (1999) Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. *Appl Environ Microbiol* 65:3805–3809
- Pinto LJ, Moore MM (2000) Release of polycyclic aromatic hydrocarbons from contaminated soils by surfactant and remediation of this effluent by *Penicillium* spp. *Environ Toxicol Chem* 19:1741–1748
- Pointing SB (2001) Feasibility of bioremediation by white-rot fungi. *Appl Microbiol Biotechnol* 57:20–33
- Pozzoli L, Gilardoni S, Perrone MG, de Gennaro G, de Rienzo M, Vione D (2004) Polycyclic aromatic hydrocarbons in the atmosphere: monitoring, sources, sinks and fate. I. Monitoring and sources. *Annali Chim* 94:17–32
- Prescott CE, Parkinson D (1985) Effects of sulphur pollution on rates of litter decomposition in a pine forest. *Can J Bot* 63:1436–1443
- Purvis OW (1996) Interactions of lichens with metals. *Sci Progr* 79:283–309
- Rasmussen G, Olsen RA (2004) Sorption and biological removal of creosote-contaminants from groundwater in soil/sand vegetated with orchard grass (*Dactylis glomerata*). *Adv Environ Res* 8:313–327
- Ravelet C, Grossot C, Krivobok S, Montuelle B, Alary J (2001) Pyrene degradation by two fungi in a freshwater sediment and evaluation of fungal biomass by ergosterol content. *Appl Microbiol Biotechnol* 56:803–808
- Reddy CA, Mathew Z (2001) Bioremediation potential of white rot fungi. In: Gadd GM (ed) *Fungi in bioremediation*. Cambridge University Press, Cambridge, pp 52–78
- Rizzo DM, Blanchette RA, Palmer MA (1992) Biosorption of metal ions by *Armillaria* rhizomorphs. *Can J Bot* 70:1515–1520
- Roberts TM, Clarke TA, Ineson P, Gray TRG (1980) Effects of sulphur deposition on litter decomposition and nutrient leaching in coniferous forest soils. In: Hutchinson TC, Hava M (eds) *Effects of acid precipitation on terrestrial ecosystems*. Dekker, New York, pp 381–393
- Romero MC, Salvioli ML, Cazau MC, Arambarri AM (2002) Pyrene degradation by yeasts and filamentous fungi. *Environ Pollut* 117:159–163
- Ruhling A, Baath E, Nordgren A, Soderstrom B (1984) Fungi in metal contaminated soil near the Gusum brass mill, Sweden. *Ambio* 13:34–36
- Saraswathy A, Hallberg R (2002) Degradation of pyrene by indigenous fungi from a former gasworks site. *FEMS Microbiol Lett* 210:227–232
- Sastad SM, Jensen HB (1993) Interpretation of regional differences. I. The fungal biota as effects of atmospheric pollution. *Mycol Res* 12:1451–1458
- Sayer JA, Cotter-Howells JD, Watson C, Hillier S, Gadd GM (1999) Lead mineral transformation by fungi. *Curr Biol* 9:691–694
- Shaw PJA, Dighton J, Poskitt J, McCleod AR (1992) The effects of sulphur dioxide and ozone on the mycorrhizas of Scots pine and Norway spruce in a field fumigation system. *Mycol Res* 96:785–791
- Singleton I (2001) Fungal remediation of soils contaminated with persistent organic pollutants. In: Gadd GM (ed) *Fungi in bioremediation*. Cambridge University Press, Cambridge, pp 79–96
- Smith WH (1977) Influence of heavy metal leaf contaminants on the *in vitro* growth of urban-tree phylloplane fungi. *Microbial Ecol* 3:231–239
- Smith SE, Read DJ (1997) *Mycorrhizal symbiosis*, 2nd edn. Academic Press, San Diego, CA
- Smith ML, Taylor HW, Sharma HD (1993) Comparison of the post-Chernobyl  $^{137}\text{Cs}$  contamination of mushrooms from Eastern Europe, Sweden, and North America. *Appl Environ Microbiol* 59:134–139
- Steffen KT, Hatakka A, Hofrichter M (2003) Degradation of benzo[a]pyrene by the litter-decomposing basidiomycete *Stropharia coronilla*: role of manganese peroxidase. *Appl Environ Microbiol* 69:3957–3964
- Sterflinger K (2000) Fungi as geologic agents. *Geomicrobiol J* 17:97–124
- Stijve T, Porette M (1990) Radiocaesium levels in wild-growing mushrooms from various locations. *Mushroom J* (Summer 1990):5–9
- Strasser H, Burgstaller W, Schinner F (1994) High yield production of oxalic acid for metal leaching purposes by *Aspergillus niger*. *FEMS Microbiol Lett* 119:365–370
- Straube WL, Jones-Meehan J, Pritchard PH, Jones WR (1999) Bench-scale optimization of bioaugmentation strategies for treatment of soils contaminated with high molecular weight polycyclic aromatic hydrocarbons. *Resources Conserv Recycling* 27:27–37
- Sutherland JB (2004) Degradation of hydrocarbons by yeasts and filamentous fungi. In: Arora DK (ed) *Fungal biotechnology in agricultural, food, and environmental applications*. Marcel Dekker, New York, pp 443–455
- Tabatabai M (1985) Effect of acid rain on soils. *CRC Crit Rev Environ Control* 15:65–109
- Thompson-Eagle ET, Frankenberger WT (1992) Bioremediation of soils contaminated with selenium. In: Lal R, Stewart BA (eds) *Advances in soil science*. Springer, Berlin Heidelberg New York, pp 261–309

- Thompson-Eagle ET, Frankenberger WT, Karlson U (1989) Volatilization of selenium by *Alternaria alternata*. *Appl Environ Microbiol* 55:1406–1413
- Tyler G (1980) Metals in sporophores of basidiomycetes. *Trans Br Mycol Soc* 74:41–49
- Veignie E, Rafin C, Woisel P, Cazier F (2004) Preliminary evidence of the role of hydrogen peroxide in the degradation of benzo[a]pyrene by a non-white rot fungus *Fusarium solani*. *Environ Poll* 129:1–4
- Verdin A, Lounès-Hadji Sahraoui A, Durand R (2004) Degradation of benzo[a]pyrene by mitosporic fungi and extracellular oxidative enzymes. *Int Biodeter Biodegrad* 53:65–70
- Verrecchia EP (2000) Fungi and sediments. In: Riding RE, Awramik SM (eds) *Microbial sediments*. Springer, Berlin Heidelberg New York, pp 69–75
- Wainwright M (1988a) Metabolic diversity of fungi in relation to growth and mineral cycling in soil – a review. *Trans Br Mycol Soc* 23:85–90
- Wainwright M (1988b) Effect of point source atmospheric pollution on fungal communities. *Proc R Soc Edinb* 94B:97–104
- Wainwright M (1992) Oligotrophic growth of fungi—stress or natural state? In: Jennings DH (ed) *Stress tolerance of fungi*. Marcel Dekker, New York, pp 127–144
- Wainwright M, Supharungsun S, Killham K (1982) Effects of acid rain on the solubility of heavy metal oxides and fluorspar added to soil. *Sci Total Environ* 23:85–90
- White C, Wilkinson SC, Gadd GM (1995) The role of microorganisms in biosorption of toxic metals and radionuclides. *Int Biodeter Biodeg* 35:17–40
- Wilkins DA (1991) The influence of sheathing (ecto-) mycorrhizas of trees on the uptake and toxicity of metals. *Agric Ecosyst Environ* 35:245–260
- Wilkinson DM, Dickinson NM (1995) Metal resistance in trees – the role of mycorrhizae. *Oikos* 72:298–300
- Wondratschek I, Roder U (1993) Monitoring of heavy metals in soils by higher fungi. In: Markert B (ed) *Plants as biomonitoring*. VCH Verlagsgesellschaft, Weinheim, pp 345–363
- Yamamoto H, Tatsuyama K, Uchiwa T (1985) Fungal flora of soil polluted with copper. *Soil Biol Biochem* 17:785–790
- Zhdanova NN, Redchitz TI, Vasilevskaya AI (1986) Species composition and sorption properties of Deuteromycetes in soils polluted by industrial wastewater (in Russian). *Mikrobiol Zhu* 48:44–50

---

## 6 Fungi in Extreme Environments

N. MAGAN<sup>1</sup>

### CONTENTS

I. General Introduction .....	85
II. Thermotolerance .....	85
A. Mechanisms of Thermotolerance .....	87
III. Psychrophiles .....	88
A. Survival at Low Temperatures .....	89
IV. Water Relations of Fungi .....	91
A. Concept of Water Availability .....	91
B. Fungal Growth and Water Potential .....	93
C. Adaptation to Water Potential .....	94
D. Yeast Physiology and Osmotic Stress .....	94
E. Xerophilic Filamentous Fungi .....	95
V. Anaerobic Fungi .....	95
VI. Acidophiles and Alkalophiles .....	97
A. Acidophiles .....	97
B. Alkalophiles .....	98
C. Mechanisms of Survival in Extreme pH Environments .....	98
VII. Irradiation and Fungi .....	98
VIII. Conclusions.....	100
References .....	100

### I. General Introduction

In nature, fungi are ubiquitous, having evolved over time to occupy a wide range of ecological niches. To occupy these niches, they must be involved in primary resource capture which is determined by the ability to germinate and become established rapidly and to produce the necessary extracellular enzymes in the immediate environment. Their activity will be further impacted upon by prevailing abiotic factors such as temperature, water availability, gas balance and pH. It has been suggested that both primary and secondary resource capture are in a state of flux and determined predominantly by prevailing biotic and abiotic interactions (Magan and Aldred 2006). Thus, niche occupation is determined by a complex of interacting factors. Certain species may occupy similar general niches because of their ecologically similar behaviour within

a community. The abiotic stress factors may be a long-lived or a permanent feature of a habitat whereas other disturbance factors may be transient, having a short-lived impact.

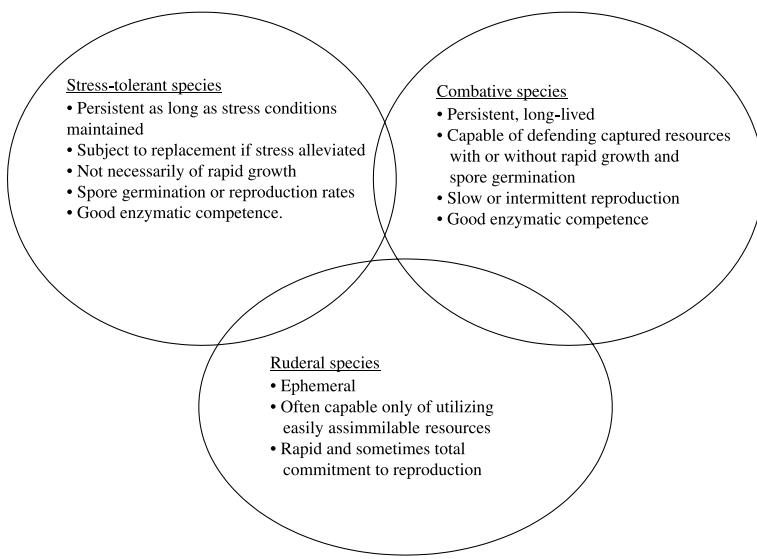
Based on work in plant communities, it has been suggested that fungi employ different primary strategies to survive and prosper in different environments. These are so-called *combative* (C-selected) strategies, which maximise occupation and exploitation of resources in relatively non-stressed and undisturbed conditions; *stress* (S-selected) strategies, which have involved the development of adaptations allowing survival and endurance under continuous environmental stress; and *ruderale* (R-selected) strategies, characterised by a short life span and high reproductive potential which often enable success under severely disturbed but nutrient-rich conditions. These three strategies can merge to give secondary strategies (C-R, S-R, C-S, C-S-R) which form part of a continuum with transition zones between them (Cooke and Whipps 1993). The main attributes of these three primary groups are summarized in Fig. 6.1.

In this chapter, we are specifically concerned with fungi which may use S-selected strategies for growth and survival in a range of so-called extreme environments. It should, however, be remembered that under natural conditions fungal activity in terrestrial ecosystems will be influenced by interactions between abiotic and biotic factors both spatially and temporally. Community structure and dominance can thus vary during succession in specific habitats.

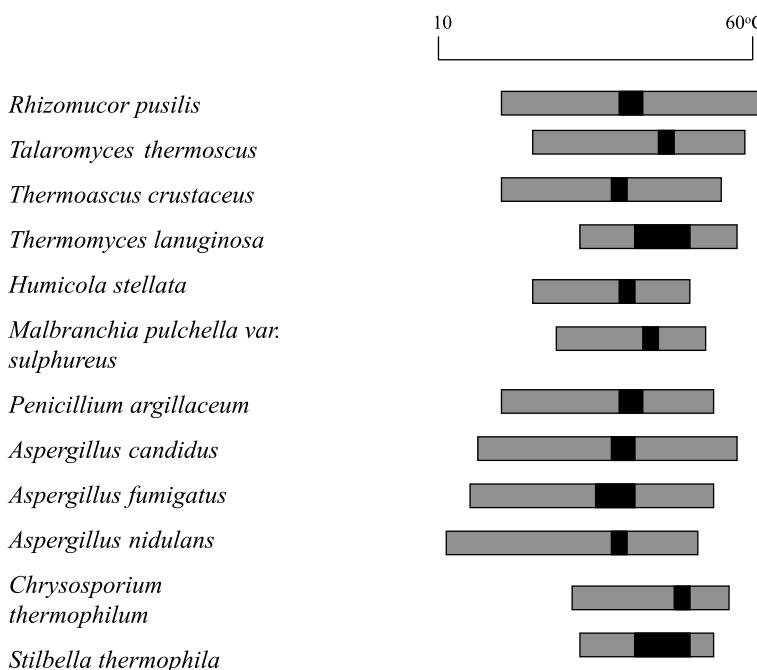
### II. Thermotolerance

Fungi have the ability to grow over a wide range of temperature conditions. Fungi, and microorganisms generally, have been classified

<sup>1</sup> Applied Mycology Group, Cranfield Health, Cranfield University, Barton Road, Silsoe, Bedford MK45 4DT, UK



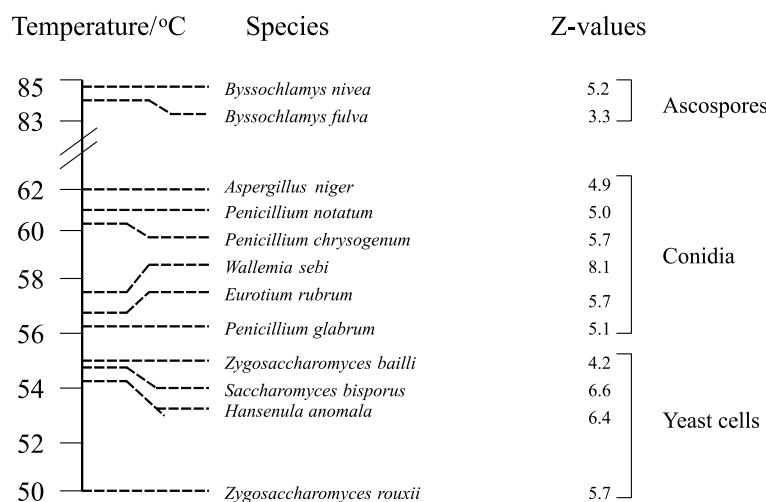
**Fig. 6.1.** Summary of attributes of fungi in relation to the three major ecological strategies (from Cooke and Rayner 1984)



**Fig. 6.2.** Optimum, minimum and maximum temperatures and range of conditions for growth of some thermotolerant and thermophilic fungi

as psychrophiles, mesophiles, thermotolerant and true thermophiles. A thermophilic fungus is defined as one which has minimum growth at 20 °C or above and a maximum growth at 50 °C or above. Optima for thermophilic fungi thus occur in the range 40–50 °C. Figure 6.2 shows the optimal, maximum and minimum temperature range for some thermotolerant and thermophilic fungi. While there are well-known examples of bacteria which are able to grow in a variety of natural environments including hot springs and geysers

where temperatures can reach 100 °C, eukaryotes are much more sensitive because, at temperatures above 65 °C, their membranes become irreparably damaged. However, many mesophilic thermotolerant fungi do exist; for example, some deuteromycetes isolated from thermal springs have maximum growth temperature of 61.5 °C (Tansey and Brock 1973). One must, however, distinguish between the ability to actively grow as a thermophile at such high temperatures and survival. Often, thermotolerant species are found



**Fig. 6.3.** Heat resistance of ascospores and conidia of different filamentous fungi and yeast cells (from Baggerman and Samson 1988). The D- and z-values were determined in phosphate buffer (0.2 ml/l, pH 5.5) containing sucrose (400 g/l)

as components of communities of fungi colonizing a range of damp organic substrates, particularly hay, straw-based composts and moist-temperature and tropical cereals, bird nests and tropical soils. They thus form important components of the succession of fungi colonizing a wide variety of substrata.

Most vegetative yeast cells, fungal mycelium and asexual conidia are killed by exposure to 80 °C for only 1 min. By contrast, sexually produced ascospores of some food-spoilage fungi are able to survive readily even for 1 h exposure at this temperature. Such fungi include *Talaromyces flavus* and *Neosartorya fischeri* var. *glaber* (Beuchat 1988; Baggerman and Samson 1988). Heat resistance has usually been described by the determination of two types of values, D and z. The D-value is defined as the decimal reduction time, and indicates the period of time required to reduce a certain number of living organisms by a factor of 10 under standard temperature and other environmental conditions. If the D-values at different temperature are plotted on a logarithmic scale, then a straight-line graph should be obtained, the slope of which is known as the z-value. This defines the increase in temperature (°C) necessary to decrease or increase the D-value by a factor of 10. Figure 6.3 shows the heat resistance of ascospores, conidia and some yeast cells at different temperatures. These data clearly demonstrate the gradation of sensitivity of yeasts and spores of filamentous fungi to increasing temperature. It should, however, be borne in mind that the D- and z-values will also be influenced by pH, water availability and the actual nature of the substratum.

#### A. Mechanisms of Thermotolerance

There have been a number of hypothesis proposed for explaining the basis of thermophily. Crisan (1973) suggested four main possibilities: (1) lipid solubilization; (2) rapid resynthesis of essential metabolites; (3) molecular thermostability and (4) ultrastructural thermostability. The latter may be of particular importance because there is the possibility that solubilization of cellular lipids can occur at high temperature to the extent that cells lose their integrity. An increase in temperature may result in cellular lipids containing more saturated fatty acids which have a higher melting point than those present in mesophiles. They would thus be able to maintain cellular integrity at higher temperatures than is the case for mesophiles, which contain markedly less saturated lipids. It has also been suggested that the increased fluidity of saturated lipids at high temperatures may enable metabolic activity and cell functioning to enable active growth at >40 °C.

Recently, attention has been focused on the ability of organisms generally to produce a specific range of proteins, so-called heat shock proteins, when exposed to extremes of environmental factors, particularly temperature. However, very little work has been carried out on heat shock proteins in fungi. These have to a large extent been carried out on *Saccharomyces cerevisiae*, *Neurospora crassa*, *Aspergillus nidulas*, *Achlya ambisexualis* and *Schizophyllum commune*. In almost all cases, studies have involved exposure of strains of these fungi to elevated temperatures of, for example, 45–55 °C for a period of 1–3 h. Such condi-

tions have been found to reduce growth but not aerial hyphal development, and often resulted in the appearance of a number of proteins resolvable by SDS-PAGE which were newly synthesised or of which the synthesis increased. In the basidiomycete *S. commune*, it was also found that proteolytic processes were significantly affected by such exposure (Higgins and Lilly 1993).

In the last 5 years, intensive investigations into the activities of heat shock proteins have produced significant advances in understanding their cellular role. It has been discovered that many of these (heat shock) proteins are in fact essential proteins which are synthesised normally by cells at temperatures optimal for growth. Sometimes, this has been accompanied by concomitant production of the low-molecular sugar alcohol, glycerol, which will be considered in more detail in Section IV. Detailed information of the type of heat shock proteins and the cellular effects of heat shock have been extensively reviewed by Plesofsky-Vig and Brambl (1993). The physiology and secretion of thermophilic enzymes by different thermophilic fungi have been reviewed more recently (Maheshwari et al. 2000). The advent of whole-genome sequences for some fungi, e.g. *Aspergillus fumigatus*, means that by using microarrays it is possible to examine the physiological pathways involved and also the number of genes which are up- and down-regulated during heat shock protein production. This will provide a more thorough understanding of the gene clusters involved in this process.

### III. Psychrophiles

There is a wide range of natural habitats where low temperatures occur continuously or intermittently due to seasonal effects. These regions include oceans, the tundra and sub-Arctic regions. For example, although the oceans have a stable temperature of  $<5^{\circ}\text{C}$  (Morita 1974), there are distinct difficulties in actually studying such ecological systems because of the effects of pressure. Also, often clear distinctions have not been made between true psychrophiles and cold-tolerant species of fungi. This group has attracted significant attention because of the interest in cold-active enzymes and also in the biogeography and ecological significance of cold-tolerant fungi. Generally, members of all classes of fungi exist in regions such as the Arctic tundra. However, most information has been gathered

on the survival and growth of fungi in sub-Arctic and dry cold desert regions of the world (Robinson 2001). These fungi may be present either because they are true psychrophiles or because they are psychrotolerant with the ability to survive but not actively grow at temperatures  $<5^{\circ}\text{C}$ . Schmidt-Nielsen (1902) proposed the term "psychophilic", defined as an organism which has optimum growth at not greater than  $16^{\circ}\text{C}$ , and a maximum at about  $20^{\circ}\text{C}$  (Morris and Clarke 1987). For example, detailed studies of the yeast community of cold dry deserts showed that species such as *Cryptococcus visnniacii* were common (Vishniac and Hempfling 1979). They also interestingly showed that many of these psychophilic yeasts in fact had temperature ranges up to  $20^{\circ}\text{C}$  or more. However, *in vitro* studies do not often give an accurate indication of the conditions over which active growth might occur in a natural habitat. This does, however, differ considerably from work in marine environments with bacteria where the temperature ranges were significantly narrower (Fuhrman and Anzam 1980).

However, species of *Aureobasidium* (dimorphic yeasts) have been isolated from rocks in Antarctica which can grow and reproduce at  $0\text{--}5^{\circ}\text{C}$  and are able to tolerate temperatures as low as  $-80^{\circ}\text{C}$ . Other stable man-made environments, such as domestic fridges, provide temperature in the range  $4\text{--}10^{\circ}\text{C}$  and allow spoilage fungi, particularly *Penicillium* spp., to grow on foodstuffs. There is also evidence that spores of common deuteromycetes such as *Cladosporium* spp. and *Sporotrichum* sp. are able to germinate and grow on meat in cold storage at temperatures below  $0^{\circ}\text{C}$  (Cochrane 1958). Some fungi, the aptly named snow moulds such as *Fusarium* spp., *Sclerotinia borealis*, *Phacidium infestans* and *Typhula* spp., are common in northern British Columbia and Sweden and able to actively colonize foliage, particularly of grasses to cause significant disease. For example, *S. borealis* in culture has optimum temperature for growth of  $0^{\circ}\text{C}$  and a minimum of  $<-7^{\circ}\text{C}$ , and does not grow at  $>15^{\circ}\text{C}$ . It is thus a true psychophile. However, *Typhula idahoensis* and *T. trifolii* grow at  $-5^{\circ}\text{C}$  and have an optimum of  $5^{\circ}\text{C}$ . They do grow at near  $20^{\circ}\text{C}$  but no growth occurs at  $25^{\circ}\text{C}$ . Some of these fungi are considered to be psychophilic whereas others, which actively grow and infect at approx.  $3^{\circ}\text{C}$  (e.g. *Monographella nivalis*), are considered to be cold-tolerant mesophiles because they are able to grow at  $20^{\circ}\text{C}$ . There may be some niche overlap between such fungi, with the latter being a very effective organism at  $3^{\circ}\text{C}$  by being at a competitive advantage

**Table 6.1.** Examples of growth, enzyme activity and viability of fungi at low temperatures (modified from Robinson 2001)

Taxon/species	Test temperatures/type of experiment	Site	Reference
54 mitosporic fungi	Growth at 0–25 °C, mainly psychrophiles/in vitro, artificial media	Arctic	Bergero et al. (1999)
<i>Phoma herbarum</i>	Faster growth at 2.5 than 20 °C/sand+glucose-mineral agar	Devon Island, Canada	Widden and Parkinson (1978)
31 mitosporic fungi	All grew at 4 °C, most optima at 15–20 °C/synthetic defined media	Antarctic, Macquarie Island	Kerry (1990)
<i>Humicola marvinii</i>	Growth at -2.5 °C, optimum at 15; no growth at 20–22 °C	Signy Island, Antarctic	Weinstein et al. (1997)
Enzyme production			
<i>Phialophora hoffmanni</i>	Occurs at 1 °C, optima between 18–20 °C/in vitro pectinase activity	Arctic sites	Flanagan and Scarborough (1974)
<i>Geomyces pannorum</i>	Occurs at -4 °C, optimum at 5 °C/in vitro cellulase activity	Arctic sites	
<i>Verticillium lecanii</i>	Active at 5, 15 and 25 °C/in vitro chitinase activity	Antarctic moss sample	Fenice et al. (1998)

in such a very specialized ecological environment. Table 6.1 summarizes some of the fungi which have been isolated from such environments and the type of experiments carried out with these.

As mentioned above, there are many climatic regions where very cold winter temperatures are followed by quite warm temperatures in the summer months, e.g. sub-Arctic zones. Here, overwintering and survival are of critical importance for effective competition in the following season. A large number of phyllosphere, endophytic and soil fungi are able to survive very severe winter temperatures of <-50 °C. They remain viable by surviving as resting structures, often thick-walled chlamydospores and sclerotia, or thin-walled conidia. Even thin-walled asexually produced conidia are resistant to freezing temperatures (Mazur 1966). For example, conidia of *Aspergillus flavus* were found to be resistant to freezing in water at -73 °C. This survival may partially be due to a very low water content, so that little or no ice formation occurs which could affect the integrity of the spore.

#### A. Survival at Low Temperatures

There are a number of key ecophysiological characteristics which are required by fungi to survive at low temperatures. These include the capacity to dehydrate, produce antifreeze compounds, high supercooling activity and chill tolerance, freezing tolerance and survival under anoxia. Cellular

processes obviously slow down significantly as temperature is reduced. This is manifested by cessation of growth, and the slowing down of enzyme activity, denaturing of proteins and their synthesis, and perhaps transport or membrane integrity. When freezing occurs, the effect depends on whether it takes place rapidly or slowly. Rapid freezing results in the cell contents becoming more concentrated and to come into equilibrium with the extracellular solution, resulting in shrinkage in the cell. Where slow cooling occurs, extensive supercooling may take place. If this happens at -39 °C or below, nucleation of ice will be homogeneous. Particles can act as a nucleus, resulting in heterogeneous nucleation before this temperature is reached. Cooling and freezing will thus affect the structure and metabolism of cells significantly. Mazur (1966) proposed a two-factor hypothesis: at relatively fast cooling rates, intracellular ice formation will cause injury whereas, at slow cooling rates, injury is caused by prolonged exposure to solution effects due to a concentration of extracellular solution or cell dehydration. By contrast, Steponkus (1984) suggests that there is no evidence that intracellular ice causes injury. Other hypotheses have been reviewed at length by Smith (1993).

It is possible to summarize the type of injury which can occur based on work on plant protoplasts (Steponkus 1984). These include expansion-induced lysis during warming, which occurs as dilution of the suspending medium allows expansion of the protoplasts; loss of osmotic responsiveness during slow cooling and warming due to changes

in electrolyte concentration, and effects on the lipid-protein membrane, thereby affecting osmotic responsiveness; leakage of intracellular solutes through the plasma membrane; and intracellular ice formation during fast cooling ( $>-3\text{ }^{\circ}\text{C/min}$ ). Thus, cryo-injury is dependent on the rate of cooling, cell type, and the internal and external composition of the cells and their substrate.

Compounds are, however, often produced within the cell to enable survival and growth at low temperature and, indeed, other stresses. Of particular importance for the survival of fungi is the production of osmoregulators or compatible solutes which are often synthesized when the fungus is placed under general environmental stress, but particularly involving water availability or low-temperature stress. These will be referred to in more detail in a section below and include arabitol, erythritol, glycerol, proline and trehalose. Trehalose has, for example, been demonstrated to effectively improve cryo-tolerance of *Saccharomyces cerevisiae*, baker's yeast, to withstand temperatures of  $-20$  to  $-70\text{ }^{\circ}\text{C}$ . They are able to reduce the amount of ice formation at low temperature, thereby reducing water loss from cells. However, during freezing significant shrinkage of the cell membrane can occur, which can influence subsequent survival. For example, it has been shown that the diameter of hyphae of *Phytophthora nicotianae* was reduced by up to 60%, compared to untreated controls. This can cause direct physical injury by dissolution of the cell membrane as ice formation results in an increase in volume of about 10%. Cryo-protectants have the effect of preventing such significant shrinkages, and reduce ice crystal formation. This can be achieved by exogenous addition of cryo-protectants to fungi prior to freezing for culture collection purposes (Smith 1993). The effects of cooling rates and storage temperatures on the recovery of strains have been studied in detail to determine the best methods of preservation (see Smith 1993). Studies of possible antifreeze proteins (AFPs) are scarce, except for a group of snow moulds (*Typhula* species; Snider et al. 2000). Isolates of *T. incarnata*, *T. ishikariensis* and *T. phacorrhiza* showed antifreeze activity in all fractions at 4 and  $10\text{ }^{\circ}\text{C}$ . However, no antifreeze activity was found in isolates with an optimum growth at  $14\text{ }^{\circ}\text{C}$  (e.g. *Microdochium nivale*). The antifreeze found in *T. phacorrhiza* was shown to originate from protein molecules. Interestingly, the ice crystal structures associated with snow mould species showed growth patterns different

from those previously observed, which suggested that these AFPs may bind to different planes of the ice crystal lattice than is the case for the AFPs isolated from fish, insects and plants.

In the 1980s, the use of cryogenic light microscopy to examine the effect of freezing and thawing on the fungal mycelium and propagules proved particularly useful. Studies of *Penicillium expansum* showed that shrinkage of hyphae occurred at slow cooling rates, and intracellular ice formation at faster rates of cooling. Interestingly, hyphal septa were found to form no barriers to such ice nucleation. Comparison of *P. expansum* with *P. nicotianae* showed that the former was extremely resistant to such freezing and thawing whereas the latter was sensitive and failed to recover from such treatment (Smith et al. 1986). Extensive and rapid shrinkage of mycelium occurred at all cooling rates up to  $-120\text{ }^{\circ}\text{C/min}$ . Culture age, growth phase, and nutrient status of the medium have all been found to influence these effects on fungi. There do appear to be two distinct groups of fungi: those which show shrinkage at slow rates of cooling but less at high rates, accompanied by intracellular ice formation; and those which shrink at all cooling rates, with no intracellular ice formation. These two subdivisions cut across taxonomic groupings. For example, *P. nicotianae* (oomycete), *Aschersonia aleurodis* (hyphomycete) and *Lentinus edodes* and *Volvariella volvacea* (basidiomycetes) all respond without any ice nucleation occurring.

Some contrasting effects have been observed with these groups of fungi. For example, fungi such as *P. nictianae* and *P. expansum* react by shrinkage of the mycelium and loss of plasma and nuclear membranes and cytoplasm between the cell wall and membrane. On subsequent thawing, the hyphae often re-expand to their original shape and size and are able to grow again. For other fungi, e.g. *L. edodes*, the hyphae do not return to their original size or shape. However, such species still retain viability. Smith (1993) has suggested that the membrane is a critical structure in tolerance to freezing and in thawing cycles. Because it is not very elastic and therefore does not fold easily, material must be lost from the structure during shrinkage due to freezing. Thus, rapid shrinkage can cause damage to the hyphal cells, although quite a number of fungi can survive during rapid cooling, even if intracellular ice formation occurs (Morris et al. 1988).

Generally, information is to a large extent available on the impact of freezing and thawing in relation to cryo-preservation. However, more data are

required on the ecology of psychrophilic fungi in natural habitats, what type of niche overlap may exist between different species, and their competence to survive such conditions and to actively grow where the cycle may involve both slow and fast cooling or thawing.

Comparisons of *Hebeloma* spp. from Arctic and temperate regions have indicated that substantial accumulation of trehalose occurred in the Arctic species when grown at low temperature (Tibbet et al. 1998a). Similarly, *Humicola marvini* and *Mortierella elongata*, psychrophiles isolated from Signy Island, Antarctica and grown at 5 and 15 °C, accumulated trehalose intracellularly to a significantly greater extent (75% more for the latter species) at 5 than 15 °C (Weinstein et al. 2000).

Studies by Weinstein et al. (1997) also suggested that sugar alcohols such as mannitol as well as trehalose were increased in isolates of *H. marvini*, compared with the non-psychrophilic species *H. fuscoatra*. They quantified glycerol, erythritol, mannitol and arabitol as well as trehalose, glucose and fructose. Table 6.2 shows a comparison between the results from this study. The significant synthesis of mannitol in the psychrophilic species and the relative increase in trehalose clearly reflect its ecological niche. In *H. fuscoatra*, by contrast, sugars are largely accumulated which have no role in cryo-protection.

Recent studies have also focused on the capacity of these fungi to produce various enzymes involved in decomposition and mycorrhizal associations with plants. Work by Weinstein et al. (1997) showed that *H. marvini* was capable of solubilizing inorganic phosphate and produce proteases. Studies by Tibbett et al. (1998a, b, 1999) have carried out a detailed examination of 12 ectomycorrhizal strains of *Hebeloma* species from the Arctic tundra

(Svalbard) and from Alaska, and compared these with some from France and Scotland. At <12 °C, the Arctic strains produced more extracellular and wall-bound acid phosphatase, although they grew more slowly than the temperate strains. Similar results were obtained for wall-bound and extracellular acid phosphomonoesterase and proteases in Arctic strains in which elevated concentrations were recorded in isolates. It has been suggested that such enzymes may be an adaptation to cold conditions. Synthesis of cryo-protectants may be a priority for survival, resulting in a few specific enzymes to only be produced by such fungi. Survival under cold conditions from year to year is of interest as well as the role of spores, sterile mycelia and septate hyphae, all very well discussed in the recent review by Robinson (2001).

#### IV. Water Relations of Fungi

All microorganisms require a source of water to enable cellular functioning to occur effectively. They all have a semi-permeable cell membrane, which allows water molecules to enter the cell through osmosis to come to equilibrium with its environment. However, often conditions prevail where water may be scarce, due to the presence of a high concentration of salts, e.g. in a marine environment or a dry, arid desert region, or in intermediate-moisture agricultural products. Because all cellular processes occur in water solutions, cells must physiologically be able to adjust to such osmotic alterations to be able to grow and reproduce in these environments. Certain groups of yeasts and filamentous fungi have over time evolved the capability of adaptation to such environments, and of exploiting niches occupied by few other microorganisms or dominating these by pre-emptive exclusion of others.

##### A. Concept of Water Availability

Scott (1957) was the first to identify the importance of water availability and try to relate this to the total water content of substrata. For example, in solid substrates such as agricultural commodities, water content consists of bound water (water of constitution), which is held in chemical union with the absorbing substrate by very strong forces, and free water which is weakly bound. Free water is more readily available for microbial growth and metab-

**Table 6.2.** Comparison of polyol and sugar accumulation (mg/100 mg dry wt) after 8 weeks at 15 °C in *Humicola marvini* from Antarctic soils and *H. fuscoatra* from a temperate climatic region (Weinstein et al. 1997)

Polyol/sugar	<i>H. marvini</i>	<i>H. fuscoatra</i>
Glycerol	0.35	0.80
Erythritol	0.27	0.11
Arabitol	0.21	Not detected
Mannitol	41.01	0.51
Trehalose	7.76	4.51
Glucose	0.18	8.07
Fructose	Not detected	5.03

**Table 6.3.** Water activity, equilibrium relative humidity (ERH) and water potentials at 25 °C

Water activity	ERH (%)	Water potential (−MPa)
1	100	0
0.99	99	1.38
0.98	98	2.78
0.97	97	4.19
0.96	96	5.62
0.95	95	7.06
0.9	90	14.5
0.85	85	22.4
0.8	80	30.7
0.75	75	39.6
0.7	70	40.1
0.65	65	59.3
0.6	60	70.3

olism than is bound water, but the ease with which it can be removed depends on the water content of the substrate. The degree of binding also varies with the type of substrate, and thus total water content is not a good indicator of water availability for microbial growth.

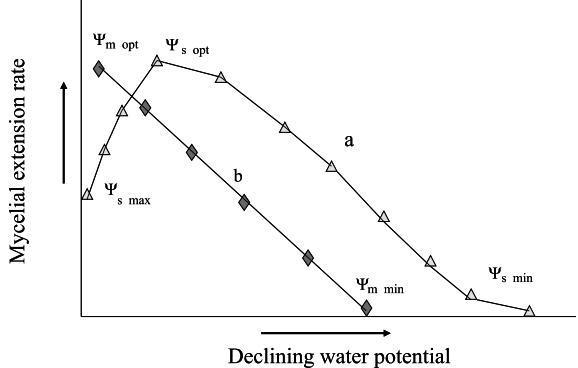
Scott (1957) suggested that water activity ( $a_w$ ) would best describe the water availability for microbial activity,  $a_w$  being the ratio between the vapour pressure of water in a substrate (P) and that of pure water ( $P_0$ ) at the same temperature and pressure; thus,  $a_w = P/P_0$ . The  $a_w$  of pure water is 1.00. A substrate containing no free water has a smaller vapour pressure than does pure water and its  $a_w$  is consequently less. An alternative measure of  $a_w$  is that of water potential ( $\Psi$ ), which is often used in soil microbiology and is measured in pascals (Pa). This is the sum of the osmotic, matrix

and turgor potentials and is related directly to  $a_w$  by the following formula:

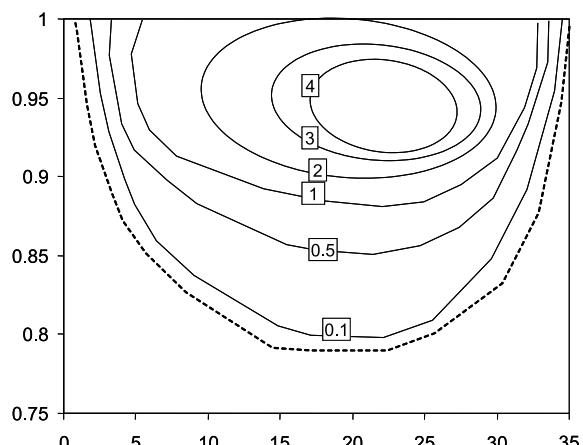
$$\text{Water potential}(\Psi) = RT/V \log_a_w (+P)$$

where R is the ideal gas constant, T the absolute temperature, P the atmospheric pressure and V the volume of 1 mole of water. The advantage of  $\Psi$  is that it is possible to partition osmotic and matric components and their influence on growth and physiological functioning of microbes. The relationship between  $a_w$  and  $\Psi$  is shown in Table 6.3. In cells, the  $\Psi$  of the environment normally almost always equals that of the cell:  $\Psi_{\text{env.}} = \Psi_{\text{cell}}$ . In most cases, the  $\Psi$  is a function of the osmotic component. In addition, because fungi have a very rigid cell wall, this prevents swelling of the cytoplasm, and the total  $\Psi$  is the combination of the osmotic and turgor pressure of the cell wall.

Microorganisms which are able to tolerate and actively grow under conditions of water stress have been described by various terms. The most common have included halophilic, osmophilic, osmotolerant, xerotolerant or xerophilic. The two most appropriate terms for fungi are probably "osmophilic", which describes specialized groups of yeasts which are able to grow in high-salt environments, and "xerophilic" (from the Greek, dry-loving). Pitt (1975) defined a xerophile as a fungus which is able to grow in some phase of its life cycle at  $-22.4$  MPa ( $0.85 a_w$ ), and this has now become generally accepted.



**Fig. 6.4.** Idealised relationship between growth and water potential: curve a solute potential, curve b matric potential, and showing  $\Psi_{\text{opt.}}$ ,  $\Psi_{\text{max.}}$ ,  $\Psi_{\text{min.}}$  (from Griffin 1981)



**Fig. 6.5.** Diagrammatic representation of the effect of water activity (Y axis) and temperature (X axis) on growth of *Penicillium versucosum*. The values on the isopleths represent growth rates in mm/day. Dotted line is absolute minimum for germination

## B. Fungal Growth and Water Potential

The effect of  $\Psi$  experimentally has been usually determined by measurement of growth responses to different steady-state osmotic and matric potentials in vitro by modifications of media using ionic or non-ionic solutes (e.g. NaCl, KCl, glycerol, and polyethylene glycol, 200–6000 mol wt). This has enabled data to be obtained on optimum, maximum and minimum conditions of  $\Psi$  for growth and survival. Figure 6.4 shows a general example of the pattern of relative growth rate in relation to  $\Psi$  of a growth medium. Osmotolerant yeasts such as *Zygosaccharomyces rugosus*, *Z. rouxii*, *Torulopsis halonitrophila* and *Saccharomyces mellis* or *S. cerevisiae* all grew in this manner with media modified with PEG 200 (Anand and Brown 1968). For these yeasts, the  $\Psi_{\text{opt.}}$  is quite close to the  $\Psi_{\text{max.}}$  Studies of a range of soil *Chytridiomycotina* species has recently shown that species and strains of these fungi can tolerate about 5% salt (−4.2 MPa) in certain complex media (Gleason et al. 2006). Generally, growth decreased and eventually ceased as  $\Psi$  was increased (drier conditions). Although these fungi are quite sensitive, compared to the xerotolerant/xerophilic species described below, this does suggest that these lower fungi will survive wetting and drying fluxes in soil.

For some xerophilic fungi, the  $\Psi_{\text{opt.}}$  may be very different from the  $\Psi_{\text{max.}}$  For example, *Europodium* spp., *Xeromyces bisporous* and *Chrysosporium fastidium* have  $\Psi_{\text{opt.}}$  values at 25 °C of −7.0, −5.6 and −2.8 MPa respectively (Pitt and Hocking 1977; Magan and Lacey 1984a). However, it should be noted that optimum  $\Psi$  for growth will also be influenced by interaction with other environmental factors, particularly temperature, pH and gas composition (Magan and Lacey 1984a, b; Magan et al. 2004). Interactions between temperature and  $\Psi$  have been studied in detail, and Figure 6.5 shows examples of the effect of  $\Psi \times$ temperature interactions on the growth rates of a xerotolerant species. The isopleths represent points of similar rates of minimum, maximum and optimum growth temperature and  $\Psi$  for growth (mm/day). This shows a two-dimensional relationship between two very important environmental factors which can influence the ecological competence and competitive ability of different fungi (Magan and Lacey 1984c). It has been found that usually germination occurs at a slightly lower  $\Psi_{\text{min.}}$  than that for growth (Magan and Lacey 1984a). Indeed, Ayerst (1969) and Smith and Hill (1982) found the reciprocal of ger-

mination time to be significantly correlated with linear growth rates. However, others have surprisingly found that for some *Fusarium* spp. growth occurred over a wider  $\Psi$  range than for germination.

It is particularly important to consider not only single- but also two- and three-way interactions between environmental factors which might affect community structure within an ecosystem. This has seldom been examined in detail. However, using the stored grain ecosystem as a model, attempts have been made to predict the dominance of individual fungi, niche overlap, and temporal changes in community structure (Magan and Lacey 1984c, 1985; Magan et al. 2004; Marin et al. 2004). These studies showed that  $a_w$ , temperature, and substrate nutrient status all have a profound influence on antagonism, competitiveness and dominance of individual fungi. The production of secondary metabolites, mycotoxins, is also similarly affected and can contribute to the success of individual fungi by pre-emptive exclusion from a common resource. However, for many mycotoxicogenic species, the two-dimensional profiles for germination and growth have been found to be wider than for mycotoxin production (Sanchis and Magan 2004).

Scott (1957) suggested that the optimum  $\Psi$  for growth of osmotolerant fungi was dependent on the predominant solute used in the medium. However, a range of filamentous spoilage fungi have been found by Pitt and Hocking (1977) and Andrews and Pitt (1987) to grow similarly with different solutes. For some soil fungi, distinct differences in germination and growth optima have been found for germination and growth in relation to ionic/non-ionic osmotic solutes and matric potential (PEG 6000) alterations (Magan 1988). Nevertheless, Scott's (1957) suggestion that absolute growth rate was related to solute type has been borne out in a number of studies. The radial growth rates of fungi over a range of  $\Psi$  have been found to vary considerably for species of decomposer fungi, with lower  $\Psi_{\text{min.}}$  for growth with glycerol as solute than with KCl to modify the medium, perhaps because of the role of glycerol as a compatible solute at lowered  $\Psi$  (Luard 1983; Magan and Lynch 1986; Marin et al. 1999). Recent studies by Hallsworth et al. (2003) have suggested that chaotropic solutes (e.g. phenols, urea, ethanol and benzyl alcohols) can impose water stress, and have shown that this may be another form of water stress, in addition to osmotic responses of microorganisms. However, very few studies have compared these types of

stress, and assessed whether physiological mechanisms overcoming these are different.

### C. Adaptation to Water Potential

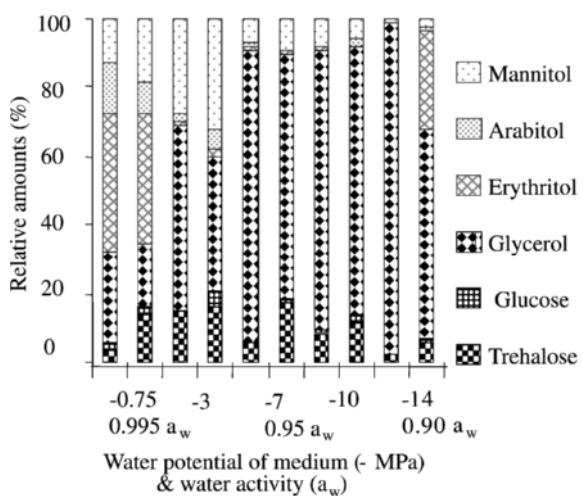
Growth under water stress, due to osmotic or matrix-potential effects, requires the maintenance of cell turgor for cell functioning, and growth and reproduction to occur. A shift to a high osmotic/water potential affects nutrient uptake, protein biosynthesis and a number of enzyme activities. To enable internal cell functioning, particularly of essential enzymes, fungi produce so-called compatible solutes, often polyhydric alcohols or organic acids. The polyols include glycerol, arabitol, erythritol and mannitol. The low-molecular weight polyol, glycerol, is particularly important as it is able to protect hydrated biopolymers and allows structural integrity under low-water potential conditions. Glycerol is more important than the other polyols because it produces a lower  $a_w$  at a given molar concentration, followed by arabitol, erythritol and then the higher-molecular weight polyol, mannitol. The physiology of osmophilic yeasts and of filamentous xerophilic fungi will be considered below in more detail, as the mechanisms for adaptation to water stress can be different.

### D. Yeast Physiology and Osmotic Stress

In yeasts, polyols are the main compatible solutes, glycerol being the predominant polyol and arabitol the minor one (Edgeley and Brown 1978). However, there are differences in the way yeast species use glycerol as a compatible solute. Sensitive isolates of *S. cerevisiae* have been found to both synthesize and secrete glycerol, thereby maintaining approx. the same ratio between intra- and extracellular glycerol concentrations (Edgeley and Brown 1978). Experiments in media containing 10% salt (approx.  $-7.0$  MPa water potential) showed almost a 40-fold increase in the enzyme glycerol-3-phosphate dehydrogenase. By contrast, the osmotolerant yeast *Zygosaccharomyces rouxii* retains a higher proportion of the same synthesized glycerol within the cell, indicative of a lower permeability of the plasma membrane. Van Zyl and Prior (1990) demonstrated that, in *Z. rouxii*, glycerol was actively transported into the cell via a carrier-mediated system with a high specificity for glycerol. An active glycerol transport system has also been demonstrated for another osmotolerant yeast, *Debaromyces hansenii*, which shows an increase

in the production and accumulation of glycerol in cells at lowered water potentials (Andre et al. 1988; Larsen et al. 1990), although the importance of the carrier system has not yet been determined. Recent studies on yeasts which are being used as biocontrol agents suggest that different polyols are accumulated by taxonomically different species. For example, *Candida sake* was shown to accumulate significant amounts of erythritol under water stress conditions (Teixido et al. 1998). By contrast, studies with a xerophilic yeast *Pichia anomala* showed that arabitol and the cryo-protectant trehalose were synthesized to much higher levels than under non-water stress conditions when a range of solutes were used (e.g. glucose, glycerol, NaCl, proline; Mokou and Magan 2002).

Studies with *S. cerevisiae* and *Z. rouxii* have shown that the trigger for glycerol synthesis may be  $K^+$  depletion, because of the transient rapid decrease when these yeasts are transferred from high-to low-osmotic potential media. By contrast, in the osmotolerant yeast *D. hansenii*,  $Na^+$  is excluded and  $K^+$  accumulated, so that the internal  $K^+ : Na^+$  ratio is much higher than that of the medium. However, glycerol accumulation is still probably more important in overcoming such stress. Work has been concentrated on the way in which yeast cells may sense and respond to osmotic stress. Mager and Varela (1993) have proposed the hypothesis that change in external osmolarity is probably sensed at the plasma membrane as a result of disturbance of ion gradients (e.g.  $Na^+$ ,  $K^+$ ,  $H^+$ ). This



**Fig. 6.6.** Impact of increased water stress imposed by NaCl on the relative accumulation of sugars and sugar alcohols by a xerophilic *Aspergillus flavus* strain (adapted from Neschi et al. 2004)

results in a loss in turgor pressure and a complex series of molecular events including the protein kinase cascade, leading to modification of enzyme activities and changes in gene expression. Part of this process may include synthesis of trehalose and certain heat shock proteins to help in recovery processes of the cell. At the same time, polyols such as glycerol are produced to restore the turgor pressure in the yeast cell.

### E. Xerophilic Filamentous Fungi

It is interesting to note that, compared with the wealth of information on physiological adaptation of yeasts to water stress, much less work has been carried out on filamentous fungi. Lower filamentous fungi have been found to lack polyols under water stress but rather to accumulate organic compounds such as proline in response to lowered water potentials. Luard (1982b) demonstrated that *Mucor hiemalis*, *Phytophthora cinnamomi* and *Pythium debaryanum* all synthesized proline when grown on media containing either ionic or non-ionic solutes to modify water potential. Although these fungi are not xerophilic and are quite sensitive to water stress, proline may act in a similar way to glycerol in yeasts, enabling enzymes to function efficiently. However, while osmotic tolerance of chytrids has been demonstrated, no information is available on the mechanisms of tolerance and whether high- or low-molecular weight polyols are synthesized by these species. For other filamentous fungi, work by Luard (1982a, b) has been particularly useful in understanding the relative importance of different polyols in enabling growth at low water potentials. This showed that the type of solute present in a medium may influence the major polyol accumulated in the mycelium of fungi. For example, the marine fungus *Dendryphiella salina* accumulates glycerol, mannitol and inositol when grown on media containing NaCl, MgCl and inositol to modify water potential. Luard showed that, for xerophilic fungi such as *Chrysosporium fastidium* and xerotolerant *Penicillium chrysogenum*, glycerol was the major polyol accumulated in the mycelium, with lower concentrations of arabitol and erythritol. As water potential was reduced, mannitol levels decreased, suggesting that it may function as a carbohydrate source or energy reserve for glycerol production. Studies by Hocking (1986) have also shown that xerophilic fungi accumulate glycerol during active growth

phases but that, when sporulation occurs, glycerol depletion can occur rapidly, suggesting that it could be acting as an energy reserve for the production of conidia. More recent work on non-xerophilic entomopathogenic fungi has demonstrated that, depending on the C:N ratio and solute used for modifying water potential, significant accumulations of glycerol, mannitol and erythritol can occur in conidia of *B. bassiana*, *M. anisopliae* and *P. farinosus* (Hallsworth and Magan 1994a, b), and can provide an ecological advantage in colonizing insects in the environment, particularly at lowered  $a_w$  (Hallsworth and Magan 1995, 1996).

Recent studies have examined whether matric stress actually does represent a bigger hurdle to overcome than does solute stress, by quantification of polyol accumulation in xerophilic mycotoxicogenic fungi such as the *Aspergillus ochraceus* and *A. flavi* group as well as pathogens such as *Fusarium graminearum* (Ramos et al. 1999; Neschi et al. 2004; Ramirez et al. 2004). Whereas growth was more sensitive to matric water stress, with slower growth rates and narrower ranges of matric potential for growth, the synthesis of polyols was not significantly different. These studies suggest that glycerol and sometimes erythritol are accumulated in significant amounts in the mycelial biomass and also in the spores under both solute and matric water stress. Figure 6.6 shows the effect of increasing water stress on relative accumulation of sugars and sugar alcohols in biomass of an *Aspergillus flavus* isolate. These results suggest that Griffin's (1981) original view that these types of stress have very different impacts on the physiology of such species may not hold for some xerophilic species. With the availability of genomic arrays, it may now be possible to confirm the similarity or differences between the impacts of solute and matric stress on the physiology of these fungi, by comparing up- and down-regulated genes under both conditions. This would also provide a better understanding of the function of these biosynthetic pathways in relation to active growth and survival in different ecosystems and in relation to secondary metabolite production. These may further enable species to maintain a competitive edge in naturally fluctuating environments.

### V. Anaerobic Fungi

In natural substrata, fungi are often exposed to atmospheric conditions which are aerobically not

ideal. Thus, many fungi are microaerophilic and able to actively colonize substrates under low-oxygen conditions. Such environments include aquatic habitats, wood, and man-made grain ecosystems where elevated CO<sub>2</sub> is sometimes used as part of controlled-atmosphere storage systems. Dematiaceus hyphomycetes, and some *Aspergillus* and *Penicillium* spp., are good examples of such fungi. Many other fungi are considered to be facultative anaerobes. Such fungi include *Saccharomyces cerevisiae*, and members of the Chytridiomycetes and Oomycetes, e.g. *Blastocladiella ramosa* and *Aqualinderella fermentans* (Held 1970; Gleason and Gordon 1989). Gleason and Gordon (1988) showed that, of four zygomycetes studied (*Mucor nevensis*, *Benjaminiella poitrasii*, *Mycotypha microspora* and *Dispira cornuta*), the former two were strict anaerobes growing under nitrogen. The others grew under microaerobic conditions under nitrogen. For such fungi to actively grow, anaerobic-specific exogenous supplies of nutrients such as fatty acids, sterols and vitamins are necessary (Bull and Bushell 1976; Gibb and Walsh 1980; Gleason and Gordon 1988).

In 1975, true anaerobic fungi, morphologically similar to Chytridiomycete fungi, were found in the rumen of sheep (Orpin 1975). Since then, several genera of anaerobic fungi have been isolated and identified from the rumen of herbivores. These fungi appear to generally have a vegetative stage and sporulate by the production of zoospores which can be mono- or polyflagellate. Because of their unusual morphological characters, including the presence of hydrogenosomes and fine structure, they have been placed in a separate family aptly called the Anaeromycetales. They are not parasites but symbiotic, as they utilize nutrients and also provide nutrients to the herbivore by the production of volatile fatty acids in the animal (Trinci et al. 1994).

Fungi such as *Neocallimastix frontalis*, *Piromonas communis* and *Spaeromonas communis* are confined to the herbivore rumen and thus need to compete effectively with a range of other microflora and fauna, including anaerobic and facultative anaerobic bacteria, anaerobic protozoa, and each other. The rumen is a very complex ecosystem and it is worthwhile comparing the populations of different organisms in the rumen to obtain a perspective on the possible interactions and competitive nature of the different components in this type of environment. The rumen contents commonly contain 10<sup>6</sup> protozoa, 10<sup>10</sup>

bacteria and 10<sup>6</sup> anaerobic fungi per gram. Thus, in this ecosystem, active competition occurs for the readily utilizable sugars which may be present in the structural components of plant material (Orpin and Ho 1992; Trinci et al. 1994). Effective competition by anaerobic fungi is also partially influenced by the pH of the rumen and its contents. The pH is usually in the range 5.7–7.0 and optimum growth of anaerobic fungi occurs at pH 6.0–7.0 (Orpin 1975) whereas the temperature range is relatively narrow and fluctuates only between 37–41 °C, depending on the herbivore species.

One of the factors which has made these fungi such effective inhabitants of this special habitat is their ability to rapidly catabolize plant material, predominantly cellulose and hemicellulose, to actively grow and multiply in the rumen. It has been suggested that approx. 8% of the rumen and hindgut microbial biomass is of fungal origin and contributes about 30% to cellulose degradation. The life cycle thus consists of two main stages: a vegetative stage when attached to fragments of digested plant material, and a sporulation stage when motile zoospores are released. These zoospores are very important in enabling anaerobic fungi to have a competitive advantage over other organisms. The zoospores, via a chemotactic response, are able to rapidly utilize the nutrients, particularly glucose, sucrose and fructose, in freshly ingested plant material. They are able to attach themselves to the material, enabling them to encyst rapidly. The hemicellulose and cellulose are utilized efficiently whereas the more recalcitrant lignified tissue is often less effectively metabolized.

Metabolism in anaerobic fungi has been studied predominantly in *Neocallimastix patriciarum*, and operates predominantly via a mixed-acid fermentation resulting in the production of volatile fatty acids and lactate. Other compounds produced include ethanol and succinate and, of course, CO<sub>2</sub> and hydrogen. There appears to be a close interrelationship between some bacteria, particularly methanogens, which can result in cross-feeding of fermentation products and more effective breakdown of the plant material.

Biotechnological exploitation of anaerobic fungi has now become important because of their ability to perform mixed-acid fermentation, which is normally carried out by bacteria. Thus, genes from anaerobic species such as *Piromyces* sp. E2 have been inserted into yeasts such as *Saccharomyces cerevisiae* for ethanol production. Recently, a beta-glucosidase gene was cloned from

the anaerobic species *Orpinomyces* PC-2; it has a significantly improved conversion of cellulose to fermentable sugars (Li et al. 2004). Because anaerobic fungi are capable of producing a range of enzymes including cellulases, cellobextrinase, xylanases, glycosidases and aryl esterases, which all enable efficient utilization of plant material in the herbivore rumen in a competitive habitat, this fungal ecological niche is being exploited for biotechnological applications. Orpin (1993) and Trinci et al. (1994) have detailed the importance of the production of these enzymes by anaerobic fungi, and which places them at a competitive advantage in this very specialized ecological niche.

## VI. Acidophiles and Alkalophiles

There are distinct ranges of hydrogen ion concentration over which biochemical and chemical processes in cells will effectively and efficiently be completed. Because the hydrogen ion concentration affects the ionic state and therefore the possible availability of inorganic ions and metabolites to the organism, it is critical in determining the metabolic activity of cells. Very high concentrations (acidic) or very low concentrations (alkaline) of hydrogen ions will have a profound effect on the activity and thus ability of organisms to effectively live in an environment. They function best at pH values close to neutrality. For example, extreme pH can result in the primary and secondary structure of proteins being irreversibly damaged. Thus, even if the external pH were to be extreme, the internal cellular pH must be maintained at close to neutrality for efficient cellular functioning. However, the possession of an osmotic barrier to the external environment can maintain the cytoplasmic components at a pH different to that of the surrounding substrate. It is this ability which has enabled fungi to become established in both extremely low- (close to 1) and high-pH (11) environments (Longworthy 1978).

### A. Acidophiles

Acidophiles have been defined as organisms which are able to grow down to pH 1.0 and are able to actively grow at pH < 4.0. Although acidophilic microorganisms are predominantly bacteria, particularly thiobacilli (Ingledeew 1990), a range of yeasts and filamentous fungi have been found to grow in very low-pH substrates. Good examples of

low-pH environments include geothermal regions with high hydrogen sulphide emissions, coal refuse tips, and acidic copper mine wastes. Most yeasts grow optimally at pH 5.5–6; some, including *Candida krusei*, *Rhodotorula mucilaginosa* and *Saccharomyces exigua*, can grow at pH 1.5–2.0 (Recca and Mrak 1952; Battley and Bartlett 1966). Species of *Saccharomyces*, *S. ellipsoideus*, *S. guttulata* and *S. cerevisiae* were demonstrated to actively grow at pH 2.5, 2.0 and 1.9 respectively (Pfaff et al. 1978).

The most acidophilic filamentous fungi reported to date are *Acontium velutum* and a *Cephalosporium* sp. which were isolated from laboratory media containing 2.5 N H<sub>2</sub>SO<sub>4</sub> (Starkey and Waksman 1943). Many other filamentous fungi have been shown to be able to grow at very low pH values. Many *Aspergillus*, *Eurotium*, *Fusarium* and *Penicillium* spp. can grow down to pH values of 2.0 but also have pH optima of up to 10. *Acontium pullulans* was isolated at pH 2.5 from acidic coal waste and acid streams by Belly and Brock (1974). Recent studies have attempted to examine metabolically active eukaryotic communities in acidic mine drainage systems by using molecular probes such as fluorescent in situ hybridization (FISH), and 18S rRNA and beta-tubulin gene phylogenies and probes for different genera (Baker et al. 2004; Lopez-Archilla et al. 2004). These studies have demonstrated that species such as *Dothideomycetes* and *Eurotiomycetes* and a number of other ascomycete fungi are present in acid mine drainage. Such culture-independent techniques are essential to obtain a better perspective on the fungal community structure in such extreme environments.

Detailed studies on intracellular pH homeostasis in xerophilic fungi (e.g. *Aspergillus niger*) are providing a better understanding of the capacity for such fungi to tolerate and remain active under extreme acidic conditions. By using real-time NMR and <sup>31</sup>P, it was possible to examine immobilized biomass in alginate pellets when exposed to different acidic conditions (Hesse et al. 2002). Vacuolar H<sup>+</sup> influx was observed in response to extreme cytoplasmic acidification, suggesting that homeostasis was operating in this organelle in *A. niger*. Interestingly, NMR spectra of citric acid-producing biomass showed that, even in the presence of a very low pH of 1.8 and a high acid-secreting capacity, the pH levels in the cytosol and vacuoles were maintained at pH 7.5 and 6.4 respectively. This provides evidence for the adaptation capacity of such fungi under extreme acidic stress.

## B. Alkalophiles

Akline environments include soda lakes, desert soils and alkaline springs where the pH can often be consistently at about pH 10. In many cases, the presence of ammonium carbonate, potassium carbonate, sodium borate or sodium orthophosphate is responsible for the alkaline nature of these environments. Alkalotolerant organisms have been defined as those which grow optimally at approx. pH 7 but are able to actively grow at pH values of up to 9–9.5. Alkalophilic organisms are defined as those which do not grow at pH < 8.5 or have optimum growth at two pH units above neutrality (Kroll 1990).

Many fungi are able to grow over a very wide range of pH values, and often between pH 2 and 11. However, many of these fungi, from 15 different genera including species of *Botrytis*, *Colletotrichum*, *Cladosporium*, *Fusarium*, *Penicillium* and *Paecilomyces*, are most probably alkalotolerant. For example, *Paecilomyces lilacinus* was described as being alkaliphilic and able to grow very well between pH 7.5–9.0. True alkaliphilic *Chrysosporium* spp. were isolated and described from bird nests, having a pH maximum for growth of pH 11. These fungi are specialized keratinolytic organisms living in a very specialized environment. Among the yeasts, *Exophila alcaliphila*, *Candida pseudotropicalis* and *Saccharomyces fragilis* have been described as being alkalotolerant. However, few examples of truly alkaliphilic yeasts exist.

## C. Mechanisms of Survival in Extreme pH Environments

The hydrogen ion ( $H^+$ ) is a very special cation because it is a proton with no electrons. In solution, it becomes hydrated to form the hydronium ion ( $H_3O^+$ ). At acid pH, this predominates whereas, at alkaline pH, the hydroxyl ion ( $OH^-$ ) is dominant. The protons and the membrane transport and bioenergetic processes are critical to the ability of acidophiles and alkalophiles to colonize such specialized environments. The ability to occupy these niches is determined largely by the ability of microorganisms, including fungi, to have pH controlling systems. This involves efficient trans-membrane transport systems, so that solutes needed to achieve intracellular modifications can be effectively utilized to maintain the membrane potential with respect to the outside environment.

This entails the efficient control of proton movement into and out of the cells, and the meeting of necessary energy requirements. It is also linked with the control of osmotic pressure, because of the involvement of cations and anions. However, practically no work has been carried out on the mechanisms involved in yeasts or filamentous fungi, having been carried out predominantly with acidophilic and alkalophilic bacteria. This aspect has been extensively reviewed by Longworthy (1978), Ingledew (1990) and Kroll (1990).

Mechanisms of internal pH control have been reported for bacteria such as *Streptococcus faecalis* where the control has been found to be completely due to the action of ATPase in increasing proton pump efficiency. For example, in extreme acidic conditions, the internal pH falls quickly, and ATP is used to rapidly pump protons out of the bacterial cells via ATPase to increase the internal pH of the cell. In alkaliphilic bacteria such as *Bacillus alcalophilus*, sodium ( $Na^+$ ) is utilized to reverse the pH gradient under extreme alkaline conditions. Adaptations of bacteria to enable growth in these environments include the possession of flagella, modifications of cell walls and membranes, and in biochemical activity including respiration and oxidative phosphorylation. The extensive studies on bacteria need to be extended to yeasts and filamentous fungi to enable a more clear understanding of their occupation of such ecological niches. However, the possible impact of pH must not be seen in isolation – organism activity will also be influenced by interactions between pH, water availability and osmotic potential, and temperature.

## VII. Irradiation and Fungi

The two types of radiation which fungi are exposed to are firstly, non-ionizing radiation due to solar radiation and ultraviolet (UV) light and secondly, ionizing radiation from natural and man-made sources. The most important component of non-ionizing radiation in the environment is UV-B light. Because of depletion of the ozone layer, exposure to UV-B light (290–320 nm) has increased and its impacts on plants and microorganisms is receiving more attention. Man-made irradiation sources include gamma-irradiation, which has been used for a long time as a method of preserving food, particularly that intended for human consumption (IAEA-FAO 1978). Microwave radiation has also

been used increasingly to enable more rapid drying of substrates and the destruction of pests and microorganisms, including fungi. However, specific groups of fungi which, for example, occupy environments where they are exposed particularly to natural radiation from UV light, such as plant surfaces (phyllosphere), often have dark pigmentation of both the mycelium and spores to increase survival potential.

Work on the effects of UV-B radiation on saprophytes and pathogens of plants has more recently received particular attention. For example, Ayres et al. (1996) showed that yeasts such as *Sporobolomyces roseus* and *Cryptococcus* spp. had different sensitivities to UV-B light. *S. roseus* was less sensitive than *Cryptococcus* sp. Spore germination of cereal plant pathogens such as *Septoria nodorum*, but not *S. tritici*, was inhibited by low doses of UV-B light. Interestingly, isolates from warmer climatic regions in North Africa were found to be less sensitive to UV-B fluxes than were UK isolates (Ayres et al. 1996). Thus, different strains of the same fungus may have evolved in very different ways in temperate and subtropical environments, where exposure to UV-B occurs for longer periods of time. While radiation damages DNA of microorganisms to some extent, some are able to repair radiation-induced damage relatively quickly to maintain essential metabolic functions. Thus, some fungi may protect themselves from natural radiation by, e.g. pigmentation; others may have developed rapid mechanisms for repair of damaged DNA.

Man-made radiation from sources such as gamma rays or microwaves splits water into free radicals which damage DNA and are highly toxic to a wide range of fungi. Therefore, the higher the water content, the more free radicals produced and the higher the level of DNA damage (Kiss and Farkas 1977). As mentioned above, a relatively large amount of information exists on the effect on fungi alone or those present on agricultural substrates exposed to various irradiation doses. Saleh et al. (1988) demonstrated that ten species of fungi from the genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium* and *Penicillium* were inactivated by doses of gamma-irradiation between 0.6 and 1.7 kGy. However, dematiaceous fungi with a high level of pigmentation were more resistant than moniliaceous species when tested in aqueous suspensions or on enriched agar media (Saleh et al. 1988). Other workers have found that the concentration required for the destruction of

fungi present on substrates, e.g. temperate and tropical grains, varied with both grain type and water content (Cuero et al. 1986; Ramakrishna 1991). On maize, barley and wheat, *Penicillium* and *Aspergillus* spp. were killed by doses of 0.3 and 1.2 kGy respectively, whereas 12 kGy was necessary to eliminate all filamentous fungi and yeasts (Cuero et al. 1986; Ramakrishna 1990; Hamer 1993). However, there are concerns about the efficacy of irradiation on mycotoxins. There are conflicting data with some studies reporting that an increase in mycotoxin formation occurs after irradiation. It has been suggested that fungal species in food matrices surviving irradiation may grow more rapidly in the absence of competitors. Other views are that irradiation can make more nutrients available and this could have an influence on the ability of surviving species to colonize such matrices rapidly (Farkas 1989; Monk et al. 1995; Shea 2000).

Microwave energy has also been used particularly in the agricultural and food industries for disinfection processes. Exposure of fungi directly or of fungi contaminating different agricultural substrata has been carried out (More et al. 1992; Cavalcante and Muchovej 1993). The microwave energy used is dependent on the frequency (often 1250 MHz) and the time period of exposure. For instance, a 30-s exposure at different power levels can represent energy inputs of between 2.25 and 18 kJ. As for gamma irradiation, moisture content of the medium can significantly influence resistance or sensitivity to the chosen energy level. In naturally contaminated sorghum grain, between 9–18 kJ of energy completely destroyed *Eurotium* spp., *Aspergillus candidus*, *A. niger*, *Penicillium* spp. and species of *Cladosporium* and *Alternaria* (More et al. 1992). Interestingly, germination of spores of *Aspergillus flavus*, *A. niger*, *Colletotrichum* spp., *Fusarium oxysporum* and *Bipolaris sorokiniana* on slides directly at 6, 9 and 18 kJ for periods of 0–7 min significantly reduced viability. Single-celled spores were more sensitive to microwave exposure than multi-celled spores, and dark-pigmented spores (*A. niger*, *B. sorokiniana*) were less affected by exposure to such concentrations of microwaves than were hyaline spores (Cavalcante and Muchovej 1993).

It may be that dark pigmentation forms sites which absorbed ionising or non-ionising radiation, thereby preventing more direct damage to DNA. However, bacterial mutants devoid of pigments were not found to be more sensitive

than wild types protected by pigments (Nasim and James 1978). It is now believed that DNA repair mechanisms are predominantly responsible for radiation resistance. The mechanisms of DNA repair involve both photo and dark repair, depending on whether visible light is involved. The reader is referred to the review by Strike and Osman (1993) for more detailed information on DNA repair mechanisms.

### VIII. Conclusions

This review has covered a wide range of highly complex stressed ecosystems which contain specialized communities of fungi. For some extreme environments, e.g. water, temperature stress, a wealth of information is available. The rapid development and availability of molecular-based techniques have allowed significant advance to be made in more detailed analyses of fungal communities based on direct measurements, as opposed to culture techniques only. The unravelling and public availability of whole genomes of yeasts and filamentous fungi, such as *Aspergillus fumigatus*, *A. flavus*, *A. nidulans* and now some *Fusarium* species, will enable much more detailed knowledge to be obtained on the function of gene clusters under different ecophysiological conditions which simulate or mimic natural fluxes in extreme environments, and will enable the switches and triggers involved in germination, growth, survival, secondary metabolite production and competitiveness in such highly complex ecosystems to be understood. An understanding of the functioning of fungi in these extreme environments could also provide a wealth of new enzymes, pigments and pharmaceutical leads which could be exploited biotechnologically.

### References

- Anand JC, Brown AD (1968) Growth patterns of so-called osmophilic and non-osmophilic yeasts in solutions of polyethylene glycol. *J Gen Microbiol* 52:205–212
- Andre L, Nilsson A, Adler L (1988) The role of glycerol in osmotolerance of the yeast *Debaryomyces hansenii*. *Arch Microbiol* 139:110–116
- Andrews S, Pitt JI (1987) Further studies on the water relations of xerophilic fungi, including the characterisation of some halophiles. *J Gen Microbiol* 133:233–238
- Ayerst G (1969) The effects of moisture and temperature on growth and spore germination in some fungi. *J Stored Prod Res* 5:127–141
- Ayres PG, Rasanayagam MS, Paul ND, Gunsekera T (1996) UV-B effects on leaf surface saprophytes and pathogens. In: Frankland JC, Magan N, Gadd GM (eds) *Fungi and environmental change*. Cambridge University Press, Cambridge, pp 32–50
- Baggerman WI, Samson RA (1988) Heat resistance of fungal spores. In: Samson RA, van Reenen-Hoekstra ES (eds) *Introduction to food-borne fungi*. Centraalbureau voor Schimmelcultures, Baarn, pp 262–267
- Baker BJ, Lutz MA, Dawson SC, Banfield PLB (2004) Metabolically active eukaryotic communities in extreme drainage. *Appl Environ Microbiol* 70:6264–6271
- Battle EM, Bartlett EJ (1966) A convenient pH-gradient method for the determination of the maximum and minimum pH for microbial growth. *Antonie Leeuwenhoek* 32:245–255
- Belly RT, Brock TD (1974) Widespread occurrence of acidophilic strains of *Bacillus coagulans* in hot springs. *J Appl Bacteriol* 37:175–177
- Bergero R, Girlanda M, Varese GC, Intili D, Luppi AM (1999) Psychrooligotrophic fungi from arctic soils of Frans Joseph Land. *Polar Biol* 21:361–368
- Beuchat LR (1988) Thermal tolerance of *Talaromyces flavus* ascospores as affected by growth medium and temperature and age and sugar content in the activation medium. *Trans Br Mycol Soc* 90:359–364
- Bull AT, Bushell ME (1976) Environmental control of fungal growth. In: Smith JE Berry DB (eds) *The Filamentous Fungi* vol II. Biosynthesis and metabolism. Arnold, London, pp 1–31
- Cavalcante MJB, Muchovej JJ (1993) Microwave irradiation of seeds and selected fungal spores. *Seed Sci Technol* 21:247–253
- Cochrane VW (1958) *Physiology of fungi*. Wiley, New York
- Cooke RC, Rayner ADM (1984) *Ecology of saprotrophic fungi*. Longman, London
- Cooke RC, Whipp JM (1993) *Ecophysiology of fungi*. Blackwell, London
- Crisan EV (1973) Current concepts of thermophilism and the thermophilic fungi. *Mycologia* 65:1171–1198
- Cuero R, Smith JE, Lacey J (1986) The influence of gamma irradiation and sodium hypochlorite sterilisation on maize microflora and germination. *Food Microbiol* 3:107–113
- Edgley M, Brown AD (1978) Response of xerotolerant and non-tolerant yeasts to water stress. *J Gen Microbiol* 104:343–345
- Farkas J (1989) Microbiological safety of irradiated foods. *Int J Food Microbiol* 9:1–15
- Fenice M, Selbmann L, Di Giambattista R, Federici F (1998) Chitinolytic activity at low temperature of an Antarctic strain (A3) of *Verticillium lecanii*. *Res Microbiol* 149:289–300
- Flanagan PW, Scarborough AM (1974) Physiological groups of decomposer fungi on tundra plant remains. In: Holding AJ, Heal OW, MacLean Jr SF, Flanagan PW (eds) *Soil organisms and decomposition in tundra*. Tundra Biome Steering Committee, Edmonton, pp 159–181
- Furhman JA, Anzam F (1980) Bacterioplankton secondary production estimates in coastal waters of British Columbia, Antarctica, and California. *Appl Environ Microbiol* 39:1085–1095

- Gibb E, Walsh JH (1980) Effect of nutritional factors and carbon dioxide on growth of *Fusarium moniliforme* and other fungi in reduced oxygen concentrations. *Trans Br Mycol Soc* 74:111–118
- Gleason FH, Gordon GLR (1988) Techniques for anaerobic growth of zygomycetes. *Mycologia* 80:249–252
- Gleason FH, Gordon GLR (1989) Further studies on anaerobic growth of zygomycetes. *Mycologia* 81:939–940
- Gleason FH, Midgely DJ, Petcher PM, McGee PA (2006) Can soil Chytridiomycota survive and grow in different osmotic potentials. *Mycol Res* 110:869–875
- Griffin DM (1981) Water and microbial stress. In: Alexander M (ed) *Advances in Microbial Ecology* vol 9. Plenum Press, New York, pp 91–136
- Hallsworth JE, Magan N (1994a) Effect of KCl concentration on accumulation of acyclic sugar alcohols and trehalose in conidia of three entomopathogenic fungi. *Lett Appl Microbiol* 18:8–11
- Hallsworth JE, Magan N (1994b) Effect of carbohydrate type and concentration on polyhydroxy alcohol and trehalose content of conidia of three entomopathogenic fungi. *Microbiology* 140:2705–2713
- Hallsworth JE, Magan N (1995) Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability. *Microbiology* 141:1109–1115
- Hallsworth JE, Magan N (1996) Culture age, temperature and pH affect the polyol and trehalose contents of fungal propagules. *Appl Environ Microbiol* 62:2435–2442
- Hallsworth JE, Heim S, Timmis MS (2003) Chaotropic solutes cause water stress in *Pseudomonas putida*. *Environ Microbiol* 5:127–1280
- Hamer A (1993) Dynamics of mould growth in stored grain. PhD Thesis, Cranfield University, Bedford
- Held AA (1970) Nutrition and fermentative energy metabolism of the water mould *Aqualinderella fermentans*. *Mycologia* 62:339–358
- Hesse SJA, Ruijter GJG, Dijkma C, Visser J (2002) Intracellular pH homeostasis in the filamentous fungus *Aspergillus niger*. *Eur J Biochem* 269:3485–3494
- Higgins SM, Lilly WW (1993) Multiple responses to heat stress by the basidiomycete *Schizophyllum commune*. *Curr Microbiol* 26:123–127
- Hocking AD (1986) Effect of water activity and culture age on the glycerol accumulation patterns in five fungi. *J Gen Microbiol* 132:269–275
- IAEA-FAO (1978) Food preservation by irradiation. IAEA-FAO Symp Proc vol 1, Wageningen
- Ingledew WJ (1990) Acidophiles. In: Edwards C (ed) *Microbiology of extreme environments*. Open University Press, Milton Keynes, pp 33–54
- Kerry E (1990) Effects of temperature on growth rates of fungi from subantarctic Macquarie Island and Casey, Antarctica. *Polar Biology* 10: 4–10
- Kiss I, Farkas J (1977) The storage of wheat and corn of high moisture content as affected by ionising radiation. *Acta Aliment* 6:93–214
- Kroll RG (1990) Alkalophiles. In: Edwards C (ed) *Microbiology of extreme environments*. Open University Press, Milton Keynes, pp 55–92
- Larsen C, Morales C, Gustafsson L, Adler L (1990) Osmoregulation of the salt-tolerant yeast *Debaryomyces hansenii* grown in a chemostat at different salinities. *J Bacteriol* 172:1769–1774
- Li X-L, Ljungdahl L, Ximenes E, Chen H, Felix C, Cotta M, Dien B (2004) Properties of a recombinant beta-glucosidase from the polycentric anaerobic fungus *Orpinomyces PC-2* and its application for cellulose hydrolysis. *J Appl Biochem Biotechnol* 113:2
- Longworthy TA (1978) Microbial life in extreme pH values. In: Kushner DJ (ed) *Microbial life in extreme environments*. Academic Press, London, pp 279–315
- Lopez-Archipilla AI, Gonzalez AE, Terron M, Amils R (2004) Ecological study of the fungal populations of the acidic Tinto River in southwestern Spain. *Can J Microbiol* 50:923–934
- Luard EJ (1982a) Accumulation of intracellular solutes by two filamentous fungi in response to growth at low steady state osmotic potential. *J Gen Microbiol* 128:2563–2574
- Luard EJ (1982b) Growth and accumulation of solutes by *Phytophthora cinnamomi* and other lower fungi in response to changes in external solute potential. *J Gen Microbiol* 128:2583–2590
- Luard EJ (1983) Activity of isocitrate dehydrogenase from three filamentous fungi in relation to osmotic and solute effects. *Arch Microbiol* 134:233–237
- Magan N (1988) Effect of water potential and temperature on spore germination and germ-tube growth in vitro and on straw leaf sheaths. *Trans Br Mycol Soc* 90:97–107
- Magan N, Aldred D (2006) Environmental fluxes and fungal interactions: maintaining a competitive edge. In: Van West P, Avery S (eds) *Stress in yeasts and filamentous fungi*. Elsevier, Amsterdam (in press)
- Magan N, Lacey J (1984a) Effect of temperature and pH on water relations of field and storage fungi. *Trans Br Mycol Soc* 82:71–81
- Magan N, Lacey J (1984b) Effect of gas composition and water activity on growth of field and storage fungi and their interactions. *Trans Br Mycol Soc* 82:305–314
- Magan N, Lacey J (1984c) Effect of water activity, temperature and substrate on interactions between field and storage fungi. *Trans Br Mycol Soc* 82:83–93
- Magan N, Lacey J (1985) Interactions between field and storage fungi. *Trans Br Mycol Soc* 85:29–37
- Magan N, Lynch JM (1986) Water potential, growth and cellulolysis of soil fungi involved in decomposition of crop residues. *J Gen Microbiol* 132:1181–1187
- Magan N, Sanchis V, Aldred D (2004) Role of spoilage fungi in seed deterioration. In: Aurora DK (ed) *Fungal biotechnology in agricultural, food and environmental applications*. Marcel Dekker, New York, pp 311–323
- Mager WH, Varela JCS (1993) Osmostress response of the yeast *Saccharomyces*. *Mol Microbiol* 10:253–258
- Maheshwari R, Bharadwaj G, Bhat MK (2000) Thermophilic fungi: their physiology and enzymes. *Microbiol Mol Biol Rev* 64:461–488
- Marin S, Sanchis V, Ramos AJ, Magan N (1999) Two-dimensional profiles of fumonisin B1 production by *Fusarium moniliforme* and *F. proliferatum* spp. in relation to environmental factors and potential for modelling toxin formation in maize grain. *Int J Food Microbiol* 51:159–167
- Marin S, Magan N, Ramos AJ, Sanchis V (2004) Fumonisin-producing strains of *Fusarium*: a review of their eco-physiology. *J Food Prot* 67:1792–1805

- Mazur P (1966) Studies of the effects of subzero temperatures on the viability of spores of *Aspergillus flavus*. I. The effect of rate of warming. *J Gen Physiol* 39:869–874
- Mokiou S, Magan N (2002) Ecophysiological manipulation of fermentation improves viability of the biocontrol yeast *Pichia anomala*. *IOBC Bull* 25:395–398
- Monk JD, Beuchat LR, Doyle MP (1995) Irradiation inactivation of food-borne microorganisms. *J Food Prot* 58:197–208
- More HG, Magan N, Stenning BC (1992) Effect of microwave heating on quality and mycoflora of sorghum. *J Stored Prod Res* 28:251–256
- Morita RY (1974) Hydrostatic pressure effects on microorganisms. In: Colwell RR, Morita RY (eds) *Effect of the ocean environment on microbial activities*. University Park Press, Baltimore, MD, pp 133–138
- Morris GJ, Clarke A (1987) Cells at low temperature. In: Grout BWW, Morris GJ (eds) *The effects of low temperatures on biological systems*. Arnold, London, pp 72–84
- Morris GJ, Smith D, Coulson GE (1988) A comparative study of the changes in the morphology of hyphae during freezing and viability upon thawing for twenty species of fungi. *J Gen Microbiol* 134:2897–2906
- Nasim A, James AP (1978) Life under conditions of high irradiation. In: Kushner DJ (ed) *Microbial life in extreme environments*. Academic Press, New York, pp 409–439
- Neschi A, Etcheverry M, Magan N (2004) Osmotic and matrial potential effects on growth and compatible solute accumulation in *Aspergillus section flavi* strains from Argentina. *J Appl Microbiol* 96:965–972
- Orpin CG (1975) Studies on the rumen flagellate *Neocallimastix frontalis*. *J Gen Microbiol* 91:249–262
- Orpin CG (1993) Anaerobic fungi. In: Jennings DH (ed) *Stress tolerance of fungi*. Marcel Dekker, New York, pp 257–273
- Orpin CG, Ho YW (1992) Ecology and function of the anaerobic rumen fungi. In: Ho YW, Wong HK, Addullah N, Tajuddin ZA (eds) *Recent advances on the nutrition of herbivores*. Malaysian Society of Animal Production, Kuala Lumpur, pp 163–170
- Pfaff HS, Miller MW, Mrak EM (1978) *The life of yeasts*, 2nd edn. Harvard University Press, Cambridge, MA
- Pitt JI (1975) Xerophilic fungi and the spoilage of food of plant origin. In: Duckworth RB (ed) *Water relations of food*. Academic Press, London, pp 273–307
- Pitt JI, Hocking AD (1977) Influence of solutes and hydrogen ion concentration on the water relations of some xerophilic fungi. *J Gen Microbiol* 101:35–40
- Plesofsky-Vig N, Brambl R (1993) Heat shock proteins in fungi. In: Jennings DH (ed) *Stress tolerance of fungi*. Marcel Dekker, New York, pp 45–68
- Ramakrishna N, Lacey J, Smith JE (1991) Effect of surface sterilisation, fumigation and gamma irradiation on the microflora and germination of barley seeds. *Int J Food Microbiol* 13:47–54
- Ramirez ML, Chulze SN, Magan N (2004) Impact of osmotic and matrial water stress on germination, growth, mycelial water potentials and endogenous accumulation of sugars and sugar alcohols by *Fusarium graminearum*. *Mycologia* 96:470–478
- Ramos AJ, Magan N, Sanchis V (1999) Osmotic and matrial potential effects on growth, sclerotial production and partitioning of polyols and sugars in colonies and spores of *Aspergillus ochraceus*. *Mycol Res* 103:141–147
- Recca J, Mrak EM (1952) Yeast occurring in citrus products. *Food Technol* 6:450–454
- Robinson CH (2001) Cold adaptation in Arctic and Antarctic fungi. *New Phytol* 151:341–353
- Saleh YG, Mayo MS, Ahearn DG (1988) Resistance of some common fungi to gamma irradiation. *Appl Environ Microbiol* 54:2134–2135
- Sanchis V, Magan N (2004) Environmental profiles for growth and mycotoxin production. In: Magan N, Olsen M (eds) *Mycotoxins in food: detection and control*. Woodhead, Cambridge, pp 174–189
- Schmidt-Nielsen S (1902) *Psychrophile Mikroorganismen und ihr Vorkommen*. Centr Bakteriol Parasitenk Abt II 9:145–147
- Scott WJ (1957) Water relations of food spoilage microorganisms. *Adv Food Res* 7:83–127
- Smith D (1993) Tolerance to freezing and thawing. In: Jennings DH (ed) *Stress tolerance of fungi*. Marcel Dekker, New York, pp 145–172
- Smith SL, Hill ST (1982) Influence of temperature and water activity on germination and growth of *Aspergillus restrictus* and *A. versicolor*. *Trans Br Mycol Soc* 49:558–560
- Smith D, Coulson GE, Morris GJ (1986) A comparative study of the morphology and viability of hyphae of *Penicillium expansum* and *Phytophthora nicotianae* during freezing and thawing. *J Gen Microbiol* 132:2013–2021
- Snider CS, Hsiang T, Zhao G, Griffith M (2000) Role of ice nucleation and antifreeze activities in pathogenesis and growth of snow molds. *Phytopathology* 90:354–361
- Starkey RL, Waksman SA (1943) Fungi tolerant to extreme acidity and high concentrations of copper sulfate. *J Bacteriol* 45:509–519.
- Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimatization. *Annu Rev Plant Physiol* 35:543–569
- Strike P, Osman F (1993) Fungal response to DNA damage. In: Jennings DH (ed) *Stress tolerance of fungi*. Marcel Dekker, New York, pp 297–338
- Tansey MR, Brock TD (1973) *Dactylaria gallopava*, a cause of avian encephalitis, in hot spring effluents, thermal soils and self-heated coal waste piles. *Nature* 242:202–203
- Teixido N, Vinas I, Usall J, Magan N (1998). Improving ecological competence and environmental stress tolerance of the biocontrol yeast *Candida sake* by modifications of intracellular polyol and sugar content. *Mycol Res* 102:1409–1417
- Tibbet M, Sanders FE, Cairney JWG (1998a) The effect of temperature and inorganic phosphorous supply on growth and acid phosphatase production in arctic and temperate strains of ectomycorrhizal *Hebeloma* spp. *Mycol Res* 102:129–135
- Tibbet M, Grantham K, Saunders FE, Cairney JWG (1998b) Induction of cold active acid phosphomonoesterase activity at low temperature in psychrophilic ectomycorrhizal *Hebeloma* spp. *Mycol Res* 102:1533–1539

- Tibbett M, Sanders FE, Cairney JWG, Leake JR (1999) Temperature regulation of extracellular proteases in ectomycorrhizal fungi (*Hebeloma* spp.) grown in axenic culture. *Mycol Res* 103:707–714
- Trinci APJ, Davies DR, Gull K, Lawrence MI, Bonde Nielsen B, Rickers A, Theodorou MK (1994) Anaerobic fungi in herbivorous animals. *Mycol Res* 98:129–152
- van Zyl PJ, Prior BA (1990) Water relations of polyol accumulation by *Zygosaccharomyces rouxii* in continuous culture. *Appl Microbiol Biotechnol* 33:231–235
- Vishniac HS, Hempfling WP (1979) Evidence of an indigenous microbiota (yeast) in the dry valleys of Antarctica. *J Gen Microbiol* 112:301–314
- Weinstein RN, Palm ME, Johnstone K, Wynn-Williams DD (1997) Ecological and physiological characterization of *Humicola marvinii*, a new psychrophilic fungus from fellfields in the maritime Antarctic. *Mycologia* 89:706–711
- Weinstein RN, Montiel PO, Johnstone K (2000) Influence of growth temperature on lipid and soluble carbohydrate synthesis by fungi isolated from fellfield soil in the maritime Antarctic. *Mycologia* 92:222–229
- Widden P, Parkinson D (1978) The effects of temperature on growth of four high Arctic soil fungi in a three-phase system. *Can J Microbiol.* 24:415–21

---

# 7 Biogeography and Conservation

E.J.M. ARNOLDS<sup>1</sup>

## CONTENTS

I. Introduction .....	105
II. Mapping of Fungi .....	105
A. Methods .....	105
B. Maps and Scales .....	106
III. Distribution Patterns .....	107
A. Global Distribution .....	107
B. Local Endemics .....	111
C. Continental Patterns .....	112
D. National and Regional Patterns .....	113
E. Local Patterns .....	114
F. Recent Changes in Distribution Patterns .....	115
IV. Expansion of Distribution Areas .....	115
A. Introductions of Plants .....	115
B. Introductions of Fungi .....	116
C. Decline and Extinction of Fungi .....	116
V. Conservation of Fungi .....	117
A. Red Listing .....	118
B. Threatened Fungi and Their Habitats .....	119
C. Mycological Reserves and Nature Management .....	120
D. Harvests of Wild Edible Mushrooms and Legal Protection .....	120
VI. Conclusions .....	121
References .....	122

## I. Introduction

Biogeography is the study of distribution patterns of organisms in space and time, including the study of factors determining these patterns. These factors comprise actual conditions such as climate, soil, the availability of hosts and substrates, and dispersal capacity of species as well as historical conditions, including geological and evolutionary processes. Biogeographical studies on fungi are relatively scarce, due mainly to methodological problems. A bibliography on distribution maps of fungi has been published by Kreisel in Feddes Repertorium from 1970 onwards. Information on distribution patterns is important for our understanding of evolutionary processes and patterns of biodiversity but also in such practical disciplines as control

of crop pests, plantation forestry and nature conservation. In view of the increasing disturbance of ecosystems by human activities and the growing public focus on applied research, conservation of fungi has become a topic of rising interest.

## II. Mapping of Fungi

### A. Methods

Mapping of fungi is basically a simple procedure: the collecting of records of a certain taxon in a certain area from different sources and plotting these on a topographical map. However, in practice, this work is hampered by a number of complications proper to fungi (Kreisel 1985; Redhead 1989; Pringle and Vellinga 2006):

1. *Problems in detection of mycelia* Ideally, mapping of fungi should be based on observations of mycelia, since mycelia are functionally the most important part of a fungus. However, mycelia are usually living within the substrate and can only rarely be identified in the field using morphological characters, e.g. some genera forming mycelial strands, such as the honey fungus. In addition, ectomycorrhizal fungi may be identified by characteristic structures on root tips of host plants (Horton and Bruns 2001). Recently, molecular techniques have been developed to identify mycelia in soil samples (Landeweert et al. 2003). Most saprotrophic microfungi can be isolated and identified only by special techniques (Gams 1992).

The above methods of detection depend on the analysis of small soil samples in the order of 100 cm<sup>3</sup>. Therefore, these methods are effective only in small-scale plots, e.g. a forest stand. Because of the extremely small size of the samples relative to the volume of the substrate, it is very

<sup>1</sup> Holthe 21, 9411 Beilen, The Netherlands

- unlikely that less-common species be detected by these techniques.
2. *Problems in detection of sporocarps* Sporocarps can be observed and identified much more easily than mycelia, in particular of macro-fungi. Therefore, most mapping programmes are based on inventories of these propagation structures. Complications are the ephemeral character of most sporocarps and the strong annual fluctuations in sporocarp production. In addition, some groups of ascomycetes and basidiomycetes produce hypogeous sporocarps which are difficult to detect for that reason (Lawrynowicz 1990). A more fundamental objection against this approach is that the distribution of sporocarps may not necessarily reflect the distribution of mycelia. Some species may be common in a vegetative state but rarely produce sporocarps, and vice versa. For instance, at the community level it has been demonstrated that 70% of the ectomycorrhizal root tips in a Sitka spruce plantation were occupied by the corticioid fungus *Tylospora fibrillosa* (Burt) Donk whereas the bulk of ectomycorrhizal sporocarps were produced by agarics (Taylor and Alexander 1991).
3. *Defective taxonomic knowledge* Many groups of fungi are in need of critical revision, and numerous taxa still have to be described. For instance, in well-investigated Europe the number of known species of the agaric genus *Entoloma* increased between 1992 and 2004 by 39%, from 246 to 342 (Noordeloos 2004). In addition, taxonomic concepts are still changing, also because of new molecular techniques. Among the 50 species mapped in Europe by Lange (1974), selected because they were "well defined and easy to identify", at least four species are now regarded as species complexes, e.g. *Armillaria mellea* (Vahl: Fr.) Kumm. This fungus proved to comprise five biological species with different distribution patterns in Europe (Kile et al. 1991). Problems on conspecificity become more complex when distant areas are compared, such as North America, Europe and East Asia (Redhead 1989).
4. *Availability of data* Distributional data come from herbarium collections, records in the literature, databases and unpublished observations in notebooks, etc. Reliable data are scanty in most parts of the world. Even in relatively well-investigated Europe, the data are often inappropriate to provide realistic distribution patterns (Lange 1974).
5. *Accessibility of data* Even if data are available, it may be problematic to trace all relevant information. This problem can be solved by establishment of central databases for records of fungi, at present realized mainly at a national level, e.g. in Germany, Great Britain, Sweden, The Netherlands, Belgium and Australia. Indeed, the gaps in our knowledge are still enormous but the situation at present is not so hopeless that "writing an essay on the geographical distribution of fungi is to attempt to accomplish an impossible task" (Pirozynski 1968).
- In practice, two different, although not clearly separated, approaches exist to mapping of fungi. In the monographic approach, specialized taxonomists collect records on a particular group of fungi, usually only herbarium collections and in combination with a taxonomic revision (Kreisel 1967; Demoulin 1971; Lawrynowicz 1990). The distribution patterns are therefore reliable in a taxonomic context but, at the same time, relatively inaccurate because of the limited data. The second approach concerns mapping programmes, which are usually carried out on a national or regional scale. Such programmes attempt to collect as many records as possible on the occurrence of selected (but not necessarily taxonomically related) species (Lange 1974) or all species in a certain area (Kriegsteiner 1991; Nauta and Vellinga 1993). The data come from different sources, including literature records and unpublished observations, and are collected by many mycologists, both professional and amateur. As a result, the maps are less reliable from a taxonomic point of view but more accurate because of the larger number of observations.

## B. Maps and Scales

Distributional studies are carried out and presented at different geographical scales: (1) global patterns may reveal relations to macroclimate, host distribution and historical factors (Fig. 7.1); (2) continental patterns may, in addition, be related to mesoclimate (e.g. mountain ranges) and large-scale edaphic patterns, e.g. zonal soils (Figs. 7.2, 7.3 and 7.4); (3) regional patterns, often studied within the political borders of a country, may express more subtle differences of the landscape – for instance, patterns of alluvial

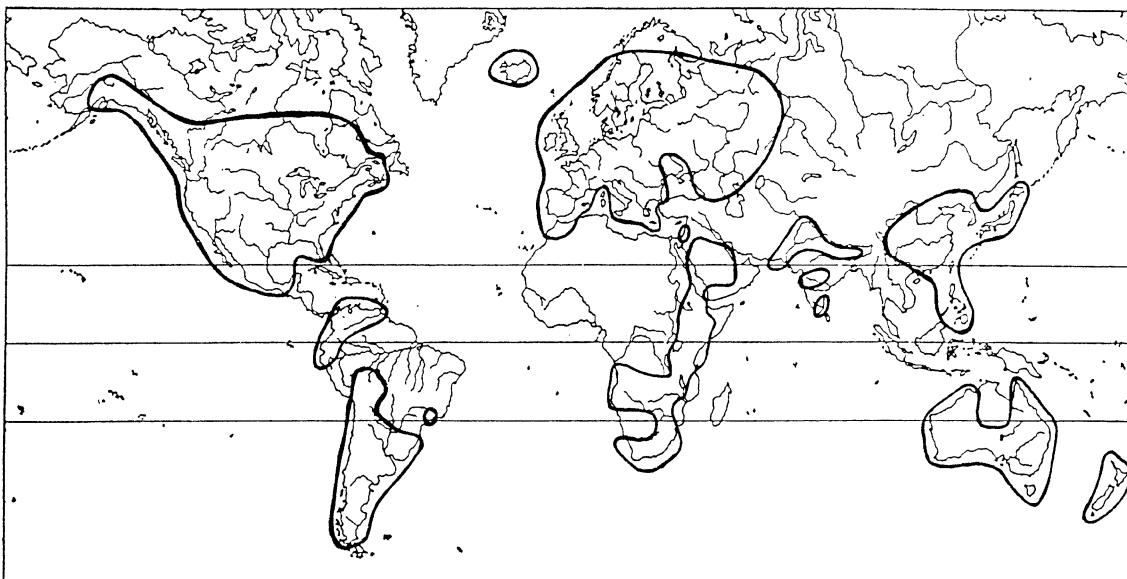


Fig. 7.1. World distribution of *Phaeosphaeria nodorum* (E. Müller) Hedjar (CAB International Mycological Institute 1992)

deposits in river valleys or human influence by agriculture or forestry (Figs. 7.5, 7.6); (4) local patterns are studies within a single landscape or stand, and may be related to microclimate, and distribution patterns of plant communities and individual plants (Fig. 7.7).

Results of biogeographical studies can be presented on different kinds of maps (Kreisel 1985):

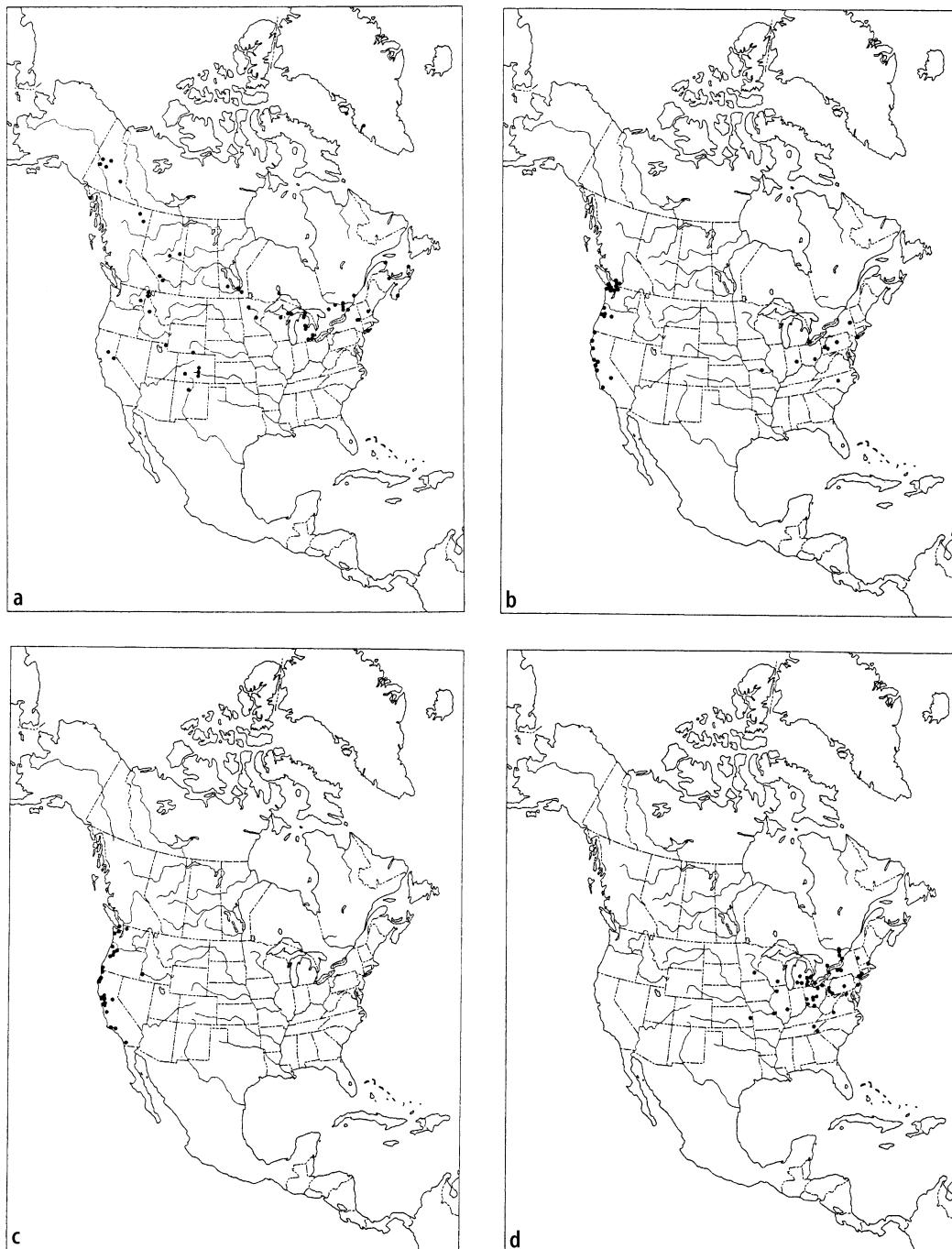
1. *Outline maps*, where borders of the known distribution areas are indicated with a line (Fig. 7.1). One disadvantage here is that the original records are not indicated. Consequently, it is impossible to detect possible differences in density inside a given area and to provide an alternative interpretation of the distribution.
2. *Dot maps*, where each record or locality is indicated by a separate sign (Fig. 7.2). This method is more accurate and objective than an outline map, the accuracy being slightly influenced by the size of the dot (Lange 1974). However, when research efforts are not evenly spread over the mapped area – which is often the case – concentrations of dots may be artefacts, marking rather the activities of mycologists than the abundance of the fungus (Kreisel 1985). Dot maps can be combined with outline maps.
3. *Grid maps*, where the records are plotted in a topographical grid with units of a given constant size (Figs. 7.3, 7.5 and 7.6). The accuracy

of grid maps is intermediate between that of dot maps and outline maps, and depends on the mesh size of the grid. It varies with the size of the investigated area: usual grid units are, for instance,  $50 \times 50$  km for Europe (Fig. 7.4),  $12 \times 13$  km for Germany (Fig. 7.5) and  $4 \times 4$  km for Belgium (Fig. 7.6). The applied grid may be a national grid indicated on topographical maps of a country (Fig. 7.5) or an international grid – for instance, in Europe the UTM grid (Figs. 7.4, 7.6), also used for the mapping of vascular plants and invertebrates. The lines of the grids can be deleted thereafter (Fig. 7.4). The different grids used in different European countries hamper an easy integration of data on a continental scale.

### III. Distribution Patterns

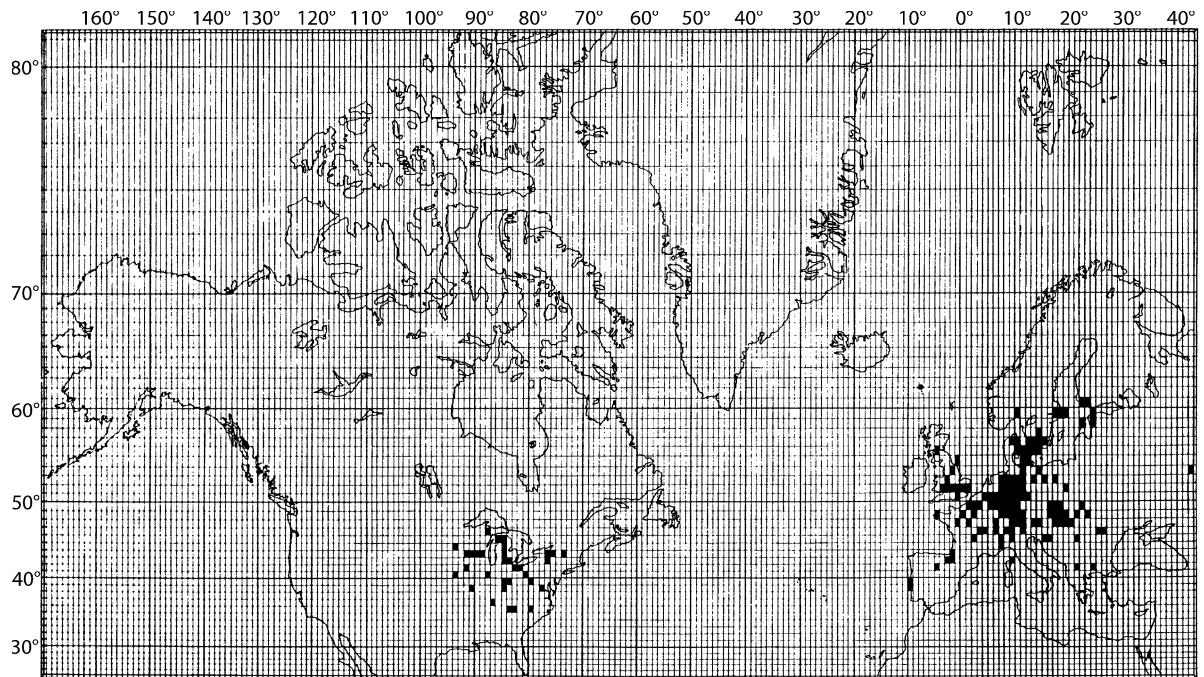
#### A. Global Distribution

Maps on the world distribution of plant pathogenic fungi are published by CMI and revised at irregular intervals – for example, the map of *Phaeosphaeria nodorum* (E. Müller) Hedjar, an ascomycete occurring on various grasses, including cereals (Fig. 7.1). Nowadays, it has a subcosmopolitan distribution, following cultivated host plants almost everywhere. The original range is difficult to trace, however, as in many other pathogens on



**Fig. 7.2.** Examples of distribution patterns of agarics in North America. **a** *Marasmius epiphyllus* (Pers.: Fr.) Fr. with boreotemperate distribution. **b** *Marasmiellus candidus* (Bolt.: Fr.) Sing. with bicoastal dis-

tribution. **c** *Marasmius plicatus* Pech with western temperate distribution. **d** *Marasmius pyrrhocephalus* Berk. with eastern temperate distribution (Redhead 1989)

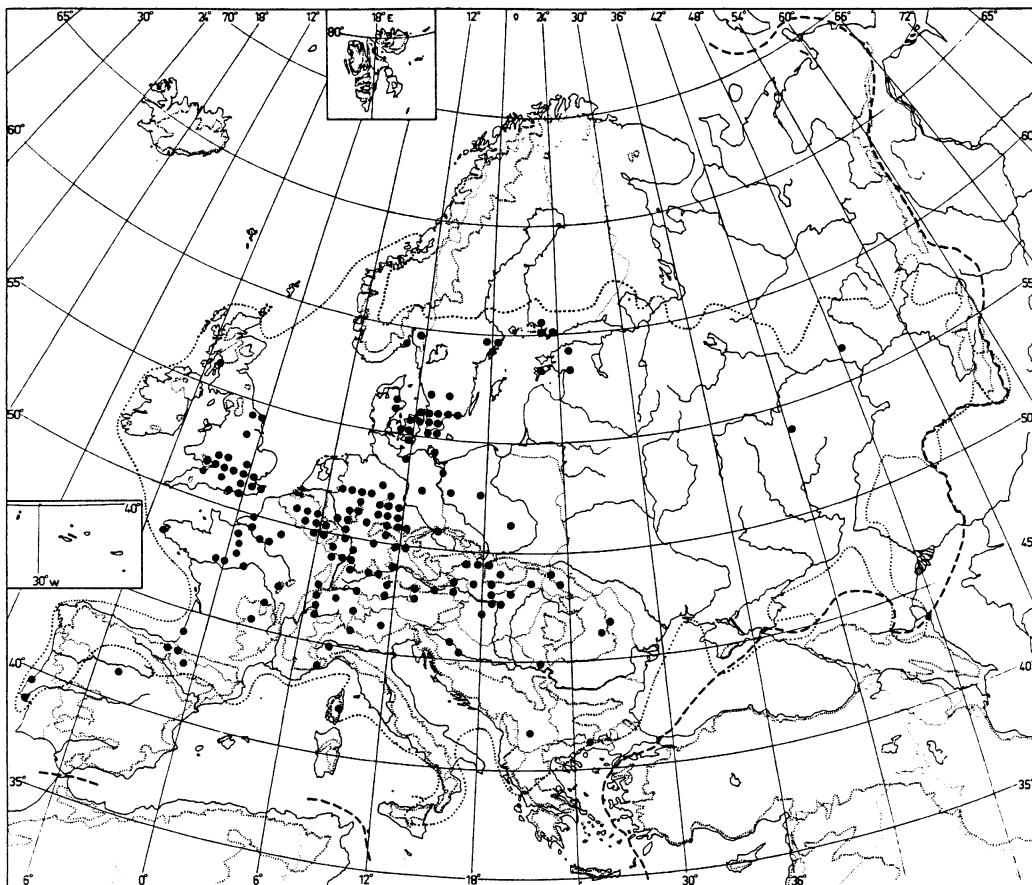


**Fig. 7.3.** World distribution of *Lycoperdon echinatum* Pers.: Pers. (in Europe) and its vicariant *L. americanum* Demoulin (in America). Each rectangle represents one degree latitude × one degree longitude (Demoulin 1987)

cultivated crops. Data on the global distribution of non-pathogenic fungi depend largely on the availability of world monographs. General ideas on distribution patterns of fungi have evolved in the course of time (Demoulin 1971). In the 19th century, it was thought that most fungal species inhabit small areas, comparable to those of phanerogams. In contrast, in the first decades of this century, it was generally assumed that most fungi are (sub)cosmopolitan. Since 1930, these opposite viewpoints have merged into the notion that few species are truly cosmopolitan but that, nevertheless, distribution areas of fungi are larger than the ranges of most vascular plants (Diehl 1937). In addition, distribution areas of soil-inhabiting and marine microfungi seem to be generally larger than those of larger fungi (Pirozynski 1968; Volkmann-Kohlmeyer and Kohlmeyer 1993).

Data on global patterns in some monographed groups of macrofungi are summarized in Table 7.1. The subdivision into distribution types is necessarily strongly simplified. Ryvarden (1991) stressed the large distribution areas of genera of polypores, being mostly either (sub)cosmopolitan (23%), circumpolar (26%) or pantropical (17%). Relatively few genera are endemic to one continent where they are usually widely distributed, in

contrast to many genera of phanerogams with very restricted distribution areas. Redhead (1989) stated that transatlantic disjunction patterns are, in general, exhibited by vascular plants at the generic or family level but by agarics at the species level, just as they are for lichenized ascomycetes and bryophytes. Hallenberg (1991) drew similar conclusions on the basis of distribution patterns of Corticiaceae. In *Lentinus* Fr., most species (78%) are tropical but only very few species (3%) are pantropical or (sub)cosmopolitan (5%; Pegler 1983a). Pantropical species seem to be lacking in *Thelephora* Ehrh.: Fr. whereas 39% of the species are endemic to Southeast Asia (Corner 1968). The proportions of supracontinental distribution areas are also low for *Bovista* Pers.: Pers. and *Scleroderma* Pers.: Pers., although these puffballs produce enormous amounts of airborne spores (Kreisel 1967; Guzman 1970). The number of (sub)cosmopolitan species among *Ascobolus* Pers. and *Saccobolus* Boud. is very high (Van Brummelen 1967). This may be due to their specialized ecology: most species grow on dung, and ascospores must pass through the intestines of grazing animals. On the other hand, dispersal of the heavy spores over large distances seems unlikely. Many species may, in fact, have spread by introduction of cattle



**Fig. 7.4.** Distribution of *Lycoperdon echinatum* Pers.: Pers. in Europe according to UTM grid. Each dot represents

a square of 50 × 50 km. The dashed line indicates the distribution area of *Quercus robur* L. (Demoulin 1987)

to other continents. In contrast, the proportion of European species is also very high; this may be due to undercollecting in other parts of the world.

Distribution patterns of fungi are, in general, so similar to the ranges of phanerogams, albeit at different taxonomic levels, that they are likely to be determined by the same environmental and historical factors. Some data supporting the importance of geological phenomena are:

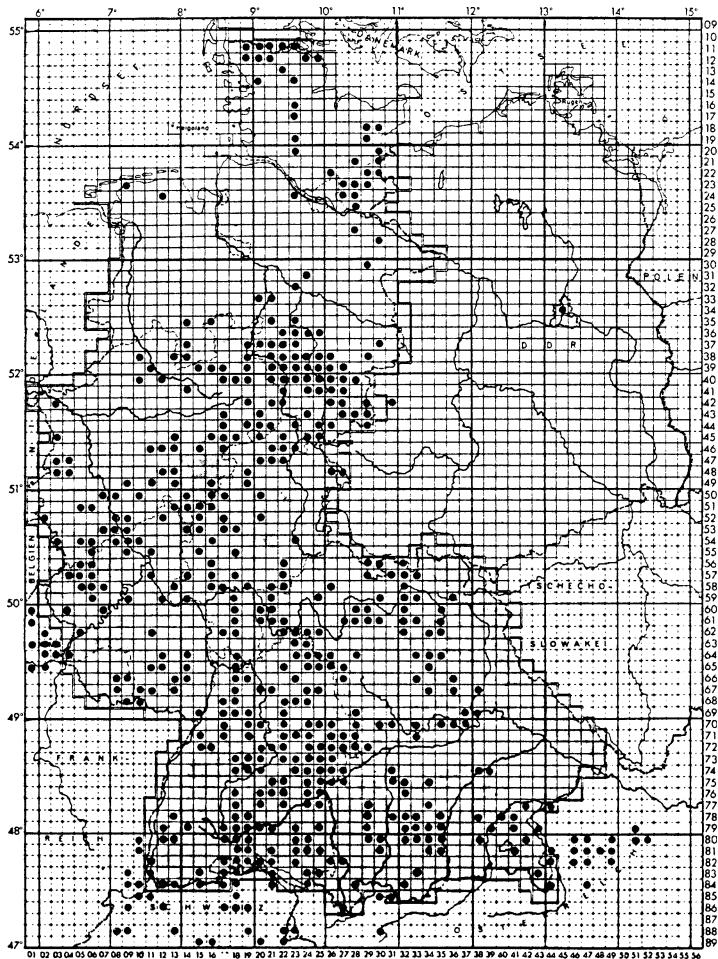
1. Some species and genera are common to southern South America and Australia or New Zealand, e.g. mycorrhizal symbionts of *Nothofagus*. They may be considered as remnants of populations fragmented by the break up of Gondwanaland approximately 100 million years ago (Horak 1983; Ryvarden 1991).
2. A number of species show a disjunct distribution in Europe and (eastern) North America, or two closely related vicariant species exist in

these areas. These patterns may be explained by the opening of the Atlantic in the Eocene (Demoulin 1973).

The species diversity of most taxonomic groups is considerably larger in North America than in Europe, which may be caused by different possibilities for reaching refugia during Pleistocene glacial periods (Redhead 1989).

The ideas on historical events are not in conflict with palaeontological evidence. Fossil basidiomycetes are known from the Middle Pennsylvanian, approximately 300 million years b.p. (Dennis 1970).

An alternative hypothesis for the explanation of supracontinental ranges of fungi is the existence of efficient long-distance spore dispersal (Redhead 1989; Hallenberg 1991; Ryvarden 1991). Arguments against this hypothesis are (1) the large majority of spores are deposited in close proximity to the



**Fig. 7.5.** Distribution of *Lycoperdon echinatum* Pers.: Pers. in Germany. Each square represents  $12 \times 13$  km (Kriegsteiner 1991)

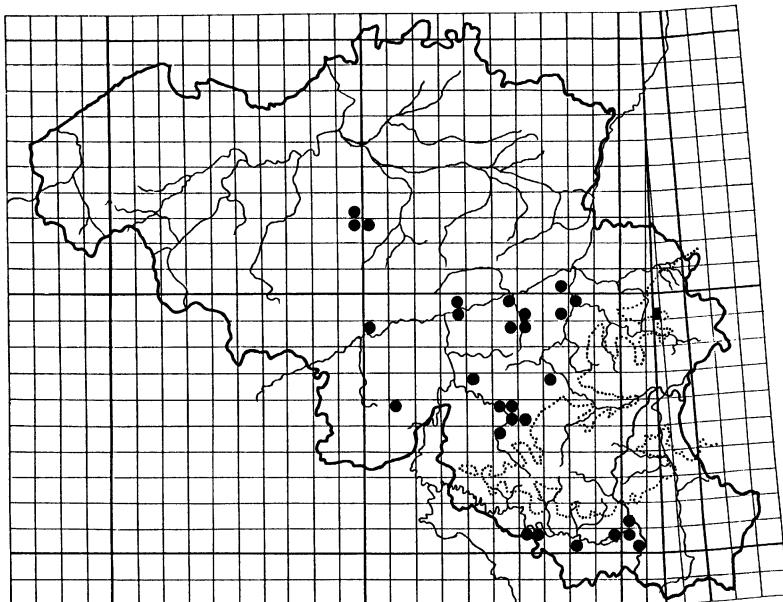
source; (2) most spores are not viable after a stay in higher atmospheric strata; (3) the chance of reaching an appropriate substrate and microhabitat is small in many cases; (4) many basidiomycetes must establish a dikaryon; the chance that two compatible colonies are formed close to each other is extremely small; (5) wind dispersal by spores does not necessarily imply genetic exchange between allopatric populations.

Consequently, also for most fungi, dispersal is likely to be restricted by geographic barriers, such as oceans and mountain ranges. Circumstantial evidence is given by some pathogenic fungi which were introduced to other continents and caused catastrophic epidemics in newly available host plants. Apparently, they were unable to spread so far without human assistance. In the cosmopolitan, wood-inhabiting fungus *Schizophyllum commune*, very limited intercontinental gene flow was found, although populations from various continents are still able to interbreed (James et al. 1999).

## B. Local Endemics

Many species and even genera of vascular plants are restricted to small areas in the order of 1 to  $1000 \text{ km}^2$ . Concentrations of such local endemics are found in isolated areas surrounded by effective barriers against dispersal – for instance, remote islands and isolated mountains. The percentage of local endemic plant species may exceed 50%. There is no evidence that this is also true for fungi.

The mycoflora of some islands has been adequately described, e.g. of the Lesser Antilles by Pegler (1983b), but comparable information on surrounding areas is not available. It is generally thought that local endemics are non-existent or scarce among fungi, except for species restricted to endemic host plants. However, the situation is rather paradoxical, since many species of fungi are known only from their type locality and, consequently, are potential local endemics. In the monographs listed in Table 7.1, the percentage



**Fig. 7.6.** Distribution of *Lycoperdon echinatum* Pers.: Pers. in Belgium according to UTM grid. Each square represents 5 × 5 km (except in the correction area). The dotted line represents the altitude of 400 m (Demoulin 1987)

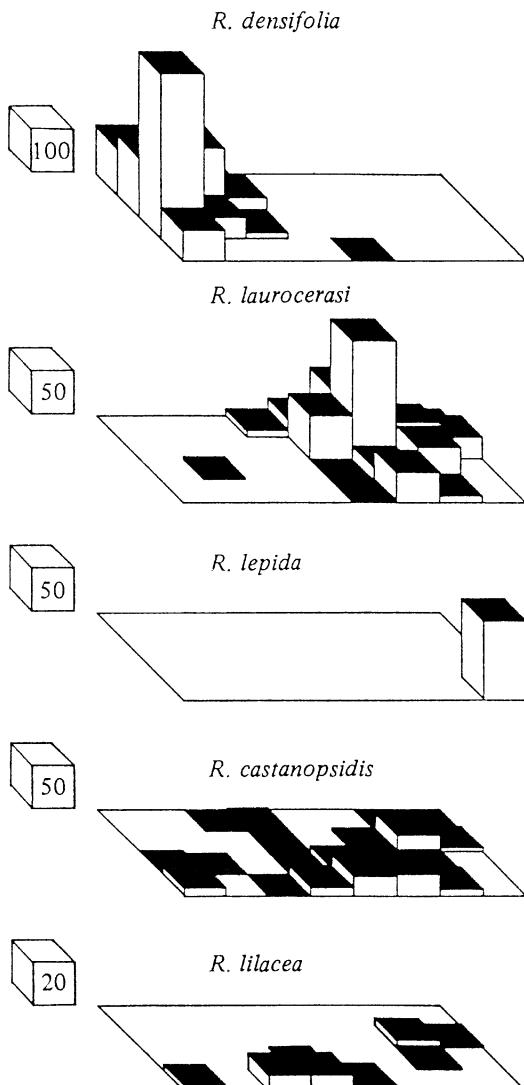
of species known from one locality ranges only from 10% in *Scleroderma* to 28% in *Ascobolus* and *Bovista*. Even the monograph of the striking genus *Hygrophorus* sensu lato (Agaricales) in relatively well-investigated North America includes 96 taxa out of 244 (39%) which are known only from the type locality. Among these, 21 species were collected exclusively before 1920 (Hesler and Smith 1963).

The following questions arise: are these species truly endemic, do they fruit sporadically, and have some simply escaped the attention of mycologists? Only more intensive field research can provide the answer. It is striking that some fungi are subsequently recorded quite far away from the original location. For instance, *Bovista verrucosa* (G.H. Cunn.) G.H. Cunn. was described by Kreisel (1967) on the basis of two collections, one from Piedmont, Italy (1857), and one from South Australia (1922). *Squamanita odorata* (Cool) Bas is a very remarkable agaric, discovered in The Netherlands in 1915 and observed there since then at approximately 30 localities. It was long thought to be endemic to The Netherlands but it seems to be spreading now in West Europe. It was reported from Denmark in 1948, Germany (1963), Norway (1968), Switzerland (1989) but, remarkably, also from the north-western United States in 1951. Such patterns may be caused by introductions but evidence is lacking. Other examples of odd disjunct distribution patterns were given by Pirozynsky (1968).

### C. Continental Patterns

Continental patterns of fungi have been studied mainly in Europe, North America and, more recently, in Australia (Grey and Grey 2005). The second Congress of European Mycologists initiated a mapping programme for 100 selected species of macrofungi. Maps of 50 species were published by Lange (1974). Lange distinguished seven climatologically determined distribution patterns. In addition, the occurrence of some species appeared to be determined by edaphic factors. Only one species showed a more or less disjunct distribution, viz. *Phallus hadriani* Vent.: Pers., with centres in the coastal dunes of West Europe and continental steppe areas in Central Europe. Some species were restricted to sub-Arctic and alpine regions in North Europe, others to the Central European mountains, although there are many similarities in climate and soil. These differences may be caused by isolation of these areas since the Pleistocene glaciations but also actual environmental factors (e.g. differences in soil temperatures in summer) may play a role.

Redhead (1989), in his overview of the distribution of Canadian fungi, provided dot maps of 78 species in North America. He distinguished 13 main types of distribution patterns, largely coinciding with those of phanerogams. Four types are shown in Fig. 7.2. Many fungi appear to be restricted to parts of the continent east and west



**Fig. 7.7.** Spatial distribution of five *Russula* species in 32 subquadrats ( $5 \times 5$  m) in a forest of *Castanopsis cuspidata* and *Pasania edulis* near Fukuoka, Japan. The numbers of the left indicate the abundance of basidiocarps, over a period of 2 years (Murakami 1987)

of the Rocky Mountains. This mountain range and the prairie area of the Midwest may be an effective barrier for numerous species. Some species occupying the entire boreal zone show subtle differences in morphology and/or ecology. Redhead (1989) suggested that these differences may be the results of survival of eastern and western populations in different refugia during Pleistocene glaciations. In Europe, Murat et al. (2004) reconstructed the postglacial colonization routes of *Tuber melanospermum* in France, using polymorphism in ribosomal DNA.

In Australia, dot maps of 100 target species were recently published by Fungimap, a national organization coordinating the activities of volunteers (Grey and Grey 2005). The maps show distinctive patterns of species restricted to native eucalypt forests (e.g. *Boletellus obscurecoccineus* (Höhn.) Sing.), coastal rainforests (e.g. *Cymatoderma elegans* Jungh.), southern temperate *Nothofagus* forests (e.g. *Cortinarius metallicus* (Bougher et al.) Peintner et al.), and arid areas in the centre of the continent (e.g. *Podaxis pistillaris* (L.: Pers.) Fr.). Patterns of some introduced species (e.g. *Amanita muscaria* (L.: Fr.) Lam.) are presented, too.

#### D. National and Regional Patterns

In principle, studies on regional distribution patterns are less complicated than those on large-scale distribution: data are easier to collect and subject to less taxonomic and nomenclatural problems. Regional studies have been carried out mainly in Europe and published in a wide variety of journals and books. In some countries, systematic mapping programmes have been initiated, e.g. in Germany, Great Britain, The Netherlands and also in Australia. The first distribution atlas on macrofungi has been published in Germany (Krieglsteiner 1991, 1993; Fig. 7.5). It contains grid maps (without accompanying text) of 5,500 taxa, based on about 3,000,000 records. An annotated atlas of 370 selected macrofungi was published in The Netherlands (Nauta and Vellinga 1995).

As an example, distribution maps of different scales are presented of the saprotrophic, well-characterized puffball *Lycoperdon echinatum* sensu lato (Figs. 7.3, 7.4, 7.5 and 7.6). They may illustrate some problems encountered with mapping and the interpretation of the results. The world distribution of *L. echinatum* sensu lato covers temperate areas in Europe and eastern North America (Fig. 7.3). However, Demoulin (1971) discovered that the North American populations are morphologically different from the European ones, and described the new species, *Lycoperdon americanum*. Consequently, what seemed to be a species with a disjunct area proved to be two vicariant species.

The distribution of *L. echinatum* in Europe is almost restricted to the nemoral zone, dominated by deciduous, broad-leaved trees, such as *Quercus robur* L., of which the area is indicated on the map (Fig. 7.4). It is tempting to interpret this pattern in terms of ecological dependence on deciduous

**Table 7.1.** Percentages of species or genera of some groups of macrofungi in different parts of the world

Reference	Ryvarden (1991)	Pegler (1983a)	Corner (1968)	Kreisel (1967)	Guzman (1970)	Van Brummelen (1967)
Taxonomic group	Polypores	Lentinus	Thelephora	Bovista	Scleroderma	Ascobolus
Principal substrate	Basidiom. Wood	Basidiom. Wood	Basidiom. Soil	Basidiom. Soil	Basidiom. Soil	Ascom. Dung
Principal way of life	Saprotoph	Saprotoph	Mycorrhizal	Saprotoph	Mycorrhizal	Saprotoph
Taxonomic units	Necrotoph Genera	Species	Saprotoph Species	Species	Species	Species
Number of taxa	132	63	49	46	20	61
Known from one locality	9	12	13	13	10	17
1. (Sub)cosmopolitan	23	5	4	2	5	28
2. Temperate/ mediterranean						
a. Bipolar	-	-	-	4	15	-
b. Boreocircumpolar	26	5	6	2	5	-
c. Southern temperate	1	2	-	11	-	-
d. Eurasia	1	-	2	11	-	-
e. Europe	2	3	10	2	-	23
f. Asia	2	3	6	13	-	1
g. Europe and N. America	-	-	4	4	20	20
h. E. Asia and N. America	3	-	4	-	-	-
i. N. America	3	4	10	22	25	10
3. Tropical						
a. Pantropical	17	3	-	4	-	5
b. Paleotropical	4	11	-	-	-	-
c. Africa	5	14	2	7	15	-
d. S.E. Asia	4	13	39	2	-	1
e. Tropical America	7	24	8	9	15	7
f. Australia/ Pacific	1	13	2	7	-	5

forests but, within the nemoral zone, the species can be found also in conifer plantations. Consequently, it is more likely to be limited by climatological factors, in particular temperature. The species seems to be much more common in north-western Europe than in the southwest, southeast and east but this might very well be an artefact due to undercollecting in these regions (Demoulin 1987).

The distribution in Belgium, on the basis of a 4×4 km grid (Fig. 7.6), provides again more detailed information. The species is lacking in the north and west of the country as well as in the Ardennes mountains above 400 m. Demoulin (1987) suggested that climatological factors caused its absence in the Ardennes. However, *L. echinatum* is found in adjacent western Germany in many areas above 500 m (Fig. 7.5). It seems that its regional

distribution is primarily determined by soil conditions, since *L. echinatum* is absent or scarce in all areas with acid, sandy or peaty soils with humus of the mor type: western Belgium, north-western Germany and also The Netherlands. Rather, it is widespread in areas with forests on calcareous, loamy soils producing mull humus. On the other hand, the occurrence of *L. echinatum* in mountains above 1000 m on the Mediterranean island of Corsica, also noted by Demoulin (1987), seems to be determined by the climate and not by soil conditions.

#### E. Local Patterns

Distribution patterns of fungal species in a stand are usually not regarded as the subject of bioge-

graphic research but, rather, as part of mycocoenological studies. For instance, Jansen (1984) studied the distribution of macrofungi in oak forests in The Netherlands using a grid of 40 subplots of  $5 \times 5$  m within  $1000 \text{ m}^2$  plots. She was able to demonstrate correlations between the occurrence of certain plants and fungi. Murakami (1987) did the same for *Russula* species in 32 subplots of  $25 \text{ m}^2$  in a Japanese *Castanopsis-Pasania* forest (Fig. 7.7). He demonstrated that the three dominant species (*R. densifolia* Gill., *R. laurocerasi* Melz., *R. lepida* Fr.) showed little spatial overlap whereas two other species (*R. castanopsidis* Hongo and *R. lilacea* Quel.) were found fruiting in much lower numbers scattered over the plot. The analysis of the distribution of individual mycelia or genets in stands is considered to belong to the domain of population biology.

#### F. Recent Changes in Distribution Patterns

Studies on changes in distribution patterns depend on the availability of accurate data on geographical ranges from different periods. Such data are known on a global scale only for some pathogenic fungi on plants. Changes in regional distribution patterns or frequencies of macrofungi have been investigated by (1) repeated mapping, (2) comparison of representative samples of the local mycoflora over the years, (3) comparison of sporocarp counts in selected stands or plots in different periods and (4) in some cases, data on the supply of edible fungi to local markets (Arnolds 1988a, b; Arnolds and Jansen 1992). Direct comparison between numbers of records or occupied grid units of a certain species during different periods (methods 1 and 2) is usually inadequate to draw conclusions on increase or decrease. The use of correction factors was described by Arnolds and Jansen (1992) and Nauta and Vellinga (1993).

### IV. Expansion of Distribution Areas

Expansion of the range of fungi may be caused by natural colonization of new areas by propagules, introduction of host plants outside their natural range, and introduction of fungi outside their natural range. Introductions may be intentional or accidental. In practice, it is often difficult to trace the origin of species because of lack of reliable historical data (Pringle and Vellinga 2006).

Few well-documented examples exist of spontaneously expanding fungi. A striking phenomenon is the recent increase of some wood-inhabiting Aphyllophorales in the Northwest European lowland, for instance, *Fomes fomentarius* (L.: Fr.) Fr., *Pycnoporus cinnabarinus* (Jacq.: Fr.) Donk (Kreisel 1985; Thoen et al. 1998), *Plicaturopsis crispa* (Pers.: Fr.) D. Reid (Arnolds and Van den Berg 2001) and *Schizophora flavigera* (Cooke) Ryvarden (Keizer 1990). The causes of these extensions of range are unknown. There have been suggestions attributing them to climatological change, changes in forestry practices, and decline of tree vitality.

Another group of increasing macrofungi in Europe comprises agarics such as *Stropharia rugosoannulata* Farlow, *S. aurantiaca* (Cooke) P.D. Orton and *Psilocybe cyanescens* Wakef (Kreisel 1985; Arnolds and Van den Berg 2005). These species are characteristic of mixtures of fertile soil and wood chips. Such habitats were rare in the past but are increasing, in particular in urban areas. The most spectacular example of this group is *Agrocybe rivulosa* Nauta, a species first found in The Netherlands in 1999 and formally described only in 2004. Nowadays, it is one of the most common species in this habitat, rapidly spreading in West Europe. The invasive character of this species suggests that it may be originally introduced, which may be true for other species of this ecological group as well.

In Europe, also some ectomycorrhizal fungi are recently spreading northwards, e.g. the sub-Mediterranean *Amanita caesarea* (Scop.: Fr.) Pers. in Belgium, probably as result of global warming (Fraiture and Walleyn 2005).

#### A. Introductions of Plants

The artificial extension of ranges of plants by introduction creates a new potential environment for associated parasites, symbionts and saprotrophs. Fungi may colonize the new area spontaneously by propagules or may be introduced together with plant material. On the other hand, the lack of compatible mycorrhizal symbionts can limit the spreading of alien plants (Richardson et al. 2000).

All over the world, many trees are planted on a large scale outside their original range. In northwest Europe, for instance, *Picea abies* (L.) Karsten and *Larix decidua* Miller are widely used in forestry. These trees are native to North Europe and the Central European mountains. Only part of the host-specific ectomycorrhizal symbionts

follow their tree outside its natural range, the number of species decreasing with increasing distance from the original habitat. For instance, in *Picea* plantations in The Netherlands, only 12 host-specific symbionts are found, most of them being rare, whereas over 50 species are native to Central Europe. Only six *Larix* symbionts occur in eastern Germany and The Netherlands (Kreisel 1985). In these cases, dispersal is probably not inhibited by geographical barriers but rather by environmental conditions. Local strains of fungi with a broad host range are apparently better adapted to these conditions, and occupy their niches.

Also North American trees were introduced into Europe on a large scale, e.g. *Pseudotsuga menziesii* (Mirbel) Franco, *Picea sitchensis* (Bong.) Carrere and *Quercus rubra* L. Only very few specific symbionts of these trees have found their way into Europe, which may at first sight indicate the effectiveness of the Atlantic as a barrier for spore dispersal. However, all introduced *Pseudotsuga* symbionts remain local and rare. Also in this case, local strains take the niches of the original symbionts.

Some ectomycorrhizal trees have been introduced in areas where suited native symbiotic fungi are lacking. Successful forestry depends in these cases either on accidental import of ectomycorrhizal fungi with nursery trees or on artificial inoculation of nursery plants with selected strains (Grove and Le Tacon 1993; Richardson et al. 2000). Diez (2005) demonstrated that successful forestation with *Eucalyptus* in Spain depends on the accidental introduction of some ectomycorrhizal symbionts from Australia. Native fungi were never found in association with *Eucalyptus*. Introduced *Pinus* spp. in New Zealand, South Africa and many tropical countries are associated with ectomycorrhizal species from the original areas in Europe and North America (Dunstan et al. 1998). The spreading of some ectomycorrhizal fungi outside their original area is well documented – for instance, of *Amanita muscaria* in Australia and New Zealand (Sawyer et al. 2001; Bagley and Orlovich 2004) and of *Amanita phalloides* outside Europe (Pringle and Vellinga 2006).

## B. Introductions of Fungi

Many pathogenic fungi have been introduced accidentally into new areas, sometimes with detrimental effects on valuable crops. A well-known example is the introduction of *Phytophthora in-*

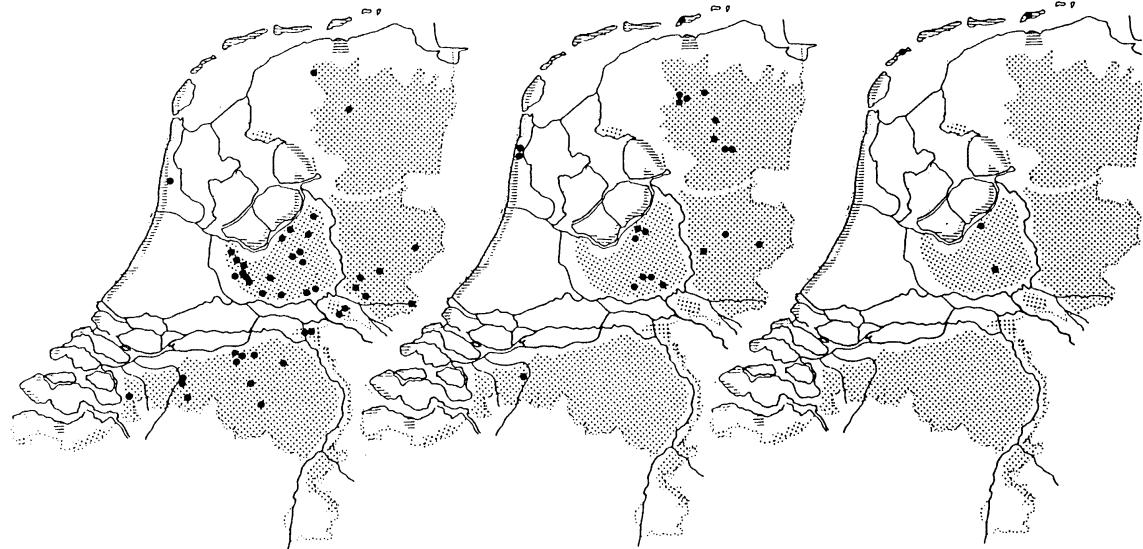
*festans* (Mont.) de Bary, the cause of late blight in potato, in Europe around 1845, resulting in the notorious famine in Ireland where 1,000,000 people died. The coffee rust, *Hemileia vastatrix* Berk. & Br., arrived in Ceylon in 1869 and destroyed 200,000 ha of coffee plantation in 20 years; it converted the British from coffee drinkers into tea drinkers. It is sometimes suggested that such epidemics are typical of monocultures. However, the introduction of *Cryptonectria parasitica* (Murr.) Barr from East Asia into North America around 1900 almost exterminated the American chestnut, *Castanea dentata* Borkh., an important component of native forests. Almost equally detrimental was the introduction from China into The Netherlands around 1915 of *Ceratocystis ulmi* (Buisman) C. Moreau, causing wilting of elm trees (Dutch elm disease; Campbell and Madden 1990).

These catastrophic introductions have led on the one hand to quarantine procedures to prevent unwanted introductions, on the other to the use of some fungi for biological control of introduced weeds. Successful examples are the suppression of the weed *Chondrilla juncea* L. by the rust fungus *Puccinia chondrillina* Bubak & Syd. in Australia and the USA, and of *Ageratina riparia* on Hawaii by an unidentified *Cercosporaella*-like fungus (Evans and Ellison 1990).

Less is known about accidental introductions of saprotrophic and mycorrhizal fungi (see also Sect. IV.A.). Most known cases of successful introductions concern *Phallales*, which combine a striking appearance with limited spore-dispersal capacity (spread by insects) and a preference for disturbed habitats – for instance, in Europe *Mutinus ravenelii* (Berk. & Curt.) E. Fischer and *M. elegans* (Mot.) E. Fischer from North America and *Anthurus archeri* (Berk.) E. Fischer from the southern hemisphere (Kreisel 1985). The extension of the latter species was described in detail by Parent et al. (2000). Some edible mushrooms were recently purposefully introduced into plantations outside their original range, in particular truffles (Yun and Hall 2004).

## C. Decline and Extinction of Fungi

The dramatic loss of natural and semi-natural habitats on earth, caused by human activities, is reflected in the rarification or extinction of many vascular plants and animals. It is inevitable that fungi are subject to the same environmental



**Fig. 7.8.** Distribution of *Sarcodon imbricatus* in the Netherlands in the periods 1890–1949 (left), 1950–1972

(centre) and 1973–1985 (right). Dotted Acid, Pleistocene sands; hatched calcareous, Holocene sands (Arnolds 1989)

changes. Nevertheless, to my knowledge, not a single species has been reported to be definitely extinct on a global scale. This is certainly in part an artefact caused by limited research efforts in this field, and the methodological complications outlined above. How do we know that a rare species has really disappeared when its ephemeral sporocarps have been collected at intervals of many years at scattered localities (see also Sect. III.B.)? Nevertheless, it is probably true that fungi are less vulnerable to extinction than vascular plants because (1) there are few species endemic to small areas; (2) their dispersal capacity is, in general, larger, at least on a continental scale; (3) it is hardly possible to purposefully exterminate fungi, e.g. by harvesting of sporocarps, as has been the case for some plants and larger animals.

However, decline and extinction of regional populations of macrofungi were observed in several parts of Europe. Evidence of a considerable decline in some ecological and taxonomic groups of fungi has stimulated efforts for fungal conservation (see section below). Here, only one example of a regionally nearly extinct fungus is described in some detail in order to illustrate some of the methodological problems encountered. It concerns the decline in The Netherlands of *Sarcodon imbricatus* (L.: Fr.) P. Karst., an obligate ectomycorrhizal species of coniferous trees, producing large epigaeous sporocarps. The species was reported as common in the

eastern Netherlands up to the 1950s, became rare in the next decade and was recorded from only four localities in the period 1973–1985 (Arnolds 1989; Fig. 7.8). Nowadays (2006), it is found at only one locality. The maps are only a weak reflection of its true decline, because of the enormous increase in mycofloristic research in The Netherlands: from the decade 1980–1989, over 200,000 records on macrofungi are available and, from all the years before 1950, only 11,000. The decrease of this species is better expressed as a proportion of the total number of fungal records, decreasing from 0.21% before 1949 to less than 0.005% since 1980.

## V. Conservation of Fungi

Conservation of fungi has only recently become an issue of major concern (Winterhoff and Krieglsteiner 1984; Arnolds 1991a). Research in this field is restricted mainly to macrofungi, and geographically to Europe and North America. Motives for the increasing attention to fungal conservation on both continents are the rapid decline of some habitats rich in rare and specialized species, and concern on the sustainability of mass collecting of edible wild mushrooms. In Europe, extensive evidence exists for severe decrease of some species in certain areas (Arnolds 1988a,

b, 1991b). In addition, international concern on decrease of biodiversity in general, culminating in 1992 in the United Nations Conference on the Environment and Development in Rio de Janeiro and the Convention on Biological Diversity ('Rio convention'), has promoted interest in the fate of fungi and other micro-organisms.

It is generally accepted that the most important strategy for conservation of fungi is *in situ* conservation of their natural environment (Staley 1997; Moore et al. 2001). For species threatened with extinction, *ex situ* cultivation may offer a (temporary) conservation strategy, e.g. for *Pleurotus nebrodensis* (Inzenga) Quél., an endemic species in Sicily (Venturella and La Rocca 2001).

The most important nongovernmental organization for global nature conservation is the International Union for Conservation of Nature (IUCN). It is, for instance, responsible for the edition of global Red Data Lists of endangered organisms and the formulation of criteria for such lists (IUCN 2005). The IUCN founded a specialist group for fungi in 1990 with representatives of most continents. Unfortunately, this group has not been very active since. In Europe, conservation issues are initiated mainly by professional and amateur mycologists, often united into mycological societies (Moore et al. 2001). Intensive cooperation takes place in a permanent European Council for Conservation of Fungi (ECCF), erected during the ninth Congress of European Mycologists in 1985. The council organizes specialist meetings devoted to conservation, at regular intervals (Senn-Irlet 2005).

### A. Red Listing

One of the main tools for the conservation of fungi is the publication of Red Data Lists, originally an acronym for 'Rarity, Endangerment and Distribution Data lists'. They consist of enumerations of species which are considered to be threatened in their long-term survival in a given area. The aims of Red Lists are (1) to inform mycologists in order to stimulate research on threatened species and in areas where many such species are present; (2) to facilitate the use of mycological data by nature conservationists and environmental planners for protection and management of nature areas; (3) to inform decision-makers and politicians, in order to develop measures and laws in favour of fungal conservation; (4) to provide data for the selection of species for monitoring programmes; (5) to

provide data for the selection of species to be protected by law.

Several categories of threatened species qualify for a Red List: (1) Extinct species which have not been observed during a considerable period in spite of extensive field surveys; (2) Critically Endangered species, facing an extremely high risk of extinction; (3) Endangered species, facing a very high risk of extinction; (4) Vulnerable species, facing a high risk of extinction. Besides these, some other categories are distinguished: Near-Threatened, when the species is likely to meet one of the threat categories in the near future; Least Concern, when a species does not qualify for one of the above categories; Data Deficient, when there is inadequate information to make a direct or indirect assessment of its risk of extinction. Three main parameters are used to determine the threat status of a species: (1) decline of the population; (2) geographical range size and (3) population size. These have recently been elaborated for each Red List category in often complicated, quantitative parameters (IUCN 2005). There are special directives for application of these criteria at regional levels (IUCN 2005).

The IUCN approach to the Red Listing procedure is designed mainly for animals with discrete individuals and well-defined populations. Application to fungi is possible only after adaptation of the criteria. A global list of threatened fungi does not exist and is unlikely to appear in near future. Virtually all species would qualify as Data Deficient on a worldwide scale. National Red Lists of fungi have been published to date in 25 European countries, in some countries already in several editions. A recent survey was published by Arnolds (2001). Most lists are restricted to macrofungi, since microfungi qualify almost automatically as data deficient. However, some microfungi are considered in some countries, mostly species associated with threatened host plants (Denchev 2005). In such cases, the qualification of the fungus as threatened is usually based on indirect evidence.

Most of the Red Lists are still based on expert judgement, rather than on numerical data and quantitative criteria, but some more recent lists use emended IUCN criteria. The number of species incorporated ranges between 17 in the former USSR to 1,655 in The Netherlands. It is evident that these values are by no means representative of the real threats to the Mycota in various countries. Indicative is the difference in the number of listed species in comparable countries such as Latvia (38 species) and Lithuania (740 species). It is clear that the crite-

ria and aims of the various lists are widely different. A total number of 4,400 species of macrofungi is included in one or more of the national lists, about 30% of the estimated total number of European species. These species belong to saprotrophic fungi on soils (35%), ectomycorrhizal species (31%), saprotrophs on wood (28%), parasitic fungi on trees (2%) and even some fungi on dung (1%). It is evident that the number of threatened species on a continental scale is much lower. A first attempt to a Red List for Europe was made by Ing (1993), enumerating 278 species. His selection was based on the number of national lists in which a species is included. However, this is not a satisfactory criterion because the threat to species with a restricted distribution area is thereby underestimated. A revised European Red List, using quantitative IUCN criteria, is in preparation by ECCF (Senn-Irlet 2005).

Outside Europe, Red Lists of fungi have been published in the USA for the states Oregon and Idaho (Molina et al. 2001).

## B. Threatened Fungi and Their Habitats

From a comparison of Red Lists, it appears that some ecological and taxonomic groups of fungi deserve special attention regarding their conservation.

1. Species largely restricted to pristine and old-growth forests, mainly lignicolous fungi on large logs and other woody debris, including many polypores. This is certainly the most widespread and important threatened ecological group throughout the world, as the result of increasing logging. Also sustainable timber harvests with selective cutting of large trees only may do much harm to the vast group of fungal species dependent on large logs and other coarse woody debris. In Europe, most of these species are at present restricted to forest reserves in remote parts of Scandinavia and the Central European mountains (Kotiranta and Niemela 1993; Parmasto 2001). In the north-western USA, conservation of old-growth forests and their characteristic organisms is a major political issue which has lead to extensive mycological research efforts (Molina et al. 2001). A monitoring programme has been initiated to survey 221 indicator species, subdivided into four categories in terms of frequency (uncommon or rare) and the possibilities of predisturbance

surveys (Castellano et al. 2003). The flagship of threatened fungi is here *Oxyporus nobilissimus* W.B. Cooke, a very rare polypore with gigantic basidiomes on huge stumps of *Abies* and *Tsuga*, and endemic to this region. Federal laws stipulate that 240 ha of habitat will be preserved at each known fruiting location of this species (Pilz and Molina 1996). In the tropics, almost no information is available on threatened fungi but the rapid loss of tropical forests must necessarily go hand in hand with unnoticed loss of mycological biodiversity. Numerous species may even become extinct before they are ever discovered and described.

2. Fungi characteristic of peat bogs, fens and other wetlands, often associated with *Sphagnum* spp. or other bryophytes, e.g. *Armillaria ectypa* (Fr.) Herink, listed on Red Lists in 10 European countries. These habitats are widespread in the northern hemisphere but, in most areas, they are subject to peat excavation, drainage activities, reclamation and/or afforestation. In Europe, extensive undamaged bogs are at present found mainly in Scandinavia, the Baltic states and Russia.
3. Saprotrophic fungi characteristic of coastal and inland sand dunes, sometimes also steppe, including many gasteromycetes, for instance, of the genera *Gastrum* Pers.: Pers., *Tulostoma* Pers.: Pers. and *Disciseda* Czern. These are threatened throughout Europe by sand excavation, recreational activities, afforestation, and natural succession on remaining relics (Winterhoff and Kriegsteiner 1984).
4. Saprotrophic fungi of old, nutrient-poor, unfertilized meadows and hayfields, including many species of *Hygrocybe* (Fr.) Kumm, *Entoloma* (Fr.) Kumm., *Dermoloma* (J. Lange) Herink, Clavariaceae and Geoglossaceae, are strongly threatened in most parts of Europe. The distribution of grassland relics rich in *Hygrocybe* species is well documented in The Netherlands (Arnolds 1988a, b), Great Britain (Newton et al. 2003) and Denmark (Raid 1985). The decline of grassland fungi in Sweden was described by Nitare (1988), who used Geoglossaceae as indicator species. Grassland fungi are threatened on the one hand by intensification of grassland use (e.g. use of dung and fertilizers), on the other by abandonment and afforestation, both processes being promoted by EEC agricultural policy.

5. Many ectomycorrhizal fungi, associated with both frondose and coniferous trees, have strongly declined since 1960 in some densely populated and industrialized parts of Europe. Reports on decline come mainly from the Czech Republic (Fellner 1993), parts of Germany (Derbsch and Schmitt 1987) and The Netherlands (Arnolds 1988a, 1991b; Nauta and Vellinga 1993). The decrease in ectomycorrhizal sporocarps is most prominent in forests on acidic soils poor in nutrients and humus, including well-known edible species such as *Cantharellus cibarius* Fr. and *Boletus edulis* Bull.: Fr. (Derbsch and Schmitt 1987; Jansen and Van Dobben 1987). The decrease in sporocarps may or may not coincide with a reduction of mycorrhizal rootlets (Dighton and Jansen 1991). The decline of ectomycorrhizal fungi is generally attributed to the effects of air pollutants on trees (reduced photosynthesis) and soil (acidification and nitrogen accumulation; Jansen and Van Dobben 1987; Arnolds 1991b; Dighton and Jansen 1991). Recently, a strong reduction in the emission of acidifying agents in some regions has lead to partial recovery of the ectomycorrhizal flora (Arnolds and Van den Berg 2001; Fellner and Landa 2003).

### C. Mycological Reserves and Nature Management

The erection of nature reserves by conservation authorities is very rarely primarily based on the significance of its mycoflora. Because it is generally accepted that fungi are optimally conserved when their habitats are preserved (Staley 1997), this situation is not necessarily dramatic. However, some habitats appear to be mycologically very important whereas their flora and fauna are not of particular interest. It is an important task for mycologists to identify these cases and to draw special attention to their fate. In Great Britain, The Netherlands and the USA, various methods have been developed to define and recognize important fungal areas (Jalink and Nauta 2001; Molina et al. 2001; Newton et al. 2003). In addition, the European organization for conservation of wild plants, Planta Europa, currently includes data on fungi in its programme for the selection of important plant areas.

An important and often neglected aspect of fungal conservation is appropriate management of

habitats in and outside nature reserves. In completely natural habitats, such as primeval forests and bogs, every form of human interference is usually disadvantageous for maintenance of biodiversity, including fungi. However, in exploited forests, semi-natural habitats and cultural landscapes, some kind of management may be useful or even necessary. In production forests, a certain proportion of living trees and coarse dead wood might be left behind after cutting in order to favour the survival of mycorrhizal and wood-inhabiting fungi. Management of poor grasslands by cutting or grazing is essential to prevent natural succession to forests (Nitare 1988; Keizer 2003). In polluted areas, removal of contaminated litter may help to restore the ectomycorrhizal flora in forests. Adequate management of roadsides may create refugia for endangered grassland fungi and ectomycorrhizal species (when planted with trees) in cultural landscapes. Surveys of the relations between management and fungi are given by Keizer (1993, 2003).

### D. Harvests of Wild Edible Mushrooms and Legal Protection

Picking of mushrooms for private consumption is a tradition since centuries in many parts of the world. Only in the last decade of the 20th century have commercial harvesting and trade of wild mushrooms become a booming business, and a major source of income for some rural communities in eastern and southern Europe, western North America, Africa and China (Arora 2001; Chiu and Moore 2001; Boa 2004). At the same time, it has become a major source of concern for mycologists and nature conservationists who query whether this harvest is sustainable in the long run. This question is not yet definitely answered.

Some authors stress that normal harvests have been intensive in many regions but without demonstrable decrease of mushroom production (Arora 2001). They believe that the generally long-living mycelia, enormous production of spores, and great dispersal capacity of fungi guarantee the long-term survival of these organisms, provided that appropriate habitats are maintained and harvesting methods are not destructive. This opinion is supported by research in two forest plots in Switzerland, where all epigaeous sporocarps were removed with weekly intervals during 29 years. No significant effects were found on species diversity and sporocarp production of

any species (Egli et al. 2006). A similar experiment on picking of *Cantharellus formosus* (Corner) during 13 years in Oregon (USA) also showed an absence of significant effects (Norvell 1995). However, trampling of the soil, a normal side-effect during collecting, reduced sporocarp production temporarily by about 30%, apparently by damage to primordia (Egli et al. 2006). Productivity recovered after termination of trampling of the soil. Destructive harvesting occurs only when the top soil is thoroughly disturbed by raking or digging in search of, e.g. truffles and very young (and very precious!) sporocarps of *Tricholoma matsutake* (S. Ito & Imai Sing. and allies, with detrimental damage to mycelia.

Other authors use additional arguments against unlimited gathering of (edible) fungi. Some stress the possible importance of sufficient spore production for the colonization of new areas. Sporocarps are food not only for humans but also for some wild animals, and they may occupy an important link in food webs in the forest. An interesting example is the importance of hypogeous sporocarps for the survival of squirrels in the north-western USA, in their turn the staple food for the endemic and endangered Great spotted owl (*Strix occidentalis*; Molina et al. 2001). Many other possible interactions, e.g. with numerous specialized insects feeding on sporocarps, are still to be discovered. In addition, not only scientific but also ethical and esthetical arguments play a role in the debate, such as the possibility for perception of undisturbed nature by naturalists and walkers.

In many European countries, harvesting of mushrooms is regulated in some way or another. However, the restrictions differ from one country to another. In general, no extraction of any natural material is allowed in national parks and other strict nature reserves. This is also true for the USA. In some countries, e.g. the Czech Republic and Hungary, some species, including edible ones, are protected by law, so that collecting is prohibited everywhere and at any time. In The Netherlands, no national regulations exist; in some municipalities, however, any collecting of any mushroom is prohibited whereas, in others, no legislation exists. In Switzerland, regional regulations exist that restrict collecting to certain quantities and certain periods. Data on the effectiveness of these and other regulations are not available.

In Europe, international legislation is becoming increasingly important for nature conservation, also on a national scale, e.g. by the Bern conven-

tion, including lists of protected species and protection of their habitats, the Habitat directive and the Natura 2000 programme, offering protection to many valuable sites. Most groups of plants and animals, including some bryophytes and lichens, are covered by this legislation but fungi not, and this despite attempts by ECCF to add some macrofungi to the species lists protected under the Bern convention. There is still a long way to go before conservation of fungi is as well regulated as that of more popular and well-known groups of organisms.

## VI. Conclusions

Biogeographical information on fungi is becoming increasingly important for the understanding of evolutionary processes and biodiversity patterns as well as for control of spread of crop pests and for nature conservation. However, this kind of information is still scanty, scattered over the literature and, therefore, often difficult to obtain. Gathering knowledge on distribution patterns of fungi is often hampered by the biological properties of these organisms and by methodological problems. Relatively little is known on global distribution patterns as well as the frequency and significance of local endemism. Programmes for mapping of (macro)fungi exist in only some European countries.

Expansion of ranges of fungi has been documented mainly for plant pathogens, usually due to accidental introductions and sometimes with profound effects on ecosystem functioning or crop production. Relatively few examples exist of saprotrophic or mycorrhizal fungi invading other continents. On the other hand, in some areas, in particular densely populated parts of Europe, a dramatic decline of many species has been recorded. These changes in the mycoflora are caused mainly by the destruction of (semi)natural habitats and environmental pollution. In particular, ectomycorrhizal species appear to be sensitive to acidification and eutrophication of forest ecosystems. It is to be expected that similar losses of biodiversity are currently taking place in other parts of the world, in particular the tropics, but escaping our attention. The exploration and documentation of biodiversity of fungi in these areas are therefore urgently needed. Effective conservation of fungi can be achieved only within the context of integral ecosystem protection and management.

## References

- Arnolds E (1988a) The changing macromycete flora in The Netherlands. *Trans Br Mycol Soc* 90:391–404
- Arnolds E (1988b) The Netherlands as an environment for agarics and boletes. In: Bas C, Kuyper ThW, Noordeloos ME, Vellinga EC (eds) *Flora Agaricina Nederlandica* 1:6–29. Balkema, Rotterdam
- Arnolds E (1989) Former and present distribution of stipitate hydnaceous fungi (Basidiomycetes) in The Netherlands. *Nova Hedwigia* 48:107–142
- Arnolds E (1991a) Mycologists and nature conservation. In: Hawksworth DL (ed) *Frontiers in mycology*. CABI, Wallingford, pp 243–264
- Arnolds E (1991b) Decline of ectomycorrhizal fungi in Europe. *Agric Ecosyst Environ* 35:209–244
- Arnolds E (2001) The future of fungi in Europe: threats, conservation and management. In: Moore D, Nauta MM, Evans SE, Rotheroe M (eds) *Fungal conservation, issues and solutions*. Cambridge University Press, Cambridge, pp 64–80
- Arnolds E, Jansen E (1992) New evidence for changes in the macromycete flora of the Netherlands. *Nova Hedwigia* 55:325–351
- Arnolds E, Van den Berg AP (2001) Trends in de paddestoelenflora op basis van karteringsgegevens. *Coolia* 44:139–152
- Arnolds E, Van den Berg AP (2005) De opkomst van snipperpaddestoelen. *Coolia* 48:131–148
- Arora D (2001) Wild mushrooms and rural economies. In: Moore D, Nauta MM, Evans SE, Rotheroe M (eds) *Fungal conservation, issues and solutions*. Cambridge University Press, Cambridge, pp 105–110
- Bagley SJ, Orlovich DA (2004) Genet size and distribution of *Amanita muscaria* in a suburban park, Dunedin, New Zealand. *N Z J Bot* 42:939–947
- Boa E (2004) Wilde edible fungi. A global overview of their use and importance to people. *Non-Wood Forest Products* 17. FAO, Rome
- Campbell LC, Madden LV (1990) An introduction to plant disease epidemiology. Wiley, New York
- Castellano MA, Cazares E, Fondrick B, Dreisbach T (2003) Handbook to Additional Fungal Species of Special Concern in the Northwest Forest Plan. USDA Forest Service, Corvallis Forest Sciences Laboratory, Corvallis, OR
- Chiu SW, Moore D (2001) Threats to biodiversity caused by traditional mushroom cultivation technology in China. In: Moore D, Nauta MM, Evans SE, Rotheroe M (eds) *Fungal conservation, issues and solutions*. Cambridge University Press, Cambridge, pp 111–119
- Corner EJH (1968) A monograph of *Thelephora* (Basidiomycetes). *Nova Hedwigia* Beih vol 27. J. Cramer, Gebr. Borntraeger, Stuttgart
- Demoulin V (1971) Le genre *Lycoperdon* en Europe et en Amérique du Nord/Étude taxonomique et phytogéographique. PhD Thesis, Université de Liège, Liège
- Demoulin V (1973) Phytogeography of the fungal genus *Lycoperdon* in relation to the opening of the Atlantic. *Nature* 242:123–125
- Demoulin V (1987) La chorologie des gastéromycètes. *Mem Soc R Bot Belg* 9:37–46
- Denchev CM (2005) Problems in conservation of fungal diversity in Bulgaria and prospects for estimating the threat status of microscopic fungi. *Mycol Balcanica* 2:251–256
- Dennis RL (1970) A Middle Pennsylvanian basidiomycete mycelium with clamp connections. *Mycologia* 62:578–584
- Derbsch H, Schmitt JA (1987) *Atlas der Pilze des Saarlandes* Teil 2: Nachweise, Vorkommen und Beschreibungen. Minister für Umwelt, Saarbrücken
- Diehl WW (1937) A basis for mycogeography. *J Wash Acad Sci* 27:244–254
- Diez J (2005) Invasion biology of Australian ectomycorrhizal fungi introduced with eucalypt plantations into the Iberian Peninsula. *Biol Invasions* 7:3–15
- Dighton J, Jansen AE (1991) Atmospheric pollutants and ectomycorrhizae: more question than answers? *Environ Pollut* 73:179–204
- Dunstan WA, Dell B, Malajezuk N (1998) The diversity of ectomycorrhizal fungi associated with introduced *Pinus* spp. in the Southern Hemisphere, with particular reference to Western Australia. *Mycorrhiza* 8:71–79
- Egli S, Peter M, Buser C, Stahel W, Ayer F (2006) Mushroom picking does not impair future harvests – results of a long-term study in Switzerland. *Biol Conserv* 129:271–276
- Evans HC, Ellison CA (1990) Classical biological control of weeds with micro-organisms: past, present, prospects. *Aspects Appl Biol* 24:39–49
- Fellner R (1993) Air pollution and mycorrhizal fungi in Central Europe. In: Pegler DN, Boddy L, Ing B, Kirk PM (eds) *Fungi of Europe: investigation, recording and conservation*. Royal Botanic Gardens, Kew, pp 239–250
- Fellner R, Landa J (2003) Mycorrhizal revival: case study from the Giant Mts. (Krkonose BR), Czech Republic. *Czech Mycol* 54:193–203
- Fraiture A, Walley R (2005) *Distributiones Fungorum Belgii et Luxemburgi* 3. Jardin Botanique National de Belgique, Meise
- Gams W (1992) The analysis of communities of saprophytic microfungi with special reference to soil fungi. In: Winterhoff W (ed) *Fungi in vegetation science. Handbook of Vegetation Science* 19(1). Kluwer, Dordrecht, pp 183–223
- Grey P, Grey E (2005). Fungi down under. Fungimap, Melbourne
- Grove TS, Le Tacon (1993). Mycorrhiza in plantation forestry. *Adv Plant Pathol* 23:191–227
- Guzman G (1970) Monografía del género *Sclerodermia* Pers. emend. Fr. (Fungi-Basidiomycetes). *Darwiniana* 16:233–407
- Hallenberg N (1991) Speciation and distribution in Corticiaceae (Basidiomycetes). *Plant Syst Evol* 177:93–110
- Hesler LR, Smith AH (1963) North American Species of *Hygrophorus*. University of Tennessee-Knoxville, Knoxville, TN
- Horak E (1983) Mycogeography in the South Pacific region: Agaricales, Boletales. *Aust J Bot Suppl Ser* 10:1–41
- Horton TR, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Mol Ecol* 10:1855–1871
- Ing B (1993) Towards a Red List of endangered European macrofungi. In: Pegler DN, Boddy L, Ing B, Kirk PM (eds) *Fungi of Europe: investigation, recording and conservation*. Royal Botanic Gardens, Kew, pp 231–237

- IUCN (2005) Guidelines for using the IUCN Red List categories and criteria. International Union for Conservation of Nature, Gland
- Jalink LM, Nauta MM (2001) Recognising and managing mycologically valuable sites in The Netherlands. In: Moore D, Nauta MM, Evans SE, Rotheroe M (eds) Fungal conservation, issues and solutions. Cambridge University Press, Cambridge, pp 89–94
- James TY, Porter D, Hamrich JL, Vilgalys R (1999) Evidence for limited intercontinental gene flow in the cosmopolitan mushroom *Schizophyllum commune*. Evolution 53:1665–1677
- Jansen AE (1984) Vegetation and macrofungi of acid oakwoods in the north-east of The Netherlands. Agricultural Research Reports 923. Pudoc, Wageningen
- Jansen E, Van Dobben HF (1987) Is decline of *Cantharellus cibarius* in The Netherlands due to air pollution? Ambio 16:211–213
- Keizer PJ (1990) The expansion of *Schizopora carneolutea* (Basidiomycetes) in Europe, in particular in The Netherlands. Persoonia 14:167–171
- Keizer PJ (1993) The influence of nature management on the macromycete flora. In: Pegler DN, Boddy L, Ing B, Kirk PM (eds) Fungi of Europe: investigation, recording and conservation. Royal Botanic Gardens, Kew, pp 251–269
- Keizer PJ (2003) Paddestoelvriendelijk natuurbeheer. KNNV, Utrecht
- Kile GA, McDonald GI, Byler JW (1991) Ecology and disease in natural forests. In: Shaw CG, Kile GA (eds) *Armillaria* root disease. US Dept Agric Agric Handb 691:102–121
- Kotiranta H, Niemela T (1993) Uhanalaiset Kaavat Suomessa (Threatened polypores in Finland). Helsinki, Vesija Ymparistohallinnon Julkaisuja-Sarja B 17:1–116
- Kreisel H (1967) Taxonomisch-Pflanzengeographische Monographie der Gattung *Bovista*. Beih Nova Hedwigia 25:1–244
- Kreisel H (1985) Geographische Verbreitung der Pilze. In: Kreisel M-H (ed) Handbuch für Pilzfreunde vol 4. Gustav Fischer, Jena, pp 48–66
- Kriegsteiner GJ (1991) Verbreitungsatlas der Grosspilze Deutschlands (West) Band 1A, B. Ulmer, Stuttgart
- Kriegsteiner GJ (1993) Verbreitungsatlas der Grosspilze Deutschlands (West) Band 2. Ulmer, Stuttgart
- Landeweert R, Leeflang P, Kuyper TW, Hoffland E, Rosling A, Wernars K, Smit E (2003) Molecular identification of ectomycorrhizal mycelium in soil horizons. Appl Environ Microbiol 69:327–333
- Lange L (1974) The distribution of macromycetes in Europe. Dan Bot Ark 30(1):1–105
- Lawrynowicz M (1990) Chorology of the European hypogeous Ascomycetes. II. Tuberales. Acta Mycol 26:7–75
- Molina R, Pilz D, Smith J, Dunham S, Dreisbach T, O'Dell T, Castellano M (2001) Conservation and management of forest fungi in the Pacific Northwestern United States: an integrated ecosystem approach. In: Moore D, Nauta MM, Evans SE, Rotheroe M (eds) Fungal conservation, issues and solutions. Cambridge University Press, Cambridge, pp 19–63
- Moore D, Nauta MM, Evans SE, Rotheroe M (eds) (2001) Fungal conservation, issues and solutions. Cambridge University Press, Cambridge
- Murakami Y (1987) Spatial distribution of *Russula* species in *Castanopsis cuspidata* forest. Trans Br Mycol Soc 89:187–193
- Murat C, Diez J, Luis P, Delaruelle C, Dupré C, Chevalier G, Bonfante P, Martin F (2004) Polymorphism at the ribosomal DNA ITS region and its relation to postglacial re-colonisation routes of the Perigord truffle *Tuber melanosporum*. New Phytol 164:401–411
- Nauta M, Vellinga EC (1993) Distribution and decline of macrofungi in the Netherlands. In: Pegler DN, Boddy L, Ing B, Kirk PM (eds) Fungi of Europe: investigation, recording and conservation. Royal Botanic Gardens, Kew, pp 21–26
- Nauta M, Vellinga EC (1995) Atlas van Nederlandse paddestoelen. Balkema, Rotterdam
- Newton AC, Davy LM, Holden E, Silverside A, Watling R, Ward SD (2003) Status, distribution and definition of mycologically important grasslands in Scotland. Biol Conserv 111:11–23
- Nitare J (1988) Jordtungor, en svampgrupp pa tillbakegang i naturliga fodermarker. Sven Bot Tidskr 82:341–368
- Noordeloos ME (2004) Entoloma s.l. Fungi Europaei vol 5a. Edizioni Candusso, Alassio
- Norvell L (1995) Loving the chanterelle to death? The 10 year chanterelle project. McIlvanea 12:6–25
- Parent GH, Thoen D, Calonge FD (2000) Nouvelles données sur la répartition de *Clathrus archeri*, en particulier dans l'ouest et le sud-ouest de l'Europe. Bull Soc Mycol Fr 116:241–266
- Parmasto E (2001) Fungi as indicators of primeval and old-growth forests deserving protection. In: Moore D, Nauta MM, Evans SE, Rotheroe M (eds) Fungal conservation, issues and solutions. Cambridge University Press, Cambridge, pp 81–88
- Pegler DN (1983a) The genus *Lentinus*, a world monograph. Royal Botanic Gardens, Kew
- Pegler DN (1983b) Agaric flora of the lesser Antilles. Royal Botanic Gardens, Kew
- Pilz D, Molina R (1996) Managing forest ecosystems to conserve fungus diversity and sustain wild mushroom harvests. US Department of Agriculture, Forest Service, Portland, OR
- Pirozynski KA (1968) Geographical distribution of fungi. In: Ainsworth GC, Sussman AS (eds) The Fungi, an Advanced Treatise vol 3. Academic Press, London, pp 487–504
- Pringle A, Vellinga EC (2006) Last chance to know? Using literature to explore the biogeography and invasion biology of the death cap mushroom *Amanita phalloides* (Vail. Ex Fr.) Link. Biol Invasions 8:1131–1144
- Rald E (1985) Vokshatte som indikatorarter for mykologisk vaerdifulde overdrevs-lokaliteter. Svampe 11:1–9
- Redhead SA (1989) A biogeographical overview of the Canadian mushroom flora. Can J Bot 67:3003–3062
- Richardson DM, Allsopp N, D'Antonio CM, Milton SJ, Rejmánek M (2000) Plant invasions – the role of mutualisms. Biol Rev Cambridge Philos Soc 75:65–93
- Ryvarden L (1991) Genera of polypores, nomenclature and taxonomy. Fungiflora, Oslo
- Sawyer NA, Chambers SS, Cairney JWB (2001) Distribution and persistence of *Amanita muscaria* genotypes in Australian *Pinus radiata* plantations. Mycol Res 105:966–970

- Senn-Irlet B (2005) The role of the ECCF in studies and conservation of fungi in Europe. *Micol Balcanica* 2:185–192
- Staley JT (1997) Biodiversity: are microbial species threatened? *Curr Opin Biotechnol* 8:340–345
- Taylor AFS, Alexander IJ (1991) Ectomycorrhizal synthesis with *Tylospora fibrillosa*, a member of the Corticiaceae. *Micol Res* 95:381–384
- Thoen D, Fraiture A, Nicolas J (1998) Chorologie et écologie de *Pycnoporus cinnabarinus* (Polyporaceae) en Belgique, au Grand-Duché de Luxembourg et dans les régions limitrophes. *Belg J Bot* 131:260–272
- Van Brummelen J (1967) A world monograph of the genera *Ascobolus* and *Saccobolus*. *Persoonia suppl* 1:1–260
- Venturella G, La Rocca S (2001) Strategies for conservation of fungi in the Madonie Park, North Sicily. In: Moore D, Nauta MM, Evans SE, Rotheroe M (eds) *Fungal conservation, issues and solutions*. Cambridge University Press, Cambridge, pp 156–161
- Volkmann-Kohlmeyer B, Kohlmeyer J (1993) Biogeographic observations on Pacific marine fungi. *Mycologia* 85:337–346
- Winterhoff W, Kriegsteiner GJ (1984) Gefährdete Pilze in Baden-Württemberg. Beih Veröff Naturschutz Landschaftspflege Bad-Württ 40:1–120
- Yun W, Hall IP (2004) Edible ectomycorrhizal mushrooms: challenges and achievements. *Can J Bot* 82:1063–1073

---

## **Fungal Interactions and Biological Control Strategies**

---

## 8 Plant Disease Biocontrol and Induced Resistance via Fungal Mycoparasites

A. VITERBO<sup>1</sup>, J. INBAR<sup>1</sup>, Y. HADAR<sup>2</sup>, I. CHET<sup>1</sup>

### CONTENTS

I. Introduction .....	127
A. Antibiosis .....	128
B. Competition .....	128
C. Mycoparasitism .....	128
II. Mycoparasites as Biocontrol Agents .....	129
A. Biotrophic Mycoparasites .....	129
1. <i>Sporidesmium sclerotivorum</i> .....	129
2. <i>Ampelomyces quisqualis</i> .....	130
B. Necrotrophic Mycoparasites .....	130
1. <i>Pythium nunn</i> .....	130
2. <i>Talaromyces flavus</i> .....	131
3. <i>Corniothyrium minitans</i> .....	131
4. <i>Gliocladium</i> and <i>Trichoderma</i> spp. ....	132
C. Induced Systemic Resistance by <i>Trichoderma</i> spp. ....	134
D. Mycoparasitism in Suppressive Environments (Soils and Composts) .....	135
III. Hyphal Interactions in Mycoparasitism .....	136
A. Biotrophs .....	136
B. Necrotrophs .....	136
IV. Molecular Aspects and Genetic Engineering in Mycoparasitism .....	137
V. Conclusions .....	140
References .....	141
	142

### I. Introduction

Due to the adverse environmental effects of pesticides that create health hazards for human and other nontarget organisms, including the pests' natural enemies, these chemicals have been the object of substantial criticism in recent years. The development of safer, environmentally feasible control alternatives has therefore become a top priority. In this context, biological control is becoming an urgently needed component of agriculture.

Biological control of plant pathogens is defined as the use of biological processes to lower inoculum density of the pathogen, with the aim of reducing its disease-producing activities (Baker and Cook 1974).

Biological control may be achieved by both direct and indirect strategies. Indirect strategies include the use of organic soil amendments that enhance the activity of indigenous microbial antagonists against a specific pathogen. Another indirect approach, cross-protection, involves the stimulation of plant self-defense mechanisms against a particular pathogen by prior inoculation of the plant rhizosphere with a nonvirulent strain or other nonpathogenic rhizo-competent bacteria or fungi. Successful protection resulting in induced resistance has been documented for viruses, bacterial pathogens, and fungi (van Loon et al. 1998; Harman et al. 2004; Haas and Defago 2005).

The direct approach involves the introduction of specific microbial antagonists into soil or plant material (Cook and Baker 1983). These antagonists have to proliferate and establish themselves in the appropriate ecological niche in order to be active against the pathogen. Antagonists are microorganisms with the potential to interfere with the growth and/or survival of plant pathogens, and thereby contribute to biological control. Antagonistic interactions among microorganisms in nature include parasitism or lysis, antibiosis, and competition. These microbial interactions serve as the basic mechanisms via which biocontrol agents operate. An elucidation of the mechanisms involved in biocontrol activity is considered to be one of the key factors in developing useful biocontrol agents. Of the numerous biocontrol agents examined, only a few have been subjected to a thorough analysis of the mechanisms involved in the suppression of the pathogen. In this chapter, we shall use examples to briefly demonstrate the different mechanisms, and will concentrate on mycoparasitism.

<sup>1</sup> Department of Plant Sciences, Weizmann Institute of Science, Rehovot 76100, Israel

<sup>2</sup> Department of Plant Pathology and Microbiology, The Hebrew University of Jerusalem, Faculty of Agriculture, Rehovot 76100, Israel

### A. Antibiosis

Handelsman and Parke (1989) restricted the definition of antibiosis to those interactions that involve a low-molecular-weight diffusible compound, or an antibiotic produced by a microorganism that inhibits the growth of another microorganism. This definition excluded proteins or enzymes that kill the target organism. Baker and Griffin (1995) extended the scope of the definition to "inhibition or destruction of an organism by the metabolic production of another." This definition includes small toxic molecules, volatiles, and lytic enzymes.

The production of inhibitory metabolites by fungal biocontrol agents has been reported in the literature over the last five decades (Bryan and McGowan 1945; Dennis and Webster 1971a, b; Ghisalberti and Sivasithamparam 1991). *Gliocladium virens* is a common example of the role of antibiotics in biological control by fungal antagonists. Gliovirin is a diketopiperazine antibiotic that appears to kill the fungus *Pythium ultimum* by causing coagulation of its protoplasm.

Howell and Stipanovic (1983) obtained gliovirin-deficient mutants of *G. virens* via ultraviolet mutagenesis. These mutants failed to protect cotton seedlings from *P. ultimum* damping-off when applied to the seeds, whereas the normal parent strain protected the seedlings. Moreover, a gliovirin-overproducing mutant provided control similar to that of the wild type, although it exhibited a much lower growth rate. A combination of *G. virens* treatment of cotton seed with reduced levels of the fungicide metalaxyl provided disease suppression equal to that of a full fungicide treatment (Howell 1991). An extensive review on antibiosis and production of *Trichoderma* secondary metabolites is provided in Howell (1998). Baker and Griffin (1995) concluded that the impact of antibiosis in biological control is uncertain. Even in cases where antifungal metabolite production by an agent reduces disease, other mechanisms may also be operating. Synergism among various lytic enzymes, and between enzymes and antibiotics has been shown to be very critical for the activity of many biocontrol agents (reviewed by Woo et al. 2002).

### B. Competition

Many plant pathogens require exogenous nutrients to successfully germinate, penetrate, and infect

host tissue (Baker and Griffin 1995). Garrett (1965) concluded that the most common cause of death in a microorganism is starvation. Therefore, competition for limiting nutritional factors, mainly carbon, nitrogen, and iron, may result in biological control of plant pathogens.

Research over the years has concentrated on competition by bacterial biocontrol agents, mainly for iron (Fe). However, fungal antagonists have received very little attention. Sivan and Chet (1989) found that a strain of *T. harzianum* (T-35) that controls *Fusarium* spp. on various crops may operate via competition for nutrients and rhizosphere colonization.

The potential of microorganisms, which are applied as a seed treatment, to proliferate and establish along the developing root system has been named rhizosphere competence (Ahmad and Baker 1987). When T-35 conidia were applied to soil enriched with chlamydospores of *F. oxysporum* f. sp. *melonis* and f. sp. *vasinfectum*, and amended with low levels of glucose and asparagine, the ability of the chlamydospores to germinate was reduced. This inhibitory effect could be reversed by adding an excess of glucose and asparagine or of seedling exudates to the soil. After its application as a seed treatment, this strain effectively colonized the rhizosphere of melon and cotton, and prevented colonization of these roots by *F. oxysporum*. Thus, competition for carbon and nitrogen in the rhizosphere, as well as rhizosphere competence itself may be involved in the biocontrol of *F. oxysporum* by *T. harzianum* strain T-35 (Sivan and Chet 1989).

### C. Mycoparasitism

Mycoparasitism is defined as a direct attack on a fungal thallus, followed by utilization of its nutrients by the parasite. The term hyperparasitism is sometimes used to describe a fungus that is parasitic on another parasitic pathogenic fungus. Barnett and Binder (1973) divided mycoparasitism into: (1) necrotrophic (destructive) parasitism, in which the relationships result in death and destruction of one or more components of the host thallus, and (2) biotrophic (balanced) parasitism, in which the development of the parasite is favored by a living, rather than a dead host structure.

Necrotrophic mycoparasites tend to be more aggressive, have a broad host range extending to wide taxonomic groups, and are relatively unspecialized in their mode of parasitism. The antag-

onistic activity of necrotrophic mycoparasites is attributed to the production of antibiotics, toxins, or hydrolytic enzymes in proportions that cause the death and destruction of their host. Biotrophic mycoparasites, on the other hand, tend to have a more restricted host range and produce specialized structures to adsorb nutrients from their host (Manocha 1990).

The parasitic relationships between fungi and their significance in biological control are the subjects of this chapter. Both types of mycoparasitism are described and discussed in the scope of their contribution to biological control. We will concentrate on the morphological, biochemical and molecular aspects of mycoparasitism in relation to biological control. The ecological aspects of this phenomenon are discussed in Jeffries (1997).

## II. Mycoparasites as Biocontrol Agents

Many comprehensive reviews on mycoparasitism in biological control in general have been published over recent years (Chet 1990; Deacon 1991; Elad and Chet 1995; Benitez et al. 2004). Due to their nature, only a few examples of biotrophic mycoparasites as biocontrol agents exist (Ayers and Adams 1981; Sztejnberg et al. 1989; Adams 1990). Necrotrophic mycoparasites, being more common, saprophytic in nature, and less specialized in their mode of action, are easier to study. As a result, the majority of the mycoparasites used as biocontrol agents in greenhouse or field trials to date have been necrotrophs. In this chapter, we will emphasize those examples in which the research that has been carried out is both applied and basic in nature.

### A. Biotrophic Mycoparasites

#### 1. *Sporidesmium sclerotivorum*

*Sporidesmium sclerotivorum* is a dermatiaceous hyphomycete that was isolated from field soil by Uecker et al. (1980). In nature, the fungus has been found to be an obligate parasite on sclerotia of *Sclerotinia sclerotiorum*, *S. minor*, *S. trifoliorum*, *Sclerotium cepivorum*, and *Botrytis cinerea* (Ayers and Adams 1981). In response to chemicals released by the host's sclerotia, macroconidia of *S. sclerotivorum* in the soil germinate and the germ tubes infect the sclerotia. When volatile compounds secreted by sclerotia of *Sclerotinia minor*, *Sclerotinia sclerotiorum*, and *Sclerotium*

*rolfsii* were tested to determine if they could stimulate germination of conidia of *S. sclerotivorum*, none of the chemicals alone or a combination of all chemicals induced germination (Fravel et al. 2002). The hyphae penetrate the intercellular matrix of the conidia, which is composed mainly of  $\beta$ -glucans (Ayers et al. 1981). The production and activity of haustoria by the mycoparasite stimulate the host sclerotia to increase their glucanase, and probably other enzyme activities, resulting in the degradation of glucan into available glucose (Bullock et al. 1986). The mycoparasite establishes itself in the sclerotia, where its mycelium grows out into the surrounding soil to infect additional sclerotia and to produce new macroconidia (Adams et al. 1984). The interaction between *S. sclerotivorum* and *Sclerotinia minor* depends on both the host and parasite density (Adams 1986). The infection process is favored by soil pH, water potential and temperature (20–22 °C) (Adams and Ayers 1980).

Under field conditions, a single application of an *S. sclerotivorum* preparation at a concentration of  $10^2$  or  $10^3$  macroconidia g<sup>-1</sup> soil caused a 75–95% reduction in the number of sclerotia of *S. minor* per plot. Control of lettuce drop caused by *S. minor* in these plots varied from 40–83% in four consecutive lettuce crops (Adams and Ayers 1982). These results were significant but not economically important (Adams 1990). Adams (1990) concluded that "one of the biggest obstacles to practical biological control is the large quantity of the agent necessary to achieve biological control when applied directly to soil in the field." He therefore suggested two alternatives: (1) to add sclerotia of *S. minor* or a nonpathogenic *Sclerotinia* that is also a host of *S. sclerotivorum* that are already infected by the mycoparasite; (2) to apply a low dosage of the mycoparasite preparation to a diseased crop, and then immediately incorporate the treated crop into the soil. This latter procedure ensures that a high percentage of the mycoparasites will be present in the soil in close contact with the sclerotia of the pathogen. Although the author assumes that these alternatives are easier and more practical, neither has been explored to any significant extent (Adams 1990). Field studies were later conducted (Del Rio et al. 2002) to evaluate the effectiveness of *S. sclerotivorum* to control *Sclerotinia* stem rot of soybean. Experimental plots were infested with *S. sclerotivorum* macroconidia at a rate of 0, 2, or 20 spores per cm<sup>2</sup>. Two years later, the disease was completely suppressed in all plots. *S. sclerotivorum* was retrieved from all infested plots at all locations 2 years

after infestation with sclerotia of *S. sclerotiorum* as bait. This paper constitutes the first report describing the biocontrol of a disease on field crops that may be employed economically.

## 2. *Ampelomyces quisqualis*

*Ampelomyces quisqualis*, a hyperparasite on Erysiphales, has been reported as a biocontrol agent of powdery mildews (Sztejnberg et al. 1989). An isolate of *A. quisqualis* obtained from an *Oidium* sp. infecting *Catha edulis* in Israel proved to be infective to several powdery mildew fungi belonging to the genera *Oidium*, *Erysiphe*, *Sphaerotheca*, *Podosphaera*, *Uncinula*, and *Leveillula*. In field trials, *A. quisqualis* parasitized the powdery mildews of cucumber, carrot, and mango, and reduced the disease. *A. quisqualis* was tolerant to many fungicides used to control powdery mildews and/or other plant diseases. Treating powdery mildew of cucumber (cv. Hazera 205) with spores of *A. quisqualis* alone significantly decreased disease severity and increased cucumber yield by approximately 50%. Combining the fungicide pyrazophos with the mycoparasite resulted in a larger increase in cucumber yield (Sztejnberg et al. 1989).

Treating powdery mildew-infected zucchini leaves with *A. quisqualis* increased the rates of photosynthesis from  $3.8 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  in untreated plants to 10.2, compared to 12.8 in uninfected healthy plants (Sztejnberg and Abo-Foul 1990). Electron micrographs of leaf sections of diseased cucumber plants revealed marked deterioration on the morphological organization of chloroplast membranes. Chloroplasts of *A. quisqualis*-treated plants seemed undamaged, like those of untreated plants (Abo-Foul et al. 1996). Fluorescence measurements (e.g., low-temperature fluorescence emission spectra, and room-temperature fluorescence transients) indicated a disease-correlated increase in levels of uncoupled chlorophyll (Abo-Foul et al. 1996). A simple, inexpensive medium based on potato dextrose broth (PDB) was developed for mass production of infective spores of *A. quisqualis* in fermentation for biological control (Sztejnberg et al. 1990), which was later developed in the commercial biofungicide AQ10.

The interaction between the hyperparasite *A. quisqualis* and its host fungi was studied by Hashioka and Nakai (1980) and Sundheim and Krekling (1982). The infection process of the cucumber powdery mildew *Sphaerotheca fuliginea*

by *A. quisqualis* was studied by scanning electron microscopy. Within 24 h after inoculation, the hyperparasite had germinated, and the germ tubes had developed appressorium-like structures at the point of contact with the powdery mildew host. Both conidia and hyphae were parasitized by penetration. Within 5 days of inoculation, the hyperparasite had developed pycnidia with conidia on the powdery mildew hyphae and conidiophores (Sunhdeim and Krekling 1982). Hashioka and Nakai (1980) used both transmission and scanning electron microscopy to study the hyphal extension and pycnidial development of the mycoparasite *A. quisqualis* Ces. inside the hyphae, and conidiophores of several species of powdery mildew fungi belonging to *Microsphaera*, *Erysiphe*, and *Sphaerotheca*. The mycoparasite cells grew normally inside the host cells, despite gradual degeneration of these latter cells. The invading hyphal cells of the mycoparasite migrated into the neighboring host cells by constricting themselves through the host cell's septal pore. The mycoparasite extended hyphae inside the conidiophores of the hosts, and formed pycnidia consisting of a unicellular outer layer and interior cells that later differentiated into conidiogenous structures (Hashioka and Nakai 1980). Recent studies based both on morphological and life cycle parameters and ribosomal DNA internal transcribed spacer region 1 sequence analysis have shown that isolates previously attributed to the genus *Ampelomyces* were actually isolates of *Phoma* spp. *Phoma glomerata* can colonize and suppress development of powdery mildew on oak, and may have utility as a mycoparasitic agent (Sullivan and White 2000).

## B. Necrotrophic Mycoparasites

### 1. *Pythium nunn*

*Pythium nunn* is a mycoparasite isolated from soil suppressive to a plant parasitic *Pythium* sp. When this mycoparasite was introduced into soil conducive to *Pythium* sp., the competitive saprophytic ability of this isolate was suppressed. An inverse relationship was found between propagule densities of the plant pathogen and of the antagonist *P. nunn* (Lifshitz et al. 1984b).

The modes of hyphal interaction between the mycoparasite *P. nunn* and several soil fungi were studied by both phase-contrast and scanning electron microscopy (Lifshitz et al. 1984a). In the zone of interaction, *P. nunn* massively coiled around

and subsequently lysed hyphae of *P. ultimum* and *P. vexans* without penetration. In contrast, *P. nunn* penetrated and eventually parasitized hyphae of *R. solani*, *P. aphanidermatum*, *Phytophthora parasitica*, and *P. cinnamomi*, forming appressorium-like structures. However, *P. nunn* was not mycoparasitic against *F. oxysporum* f. sp. *cucumerinum* or *Trichoderma koningii*, and was destroyed by *T. harzianum* and *T. viride*. The authors concluded that *P. nunn* is a necrotrophic mycoparasite with a limited host range and differential modes of action among susceptible organisms (Lifshitz et al. 1984a).

Lysis and penetration of the host cell wall at the site of interaction with the mycoparasite were demonstrated by Elad et al. (1985). Calcofluor White M2R binds to the edges of polysaccharide oligomers (Kritzman et al. 1978). Using this reagent, the appearance of fluorescence indicated localized lysis of the host cell wall by *P. nunn*.

The cell walls of Oomycota are composed of  $\beta$ -glucan, cellulose, and less than 1.5% chitin. Basidiomycota and Ascomycota contain mainly  $\beta$ -glucan and chitin but no cellulose. *P. nunn* produced large amounts of  $\beta$ -1-3-glucanase and chitinase in liquid cultures containing cell walls of pathogenic fungi belonging to the class Basidiomycota. This mycoparasite produced cellulase but no chitinase when grown on culture containing cell walls of two pathogens belonging to the Oomycota (Elad et al. 1985). These extracellular hydrolytic enzymes were detected in *P. nunn* when grown in dual culture with six host fungi but not with ten nonhost fungi, indicating specificity in the antagonistic activity of *P. nunn* (Baker 1987).

## 2. *Talaromyces flavus*

*Talaromyces flavus* (the perfect stage of *Penicillium dangeardii*; synonym: *P. vermiculatum*) is a mycoparasite of several soil-borne plant pathogenic fungi including *R. solani* (Boosalis 1956), *S. sclerotiorum* (McLaren et al. 1986) and *Verticillium* spp. (Fahima and Henis 1990). Laboratory investigations using light and electron microscopy indicate that *T. flavus* is a destructive hyperparasite of *S. sclerotiorum*. In dual culture, hyphae of *T. flavus* grew toward, and coiled around the host hyphal cells. The coiling effect intensified as the hyphae of *T. flavus* branched repeatedly on the host surface. Tips of the hyphal branches often invaded the host by direct penetration of the cell wall without formation of appressoria. Infection of host cells by

*T. flavus* resulted in granulation of the cytoplasm and collapse of the cell walls (McLaren et al. 1986).

Direct invasion of *R. solani* hyphae via the production of penetration pegs by *T. flavus* was observed by Boosalis (1956). These pegs developed from either a mycelium coiling around the host hyphae or from a hypha in direct contact with the host. Fahima and Henis (1990) applied *T. flavus* as an ascospore suspension to soil naturally infested with *Verticillium dahliae*, the causal agent of *Verticillium* wilt in eggplant. Twelve weeks after transplanting, 77% disease reduction was achieved, compared with the untreated control.

Scanning electron micrographs showed heavy fungal colonization and typical *T. flavus* conidia on the surface of the microsclerotia buried in the treated soil, but not in control soils. Transmission electron micrographs of microsclerotia incubated with *T. flavus* on agar revealed parasitism involving invasion of some host cells by means of small penetration pegs; the host cell walls were lysed mainly at their site of contact with the parasite hyphal tips. Further colonization of the microsclerotial cells occurred simultaneously with the degradation of the invaded host cell contents, rather than the cell walls (Fahima et al. 1992). It was suggested that mycoparasitism of *V. dahliae* microsclerotia by *T. flavus* hyphae may be involved in the biological control of *Verticillium* wilt disease. Fravel and Keinath (1991), however, claimed that *T. flavus* is known to produce compounds that mediate antibiosis, which is therefore suspected of being involved in the control of *Verticillium* wilt of eggplant and potato. Similarly, McLaren et al. (1986) observed that hyphal cells of *S. sclerotiorum* eventually collapse as a result of infection by *T. flavus*, but host cell walls remain intact. They suggested that cell wall-degrading enzymes may not play a major role in the control of *S. sclerotiorum* by *T. flavus*, and that antibiotics produced by the parasite may be involved in the deterioration of the host's hyphae (McLaren et al. 1986). In a recent work (Duo-Chuan et al. 2005), two chitinases (CHIT41 and CHIT32) were isolated from *T. flavus* and were shown to be able to decompose chitin in the cell walls of *V. dahliae*, *S. sclerotiorum* and *R. solani*, thus indicating that these enzymes may play an important role in the mycoparasitic behavior of *T. flavus*.

## 3. *Corniothyrium minitans*

*Corniothyrium minitans* has been found to be a natural mycoparasite of sclerotia of the plant

pathogenic fungus *S. sclerotiorum*. In Canada, Huang (1977) found that sclerotia of *S. sclerotiorum* in roots and stems of sunflower, at the end of the season, became infected with the parasite *C. minitans*. This infection actually provided natural biological control of this pathogen in the field. Applying *C. minitans* to the seed furrow in field trials, in soil naturally or artificially infested with *S. sclerotiorum*, produced 42–78% disease control of sunflower wilts over 2 successive years (Huang 1980). *C. minitans* is a destructive parasite that kills both hyphae and sclerotia of *S. sclerotiorum*. By using scanning electron microscopy, it was shown that hyphae of *C. minitans* grow intracellularly in the infected sclerotia (Phillips and Price 1983; Tu 1984). Phillips and Price (1983), based on transmission electron microscopic studies, concluded that penetration of the rind cells of *S. sclerotiorum* sclerotia by *C. minitans* is due to physical pressure, rather than enzymatic lysis of the cell wall. In a later study, Huang and Kokko (1987) found, by transmission electron microscopy, that there was destruction and disintegration of the sclerotial tissues, caused by penetration of the parasitic hyphae. Evidence from cell-wall etching at the penetration site suggests that chemical activity is indeed required for hyphae of *C. minitans* to penetrate the thick, melanized rind walls. The medullary tissue infected by *C. minitans* showed signs of plasmolysis, aggregation and vacuolization of the cytoplasm, and dissolution of the cell walls. The authors concluded that cell wall-lysing enzymes, responsible for the degradation of *S. sclerotiorum* hyphae, may also play a significant role in the dissolution and degradation of the sclerotial rind wall at the penetration site and other affected areas (Huang and Kokko 1987). Glucanase, chitinase, cellulase, and xylanase enzyme activities were recently reported in sclerotia-containing cultures of *C. minitans* (Kaur et al. 2005).

Infection of *S. sclerotiorum* hyphae by the hyperparasite *C. minitans* has been reported by several workers (Huang and Hoes 1976; Tu 1984). However, researchers are not in complete agreement on the mode of hyperparasitism. Using light microscopy, Huang and Hoes (1976) observed that hyphal tips of *C. minitans* invade hyphae of *S. sclerotiorum* by direct penetration, without forming any special structure. Host cytoplasm disintegrates and cell walls collapse as a result of infection. Macroconidia and intrahyphal hyphae were produced by *S. sclerotiorum* in infected colonies.

Production of appressoria by *C. minitans* when it comes into contact with the undamaged hyphae of *S. sclerotiorum* in dual culture on potato dextrose agar (PDA) was observed by Tu (1984). He stated that hyphal penetration by the hyperparasite sometimes occurs without the formation of appressoria, but only on damaged host cells.

Huang and Kokko (1988), using scanning electron microscopy, confirmed previous reports from light microscopic studies that hyphal tips of *C. minitans* invade the host hyphae by direct penetration, without developing appressoria, and that indentation of the host cell wall at the point of penetration is often evident. No functional distinction between main branch and side branch hyphae of the hyperparasite was found, and tips of either type of hypha are capable of invading host hyphae by direct penetration.

#### 4. *Gliocladium* and *Trichoderma* spp.

The morphological borders between *Trichoderma* and *Gliocladium* are blurred. Therefore, in recent years molecular methods have been applied as an aid to resolving the taxonomy and systematic of *Trichoderma* and *Gliocladium*. *Gliocladium virens* is now generally recognized as belonging to the genus *Trichoderma* (Gams and Bisset 1998).

Particular attention has been paid to species identification of the genus *Hypocrea/Trichoderma* that has proved problematic when traditional methods are used. An update on the taxonomy and phylogeny of the 88 taxa (which occur as 14 holomorphs, 49 teleomorphs and 25 anamorphs in nature) of *Hypocrea/Trichoderma*, confirmed by a combination of morphological, physiological and genetic approaches, is presented in Druzhinina and Kubicek (2005).

Several species of *Gliocladium* have been reported to be hyperparasites of many fungi. The biology, ecology, and potential of this genus for biological control of plant pathogens have been extensively reviewed in a comprehensive treatise by Papavizas (1985). Huang (1978) reported that *G. catenulatum* parasitizes *S. sclerotiorum* and *Fusarium* spp. It kills the host by direct hyphal contact, causing the affected cells to collapse or disintegrate. Pseudoappressoria are formed by the hyperparasite, but hyphae derived from these do not penetrate the host cell walls. Vegetative hyphae of all species tested, and macroconidia of *Fusarium* spp. are susceptible to this hyperparasite, but chlamydospores of *Fusarium equiseti* are resistant.

Phillips (1986) studied aspects of the biology of *G. virens* and its parasitism of sclerotia of *S. sclerotiorum* in soil. *G. virens* parasitized and decayed sclerotia of *S. sclerotiorum*, *S. minor*, *Botrytis cinerea*, *Sclerotium rolfsii*, and *Macrophomina phaseolina* on laboratory media, and caused a reduction in the survival of sclerotia of *S. sclerotiorum* in soil. However, parasitism of the mycelium was not detected.

A strain of *G. virens* isolated from the parasitized hyphae of *R. solani* by Howell (1982) significantly suppressed damping-off in cotton seedlings by this pathogen and by *Pythium ultimum*. Treatment with *G. virens* more than doubled the number of surviving cotton seedlings grown in soil infested with either pathogen. *G. virens* parasitized *R. solani* by coiling around, and penetrating the hyphae. *P. ultimum* was not parasitized by *G. virens*, but was strongly inhibited by antibiosis. Treatment of soil infested with propagules of *R. solani* or *P. ultimum* with *G. virens* resulted in a 63% reduction in the number of viable *R. solani* sclerotia after 3 weeks of incubation, whereas oospores of *P. ultimum* were unaffected. Strains of *G. virens* were separated into two distinct groups, P and Q, on the basis of secondary metabolite production in vitro (Howell et al. 1993).

Gliovirin was very inhibitory to *P. ultimum*, but exhibited no activity against *R. solani*, and strains that produced it (P group) were more effective seed-treatment biocontrol agents of disease incited by *P. ultimum*. Conversely, gliotoxin was more active against *R. solani* than against *P. ultimum*, and strains that produced it (Q group) were more effective seed treatments for controlling disease incited by *R. solani*. Based on these results, the authors suggested that it may be necessary to treat seeds with a combination of strains in order to broaden the disease control spectrum.

Howell (1987) isolated mutants of *G. virens*, obtained by irradiation with ultraviolet light, that showed no mycoparasitic activity. The selected mutants retained the same antibiotic complement as the parent strains. Peat moss-Czapek's broth cultures of parent and mutant strains were similarly effective as biocontrol agents of cotton seedling disease induced by *R. solani*, and as antagonists of *R. solani* sclerotia in soil. In the light of these results, Howell (1987) concluded that mycoparasitism is not a major mechanism in the biological control of *R. solani*-incited seedling disease by *G. virens*.

In addition, Pachanari and Dix (1980) concluded that *G. virens* need not make intimate

contact with *Botrytis allii* to cause severe internal disorganization of host cells, coagulation of cytoplasm, vacuolation, and loss of contents from organelles. Cultures of *B. allii* parasitized by *G. roseum* contained considerable  $\beta$ -(1-3)-glucanase and chitinase, and the cytoplasm coagulated without physical contact. *G. virens* isolate G1-21 was grown on various solid and liquid media: wheat bran and peanut hull meal (PHM), as well as spent glucose tartrate broth (GTB), Czapek-Dox broth (CDB), and potato dextrose broth (PDB) (Lewis et al. 1991). Aqueous extracts of these media caused leakage of carbohydrates and electrolytes from hyphae of the soil-borne plant pathogen *R. solani*, and its mycelial weight was reduced. Size fractionation experiments indicated that it was a combination of factors associated with *G. virens*, rather than a single one, which induced this phenomenon. Gliotoxin was detected in culture filtrates from *G. virens* grown on bran and PHM media. Gliotoxin preparations induced leakage of carbohydrates and electrolytes from *R. solani*, and caused a concomitant reduction in mycelial weight, which suggests the action of a leakage factor (Lewis et al. 1991). The authors speculated that hydrolytic enzymes such as  $\beta$ -1-3-glucanase,  $\beta$ -1-4-glucanase, chitinase, and protease, shown to be produced by isolates of *G. virens* (Roberts and Lumsden 1990), have the potential to act on *R. solani* cell walls and membranes. The role of extracellular chitinase in the biocontrol activity of *Trichoderma virens* was later examined using genetically manipulated strains of this fungus. The *T. virens* strains in which the chitinase gene (*cht42*) was disrupted (KO) or constitutively overexpressed (COE) were constructed through genetic transformation. Biocontrol activity of the KO and COE strains were significantly decreased and enhanced, respectively against cotton seedling disease incited by *Rhizoctonia solani* when compared with the wild-type strain (Baek et al. 1999).

More than 60 years ago, Weindling (1932) was the first to demonstrate the mycoparasitic nature of fungi from the genus *Trichoderma*. He suggested their potential use as biocontrol agents of plant pathogenic fungi. However, the first report on a biological control experiment using *Trichoderma* spp. under natural field conditions came 40 years later, by Wells et al. (1972) who used *T. harzianum* grown on an autoclaved mixture of ryegrass seeds and soil to control *Sclerotium rolfsii* Sace. Since then, more *Trichoderma* isolates have been obtained from natural habitats, and used in biocontrol trials against

several soil-borne plant pathogenic fungi under both greenhouse and field conditions (Chet 1990; Harman and Lumsden 1990; Harman 2006).

A seed treatment was developed by Harman et al. (1980) to reduce the amount of *Trichoderma* added to the soil to control soil-borne plant pathogenic fungi. *T. hamatum* conidia applied in the laboratory, to seeds of pea and radish as a Methocel slurry, provided protection to seeds and seedlings from *Pythium* spp. and *R. solani*, respectively, almost as effectively as fungicide seed treatment. Establishment of the mycoparasite and long-term action were demonstrated, as the propagules of *T. hamatum* increased approximately 100-fold in soils planted with treated seeds. Population densities of *R. solani* and *Pythium* spp. were lower in soils containing *T. hamatum* than in soils lacking this antagonist. Replanting these soils once, or even twice with untreated seeds yielded lower disease incidence than in soils originally planted with untreated seeds. Addition of chitin or *R. solani* cell walls to the coating of seeds previously treated with a conidial suspension increased both the ability of *T. hamatum* to protect the seeds against *Pythium* spp. or *R. solani*, and the population density of *Trichoderma* in the soil. *T. hamatum* with chitin, but without *R. solani* cell walls, effectively reduced damping-off caused by *Pythium* spp., compared to seed treatment containing only *T. hamatum* (Harman et al. 1980). Sivan et al. (1984) applied a peat-bran mixture (1:1 v/v) preparation of *T. harzianum* (isolate 315) to either soil or rooting mixture, and efficiently controlled damping-off induced by *Pythium aphanidermatum* in pea, cucumber, tomato, pepper, and gypsophila. Several isolates of *T. harzianum* and *T. hamatum* were found to antagonize and control *Macrophomina phaseolina* in beans and melon (Elad et al. 1986). Isolates of *T. harzianum* and *T. hamatum* antagonized and controlled *Rosellinia necatrix* in almond seedlings (Freeman et al. 1986). Sztejnberg et al. (1987) combined sublethal soil heating with an application of *T. harzianum* to yield better control of *R. necatrix* than that achieved by either treatment alone.

Sivan and Chet (1986) isolated a new *Trichoderma harzianum* isolate (T-35) from the rhizosphere of cotton plants grown in fields infested with *Fusarium*. In a further study, the isolate was tested in biological control trials over two successive growing seasons against *Fusarium* crown rot of tomato in fields naturally infested with *F. oxysporum* f. sp. *radici lycopersici* (Sivan et al. 1987).

*T. harzianum* was applied as a seed coating or as a wheat branpeat (1:1, v/v) preparation introduced into the tomato rooting mixture. *Trichoderma*-treated transplants were better protected against *Fusarium* crown rot than untreated controls when planted in MB-fumigated or nonfumigated infested fields. The total yield of tomatoes in the *T. harzianum*-treated plots increased as much as 26.2% over the controls. Integrated control of *Verticillium dahliae* in potato by *T. harzianum* and the fungicide Captan was reported by Ordentlich et al. (1990).

### C. Induced Systemic Resistance by *Trichoderma* spp.

Some *Trichoderma* rhizosphere-competent strains colonize entire root surfaces with morphological features reminiscent of those seen during mycoparasitism (Yedidia et al. 1999). Penetration of the root tissue is usually limited to the first or second layers of cells, and occurs only in the intercellular spaces. *Trichoderma* strains capable of establishing such interaction induce metabolic changes in plants that increase resistance to a wide range of plant-pathogenic microorganisms and viruses (Harman et al. 2004; Fig. 8.1). This response seems to be broadly effective for many plants, which indicates that there is little or no plant specificity.

At least three classes of substances that elicit plant defense responses have been identified. These elicitors include proteins, peptides, and low-molecular-weight compounds (Harman et al. 2004; Viterbo et al. 2004). The systemic response in plants occurs through the jasmonic

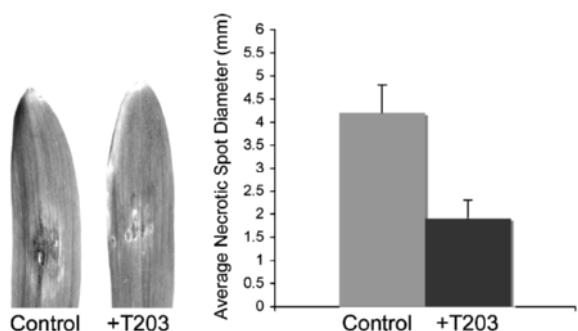


Fig. 8.1. Induced resistance toward the leaf pathogen *Cochliobolus heterostrophus* in maize. Seedling roots were infected with germinated *Trichoderma* spores ( $10^5 \text{ ml}^{-1}$ ) 48 h prior to pathogen leaf infection (800 spores). The symptoms were recorded 72 h after infection

acid/ethylene signaling pathway in a way similar to the rhizobacteria-induced systemic resistance (van Loon et al. 1998; Shores et al. 2005). Several studies have shown that root colonization by *Trichoderma* strains results in massive changes in plant gene expression patterns and metabolome. Changes in plant metabolism lead to the accumulation of antimicrobial compounds. In cucumber, root colonization by *T. asperellum* strain T-203 causes an increase in phenolic glucoside levels in leaves, which are strongly inhibitory to a range of bacteria and fungi (Yedidia et al. 2003). The protection afforded by the biocontrol agent is associated with the accumulation of mRNA of two defense genes: the phenylpropanoid pathway gene phenylalanine ammonia lyase (*PAL*) and the lipoxygenase pathway gene hydroxyperoxide lyase (*HPL*) (Yedidia et al. 2003). Increased levels of other defense-related plant enzymes, such as peroxidases, chitinases, and  $\beta$ -1,3-glucanases, have been recorded in *Trichoderma*-treated cucumber seedlings upon pathogen challenge (Shores et al. 2005). This potentiation in the gene expression enables *Trichoderma*-treated plants to be more resistant to subsequent pathogen infection. The MAPK signal transduction pathways, both of the plant and of *Trichoderma*, are important for the induction of systemic resistance (Viterbo et al. 2005; Shores et al. 2006).

#### D. Mycoparasitism in Suppressive Environments (Soils and Composts)

Suppression of soil-borne plant pathogens occurs in environments such as field soils, and soils amended with organic matter or compost as the organic component in media for container-grown plants. A review on this topic for field soils was recently published by Stone et al. (2004).

Pathogen suppressiveness has been defined by Cook and Baker (1983) as "soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil."

Baker and Cook (1974) divided suppression mechanisms into two broad categories defined as general and specific. General suppression is a result of total microbial activity. In contrast, specific suppression applies when bacteria or fungi, indi-

vidually or as a group, are responsible for the suppression effect. Mycoparasitism, the focus of this chapter, is a major mechanism of specific suppression. For example, Chet and Baker (1981) reported on a soil which was suppressive to *R. solani* of carnation near Bogota, Colombia. This soil contained high levels of organic matter (35%), was highly acidic (pH 5.1), and its main microbiological component was the antagonistic fungus *T. hamatum* at a population density of  $8 \times 10^5$  propagules g<sup>-1</sup>. The level of this mycoparasite in mineral-conducive soil was four orders of magnitude lower.

In another, related study, Henis et al. (1978) showed the effect of successive plantings on the development of suppression. A soil sown with radish every week became suppressive to *R. solani* by the fourth sowing, and was even more suppressive by the fifth and subsequent sowings. The population of *T. harzianum*, antagonistic to *R. solani*, increased with successive sowings of radish (Liu and Baker 1980), possibly in response to increases in the amount of *R. solani* in the soil resulting from its parasitism of the radish seedlings. The addition of *T. harzianum* spores to a conducive soil at the same density as that found in the suppressive soil caused the conducive soil to become suppressive. *Trichoderma* spp. were also reported to be responsible for the suppression of the take-all disease caused by *Gaeumannomyces graminis*. Low pH conditions were found to be favorable to *Trichoderma*, and to enhance suppression (Simon and Sivasithamparam 1990).

A practical approach to utilizing suppression in agriculture is the use of suppressive composts, mainly in container media. Composting is the breakdown of organic waste material by a succession of mixed populations of microorganisms in a thermophilic aerobic environment. The final product is compost or humus, which is the stabilized organic matter populated by microorganisms capable of suppressing soil-borne plant pathogens. Disease-suppressive effects of composts have been investigated intensively over the past two decades, and were recently reviewed by Noble and Coventry (2005) and Zinati (2005). Compost of a wide variety of waste materials (hardwood or pine bark, municipal sludge, grape marc, or cattle manure) is an economically and ecologically sound alternative to pesticides.

The mechanisms of suppression in composts do not differ substantially from those described for soils, and can be either general or specific. Physiological profiling, and the use of DNA-based

techniques such as denaturing gradient gel electrophoresis (DGGE) may lead to an improved understanding of the changes in microbial communities associated with disease control resulting from compost amendment of soil, sand, or peat. Nelson et al. (1983) identified specific strains of four *Trichoderma* spp. and isolates of *Gliocladium virens* as the most effective fungal hyperparasites of *R. solani* present in bark compost. A few of the 230 other fungal species also showed activity, but most were ineffective. Kwok et al. (1987) described synergistic interactions between *T. hamatum* and *Flavobacterium balustinum*. Several other bacterial strains, including *Enterobacter*, *Pseudomonas*, and *Xanthomonas* spp., also interacted with the *Trichoderma* isolate in suppression of *Rhizoctonia* damping-off (Kwok et al. 1987). Composted grape marc was effective in suppressing disease caused by *S. rolfsii* in beans and chickpeas (Gorodecki and Hadar 1990). Hadar and Gorodecki (1991) placed sclerotia of *S. rolfsii* on composted grape marc to isolate hyperparasites of this pathogen. Viability of sclerotia decreased from 100% to less than 10% within 40 h. It remained close to 100% for sclerotia placed on a conducive peat mix. *Penicillium* spp. and *Fusarium* spp. were observed by scanning electron microscopy to colonize the sclerotia. *Trichoderma* populations in the grape marc compost were at very low levels ( $10^2$  cfu g<sup>-1</sup> dry weight). The hyperparasites present in this compost are therefore quite different from those isolated from tree bark compost, where *Trichoderma* and *Gliocladium* isolates predominate.

In conclusion, suppression of soil-borne plant pathogens in field soil or container media is brought about by antagonistic microorganisms. Such systems could be a source for mycoparasites to be used in biocontrol, or to be incorporated into integrated disease control programs. The inoculation of composts with biological control agents may improve the efficacy and reliability of disease control obtained.

### III. Hyphal Interactions in Mycoparasitism

#### A. Biotrophs

*Piptocephalis virginiana* is a haustorial biotrophic mycoparasite that parasitizes fungi belonging to the order Mucorales exclusively (Manocha

1981). Attachment of a biotrophic mycoparasite to its host surface is considered to be an essential prerequisite step for further penetration of the host by the parasite (Manocha and Chen 1990). *P. virginiana* attaches to the surface of both the compatible *Choanephora cucurbitarum* and *Mortierella pusilla*, and the incompatible *Phascolomyces articulosus* hosts, but not to the surface of the nonhost *Mortierella candelabrum* (Manocha 1985; Manocha et al. 1986). Comparative research was performed by Manocha and his coworkers in an attempt to unravel the molecular basis for specificity and recognition in this system. Cytological and biochemical investigations were carried out to study the structure and chemical composition of cell walls of host and nonhost species (Manocha 1981, 1987). The germ tubes of the biotrophic mycoparasite *P. virginiana* were found to attach to the cell-wall surface of the host, but not to that of the nonhost (Manocha 1985; Manocha et al. 1986). This attachment could be specifically inhibited by chitobiose and chitotriose. The authors therefore suggested a possible involvement of carbohydrate-binding proteins in the specificity of this interaction. A comparison of protein and glycoprotein profiles of cell-wall extracts revealed marked differences between host and nonhost species. Two high-molecular-weight glycoproteins were observed only in the extract of host cell walls, being absent in that of the nonhost (Manocha 1985; Manocha et al. 1986). Further isolation and characterization of the host cell surface proteins revealed that attachment and appressorium formation by the parasite germ tubes could be inhibited by treating host cell-wall fragments with 0.1 M NaOH or pronase E. Furthermore, the two purified glycoproteins were able to agglutinate both nongerminated and germinated spores of the mycoparasite. Arabinose, glucose, and N-acetylglucosamine could totally inhibit this agglutination. These glycoproteins were suggested to be two subunits of a carbohydrate-binding agglutinin present on the host cell surface, and to be involved in agglutination and attachment of the mycoparasite germ tubes (Manocha and Chen 1991).

Using fluorescein isothiocyanate-labeled lectin-binding techniques, Manocha et al. (1990) were able to show differences in the distribution pattern of glycosyl residues at the level of the cell wall between fungi that are hosts and those that are nonhosts of the mycoparasite *P. virginiana*, and at the protoplast level between compatible and incompatible hosts.

The cell walls of the compatible hosts (*C. cucurbitarum* and *M. pusilla*) and the incompatible host (*P. articulosus*), as well as that of the mycoparasite itself, contain glucose and N-acetylglucosamine. In the nonhost (*M. candelabrum*), however, other sugars such as fucose, N-acetylgalactosamine, and galactose could also be detected. These latter sugars could be detected on both the host and the parasite surface after mild treatment with proteinase or when grown in liquid medium. The researchers speculated that the failure of the mycoparasite to attach to the host cells after proteinase treatment or in liquid culture may be due to the appearance of galactose and galactosamine at the host cell surface. The idea that N-acetylglucosamine and glucose may be involved in the attachment of *P. virginiana* to its host cell surface was supported by the observation that pretreatment of the mycoparasite germ tubes with N-acetylglucosamine or glucose inhibited their attachment to the host cells. In addition, the germ tubes attached to agarose beads coated with glucose or with N-acetylglucosamine, but not with N-acetylgalactosamine (Manocha et al. 1990).

The protoplast surfaces of compatible hosts contained all of the above-listed sugars, and these protoplasts could attach to the germ tube of the mycoparasite. Only lectins specific for N-acetylglucosamine and glucose were bound at the protoplast surface of the incompatible host; these protoplasts did not attach to the mycoparasite germ tubes. Indications were found for different factors being responsible for attachment and for appressorium formation, as pretreatment of the mycoparasite with glucose and N-acetylglucosamine inhibited its attachment to the host cell surface, but had no obvious effect on appressorium formation. On the other hand, appressorium formation was inhibited by heat treatment of host cell-wall fragments that still permitted attachment (Manocha et al. 1990). The authors therefore suggested a model for the recognition between *P. virginiana* and its host fungi that operates at two levels at least: the cell wall, and the protoplast surface. At the cell-wall level, the attachment probably involves carbohydrate-binding agglutinins that recognize specific sugar residues on the host but not on the nonhost cell wall. After the initial recognition and attachment, at the protoplast level, the parasite distinguishes compatible from incompatible hosts. The mechanism of this distinction is not clear. Yet, it seems that protoplast membrane sugars

are not a major factor in recognition at this level (Manocha et al. 1990). Immunofluorescence microscopy was used to detect, in the mycoparasite *P. virginiana*, the presence of a complementary glycoprotein that binds specifically to the host cell surface glycoproteins. This technique revealed surface localization of the protein on the germ tubes of *P. virginiana*. Fluorescence was also observed at the surface of the germinated spores and hyphae of the host *M. pusilla*, after treatment with complementary protein from *P. virginiana*, and with primary antibody prepared against the complementary protein (Manocha et al. 1997).

## B. Necrotrophs

As early as 1932, Weindling reported the coiling of *Trichoderma* spp. hyphae around hyphae of other fungi. These strains were later shown to actually be a species of *Gliocladium* (Webster and Lomas 1964). Dennis and Webster (1971a, b, c) published an extensive report on the antagonistic properties of species groups of *Trichoderma*. The hyphal interaction between *Trichoderma* and plant pathogenic fungi was first comprehensively studied in their work. Since then, numerous studies on the hyphal interaction and coiling phenomenon of *Trichoderma* around its host hyphae have been carried out with the use of light and electron microscopy (Chet et al. 1981; Elad et al. 1983a; Baker 1987; Inbar and Chet 1992; Omero et al. 1999; Rocha-Ramirez et al. 2002; Fig. 8.2).

The destructive mode of parasitism in *Trichoderma* appears to be a process consisting of several consecutive events initiated by attraction and directed growth of *Trichoderma* toward its host, probably by chemotropism. Positive chemotropism was found in *Trichoderma* (Chet et al. 1981), as it could detect its host from a distance and begin to branch in an atypical way. These branches grew toward the pathogenic host fungi. Similar behavior was also found in *Pythium nunn* (Lifshitz et al. 1984a), *P. oligandrum* (Lewis et al. 1989), and in *Gliocladium* spp. (Huang 1978). This event is presumably a response of the antagonist to the chemical gradient of an attractant coming from the host. However, no specific stimuli other than amino acids and simple sugars have thus far been detected (R. Barak and I. Chet, unpublished data). Hence, the specificity of the phenomenon is not clear. Apparently, it is not an essential step for mycoparasitism, although it may hold some advantage for



**Fig. 8.2.** A mycoparasitic relationship. Scanning electron micrograph of *T. harzianum* hyphae coiling around those of the plant pathogenic fungus *S. sclerotiorum*. Bar 10  $\mu\text{m}$

the antagonist. Subsequently, contact is made, and in some cases, *Trichoderma* coils around or grows along the host hyphae and forms hook-like structures, presumably appressoria, which probably aid in penetrating the host hyphal cell wall (Chet et al. 1981; Elad et al. 1983b). The coiling phenomenon and appressoria formation have been reported for other mycoparasites as well (Tu 1984; Lifshitz et al. 1984a). However, Deacon (1976) concluded that in the case of *P. oligandrum*, coiling of the antagonist around its host hyphae indicates temporary host resistance, rather than susceptibility. Nevertheless, in *Trichoderma*, this reaction was found to be rather specific, and *Trichoderma* attacks only a few fungi. Moreover, Dennis and Webster (1971c), using plastic threads of a diameter similar to that of *P. ultimum* hyphae, concluded that the coiling of the *Trichoderma* is not merely a thigmotropic response. The *Trichoderma* hyphae never coiled around the threads, but rather grew over or followed them in a straight course. This led to the idea that there is a molecular basis for the specificity. However, despite the fact that first observations and reports of this phenomenon were published decades ago, we are only now on the verge of being able to understand it. Reviews dealing with cellular interactions

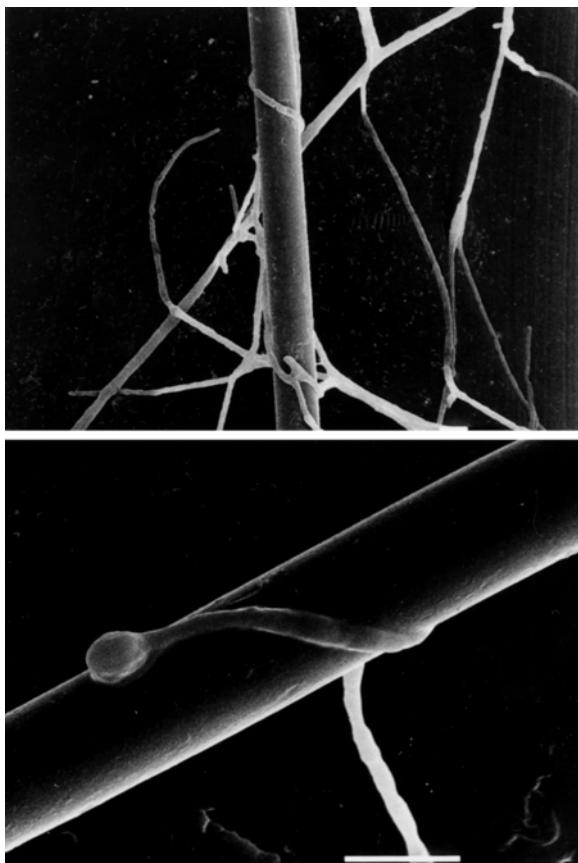
in fungi (Tunlid et al. 1992; Manocha and Sahai 1993), and the specificity of attachment of fungal parasites to their hosts (Manocha and Chen 1990) have been published. The physiology and biochemistry of biotrophic mycoparasitism in particular have been extensively reviewed by Manocha (1990).

Attachment and “recognition” between the mycoparasite and its host appears to be essential, and a crucial stage for successful continuation of the process. Lectins are sugar-binding proteins or glycoproteins of nonimmune origin that agglutinate cells and/or precipitate glycoconjugates (Goldstein et al. 1980). First discovered in plants and later in other organisms, they are involved in interactions between the cell surface and its extracellular environment (Barondes 1981). Indeed, lectins were found to be produced by some soil-borne plant pathogenic fungi such as *R. solani* and *S. rolfsii* (Barak et al. 1985, 1986), and by different members of the Sclerotiniaceae (Kellens et al. 1992).

Therefore, a role for lectins in the recognition and specificity of attachment between *Trichoderma* and its host fungi was suggested. However, no conclusive evidence to support this hypothesis was available at the time. In an attempt to test this hypothesis, Inbar and Chet (1992) used a novel approach based on the binding of lectins to a surface of nylon fibers. This biomimetic system imitates the host hyphae, and enables an examination of the role of lectins in mycoparasitism. Inert nylon fibers were chemically activated to enable the covalent binding of the lectins (Inbar and Chet 1992).

Concanavalin A, a plant lectin that is similar to the lectin of *S. rolfsii* (LSR) in its carbohydrate specificity (cf. they are both specific to D-glucose and D-mannose), was used first to establish the system. The *Trichoderma* recognized the LSR-treated fibers as a host, and attached and coiled around them in a pattern similar to that seen with real host hyphae (Inbar and Chet 1992; Fig. 8.3). In contrast, in the untreated control, no interaction could be observed – the *Trichoderma* grew uninterruptedly over and along the fibers, exactly as outlined by Dennis and Webster (1971c). These findings provided the first direct evidence for the role of lectins in mycoparasitism. The researchers were able to show that inert nylon fibers coated with fungal lectins mimic the real host hyphae, and can stimulate the parasite to coil around them.

A novel lectin was isolated and purified from the culture filtrate of the soil-borne plant pathogenic fungus *S. rolfsii* (Inbar and Chet 1994). Agglutination of *E. coli* cells by the purified lectin



**Fig. 8.3.** Biomimetic systems for simulating the interaction between *Trichoderma* and plant pathogenic fungi. **a** Scanning electron micrograph of *T. harzianum* hyphae coiling around inert nylon fibers coated with a surface lectin from the plant pathogenic fungus *S. rolfsii*. Bar 10  $\mu\text{m}$ . **b** Appressorium formation by *T. harzianum* grown on nylon fibers coated with the *S. rolfsii* lectin. Bar 10  $\mu\text{m}$

could be inhibited by the glycoproteins mucin and asialomucin. Proteases, as well as  $\beta$ -1,3-glucanase, were found to be totally destructive to the agglutination activity, indicating that both protein and  $\beta$ -1,3-glucan are necessary for agglutination. Using the biomimetic system, it was apparent that the presence of the purified agglutinin of the surface of the fibers significantly induces mycoparasitic behavior in *T. harzianum*, compared with the untreated ones or with those treated with nonagglutinating extracellular proteins from *S. rolfsii* (Inbar and Chet 1994).

It was later demonstrated that induction of chitinolytic enzymes in *Trichoderma* is elicited by the recognition signal (i.e., lectin–carbohydrate interactions). It was postulated that recognition is the first step in a cascade of antagonistic events trig-

gering the parasitic response in *Trichoderma* (Inbar and Chet 1995).

The same biomimetic system was used to test the involvement of signal transduction pathways in the induction of coils in *T. harzianum* (Omero et al. 1999). Two activators of G protein-mediated signal transduction induced coiling of hyphae around nylon fibers. The peptide toxin mastoparan increased coiling more than twofold in comparison with controls. The activator fluoroaluminate (A1F4) had a similar effect, whereas aluminum ions alone were ineffective; cAMP increased coiling about threefold. Although the two G-protein activators, mastoparan and fluoroaluminate, have very different modes of action, they share the Ga subunit as a target. Based on these results, it was proposed that a signal for mycoparasitic behavior from the host cell surface is transduced by heterotrimeric G protein(s) and mediated by cAMP.

Rocha-Ramirez et al. (2002) isolated a *T. atroviride* G-protein alpha-subunit (Ga) gene (*tga1*). Transgenic lines overexpressing *tga1* showed a delayed sporulation and coiled at a higher frequency, compared to the wild type. Likewise, transgenic lines that expressed an activated mutant protein with no GTPase activity did not sporulate and coiled at a higher frequency. Lines that expressed an antisense version of the gene were hypersporulating and coiled at a much lower frequency in the biomimetic assay. The loss of *tga1* in these mutants correlated with the loss of GTPase activity stimulated by the peptide toxin Mas-7. The application of Mas-7 to growing mycelia raises intracellular cAMP levels, suggesting that *tga1* can activate adenylyl cyclase. In contrast, cAMP levels and cAMP-dependent protein kinase activity drop when diffusible host signals are encountered and the mycoparasitism-related genes *ech42* and *prb1* are highly expressed. These results demonstrated that the product of the *tga1* gene is involved in both coiling and conidiation.

Penetration and degradation of the host cell wall under the coiling and interaction sites are evident by visual observation, fluorescent indicators, and enzymatic studies. Using scanning electron microscopy, lysed sites and penetration holes were found in hyphae of *R. solani* and *S. rolfsii* following removal of *Trichoderma* spp. hyphae (Elad et al. 1983b). The cell walls of Basidiomycota and Ascomycota contain chitin and laminarin (P glucan) but no cellulose. Oomycota contain  $\beta$ -glucans and cellulose and relatively small

amounts of chitin (<1.5%). Therefore, to penetrate the host cell wall, mycoparasites should have a system of hydrolytic enzymes that can degrade these components. Enzymatic degradation of fungal cell walls occurs mainly via the excretion of the extracellular enzymes  $\beta$ -1-3-glucanase and chitinase. Indeed, high  $\beta$ -1-3-glucanase and chitinase activities were detected in dual cultures when *T. harzianum* parasitized *S. rolfsii*, contrasting with the low levels found with either fungus alone. Cycloheximide prevented antagonism, and enzymatic activity was diminished (Elad et al. 1983b). Using gold cytochemistry, *T. harzianum* hyphae were shown to coil around and penetrate cells of *R. solani*, causing extensive damage such as cell-wall alteration, plasma membrane retraction, and cytoplasm aggregation (Benhamou and Chet 1993).

The involvement and importance of lytic enzymes, mainly chitinase, in the biological control of plant pathogens by both fungi and rhizobacterial agents (Ordentlich et al. 1988; Inbar and Chet 1991; Sahai and Manocha 1993; Viterbo et al. 2002), as well as their involvement in the defense of plants against pathogenic infection (Boller 1985; Broglie et al. 1991) is well documented.

#### IV. Molecular Aspects and Genetic Engineering in Mycoparasitism

*Trichoderma* is one the most frequently used biocontrol agents in agriculture. The role of lytic enzymes in its mycoparasitic activity has recently been largely reviewed (Viterbo et al. 2002; Benítez et al. 2004). The sensing of the host in the *Trichoderma* mycoparasitic interaction and gene activation has been the subject of extensive studies in the last few years (Inbar and Chet 1995; Zeilinger et al. 1999; Brunner et al. 2003). The pattern of induction of different cell wall-degrading enzymes differs from one *Trichoderma* strain to another. It is believed that *Trichoderma* secrete exochitinases constitutively at low levels. When chitinases degrade fungal cell walls, they release oligomers that induce other chitinases, and attack begins.

In the last decade, the significance of several newly isolated lytic enzymes has been demonstrated by overexpression and deletion of the respective genes (Pozo et al. 2004; Hoell et al. 2005). Molecular approaches and genetic engineer-

ing techniques have been applied to gain a better and more basic understanding of the system, as well as to develop superior and improved strains of biocontrol agents with enhanced activity (Mendoza et al. 2003; Brunner et al. 2005). The chitinase gene *chiA*, encoding one of the chitinases from *Serratia marcescens*, a well-known biocontrol agent, was isolated and cloned into *E. coli* (Shapira et al. 1989). *E. coli* transformed by the *chiA* gene, under the oLpL operator and promoter of bacteriophage, expressed and excreted the corresponding protein into the growth medium. Almost pure *S. marcescens* chitinase from *E. coli* or whole viable cells were used in greenhouse experiments against *S. rolfsii* in beans, and *R. solani* in cotton. Using the chitinase preparation in the irrigation water effectively reduced the number of diseased plants. Whole viable cells of transformed *E. coli* were also effective in inhibiting *S. rolfsii*, but to a lesser degree. The genetically engineered *E. coli*, a nonsoil bacterium, served here as a model system to demonstrate the role of chitinase in controlling a chitin-containing plant pathogen.

It is suggested that the introduction of such engineered genes into soil bacteria will increase control efficiency by combining high expression of a gene coding for a lytic enzyme with rhizosphere competence. Southern blot analysis of the *chiA* gene cloned from *S. marcescens* showed homology to one of the *Trichoderma* chitinase genes. Based on this, the *chiA* gene was used as a probe to isolate a chitinase gene from a cDNA library prepared from *T. harzianum* (T-35) grown on chitin (Chet et al. 1993). The chitinase gene from *T. harzianum* (T-35) was cloned in a Bluescript plasmid under the lac promoter. When the transformed *E. coli* was plated on LB+0.2% chitin plates and induced by 1 mM IPTG, the bacteria showed chitinolytic activity. In greenhouse experiments, irrigation of bean seedlings with  $10^7 \text{ cfu g}^{-1} \text{ soil day}^{-1}$  of *E. coli* XLIBlue, transformed with the *Trichoderma* chitinase gene induced by 1 mM IPTG, resulted in significant biocontrol activity. Suppression of the disease caused by *S. rolfsii* was obvious. The treated plants exhibited a better growth rate than untreated controls. After 18 days, the growth rate of the plants irrigated with the transformed bacteria was similar to that of uninfected plants (Chet et al. 1993).

In an attempt to increase its effectiveness, *T. harzianum* protoplasts were cotransformed using two plasmids: pSL3chiAII, containing a bacterial chitinase gene from *S. marcescens* under

the control of a constitutive viral promotor, and p35SR2, a marker for selection after transformation, encoding for acetamidase. Two transformants showed increased constitutive chitinase activity (specific activity 11 and 5 times higher than the recipient; Fig. 8.4), and excreted a protein of ca. 58 kDa, the expected size of *S. marcescens* chitinase, when grown on synthetic medium. Antagonistic activity of the transformants was significantly higher than that of the wild-type *T. harzianum*, as evaluated by testing their ability to overgrow the plant pathogen *S. rolfsii* in dual culture (Haran et al. 1993). The major advantage of such genetic manipulations is the ability to isolate genes from one strain and introduce them into other varieties of fungi, bacteria, or plants. This enhances the potency of biocontrol agents and makes a single strain consistently effective against more than one plant pathogenic fungus, without the hazardous effects of chemical pesticides.

This approach was taken by Broglie et al. (1991) who, in a pioneering work, produced seedlings constitutively expressing a bean chitinase gene under the control of the cauliflower mosaic virus 35S promoter. The timing of the natural host defense mechanism was modified to produce fungus-resistant plants with increased ability to survive in soil infested with the fungal pathogen *R. solani*, delaying the development of disease symptoms.

Since then, genetic manipulations of valuable crop plants with one or more cell wall-degrading enzymes from mycoparasitic fungi have been considered a potent tool for improving plant resistance

to fungal pathogens. Transgenic apple plants expressing *T. atroviride* endochitinase and exochitinase, singly or in combination, were produced and screened for resistance to *Venturia inaequalis*, the causal agent of apple scab (Bolar et al. 2001). Plants expressing both enzymes at the same time were more resistant, demonstrating for the first time in planta synergism between the two enzymes. Constitutive expression of *Trichoderma* endochitinase can be exploited to enhance resistance to fungal pathogens in important forest tree species. The *ech42* from *T. harzianum* was introduced into forest trees, black spruce (*Picea mariana*) and hybrid poplar (*Populus nigra* x *Populus maximowiczii*), by *Agrobacterium*-mediated transformation. In vitro assays demonstrated that the transgenic poplars had increased resistance to the leaf rust pathogen *Melampsora medusae*. Seedlings of transgenic spruce lines showed an increased resistance to the spruce root pathogen *Cylindrocladium floridanum* (Noël et al. 2006).

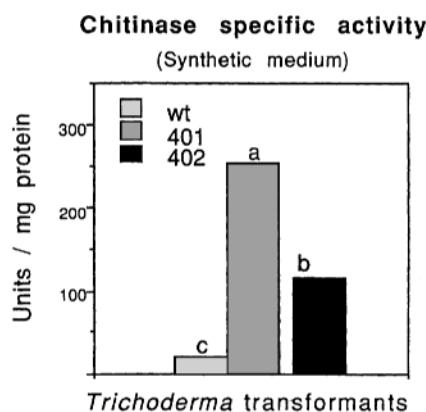
## V. Conclusions

Mycoparasitism is a quite common, and yet exciting phenomenon. It appears to play an important role in biological control, even though it should be pointed out that mycoparasitism is only one specific aspect in the whole complex system of biological control of plant diseases. For example, the direct effects of root-colonizing *Trichoderma* spp. on plants are at least as important as the direct effects on pathogens – or perhaps more so. These fungi have profound impacts on plant growth and development, and they also induce resistance to a variety of classes of plant pathogens.

Mycoparasitism is a complex process that includes the following steps: (1) chemotrophic growth of the antagonist toward the host; (2) recognition of the host by the mycoparasite; (3) attachment; (4) excretion of extracellular enzymes; and (5) lysis and exploitation of the host.

Mycoparasitism occurs under appropriate ecological conditions. The population and activity of the mycoparasite can be increased by relatively specific substances, such as chitin. The gene coding for chitinase is only one example of genes with mycoparasitic activity. Other potential genes are those coding for  $\beta$ -1,3-glucanase, protease, and lipase.

Engineering various chitinases together with other genes that may act as antifungal agents



**Fig. 8.4.** Chitinase-specific activity of crude enzyme (units per mg protein) excreted by the wild-type *T. harzianum* (wt) and transformants (401 and 402), after 5 days on synthetic medium. Columns headed by different letters are significantly different ( $p = 0.05$ ) according to Duncan's multiple range test (Haran et al. 1993)

may lead to better protection of plants against pathogenic fungi. It may therefore be possible to improve mycoparasitism, and to enhance the plants resistance response by integrating cloned chitinase with different lytic enzymes and other available antifungal polypeptides. By understanding the mode of action of biocontrol mycoparasitic fungi, we should be able to manipulate the fungal agent, the plant, and their interactions to achieve more effective and safer plant resistance to various biotic and abiotic stresses.

**Acknowledgements.** This work was supported by *The Dr. Alexander and Myrna Strelinger Endowment Fund*, *The Judith and Avraham Goldwasser Foundation*, and a United States-Israel Binational Agricultural Research and Development Fund (BARD) (US-3507-04).

## References

- Abo-Foul S, Raskin VI, Sztejnberg A, Marder JB (1996) Disruption of chlorophyll organization and function in powdery mildew-diseased cucumber leaves and its control by the hyperparasite *Ampelomyces quisqualis*. *Phytopathology* 86:195–199
- Adams PB (1986) Effect of soil temperature, moisture, and depth on survival and activity of *Sclerotinia minor*, *Sclerotium cepivorum* and *Sporidesmium sclerotivorum*. *Plant Disease* 71:170
- Adams PB (1990) The potential of mycoparasites for biological control of plant diseases. *Annu Rev Phytopathol* 28:59–72
- Adams PB, Ayers WA (1980) Factors affecting parasitic activity of *Sporidesmium sclerotivorum* on sclerotia of *Sclerotinia minor* in soil. *Phytopathology* 70:366–368
- Adams PB, Ayers WA (1982) Biological control of *Sclerotinia* lettuce drop in the field by *Sporidesmium sclerotivorum*. *Phytopathology* 72:485–488
- Adams PB, Marois JJ, Ayers WA (1984) Population dynamics of the mycoparasite, *Sporidesmium sclerotivorum*, and its host, *Sclerotinia minor* in soil. *Soil Biol Biochem* 16:627–633
- Ahmad JS, Baker R (1987) Rhizosphere competence of *Trichoderma harzianum*. *Phytopathology* 77:182–189
- Ayers WA, Adams PB (1981) Mycoparasitism and its application to biological control of plant diseases. In: Papavizas GC (ed) *Biological control in crop protection*, vol 15. Allanheld, Osmun Totowa, NJ, pp 91–105
- Ayers WA, Barnett EA, Adams PB (1981) Germination of macroconidia and growth of *Sporidesmium sclerotivorum* in vitro. *Can J Microbiol* 27:664–669
- Baek JM, Howell CR, Kenerley CM (1999) The role of an extracellular chitinase from *Trichoderma virens* Gv29-8 in the biocontrol of *Rhizoctonia solani*. *Curr Genet* 35:41–50
- Baker R (1987) Mycoparasitism: ecology and physiology. *Can J Plant Pathol* 9:370–379
- Baker RF, Cook RJ (1974) Biological control of plant pathogens. The American Phytopathological Society, St Paul, MN
- Baker R, Griffin GJ (1995) Molecular strategies for biological control of fungal plant pathogens. In: Reuveni R (ed) *Novel approaches to integrated pest management*. Lewis, CRC Press, Boca Raton, FL, pp 153–182
- Barak R, Elad Y, Mirelman D, Chet I (1985) Lectins: a possible basis for specific recognition in *Trichoderma-Sclerotium rolfsii* interaction. *Phytopathology* 75:458–462
- Barak R, Elad Y, Chet I (1986) The properties of L-fucose binding agglutinin associated with the cell wall of *Rhizoctonia solani*. *Arch Microbiol* 144:346–349
- Barnett HL, Binder FL (1973) The fungal host-parasite relationship. *Annu Rev Phytopathol* 11:273–292
- Barondes SH (1981) Lectins: their multiple endogenous cellular functions. *Annu Rev Biochem* 50:207–231
- Benhamou N, Chet I (1993) Hyphal interactions between *Trichoderma harzianum* and *Rhizoctonia solani*: ultrastructure and cytochemistry of the antagonistic process. *Phytopathology* 83:1062–1071
- Benitez T, Rincon AM, Limon MC, Codon AC (2004) Biocontrol mechanisms of *Trichoderma* strains. *Int Microbiol* 7:249–60
- Bolar JP, Norelli JL, Harman GE, Brown SK, Aldwinckle HS (2001) Synergistic activity of endochitinase and exochitinase from *Trichoderma atroviride* (*T. harzianum*) against the pathogenic fungus (*Venturia inaequalis*) in transgenic apple plants. *Transgenic Res* 10:533–43
- Boller T (1985) Induction of hydrolases as a defence reaction against pathogens. In: Key JL, Kosuge T (eds) *Cellular and molecular biology of plant stress*. Alan R Liss, New York, pp 247–262
- Boosalis MG (1956) Effect of soil temperature and green-manure amendment of unsterilized soil on parasitism of *Rhizoctonia solani* by *Penicillium vermiculatum* and *Trichoderma* spp. *Phytopathology* 46:473–478
- Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Broglie R (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* 254:1194–1197
- Brunner K, Peterbauer CK, Mach RL, Lorito M, Zeilinger S, Kubicek CP (2003) The Nag1 N-acetylglucosaminidase of *Trichoderma atroviride* is essential for chitinase induction by chitin and of major relevance to biocontrol. *Curr Genet* 43:289–95
- Brunner K, Zeilinger S, Ciliento R, Woo SL, Lorito M, Kubicek CP, Mach RL (2005) Improvement of the fungal biocontrol agent *Trichoderma atroviride* to enhance both antagonism and induction of plant systemic disease resistance. *Appl Environ Microbiol* 71:3959–3965
- Bryan PW, McGowan JC (1945) Viridin: a highly fungistatic substance produced by *Trichoderma viride*. *Nature* 151:144–145
- Bullock S, Adams PB, Willetts HJ, Ayers WA (1986) Production of haustoria by *Sporidesmiacm sclerotivorum* in sclerotia of *Sclerotinia minor*. *Phytopathology* 76:101–103
- Chet I (1990) Mycoparasitism – recognition, physiology and ecology. In: Baker R, Dunn P (eds) *New directions in biological control: alternatives for suppressing agricultural pests and diseases*. Alan R Liss, New York, pp 725–733
- Chet I, Baker R (1981) Isolation and biocontrol potential of *Trichoderma hamatum* from soil naturally suppressive to *Rhizoctonia solani*. *Phytopathology* 71:286–290

- Chet I, Harman GE, Baker R (1981) *Trichoderma hamatum*: its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. *Microbial Ecol* 7:29–38
- Chet I, Barak Z, Oppenheim A (1993) Genetic engineering of microorganisms for improved biocontrol activity. In: Chet I (ed) Biotechnology in plant disease control. Wiley-Liss, New York, pp 211–235
- Cook RJ, Baker KF (1983) The nature and practice of biological control of plant pathogens. American Phytopathological Society, St Paul, MN
- Deacon JW (1976) Studies on *Pythium oligandrum*, an aggressive parasite of other fungi. *Trans Br Mycol Soc* 66:383–391
- Deacon JW (1991) Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens. *Biocontrol Sci Technol* 1:5–20
- Del Rio LE, Martinson CA, Yang XB (2002) Biological control of *Sclerotinia* stem rot of soybean with *Sporidesmium sclerotivorum*. *Plant Disease* 86:999–1004
- Dennis C, Webster J (1971a) Antagonistic properties of species-groups of *Trichoderma*. I. Production of non volatile antibiotics. *Trans Br Mycol Soc* 57:25–39
- Dennis C, Webster J (1971b) Antagonistic properties of species-groups of *Trichoderma*. II. Production of volatile antibiotics. *Trans Br Mycol Soc* 57:41–48
- Dennis C, Webster J (1971c) Antagonistic properties of species-groups of *Trichoderma*. III. Hyphal interaction. *Trans Br Mycol Soc* 57:363–369
- Druzhinina I, Kubicek CP (2005) Species concepts and biodiversity in *Trichoderma* and *Hypocreales*: from aggregate species to species clusters? *J Zhejiang Univ Sci B* 6:100–112
- Duo-Chuan LI, Chen S, Jing LU (2005) Purification and partial characterization of two chitinases from the mycoparasitic fungus *Talaromyces flavus*. *Mycopathologia* 159:223–229
- Elad Y, Chet I (1995) Practical approaches for biocontrol agents implementation. In: Reuveni R (ed) Novel approaches to integrated pest management. Lewis, CRC Press, Boca Raton, FL, pp 323–338
- Elad Y, Barak R, Chet I, Henis Y (1983a) Ultrastructural studies of the interaction between *Trichoderma* spp. and plant pathogenic fungi. *Phytopathol Z* 107:168–175
- Elad Y, Chet I, Boyle P, Henis Y (1983b) Parasitism of *Trichoderma* spp. on *Rhizoctonia solani* and *Sclerotium* – scanning electron microscopy and fluorescence microscopy. *Phytopathology* 73:85–88
- Elad Y, Lifshitz R, Baker R (1985) Enzymatic activity of the mycoparasite *Pythium nunn* during interaction with host and non-host fungi. *Physiol Plant Pathol* 27:131
- Elad Y, Zvieli Y, Chet I (1986) Biological control of *Macrophomina phaseolina* (Tassi) by *Trichoderma harzianum*. *Crop Protect* 5:288–292
- Fahima T, Henis Y (1990) Interaction between pathogen, host and biocontrol agent: multiplication of *Trichoderma hamatum* and *Talaromyces flavus* roots of diseased and healthy hosts. In: Hornby D (ed) Biological control of soil-borne plant pathogens. CABI, Wallingford, pp 165–180
- Fahima T, Madi L, Henis Y (1992) Ultrastructure and germinability of *Verticillium* microsclerotia parasitized by *Talaromyces flavus* on agar medium and in treated soil. *Biocontrol Sci Technol* 2:69–78
- Fravel DR, Keinath AP (1991) Biocontrol of soilborne plant pathogens with fungi. In: Keister DL, Cregan PB (eds) The rhizosphere and plant growth. Kluwer, Dordrecht, pp 237–243
- Fravel DR, Connick WJ Jr, Grimm CC, Lloyd SW (2002) Volatile compounds emitted by sclerotia of *Sclerotinia minor*, *Sclerotinia sclerotiorum*, and *Sclerotium rolfsii*. *J Agric Food Chem* 50:3761–3764
- Freeman S, Sztejnberg A, Chet I (1986) Evaluation of *Trichoderma* as a biocontrol agent for *Rosellinia necatrix*. *Plant Soil* 94:163–170
- Gams WX, Bisset JX (1998) Morphology and identification of *Trichoderma*. In: Kubicek CP, Harman GE (eds) *Trichoderma and Gliocladium*. Taylor and Francis, Padstow, pp 3–25
- Garrett SD (1965) Toward biological control of soil-borne plant pathogens. In: Baker KF, Synder WC (eds) Ecology of soil-borne plant pathogens. University of California Press, Berkeley, CA, pp 4–17
- Ghisalberti EL, Sivasithamparam K (1991) Antifungal antibiotics produced by *Trichoderma* spp. *Soil Biol* 23:1011–1020
- Goldstein IJ, Hughes RC, Monsigny M, Osawa T, Sharon N (1980) What should be called a lectin? *Nature* 285:66
- Gorodecki B, Hadar Y (1990) Suppression of *Rhizoctonia solani* and *Sclerotium rolfsii* in container media containing composted separated cattle manure and composted grape marc. *Crop Protect* 9:271
- Haas D, Defago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Rev Microbiol* 3(4):307–319
- Hadar Y, Gorodecki B (1991) Suppression of germination of sclerotia of *Sclerotium rolfsii* in compost. *Soil Biol Biochem* 23:303–306
- Handelsman J, Parke JL (1989) Mechanism in biocontrol of soilborne plant pathogens. In: Kosuge T, Nester EW (eds) Plant microbe interactions, vol 3. McGraw-Hill, New York, pp 27–61
- Harari S, Schickler H, Pe'er S, Logemann S, Oppenheim A, Chet I (1993) Increased constitutive chitinase activity in transformed *Trichoderma harzianum*. *Biol Control* 3:101–108
- Harman GE (2006) Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology* 96:190–194
- Harman GE, Lumsden RD (1990) Biological disease control. In: Lynch JM (ed) The rhizosphere. Wiley, New York, pp 259–280
- Harman GE, Chet I, Baker R (1980) *Trichoderma hamatum* effects on seed and seedling disease induced in radish and pea by *Pythium* spp. or *Rhizoctonia solani*. *Phytopathology* 70:1167–1172
- Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004) *Trichoderma* species-opportunistic, avirulent plant symbionts. *Nature Rev Microbiol* 2(1):43–56
- Hashioka Y, Nakai Y (1980) Ultrastructure of pycnidial development and mycoparasitism of *Ampelomyces quisqualis* parasitic on *Erysiphales*. *Trans Mycol Soc Jpn* 21:329–338
- Henis Y, Ghaffar A, Baker R (1978) Integrated control of *Rhizoctonia solani* damping-off of radish: effect of successive plantings, PCNB, and *Trichoderma harzianum* on pathogen and disease. *Phytopathology* 68:900–907

- Hoell IA, Klemsdal SS, Vaaje-Kolstad G, Horn SJ, Eijsink VG (2005) Overexpression and characterization of a novel chitinase from *Trichoderma atroviride* strain P1. *Biochim Biophys Acta* 15:180–190
- Howell CR (1982) Effect of *Gliocladium virens* on *Pythium ultimum*, *Rhizoctonia solani* and damping-off of cotton seedling. *Phytopathology* 72:496–498
- Howell CR (1987) Relevance of mycoparasitism in the biological control of *Rhizoctonia solani* by *Gliocladium virens*. *Phytopathology* 77:992–994
- Howell CR (1991) Biological control of *Pythium* damping-off of cotton with seed coating preparation of *Gliocladium virens*. *Phytopathology* 81:738–741
- Howell CR (1998) The role of antibiosis in biocontrol. In: Harman GE, Kubicek CP (eds) *Trichoderma and Gliocladium*, vol 2. Taylor and Francis, Padstow, pp 173–184
- Howell CR, Stipanovic RD (1983) Gliovirin, a new antibiotic from *Gliocladium virens* and its role in the biological control of *Pythium ultimum*. *Can J Microbiol* 29:321–324
- Howell CR, Stipanovic RD, Lumsden RD (1993) Antibiotic production by strains of *Gliocladium virens* and its relation to the biocontrol of cotton seedling diseases. *Biocontrol Sci Technol* 3:435–441
- Huang HC (1977) Importance of *Coniothyrium minitans* in survival of sclerotia of *Sclerotinia sclerotiorum* in wilted sunflower. *Can J Bot* 55:289–295
- Huang HC (1978) *Gliocladium catenulatum*: hyperparasite of *Sclerotinia sclerotiorum* and *Fusarium* species. *Can J Bot* 56:2243–2246
- Huang HC (1980) Control of *Sclerotinia* wilt of sunflower by hyperparasites. *Can J Plant Pathol* 2:26–32
- Huang HC, Hoes JA (1976) Penetration and infection of *Sclerotinia sclerotiorum* by *Coniothyrium minitans*. *Can J Bot* 54:406–410
- Huang HC, Kokko EG (1987) Ultrastructure of hyperparasitism of *Coniothyrium minitans* on sclerotia of *Sclerotinia sclerotiorum*. *Can J Bot* 65:2483–2489
- Huang HC, Kokko EG (1988) Penetration of hyphae of *Sclerotinia sclerotiorum* by *Coniothyrium minitans* without the formation of appressoria. *J Phytopathol* 123:133–139
- Inbar J, Chet I (1991) Evidence that chitinase produced by *Aeromonas caviae* is involved in the biological control of soil plant pathogens by this bacterium. *Soil Biol Biochem* 23:973–978
- Inbar J, Chet I (1992) Biomimics of fungal cell-cell recognition by use of lectin-coated nylon fibers. *J Bacteriol* 174:1055–1059
- Inbar J, Chet I (1994) A newly isolated lectin from the plant pathogenic fungus *Sclerotium rolfsii*: purification, characterization and its role in mycoparasitism. *Microbiology* 140:651–657
- Inbar J, Chet I (1995) The role of recognition in the induction of specific chitinases during mycoparasitism by *Trichoderma harzianum*. *Microbiology* 141:2823–2829
- Jeffries P (1997) Mycoparasitism. In: Wicklow DT, Söderström B (eds) *The Mycota* vol IV, 1st edn. Environmental and microbial relationships. Springer, Berlin Heidelberg New York, pp 149–164
- Kaur J, Munshi GD, Singh RS, Koch E (2005) Effect of carbon source on production of lytic enzymes by the sclerotial parasites *Trichoderma atroviride* and *Coniothyrium minitans*. *J Phytopathol* 153:274–279
- Kellens JTC, Goldstein IJ, Peumans WJ (1992) Lectins in different members of the Sclerotiniaceae. *Mycol Res* 96:495–502
- Kritzman G, Chet I, Henis Y, Huttermann A (1978) The use of the brightener Calcofluor White M2R New in the study of fungal growth. *Isr J Bot* 27:138–146
- Kwok OCH, Fahy PC, Hoitink HAJ, Kuter FA (1987) Interaction between bacteria and *Trichoderma hamatum* in suppression of *Rhizoctonia* damping-off in bark compost media. *Phytopathology* 77:1206–1212
- Lewis K, Whipps JM, Cooke RC (1989) Mechanisms of biological disease control with special reference to the case study of *Pythium oligandrum* as an antagonist. In: Whipps JM, Lumsden RD (eds) *Biotechnology of fungi for improving plant growth*. Cambridge University Press, Cambridge, pp 191–217
- Lewis JA, Roberts DP, Hollenbeck MD (1991) Induction of cytoplasmic leakage from *Rhizoctonia solani* hyphae by *Gliocladium virens* and partial characterization of a leakage factor. *Biocontrol Sci Technol* 1:21–29
- Lifshitz R, Dupler M, Elad Y, Baker R (1984a) Hyphal interactions between *Pythium nunn* and several soil fungi. *Can J Microbiol* 30:1482–1487
- Lifshitz R, Stanghellini ME, Baker R (1984b) A new species of *Pythium* isolated from soil in Colorado. *Mycotaxon* 20:373–379
- Liu SY, Baker R (1980) Mechanism of biological control in soil suppressive to *Rhizoctonia solani*. *Phytopathology* 70:404–412
- Manocha MS (1981) Host specificity and mechanism of resistance in a mycoparasitic system. *Physiol Plant Pathol* 18:257–267
- Manocha MS (1985) Specificity of mycoparasite attachment to the host cell surface. *Can J Bot* 63:772–778
- Manocha MS (1987) Cellular and molecular aspects of fungal host-mycoparasite interaction. *J Plant Diseases Protect* 94:431–444
- Manocha MS (1990) Cell-cell interaction in fungi. *J Plant Diseases Protect* 97:655–669
- Manocha MS, Chen Y (1990) Specificity of attachment of fungal parasites to their hosts. *Can J Microbiol* 36:69–76
- Manocha MS, Chen Y (1991) Isolation and partial characterization of host cell surface agglutinin and its role in attachment of a biotrophic mycoparasite. *Can J Microbiol* 37:377–383
- Manocha MS, Sahai AS (1993) Mechanisms of recognition in necrotrophic and biotrophic mycoparasites. *Can J Microbiol* 39:269–275
- Manocha MS, Balasubramanian R, Enskat (1986) Attachment of a mycoparasite with host but not with nonhost *Mortierella* species. In: Bailey J (ed) *Biology and molecular biology of plant pathogen interactions*. Springer, Berlin Heidelberg New York, pp 59–69
- Manocha MS, Chen Y, Rao N (1990) Involvement of cells surface sugars in recognition attachment and appressorium formation by a mycoparasite. *Can J Microbiol* 36:771–778
- Manocha MS, Xiong D, Govindsamy V (1997) Isolation and partial characterization of a complementary protein from the mycoparasite *Piptocephalis virginiana* that specifically binds to two glycoproteins at the host cell surface. *Can J Microbiol* 43:625–632

- McLaren DL, Huang HC, Rimmer SR (1986) Hyperparasitism of *Sclerotinia sclerotiorum* by *Talaromyces flavus*. Can J Plant Pathol 8:43–48
- Mendoza-Mendoza A, Pozo MJ, Grzegorski D, Martinez P, Garcia JM, Olmedo-Monfil V, Cortes C, Kenerley C, Herrera-Estrella A (2003) Enhanced biocontrol activity of *Trichoderma* through inactivation of a mitogen-activated protein kinase. Proc Natl Acad Sci USA 23:15965–15970
- Nelson EB, Kuter GA, Hoitink HAJ (1983) Effect of fungal antagonists and compost age on suppression of *Rhizoctonia* damping-off in container media amended with composted hardwood bark. Phytopathology 73:1457–1462
- Noble R, Coventry E (2005) Suppression of soil-borne plant diseases with composts: a review. Biocontrol Sci Technol 15:3–20
- Noël A, Levasseur C, Le VQ, Séguin A (2006) Enhanced resistance to fungal pathogens in forest trees by genetic transformation of black spruce and hybrid poplar with a *Trichoderma harzianum* endochitinase gene. Physiol Mol Plant Pathol 67:92–99
- Omero C, Inbar J, Rocha-Ramirez V, Herrera-Estrella A, Chet I, Horwitz BA (1999) G protein activators and cAMP promote mycoparasitic behaviour in *Trichoderma harzianum*. Mycol Res 103:1637–1642
- Ordentlich A, Elad Y, Chet I (1988) The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. Phytopathology 78:84–88
- Ordentlich A, Nachmias A, Chet I (1990) Integrated control of *Verticillium dahliae* in potato by *Trichoderma harzicnum* and captan. Crop Protect 9:363–366
- Pachenari A, Dix NJ (1980) Production of toxins and wall degrading enzymes by *Gliocladium roseum*. Trans Br Mycol Soc 74:561–566
- Papavizas GC (1985) *Trichoderma* and *Gliocladium*: biology, ecology and the potential for biocontrol. Annu Rev Phytopathol 23:23–54
- Phillips AJL (1986) Factors affecting the parasitic activity of *Gliocladium virens* on the sclerotia of *Sclerotinia sclerotiorum* and a note on its host range. J Phytopathol 116:212–220
- Phillips AJL, Price K (1983) Structural aspects of the parasitism of sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Coniothyrium minitans* Campb. Phytopathol Z 107:193–203
- Pozo MJ, Baek JM, Garcia JM, Kenerley CM (2004) Functional analysis of *tvsp1*, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens*. Fungal Genet Biol 41:336–348
- Roberts DP, Lumsden RD (1990) Effect of extracellular metabolites from *Gliocladium virens* on germination of sporangia and mycelial growth of *Pythium ultimum*. Phytopathology 80:461–465
- Rocha-Ramirez V, Omero C, Chet I, Horwitz BA, Herrera-Estrella A (2002) *Trichoderma atroviride* G-protein alpha-subunit gene *tga1* is involved in mycoparasitic coiling and conidiation. Eukaryot Cell 1:594–605
- Sahai AS, Manocha MS (1993) Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. FEMS Microbiol Rev 11:317–338
- Shapira R, Ordentlich A, Chet I, Oppenheim AB (1989) Control of plant diseases by chitinase expressed from cloned DNA in *Escherichia coli*. Phytopathology 79:1246–1249
- Shoresh M, Yedidia I, Chet I (2005) Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by *Trichoderma asperellum* T203. Phytopathology 95:76–84
- Shoresh M, Gal-On A, Leibman D, Chet I (2006) Characterization of a mitogen-activated protein kinase gene from cucumber required for trichoderma-conferred plant resistance. Plant Physiol 142:1169–1179
- Simon A, Sivasithamparam K (1990) Effect of crop rotation, nitrogenous fertilizer and lime on biological suppression of the take-all fungus. In: Hornby D (ed) Biological control of soil-borne plant pathogens. CABI, Wallingford, pp 215–226
- Sivan A, Chet I (1986) Biological control of *Fusarium* spp. in cotton, wheat and muskmelon by *Trichoderma harzianum*. Phytopathol Z 116:39–47
- Sivan A, Chet I (1989) The possible role of competition between *Trichoderma harzianum* and *Fusarium oxysporum* on rhizosphere colonization. Phytopathology 79:198–203
- Sivan A, Elad Y, Chet I (1984) Biological control effects of a new isolate of *Trichoderma harzianum* on *Pythium aphanidermatum*. Phytopathology 74:498
- Sivan A, Ucko O, Chet I (1987) Biological control of *Fusarium* crown rot of tomato by *Trichoderma harzianum* under field conditions. Plant Disease 71:587–592
- Stone AG, Scheurell SJ, Darby HM (2004) Suppression of soilborne diseases in field agricultural systems: organic matter management, cover cropping and other cultural practices. In: Magdoff F, Weil RR (eds) Soil organic matter in sustainable agriculture. CRC Press LLC, Boca Raton, FL, pp 131–177
- Sullivan RF, White JF (2000) *Phoma glomerata* as a mycoparasite of powdery mildew. Appl Environ Microbiol 66:425–427
- Sundheim L, Krekling T (1982) Host-parasite relationships of the hyperparasite *Ampelomyces quisqualis* and its powdery mildew host *Sphaerotheca fuliginea*. Phytopathol Z 104:202–210
- Sztejnberg A, Abo-Foul S (1990) The hyperparasite *Ampelomyces quisqualis* increases yield and photosynthesis of powdery mildew-infected cucumber and zucchini. In: Proc APS/CPS Annu Meet, Grand Rapids, MI, August 1990
- Sztejnberg A, Freeman S, Chet I, Katan J (1987) Control of *Rosellinia necatrix* in soil and apple orchard by solarization and *Trichoderma harzianum*. Plant Disease 71:365–369
- Sztejnberg A, Galper S, Mazar S, Lisker N (1989) *Ampelomyces quisqualis* for biological and integrated control of powdery mildews in Israel. J Phytopathol 124:285–295
- Sztejnberg A, Galper S, Lisker N (1990) Conditions for pycnidial production and spore formation by *Ampelomyces quisqualis*. Can J Microbiol 36:193–198
- Tu JC (1984) Mycoparasitism by *Coniothyrium minitans* and its effects on sclerotia germination. Phytopathol Z 109:261–268
- Tunlid A, Jansson HB, Nordbring-Hertz B (1992) Fungal attachment to nematodes. Mycol Res 96:401–412

- Uecker FA, Ayers WA, Adams PB (1980) *Teratosperma glio-cladum* – a new hyphomycetous mycoparasite on sclerotia of *Sclerotinia sclerotiorum*, *S. trifolium* and *S. minor*. Mycotaxon 10:421–427
- van Loon LC, Bakker PA, Pieterse CM (1998) Systemic resistance induced by rhizosphere bacteria. Annu Rev Phytopathol 36:453–483
- Viterbo A, Ramot O, Chernin L, Chet I (2002) Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. Antonie Van Leeuwenhoek 81:549–556
- Viterbo A, Harel M, Chet I (2004) Isolation of two aspartyl proteases from *Trichoderma asperellum* expressed during colonization of cucumber roots. FEMS Microbiol Lett 238:151–158
- Viterbo A, Harel M, Horwitz BA, Chet I, Mukherjee PK (2005) *Trichoderma* mitogen-activated protein kinase signaling is involved in induction of plant systemic resistance. Appl Environ Microbiol 71:6241–6246
- Webster J, Lomas N (1964) Does *Trichoderma viride* produce gliotoxin and viridin? Trans Br Mycol Soc 47:535–540
- Weindling R (1932) *Trichoderma lignorum* as a parasite of other fungi. Phytopathology 22:837
- Wells HD, Bell DK, Jaworski CA (1972) Efficacy of *Trichoderma harzianum* as a biocontrol agent for *Sclerotium rolfsii*. Phytopathology 62:442
- Woo S, Fogliano V, Scala F, Lorito M (2002) Synergism between fungal enzymes and bacterial antibiotics may enhance biocontrol. Antonie Van Leeuwenhoek 81:353–356
- Yedidia I, Benhamou N, Chet I (1999) Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Appl Environ Microbiol 65:1061–1070
- Yedidia I, Shores M, Kerem Z, Benhamou N, Kapulnik Y, Chet I (2003) Concomitant induction of systemic resistance to *Pseudomonas syringae* pv. *lachrymans* in cucumber by *Trichoderma asperellum* (T-203) and accumulation of phytoalexins. Appl Environ Microbiol 69:7343–7353
- Zeilinger S, Galhaup C, Payer K, Woo SL, Mach RL, Fekete C, Lorito M, Kubicek CP (1999) Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. Fungal Genet Biol 26:131–140
- Zinati GM (2005) Compost in the 20th century: a tool to control plant diseases in nursery and vegetable crops. HortTechnology 15:61–66

---

## 9 Antagonism of Plant Parasitic Nematodes by Fungi

S. CASAS-FLORES<sup>1</sup>, A. HERRERA-ESTRELLA<sup>2</sup>

### CONTENTS

I. Introduction .....	147
II. Biological Control of Nematodes .....	148
III. Virulence Factors .....	149
A. Serine Proteases .....	150
B. Chitinases .....	153
C. Other Enzymes .....	154
D. Toxins or Inhibitory Metabolites .....	154
IV. Improvement of Nematode-Trapping Fungi by Genetic Engineering .....	155
V. Concluding Remarks .....	155
References .....	155

### I. Introduction

The continuing growth of the human population and the consequent foodstuffs demand require the implementation of novel alternatives to increase food production. A reduction in crop damage caused by pests and diseases will be the best choice to solve food problems worldwide. Viruses, bacteria, fungi and nematodes are responsible for major losses in economically important crops. Plant parasitic nematodes represent a serious problem in agriculture around the world. The global loss per year in agricultural production due to damage generated by nematodes has been estimated as 100 billion US dollars worldwide (Nordmeyer 1992).

Nematodes have successfully established in nearly all-ecological niches. Plant parasitic nematodes obtain their food from the plant foliage and roots. The nematodes that obtain their food from plant roots are migratory ectoparasites, migratory endoparasites, semi-endoparasites and sedentary endoparasites. Many nematodes that directly or indirectly affect plant growth have

developed parasitic strategies that make them more efficient in exploiting their source of food (Sijmons et al. 1994). The damage provoked by nematodes cannot be recognized at first sight, because their ecological niche is mostly under soil. Plants infected by nematodes resemble those suffering from water or nutrients stress, loosing vigor and showing chlorosis.

Plant parasitic nematodes affect the plant directly by altering the morphology of the root system as a result of their feeding activities or by invasion of the plant tissue. This damage can be generated by the migratory stages of endo- and ectoparasitic nematodes. The most specialized level has been reached by the sedentary endoparasitic nematodes that invade the root, and partially reorganize the root function to satisfy their own demand of nutrients (Jung and Wyss 1999). In order to carry out these modifications, nematodes transform some of their cells into highly specialized feeding structures such as the stylet and feeding tubes, which provide a permanent source of nutrients enabling them to settle essentially indefinitely in the place of infection (Jung and Wyss 1999). The nematode penetrates the root tissues only by means of its stylet and injects secretory fluids, produced in esophageal glands; these fluids modify the plant cytoplasm prior to food removal. Some nematode species feed on the root tips of their host plants, which become transformed into terminal galls. Galls contain necrotic cells and enlarged multinucleated cells that are metabolically highly active and essential for nematode development, growth and reproduction. The most dramatic alterations in root architecture are generated by cyst and root-knot nematodes. The most important nematodes in agriculture are the root-knot nematode (*Meloidogyne*), and the cyst nematodes (*Heterodera* and *Globodera*), due to the extensive damage they cause to crops. Root-knot nematodes are generally polyphagous and each species can infect a large variety of plants species, from grasses to trees, by generating galls

<sup>1</sup> División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, Camino a la Presa de San José 2055, Lomas 4a sección, CP 78216, San Luis Potosí SLP, México

<sup>2</sup> Laboratorio Nacional de Genómica para la Biodiversidad, Cinvestav Campus Guanajuato, Km 9.6 Libramiento Norte Carretera Irapuato-León, A.P. 629, Irapuato 36500, Guanajuato, México

in the root. Due to their broad host range, these nematodes cannot be controlled by crop rotation. In contrast, cyst nematodes are highly host-specific parasites, and can be effectively controlled by crop rotation using non-host plants.

Since the origins of agriculture, man has used diverse strategies to eliminate pests that attack crop plants. Centuries ago, the Chinese used natural pest enemies to eliminate these, introducing ants in cultivated lands in order to eradicate hornets, worms and insects (Doutt 1964). Similarly, in the 19th century, American and European scientists used natural predators and pathogens to protect crops and forests, obtaining successful results (Doutt 1964). The work of those researchers lost impact at the beginning of the past century, with the discovery of chemical pesticides that resulted more efficient, cheaper, and with wider action spectra (Spiegel and Chet 1998). Since the second half of the last century, the use of pesticides has increased alarmingly with the aim of increasing agricultural production. Today the most common plant parasitic nematodes are controlled with chemical nematicides, cultural practices and by the use of resistant cultivars. Chemical nematicides can directly or indirectly reduce the density of nematode populations in soil (Johnson and Feldmesser 1987). These chemicals are applied as fumigants; root dips, foliarly or as seed treatments, and can be formulated as gases, volatile liquids, emulsifiable concentrates, etc. (Akhtar 1997). Most nematicide fumigants also show phytotoxic activity (Akhtar 1997). The use of chemical pesticides has provided good solutions, but only in the short term.

In spite of the "successful" use of chemical pesticides to efficiently control plant pests, it has been determined that these compounds are highly hazardous to human health and the environment. Another disadvantage of chemical pesticides is their persistence in the environment, which favors the selection of resistant pests, leading to the use of more toxic pesticides. These actions have generated concern around the world. Consequently, there is a heightened scientific interest in the establishment of integrated pest management strategies in order to reduce the application of chemical pesticides; these should be more effective, and less pollutant, such as traps, and other means of biological control.

The term biological control, in the classical sense, was defined by De Bach (1964) as the action of parasites, predators or pathogens in maintaining the population density of another organism at a lower average than would occur in their absence.

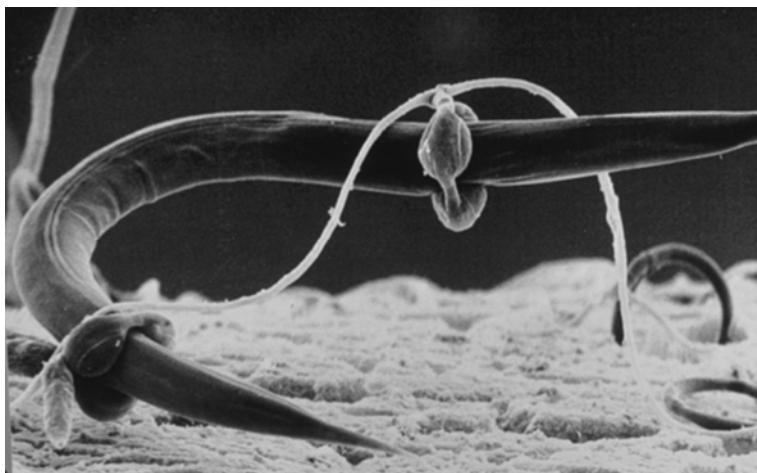
There is a plethora of natural enemies of plant pests, including bacteria, protozoa, predatory nematodes, and fungi, which could be used to reduce populations of phytophagous nematodes. During the second half of the 20th century, few research areas in plant pathology attracted more interest than the use of microorganisms to control plant pathogens. The great interest generated by the use of biological control against plant pathogens is a response to the growing concern of society regarded the unconstrained use of chemical pesticides.

## II. Biological Control of Nematodes

Biological control agents frequently use several modes of action such as antibiosis, parasitism, and competition for nutrients and space. It is important to highlight that, in addition to their antagonistic activity, a good control agent should have the ability to survive in the habitat where it is going to be applied. Nematodes have a large variety of natural enemies, including bacteria, protozoa, other nematodes, insects, mites, and fungi (Stirling 1991; Stirling and Smith 1998). Some antagonists of plant parasitic nematodes have been shown to be useful as biological control agents (Stirling 1991; Stirling and Smith 1998). Natural enemies of nematodes are often found in nematode-suppressive soils. Additionally, the introduction of natural enemies in soil has resulted in an efficient method for the biological control of nematodes. Most research on the interaction of nematodes with their natural enemies has been focused mainly on plant parasitic nematode species, due to their economic importance as crop pests (Akthar 1997).

The fungal antagonists of nematodes include nematode-trapping fungi, predacious fungi, endoparasitic fungi, egg parasitic fungi, cyst parasitic fungi and fungi that produce nematotoxic metabolites (Mankau 1980). Furthermore, these types of fungi play a key role in recycling elements such as carbon, nitrogen, and other important elements from the biomass found in soil, these activities positioning them as important microbial decimators in trophic chains.

General parasites such as the nematode-trapping fungi can attack plant parasitic nematodes and free-living nematodes. Since the description of the nematode-trapping fungus *Arthrobotrys oligospora* in 1988 (Zopf 1888),



**Fig. 9.1.** Nematode captured by the constricting rings of the predatory fungus *Arthrobotrys anchonia*. Note that the ring cells “cushion” around the body of the victim, but have not yet constricted the body. This is a very early stage after capture (scanning electron micrograph reprinted with permission from Barron)

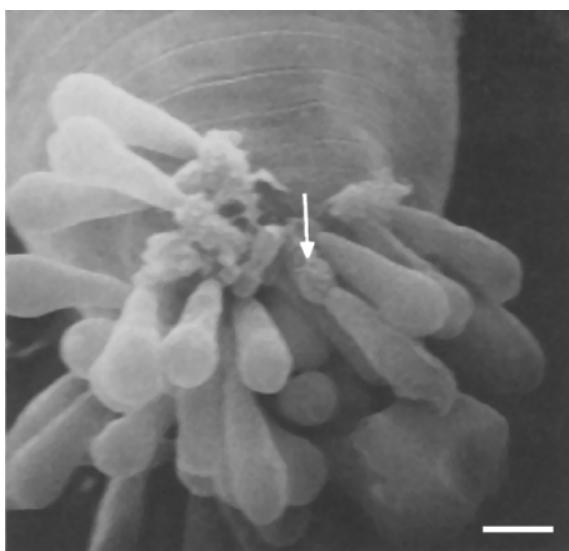
nematode-trapping fungi have become the most studied group with a high potential for use in biological control (Kerry 2000). Members of this group produce diverse structures to capture their preys (Barron 1977, 1979), such as hyphal traps, adhesive trapping nets or constricting rings (Fig. 9.1). Traps can be spontaneously produced, or their formation induced by nematodes or peptides (Nordbring-Hertz 1973; Barron 1977). Similarly, adhesive conidia of endoparasitic nematophagous fungi strongly attract nematodes, whereas non-adhesive conidia do not (Jansson 1982). Adhesive conidia of *M. coniospora* adhere specifically to the

head of female *P. reredivivus*, and the head and tail of the male (Fig. 9.2; Jansson et al. 1984).

### III. Virulence Factors

Nematophagous fungi are classified, according to their role as biological control agents, into free-living nematode parasites, and egg parasitic fungi (Kerry and Jaffe 1997). These can also be classified as facultative and obligate parasites. It is considered that facultative parasitic fungi use the nematodes as a supplementary source of nutrients, rather than a primary source. However, the construction of nematode-trapping nets by nematophagous fungi shows that nematophagy is an important trophic state (Jaffe 1992; Jaffe et al. 1992). The egg parasitic fungus *P. chamydosporia* grows much better on nematode-infested roots than on healthy roots or in the soil (Kerry 2000), and shows a genetic variability closely related to the host from which they were isolated (Morton et al. 2003). These observations suggest that nematodes may be more important to these fungi than being simply an eventual source of nutrients. In the case of obligate parasites, nematode infection initiates through the ingestion of spores or their attachment to the cuticle (Morton et al. 2003). Some endoparasites produce zoospores that are attracted to the nematodes before adhesion and encystment on the cuticle surface.

The parasitic fungi of free-living nematodes are similar to the egg parasitic fungi in some aspects. Nematode-trapping fungi grow in the soil and in the rhizosphere where they form nets of hyphae with trapping structures to capture nema-



**Fig. 9.2.** Scanning electron micrograph of the head region of a nematode, fixed in glutaraldehyde, heavily infected with conidia. Arrow Adhesive bud of conidia, bar 2  $\mu\text{m}$  (taken from Jansson et al. 1984)

todes. Some species produce consistent constricting rings, whereas others have adhesive hyphae.

The outer-most layer of a nematode is constituted by carbohydrates and proteins, and is considered to be of great importance in recognition events with the host plants and their antagonists (Bird and Bird 1991). The cuticle surface influences the specific interaction between the nematode and the fungus, including nematode-trapping fungi (Mendoza de Gives et al. 1999). Nematophagous fungi penetrate the nematode cuticle via trapping organs in predacious fungi, or conidia in endoparasitic fungi. The nematodes not only serve as prey, but also induce the formation of trapping structures of the fungus (Barron 1977; Nordbring-Hertz 1977). Cuticle penetration takes place only in living nematodes, with dead individuals being invaded through the mouth, anus, or other natural openings (Nordbring-Hertz and Stalhammar-Carlemalm 1978).

The initial phase of penetration is believed to be associated with recognition mediated by a lectin-carbohydrate interaction (Nordbring-Hertz 1983). Lectins located on fungal traps or adhesive conidia bind specifically to carbohydrates on the nematode cuticle. Further studies on the role of lectins in this interaction led to the proposal that the recognition event allows the release of enzymes, to proceed with the attachment of the fungus onto the nematode (Tunlid et al. 1992). However, disruption of the gene that encodes the lectin in *A. oligospora* did not affect the virulence of the fungus on the nematode (Balogh et al. 2003). Nevertheless, the possibility that another lectin could compensate for the loss of the product of the disrupted gene can not be discarded.

It has been suggested that after the recognition event, the fungus immobilizes the nematode and secretes extra-cellular enzymes at the point of contact that allow posterior parasitism (Tunlid et al. 1994). The endoparasitic fungi *D. coniospora* and *Hirsutella rhossiliensis*, through their adhesive spores, attack the anteroposterior part of the body of juvenile nematodes. Similarly, when the hyphae from nematophagous fungi reach the eggshell, they form appresoria from which extra-cellular enzymes are secreted (Lopez-Llorca and Robertson 1992). The formation of appresoria depends on the recognition of the host surface. Surface hydrophobicity is considered an important recognition factor (Lopez-Llorca et al. 2002).

Several studies have revealed that extra-cellular enzymes play an important role as virulence factors

in entomopathogenic fungi and nematophagous fungi (St. Leger 1995; Clarkson and Charnley 1996). In the case of nematophagous fungi, these enzymes are induced by the presence of nematode eggs. The set of enzymes of which the production is stimulated by eggs has been found to be directly related to the structure of the eggshell, which is formed by several layers (Wharton 1980), including a chitinous layer composed of a protein matrix (50–60%) embedding chitin microfibrils. The main enzymes induced are chitinases and proteases, and are considered virulence factors; some appear to be important determinants of host specificity (Åhman et al. 1996).

#### A. Serine Proteases

Alkaline serine proteases are produced by a wide variety of fungi that digest proteins under diverse nutritional conditions. The role of serine proteases in invertebrate pathogenesis was initially characterized in the entomopathogenic fungi *Metarrhizium anisopliae* (St. Leger et al. 1987), and *Bauveria bassiana* (Bidochka and Khachatourians 1987). A 30-kDa serine protease (Pr1) was found to play an important role in the infection process (Morton et al. 2004). Proteases from entomopathogenic fungi sharing characteristics with Pr1, including size, reaction to inhibitors and substrate utilization, called Pr1-like, were purified and characterized from the nematophagous fungi *Paecilomyces lilacinus* (Bonants et al. 1995), *P. rubescens* (Lopez-Llorca 1990), and *P. chlamydospora* (Segers et al. 1994). Consequently, research on proteases from entomopathogenic fungi has been closely followed by scientists studying the role of proteases in the infection process in nematodes.

The first report on protease production in nematophagous fungi came from nematode-trapping species (Schenck et al. 1980). The extra-cellular protease P32 from the egg parasite *Verticillium suchlasporium* was the first protease purified and characterized from a nematophagous fungus (Lopez-Llorca 1990). A similar protease, also named P32, was immunolocalized in appresoria of the fungus *P. rubescens*, which infects eggs of the beet cyst nematode *Heterodera schachtii*. These results pointed to the involvement of this protease in the infection process (Lopez-Llorca and Robertson 1992). In further research, the use of the protease inhibitors PMSF and DFP reduced egg penetration by the fungi *Lecanicillium lecanni*

and *P. chlamydosporia*, showing the relevance of proteases at the early stages of the infection process (Lopez-Llorca et al. 2002).

Those fungi that infect nematode eggs form specialized structures called appresoria; these structures adhere to eggshells through mucigens, where the infection develops to penetrate the eggshell (Morton et al. 2004). The fungus *Pochonia chlamydosporia* produces an alkaline serine protease, VCP1, during the infection of nematode eggs. The incubation of nematodes eggs with purified VCP1 resulted in the removal of the outer vitellin membrane from eggs of *Meloidogyne incognita* (Segers et al. 1994). Subsequent infections of these eggs by *P. chlamydosporia* extensively degraded the eggshell, to the degree of generating large holes in the structure, with no evident appresorium formation (Morton et al. 2004). Contrasting results were obtained when eggs of *G. pallida* were treated with VCP1, which might be due to the different composition of nematode eggshells (Morton et al. 2004). Based on these observations, it may be concluded that the outer vitellin membrane is a barrier that helps protect against infections, and which is overcome by the action of secreted proteases; furthermore, the vitellin membrane may be involved in host recognition (Morton et al. 2004).

Further research on the role of proteases in the process of infection by nematode egg parasitic fungi showed that incubation of *M. incognita* eggs with *P. lilacinus* culture filtrates disrupts egg development. This effect was not observed with eggs containing mature juveniles, but hatching appeared to be stimulated (Bonants et al. 1995). In yet another example, a serine protease belonging to the subtilisin family, designated PII, which immobilizes the active stages of *Panagrellus redivivus* and hydrolyzes its cuticle, was described for the fungus *A. oligospora* (Tunlid et al. 1994). The enzyme is expressed under starvation conditions and is repressed by primary glucose and nitrogen sources, which are more easily assimilated than the nematode cuticle (Åhman et al. 1996). Similarly, infection of nematode eggs by *D. coniospora* was blocked by the addition of the protease inhibitor chymostatin, indicating the possible role of chymotrypsin-like proteases in the infection process (Jansson and Frimen 1999).

Data obtained on the role of a neutral serine protease designated Aoz1 from the nematophagous fungus *A. oligospora* point in the same direction. The purified protein showed a molecular mass of

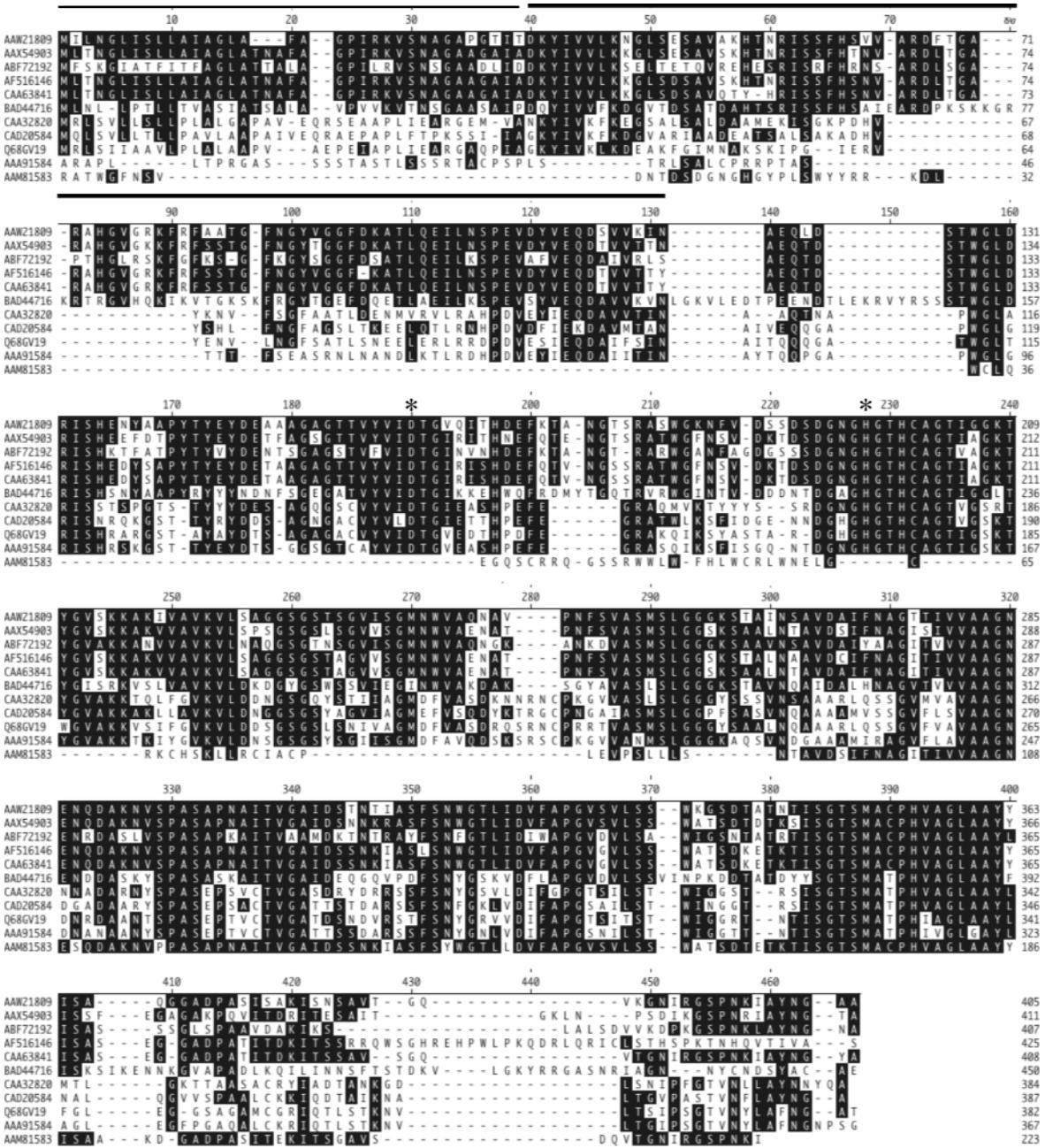
approximately 38 kDa. The expression of this protein is enhanced by addition of gelatine to the culture medium. Treatment of nematodes with purified enzyme showed dramatic structural changes in the nematode cuticle (Minglian et al. 2004). Sequence analysis from the cDNA and genomic clones revealed 97% similarity with PII from *A. oligospora* (Minglian et al. 2004). These data suggest that Aoz1 is likely a PII ortholog (Minglian et al. 2004).

Recently, the neutral serine protease Mlx from the nematophagous fungus *Monacrosporium microscaphoides* was purified and cloned (Wang et al. 2006). The protease could immobilize the nematode *Penegrellus redivivus* in vitro and degrade its purified cuticle, suggesting that Mlx could serve as a virulence factor during infection (Wang et al. 2006).

Almost all identified proteases from entomopathogenic and nematophagous fungi belong to the K subtilisin family, a large family of endopeptidases found only in fungi and bacteria. Proteins belonging to this family share a high degree of homology at the sequence level, showing only minor deletions or insertions in the sequence of its members (Siezen and Leunissen 1997; Fig. 9.3). The protease-encoding genes that have been cloned and sequenced are: the serine protease PIP from *P. lilacinus* (Bonants et al. 1995), the PII and Aoz1 genes from *A. oligospora* (Åhman et al. 1996; Minglian et al. 2004), the Vcp1 gene from *P. chlamydosporia* (Segers et al. 1994), the Ver112 gene from *Lecanicillium psalliotae* (Yang et al. 2005), and the neutral serine protease-encoding gene Mlx from *M. microscaphoides* (Wang et al. 2006).

Sequence analysis of the promoters of these protease-encoding genes has allowed the identification of regulatory elements described in other fungal systems that participate in responses to specific nutritional conditions. The promoter sequence from these genes include TATA boxes, GATA boxes, and CREA boxes that are involved in nitrogen and carbon catabolic repression (Screen et al. 1997).

Sequence analysis of these proteases shows the typical characteristics of subtilisins, such as the three catalytic amino acids Asp-His-Ser (Siezen and Leunissen 1997; Fig. 9.3). In addition, few variations were detected in the sequences, and these differences might be related to observed differences in substrate specificity (St. Leger et al. 1991). This group of proteases has a wide spectrum of peptidic substrates, but with preferences for specific substrates. Furthermore, Morton et al. (2003) ob-



**Fig. 9.3.** Multiple sequence alignment of proteases from different nematophagous fungi containing the conserved catalytic domains, performed by means of the ClustalW program. A *thin line* (*top*) indicates the region spanning the signal peptide, followed by the propeptide indicated with a *thicker line*. A high level of similarity among the different sources of proteases can be observed. Residues in *dark* indicate amino acids identical among all sequences. The putative catalytic triad (D-H-S) of the subtilase active site is marked by *asterisks*. The GenBank accession numbers AAX54903, ABF72192, CAD20584, AAA91584, AAM81583,

BAD44716, CAA32820, Q68GV19, CAA63841, AF516146, and AAW21809 correspond to the following organisms and proteases, respectively: *Arthrobotrys conoides* (cuticle-degrading protease), *Dactylorella avrietas* (cuticle-degrading protease), *Cordyceps chlamydosporia* (VCP1), *Paecilomyces lilacinus* (pSP-3), *Dactylaria parvispora* (cuticle-degrading protease), *Monacrosporium megalosporium* (cuticle-degrading protease), *Tritirachium album* (cuticle-degrading protease), *Lecanicillium psaliotiae* (Ver12), *Arthrobotrys oligospora* (PII), *Arthrobotrys oligospora* (Aoz1), and *Monacrosporium microscaphoides* (Mlx).

served strong variations at the sequence level and for substrate utilization in isolates from *P. chlamydosporia* in different nematode hosts.

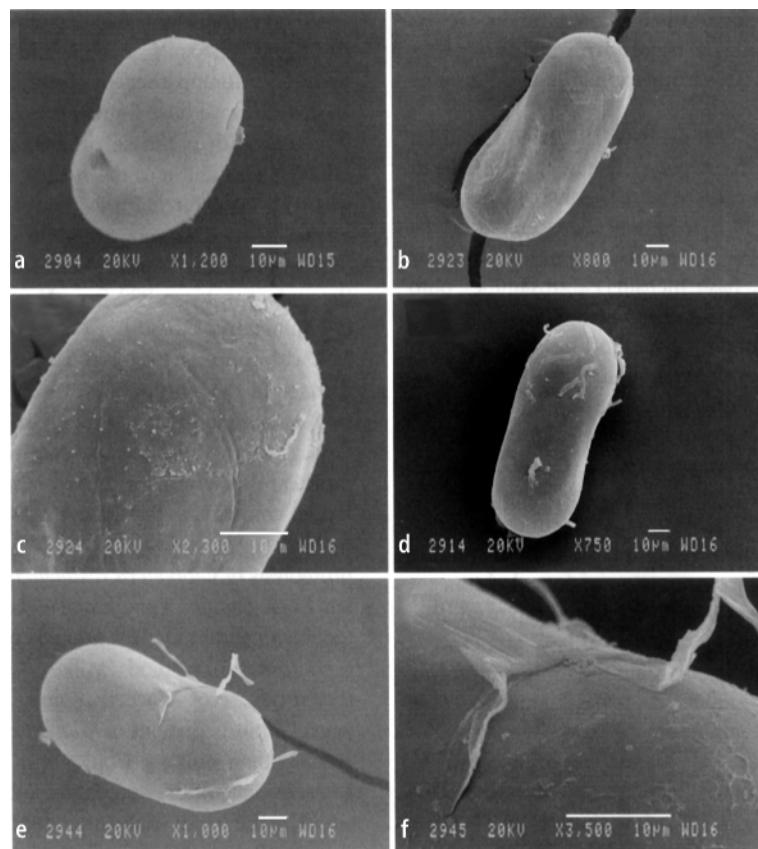
## B. Chitinases

Chitinases have been detected in a great variety of organisms that do not contain chitin, such as bacteria, higher plants and vertebrates, and also in those that contain chitin, such as insects, crustaceans and fungi (Herrera-Estrella and Chet 1999). Chitin is a structural polymer found in the cell wall of fungi, and constitutes the exoskeletons of invertebrates; it is an important component of the middle layer of nematode eggshells. Chitin is degraded by the combined action of endo- and exo-chitinases. All fungi analyzed to date produce intracellular chitinases necessary for the apical growth of hyphae (Takaya et al. 1998), while extra-cellular chitinases play an important role in parasitism (Chet et al. 1997). The chitinases and proteases of the biological control agents *Trichoderma* spp. are very similar to those of nematophagous fungi, and have been shown to

have a great potential for their use in the biological control of nematodes (Sharon et al. 2001).

Nematophagous fungi that parasitize eggs must penetrate the eggshell during the infection (Lysek and Krajci 1987; Lopez-Llorca and Duncan 1988). As mentioned above, the structure of the eggshell is formed by several layers, including a chitinous one (Wharton 1980). This is the thickest layer, and is likely to be the major barrier for infection (Bird and Bird 1991).

The first report for chitinase activity in nematophagous fungi was in *Verticillium* spp., isolated from infected nematode eggs, both in screening on solid media with colloidal chitin and in liquid media (Dackman et al. 1989). More recently, several chitinases produced by the egg parasitic fungi, *P. chlamydospora* and *P. rubescens*, have been purified. Culture filtrates of *P. rubescens* show higher activity of N-acetyl- $\beta$ -D-glucosamidase than culture filtrates of *P. chlamydospora* (Bird and Bird 1991). This might be due to the fact that eggshells from their preferred hosts are different. *Globodera* eggshells are thicker than those of *Meloidogyne*, and *P. rubescens*



**Fig. 9.4.** Scanning electron microscope (SEM) images of eggs of the nematode *Globodera*, treated with various enzymes and enzyme combinations. **a** Untreated eggs (control). **b** Treated with purified chitinase from *V. suchlasporium* (CHI43). **c** Close-up of **b** to show scars on eggshell. **d** Treatment with *V. suchlasporium* protease (P32). **e** Treatment with both P32 and CHI43. **f** Close-up of **e** to show extensive peeling of eggshell (modified from Tikhonov et al. 2002)

appears to prefer *Globodera* eggs. *G. pallida* eggs were treated with the purified chitinase CHI43, provoking scars on the egg surface; the addition of the protease P32 to the mix generated peeling on the eggshell membrane (Fig. 9.4; Tikhonov et al. 2002). These results show the importance of both enzymes in the infection, and contrast with the failure of purified VCP1 to produce similar effects on *Globodera* (Morton et al. 2004).

Unfortunately, current knowledge on the role of chitinases in the infection process is still very limited, especially at the level of gene expression. Fortunately, the situation is likely to change in the near future, with the study of more fungal genomes. In this sense, recently, the cDNAs from an acidic chitinase gene, *chi1*, and a basic chitinase gene, *chi2*, from *Verticillium lecanii* were isolated and the deduced protein sequence analyzed. The *chi1* cDNA encodes a predicted protein of 370 aa, while the cDNA of *chi2* encodes one of 423 aa. The basic chitinase gene (*chi2*) was successfully expressed in *Pichia pastoris*, where CHI2 was shown to be a functional enzyme that can hydrolyze chitinous substrates (Lu et al. 2005).

### C. Other Enzymes

Most studies of the chemical composition of the nematode cuticle have been performed with the mammal parasitic nematode, ascarids (Schenck et al. 1980). The ascarid cuticle is a three-layered, fibrous structure that contains collagen and keratin of types unique to the Nematoda (Bird 1971). Collagens are among the most complex of proteins, and are slowly degraded in natural soils and waters (Weiss 1976). Collagenolytic enzymes have been isolated from vertebrate (Gross and Lapierre 1962; Gross and Nagai 1965) and invertebrate (Phillips and Dresden 1973) animal tissues and from bacteria, but reports of production of collagenase by fungi are relatively rare (Hurion et al. 1979). Collagenase was defined as an enzyme that catalyzes the hydrolysis of collagen and gelatin, rather than other protein substrates (Mandl et al. 1953).

During the infection of nematodes, nematophagous fungi must penetrate the nematode cuticle; it is believed that collagenase is an important enzyme involved in the pathogenicity of nematophagous fungi (Dackman et al. 1992; Tunlid et al. 1994). One of the first attempts to determine whether nematode-trapping fungi produced extra-cellular collagenase and keratinase

was made by Schenck et al. (1980), who observed that a group of eight nematode-trapping fungi produced collagenase in the growth media of all tested species. All the tested species could secrete extra-cellular collagenase with acceptable collagen-hydrolyzing activities. In contrast, keratinase activity was not found in the culture media.

More recently, Tosi et al. (2002) reported results of a screening process comparing the Antarctic nematophagous fungus *Arthrobotrys tortor* with other *Arthrobotrys* species in the production of extra-cellular collagenases. To carry out this research, they used the nematode *Caenorhabditis elegans*. Collagenase activity was determined using insoluble collagen from bovine Achilles tendon, and measuring the amount of solubilized hydroxyproline produced. The results showed that the total amount of collagenase produced by the Antarctic strain of *A. tortor* was about three-fold higher than that observed for the other species. In the Antarctic strain, collagenase was shown to be a constitutive enzyme. The level of collagenase production in nematode-trapping fungi could be related to their virulence. Certain organisms producing collagenase are highly invasive; presumably, these collagenases contribute to their virulence.

### D. Toxins or Inhibitory Metabolites

In order to eliminate their competitors, many microorganisms produce toxic metabolites such as antibiotics. Toxins are also important for parasitic microorganisms, because they facilitate infection by debilitating the host (Morton et al. 2004). Organisms that can produce metabolites similar to those of nematicides have been investigated and considered as possible biocontrol agents.

Nematophagous fungi are not an exception in the production of toxins. The fungus *P. lilacinus* produces acetic acid that paralyzes juvenile nematodes (Djian et al. 1991). *Fusarium equiseti* produces compounds that reduce hatching of root-knot nematode eggs and immobilizes infective juveniles (Nitao et al. 1999). Fungal filtrates from several fungi grown in malt extract broth were toxic to infective juveniles and eggs (Chen et al. 2000). A metabolite with nematicidal activity against infective juveniles, phomalactone, was isolated from *P. chlamydosporia* (Khambay et al. 2000). Research into finding new metabolites with nematicidal activity by nematophagous fungi is practically a new

field to be exploited. Genetic and molecular analysis on the metabolic pathways to synthesize these metabolites could help to manipulate the production of these compounds in order to use them in fields infested by nematodes.

#### IV. Improvement of Nematode-Trapping Fungi by Genetic Engineering

Although many microbial antagonists of nematodes have been found and tested for their activity against nematodes, they have not led to the development of commercial products as cost-effective as chemical nematicides (Oka et al. 2000).

The most common strategy to control plant parasitic nematodes by the use of nematode-trapping fungi has been to mass-produce the fungus that infects the nematodes on solid substrates, followed by application to the soil. There are several reports of successful biological control of plant parasitic nematodes by means of nematophagous fungi; however, these are perceived as inefficient, compared to chemical nematicides. Based on this has emerged the demand for new strategies in order to combat phytophagous nematodes, one of these being the generation of improved fungal strains.

Improvement of biological control agents has involved the overexpression of lytic enzymes. In an attempt to obtain improved strains from the entomopathogenic fungus *A. oligospora*, Åhman et al. (2002) investigated the potential roles of protease II in host infection by generating several *PII* mutants, including mutants in which the corresponding gene had been disrupted, and transfromants that over-expressed it. Deletion of the *PII* gene had a limited effect on pathogenicity, including decreased percentages of adhesion and immobilization of nematodes, while overexpression of the gene resulted in a higher capacity to kill nematodes. Other interesting observations were that the deletion mutant produced less traps, while the multicopy transfromants produced more (Åhman et al. 2002).

#### V. Concluding Remarks

Despite the great amount of knowledge accumulated on the structural characteristics of specialized structures produced by nematode-trapping fungi during interaction with their hosts, and even

on the life cycle and ecology of this organisms, our understanding of the host-fungus interaction is poor at the molecular level, and it is necessary to gain further knowledge in this domain. All studies on lytic enzymes reviewed in this chapter undoubtedly indicate that they are important virulence factors in the infection process. However, as mentioned above, present understanding of their mode of action and their regulation at the molecular level is still limited. More research on virulence factors and their regulation is necessary in order to better understand the mechanisms underlying the nematode infection process by fungi, which in turn could be used to generate improved biological control fungi against nematodes by genetic engineering. It must be noted that lytic enzymes are unlikely to be the only factors involved in the infection process. In addition, the analysis of different isolated structures such as knobs, trapping nets, appresoria, and other structures will help to understand their role in the parasitic process as infection structures. Identification of new potential virulence factors is important, and new technologies such as functional genomics, proteomics, and metabolomics should enable us to identify the players involved in the infection process and to elucidate the signals that switch on the process in the fungus.

#### References

- Åhman JB, Ek B, Rask L, Tunlid A (1996) Sequence analysis and regulation of a gene encoding a cuticle-degrading serine protease from the nematophagous fungus *Arthrobotrys oligospora*. *Microbiology* 142:1605–1616
- Åhman JB, Johansson T, Olsson M, Punt PJ, Van Den Hondel C, Tunlid A (2002) Improving the pathogenicity of a nematode-trapping fungus by genetic engineering of a subtilisin with nematotoxic activity. *Appl Environ Microbiol* 68:3408–3415
- Akhtar M (1997) Current options in integrated management of plant-parasitic nematodes. Chapman and Hall, London
- Balogh J, Tunlid A, Rosen S (2003) Deletion of a lectin gene does not affect the phenotype of the nematodetrapping fungus *Arthrobotrys oligospora*. *Fungal Genet Biol* 39:128–135
- Barron GL (1977) The nematode-destroying fungi. Topics in Mycobiology 1, Canadian Biological Publications; Guelph
- Barron GL (1979) Observations on predatory fungi. *Can J Bot* 57:187–193
- Bidochka MJ, Khachatourians GG (1987) Purification and properties of an extracellular protease produced by the entomopathogenic fungus *Beauveria bassiana*. *Appl Environ Microbiol* 53:1679–1684

- Bird AF (1971) The structure of nematodes. Academic Press, New York
- Bird AF, Bird J (1991) The structure of nematodes. Academic Press, San Diego, CA
- Bonants PJ, Fitters MPFL, Thijs H, Den-Belder E, Waalwijk C, Henfling JWDM (1995) A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. *Microbiology* 141:775–784
- Chen SY, Dickson DW, Mitchel DJ (2000) Viability of *Heterodera glycines* exposed to fungal filtrates. *J Nematol* 32:190–197
- Chet I, Inbar J, Hadar Y (1997) Fungal antagonist and mycoparasites. In: Wicklow DT, Söderström B (eds) The Mycota IV, 1st edn. Environmental and microbial relationships. Springer, Berlin Heidelberg New York, pp 165–184
- Clarkson JMC, Charnley AK (1996) New insights into the mechanisms of fungal pathogenesis in insects. *Trends Microbiol* 4:197–203
- Dackman C, Chet I, Nordbring-Hertz B (1989) Fungal parasitism of the cyst nematode *Heterodera schachtii*: infection and enzymatic activity. *FEMS Microbiol Ecol* 62:201–208
- Dackman C, Jansson HB, Nordbring-Hertz B (1992) Nematophagous fungi and their activities in soil. In: Stotzky G, Bollag JM (eds) Soil biochemistry. Marcel Dekker, New York, pp 95–130
- De Bach P (1964) Biological control of insect pests and weeds. Chapman and Hall, London
- Djian C, Pijarowski L, Ponchet M, Arpin N, Favrebonvin J (1991) Acetic-acid a selective nematocidal metabolite from culture filtrates of *Paecilomyces lilacinus* (Thom) Samson and *Trichoderma longibrachiatum* Rifai. *Nematologica* 37:101–112
- Doutt RL (1964) The historical development of biological control. In: DeBach P (ed) Biological control of insect pests and weeds. Chapman and Hall, London
- Gross J, Lapierre CM (1962) Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proc Natl Acad Sci USA* 48:1014–1022
- Gross J, Nagai Y (1965) Specific degradation of the collagen molecule by tadpole collagenolytic enzyme. *Proc Natl Acad Sci USA* 54:1197–2004
- Herrera-Estrella A, Chet I (1999) Chitinases in biological control. In: Jolles P, Muzzarelli RAA (eds) Chitin and chitinases. Birkhäuser, Basel, pp 171–184
- Hurion N, Fromentin H, Keil B (1979) Specificity of the collagenolytic enzyme from the fungus *Entomophthora coronata*: comparison with the bacterial collagenase from *Achromobacter iophagus*. *Arch Biochem Biophys* 192:438–445
- Jaffe BA (1992) Population biology and biological control of nematodes. *Can J Microbiol* 38:359–364
- Jaffe BA, Muldoon AE, Tedford EC (1992) Trap production by nematophagous fungi growing from parasitised nematodes. *Phytopathology* 82:615–620
- Jansson HB (1982) Predacity by nematophagous fungi and its relation to the attraction of nematodes. *Microbial Ecol* 8:233–240
- Jansson HB, Friman E (1999) Infection-related surface proteins on conidia of the nematophagous fungus *Drechmeria coniospora*. *Mycol Res* 103:249–256
- Jansson HB, Nordbring-Hertz B (1983) The endoparasitic nematophagous fungus *Meria coniospora* infects nematodes specifically at the chemosensory organs. *J Gen Microbiol* 129:1121–1126
- Jansson HB, Hofsten AV, Mecklenburg CV (1984) Life cycle of the endoparasitic nematophagous fungus *Meria coniospora*: a light and electron microscopic study. *Antonie van Leeuwenhoek* 50:321–327
- Johnson AW, Feldmesser J (1987) Nematicides: a historical review. In: Veech JA, Dickson DW (eds) *Vistas on nematology*. Society of Nematologists, Hyattsville, MD, pp 448–454
- Jung C, Wyss U (1999) New approach to control plant parasitic nematodes. *Appl Microbiol Biotechnol* 51:439–446
- Kerry BR (2000) Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant parasitic nematodes. *Annu Rev Phytopathol* 38:423–441
- Kerry BR, Jaffe BA (1997) Fungi as biological control agents for plants parasitic nematodes. In: Wicklow DT, Söderström B (eds) The Mycota IV, 1st edn. Environmental and microbial relationships. Springer, Berlin Heidelberg New York, pp 203–218
- Khambay BSP, Bourne JM, Cameron S, Kerry BR, Zaki MJ (2000) A nematicidal metabolite from *Verticillium chlamydosporium*. *Pest Manage Sci* 56:1098–1099
- Lopez-Llorca LV (1990) Purification and properties of extracellular proteases produced by the nematophagous fungus *Verticillium suchlasporium*. *Can J Microbiol* 36:530–537
- Lopez-Llorca LV, Duncan GH (1988) A study of fungal endoparasitism of the cereal cyst nematode (*Heterodera avenae*) by scanning electron microscopy. *Can J Microbiol* 34:613–619
- Lopez-Llorca LV, Robertson WM (1992) Immunocytochemical localization of a 32-kDa protease from the nematophagous fungus *Verticillium suchlasporium* in infected nematode eggs. *Exp Mycol* 16:261–267
- Lopez-Llorca LV, Olivares-Bernabeu C, Salinas J, Jansson HB, Kolattukudy PE (2002) Prepenetration events in fungal parasitism of nematode eggs. *Mycol Res* 106:499–506
- Lu ZX, Laroche A, Huang HC (2005) Isolation and characterization of chitinases from *Verticillium lecanii*. *Can J Microbiol* 51:1045–1055
- Lysek H, Krajci D (1987) Penetration of ovicidal fungus *Verticillium chlamydosporium* through the *Ascaris lumbricoides* egg-shells. *Folia Parasitol (Praha)* 34:57–60
- Mandl I, MacLennan JD, Howes EL (1953) Isolation and characterization of proteinase and collagenase from *Cl. histolyticum*. *J Clin Invest* 32:1323–1329
- Mankau R (1980) Biocontrol: fungi as nematode control agents. *J Nematol* 12:244–252
- Mendoza de Gives PM, Davies KG, Clark SJ, Behnke JM (1999) Predatory behaviour of trapping fungi against srf mutants of *Caenorhabditis elegans* and different plant and animal parasitic nematodes. *Parasitology* 119:95–104
- Minglian Z, Minghe M, Keqin Z (2004) Characterization of a neutral serine protease and its full-length cDNA from the nematode-trapping fungi *Arthrobotrys oligospora*. *Mycologia* 96:16–22

- Morton CO, Hirsch PR, Peberdy JP, Kerry BR (2003) Cloning of and genetic variation in protease VCP1 from the nematophagous fungus *Pochonia chlamydosporia*. Mycol Res 107:38–46
- Morton CO, Hirsch PR, Kerry BR (2004) Infection of plant-parasitic nematodes by nematophagous fungi – a review of the application of molecular biology to understand infection processes and to improve biological control. Nematology 6:161–170
- Nitao JK, Meyer SLF, Chitwood DJ (1999) *In vitro* assays of *Meloidogyne incognita* and *Heterodera glycines* for detection of nematode antagonistic fungal compounds. J Nematol 31:172–183
- Nordbring-Hertz B (1973) Peptide induce morphogenesis in the nematode trapping fungus *Arthrobotrys oligospora*. Physiol Plant 29:223–233
- Nordbring-Hertz B (1977) Nematode-induced morphogenesis in the predacious fungus *Arthrobotrys oligospora*. Nematologica 23:443–451
- Nordbring-Hertz B (1983) Mycelial development and lectin carbohydrate interactions in nematode-trapping fungi. In: Jennings DH, Rayner ADM (eds) Ecology and physiology of the fungal mycelium. Cambridge University Press, London
- Nordbring-Hertz B, Stalhammar-Carlemalm M (1978) Capture of nematode by *Arthrobotrys oligospora*, an electron microscope study. Can J Bot 56:1297–1307
- Nordmeyer D (1992) The search for novel nematicidal compounds. In: Gommers FJ, Maas WTh (eds) Nematology from molecules to ecosystems. European Society of Nematologists, Invergowrie, Dundee, pp 281–293
- Oka Y, Koltai H, Bar-Eyal M, Mor M, Sharon E, Chet I, Spiegel Y (2000) New strategies for the control of plant-parasitic nematodes. Pest Manage Sci 56:983–988
- Phillips J, Dresden MH (1973) A collagenase in extracts of the invertebrate *Bipalium kewense*. Biochem J 133:329–334
- Schenck S, Chase T Jr, Rosenzweig WD, Pramer D (1980) Collagenase production by nematode-trapping fungi. Appl Environ Microbiol 40:567–570
- Screen S, Bailey A, Charnley AK, Cooper RM, Clarkson JM (1997) Carbon regulation of the cuticledegrading enzyme PR1 from *Metarhizium anisopliae* may involve a trans-acting DNA-binding protein CRR1, a functional equivalent of the *Aspergillus nidulans* CREA protein. Curr Genet 31:511–518
- Segers R, Butt TM, Kerry BR, Peberdy JF (1994) The nematophagous fungus *Verticillium chlamydosporium* Goddard produces a chymoelastase-like protease which hydrolyses host nematode proteins *in situ*. Microbiology 140:2715–2723
- Sharon E, Bar-Eyal M, Chet I, Herrera-Estrella A, Kleifeld O, Spiegel Y (2001) Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Phytopathology 91:687–693
- Siezen RJ, Leunissen JAM (1997) Subtilases: the superfamily of subtilisin-like serine proteases. Protein Sci 6:501–523
- Sijmons PC, Atkinson HJ, Wyss U (1994) Parasitic strategies of root nematodes and associated host cell response. Annu Rev Phytopathol 32:235–259
- Spiegel Y, Chet I (1998) Evaluation of *Trichoderma* spp. as a biological control agent against soil borne fungi and plant-parasitic nematodes in Israel. Integr Pest Manage Rev 3:1–7
- Stirling GR (1991) Biological control of plant parasitic nematodes: progress, problems and prospects. CABI, Wallington
- Stirling GR, Smith LJ (1998) Field tests of formulated products containing either *Verticillium chlamydosporium* or *Arthrobotrys dactyloides* for biological control of root-knot nematodes. Biol Control 11:231–239
- St. Leger RJ (1995) The role of cuticle-degrading proteases in fungal pathogenesis of insects. Can J Bot 73:S1119–S1125
- St. Leger RJ, Charnley AK, Cooper RM (1987) Characterization of cuticle-degrading proteases produced by the entomopathogen *Metarhizium anisopliae*. Arch Biochem Biophys 253:221–232
- St. Leger RJ, Charnley AK, Cooper RM (1991) Kinetics of the digestion of insect cuticles by a protease (Pr1) from *Metarhizium anisopliae*. J Invertebr Pathol 57:146–147
- Takaya N, Yamakazi D, Horiuchi H, Ohta A, Takagu M (1998) Intracellular chitinase gene from *Rhizopus oligosporus*: molecular cloning and characterization. Microbiology 144:2647–2654
- Tikhonov VE, Lopez-Llorca LV, Salinas J, Jansson HB (2002) Purification and characterization of chitinases from the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium*. Fungal Genet Biol 35:67–78
- Tosi S, Aannovazzi L, Tosi I, Iadarola P, Caretta G (2002) Collagenase production in an Antarctic strain of *Arthrobotrys tortor* Jarowaja. Mycopathologia 153:157–162
- Tunlid A, Jansson HB, Nordbring-Hertz B (1992) Fungal attachment to nematodes. Mycol Res 96:401–412
- Tunlid A, Rosen S, Ek B, Rask R (1994) Purification and characterisation of an extracellular serine protease from the nematode-trapping fungus *Arthrobotrys oligospora*. Microbiology 140:1687–1695
- Wang M, Yang J, Zhang KQ (2006) Characterization of an extracellular protease and its cDNA from the nematode-trapping fungus *Monacrosporium microscaphoides*. Can J Microbiol 52:130–139
- Wharton DA (1980) Nematode egg-shells. Parasitology 81:447–463
- Weiss J (1976) Enzymatic degradation of collagens. Int Rev Connect Tissue Res 7:101
- Yang J, Huang X, Tian B, Wang M, Niu Q, Zhang K (2005) Isolation and characterization of a serine protease from the nematophagous fungus, *Lecanicillium psalliotae*, displaying nematicidal activity. Biotechnol Lett 27:1123–1128
- Zopf W (1888) Zur Kenntnis der Infektions-Krankheiten niederer Tiere und Pflanzen. Nova Acta Leopold Carol 52:314–376

---

# 10 Entomopathogenic Fungi and Their Role in Pest Control

A.K. CHARNLEY<sup>1</sup>, S.A.COLLINS<sup>1</sup>

## CONTENTS

I. Introduction .....	159
II. Taxonomy .....	159
III. Infection Process .....	160
A. Fungal Invasion of the Host .....	160
B. Host Response to Fungal Invasion .....	162
IV. Epizootiology of Fungal Diseases in Insects .....	162
V. Pest Control .....	163
A. Approaches to the Use of Insect Pathogenic Fungi for Pest Control .....	163
B. Current Use .....	165
1. Status of Mycoinsecticides .....	165
2. Constraints on Efficiency .....	170
3. Integration in Pest Management Schemes .....	171
C. Development of a Mycoinsecticide .....	171
1. Isolate Selection .....	171
2. Production and Formulation .....	172
3. Application .....	175
4. Safety .....	176
5. Registration .....	177
VI. Future Developments .....	178
A. Potential Targets .....	178
B. Constraints on the Commercial Use of Entomopathogenic Fungi .....	178
C. Strain Improvement .....	179
VII. Conclusions .....	180
References .....	181

## I. Introduction

Synthetic chemical pesticides have been the mainstay of insect pest control for over 50 years. However, insecticide resistance, pest resurgence and concern over the environmental impact of agricultural inputs give urgency to the search for alternative, biologically based forms of pest control. The impact on insect populations of natural epizootics caused in particular by fungal and viral pathogens demonstrates the potential of microbial pest control. Seminal attempts towards the end of the 19th Century to use the Ascomycota pathogen *Metarhizium anisopliae* for pest control (described in Gillespie 1988) inspired more recent extensive

efforts to harness entomopathogenic fungi for biocontrol. This review outlines the current state of knowledge of insect fungal pathogens as it relates to their present use and future potential as mycoinsecticides.

## II. Taxonomy

Relationships between fungi and insects may be mutualistic, through commensal to obligately pathogenic. The term entomogenous is often used to encompass all types of association between insects and fungi, with disease-causing fungi being referred to as entomopathogenic. A further distinction can be made between fungi which are aggressively pathogenic, like *Metarhizium anisopliae*, and opportunists, like the wound pathogen *Mucor haemalis* (McCoy et al. 1988; Samson et al. 1988; Tanada and Kaya 1993). The system of classification adopted here accords with that in the Index Fungorum ([www.speciesfungorum.org](http://www.speciesfungorum.org)).

Entomopathogens are to be found in most taxonomic groupings in the fungal kingdom, apart from the higher Basidiomycota. The phylum Oomycota, in the kingdom Chromista (Leipe et al. 1994), originally classified as fungi, includes the mosquito pathogen *Lagenidium giganteum*. Of the fungi, the most common insect pathogens in phylum Chytridiomycota are found in the genus *Coelomomyces*, e.g. *C. psorophorae*, a mosquito pathogen with an obligate copepod secondary host. Entomophthorales, e.g. genera *Conidiobolus*, *Entomophaga*, *Erynia* and *Pandora*, are widespread members of the phylum Zygomycota. Mummified aphids stricken by fungi of this group are familiar features of cereal crops in temperate regions (Fig. 10.1f). Among the members of the phylum Ascomycota, *Cordyceps* spp. have fruiting structures or perithecia which can dwarf the cadavers of their insect victims (Fig. 10.1b). The most widespread insect pathogenic fungal genera are found in

<sup>1</sup> Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK

the order Hypocreales of the phylum Ascomycota, viz. *Beauveria* and *Metarhizium*. *Beauveria bassiana* (Fig. 10.1g, h) and *Metarhizium anisopliae* (Fig. 10.1g) have broad host ranges, though considerable specificity occurs among individual isolates. Subspecies *M. anisopliae* var. *majus* and *M. anisopliae* var. *acridum* are specific for Scarabid beetles and grasshoppers/locusts respectively (Samson et al. 1988; Driver et al. 2000). Other important entomopathogens in the phylum Ascomycota include *Lecanicillium* (Fig. 10.1d) (formerly *Verticillium*), *Nomuraea* (both Incertae sedis: Ascomycota) (Fig. 10.1c), *Paecilomyces* (Eurotiales: Ascomycota) and *Aschersonia* (Hypocreales: Ascomycota) (Fig. 10.1a). The fungi described above are all destructively pathogenic. Laboulbeniales (Ascomycota), on the other hand, are biotrophic. They remain largely external to their hosts, gaining nutrition via a penetrant haustoria while apparently causing little harm. Most Trichomycetes (Zygomycota) have a commensal existence in the guts of their Dipteran hosts (Misra 1998).

### III. Infection Process

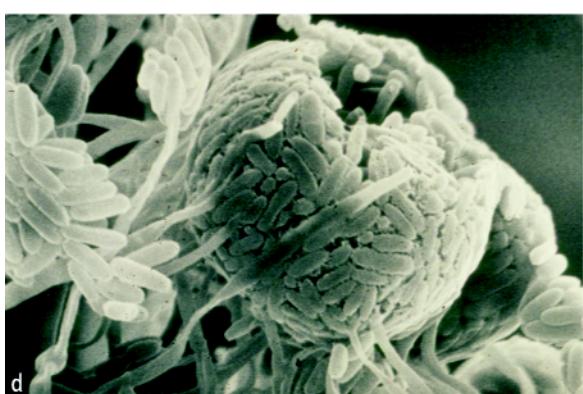
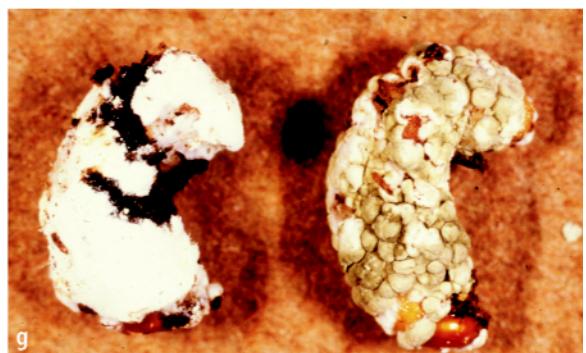
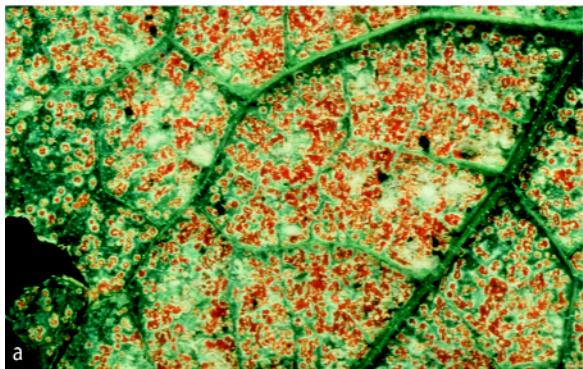
Unique among entomopathogens, fungi do not have to be ingested and can invade their hosts directly through the exoskeleton or cuticle. Therefore, they can infect non-feeding stages such as eggs and pupae. The site of invasion is often between the mouthparts, at intersegmental folds or through spiracles, where locally high humidity promotes germination and the cuticle is non-sclerotised and more easily penetrated (Hajek and St Leger 1994; Clarkson and Charnley 1996). Members of the Hypocreales such as *Metarhizium* spp. and *Beauveria* spp. are opportunistic hemibiotrophs with a parasitic phase in the live host and saprotrophic phase during post-mortem growth on the cadaver. These fungi may use toxins to help overcome host defences (Gillespie et al. 2000; Freimoser et al. 2003a). By contrast, Entomophthorales are biotrophs with little or no saprotrophism; they kill by tissue colonisation without the help of toxins (Charnley 2003; Freimoser et al. 2003b).

#### A. Fungal Invasion of the Host

*M. anisopliae* and *B. bassiana* have hydrophobic spores which appear to bind to insect cuticle by non-specific interactions, though failure to adhere

to particular insect species may help to define isolate host range (Holder and Keyhani 2005). Zoospores of *Lagenidium giganteum* are host selective. Cuticle-degrading enzymes are present on the surface of conidia of *M. anisopliae* and, therefore, there is the potential for the fungus to modify the cuticle surface to aid attachment. Host and fungal lectins have been implicated also in the process of attachment. Germination in vitro of nutrient-dependent spores of *M. anisopliae* and *B. bassiana* is consequent upon a non-specific, accessible source of carbon and nitrogen, though in vivo isolate specificity may depend on response to qualitative and quantitative differences in available nutrients on the host cuticle. More selective pathogens appear to have more specific requirements, e.g. strains of *M. anisopliae* which are specialist for Scarabid beetles. Ability to withstand antifungal compounds in the cuticle, such as short chain fatty acids, is a prerequisite for successful invasion (see Boucias and Pendland 1991). The importance of signal exchange between host and pathogen is seen in particular in the cues which cause the fungus to stop horizontal growth on the

Fig. 10.1. a *Aschersonia aleyrodis* (Ascomycota: Hypocreales), sporulating on cadavers of the glasshouse whitefly *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae) (with permission of HRI at the University of Warwick, UK). b Stroma of *Cordyceps* sp. (Ascomycota: Hypocreales: Clavicipitaceae) emerging from a locust (Orthoptera: Acrididae). They each contain many ascospores (with permission of H. Evans, CABI Bioscience, Egham, UK). c Dead caterpillar (Lepidoptera) covered in the green conidia of *Nomuraea rileyii* (Ascomycota: Hypocreales) (with permission of HRI at the University of Warwick, UK). d Scanning electron micrograph of conidia of *Lecanicillium longisporum* (Ascomycota: Incertae sedis) (with permission of HRI at the University of Warwick, UK). e A germinating conidium of *Metarhizium anisopliae* var. *acridum* (Ascomycota: Hypocreales), on the surface of the wing of a desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae). The germ tube terminates in an appressorium. f *Pandora neoaphidis* (Zygomycota: Entomophthorales) on the aphid, *Macrosiphum euphorbiae* (Homoptera: Aphididae). Note white halo of spores on the leaf around the dead aphids (brown, centre-right). g Dead larvae of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae); the one on the left is covered in conidia of *Beauveria bassiana* (Ascomycota: Hypocreales), the one on the right is covered in conidia of *Manisopliae* var. *anisopliae* (Ascomycota: Hypocreales) (with permission of HRI at the University of Warwick, UK). h *Beauveria bassiana* (Ascomycota: Hypocreales) sporulating on cadavers of blowflies, *Phormia regina* (Diptera: Calliphoridae) (with permission of D. Steinkraus, University of Arkansas, USA)



surface of the cuticle, produce an appressorium and initiate penetration. Differentiation of the germ tube to produce the holdfast structure, or appressorium, is most completely understood for *M. anisopliae*. Isolate ARSEF2575 requires low concentrations of a complex carbon and nitrogen source, and a hard surface. *Metarhizium* isolates from Homoptera form appressoria in media (high concentrations of simple sugars) which are repressive for isolates from Coleoptera. This is probably an adaptation to parasitism, as the cuticle of plant-sucking bugs (Homoptera) is contaminated with sugars from their copious excreta (St Leger et al. 1992a). The specificity for locusts of at least one isolate of *M. anisopliae* var. *acridum* appears in part to be due to cues for appressorium formation (Wang and Leger 2005; Fig. 10.1e).

Numerous light and electron microscope studies on the invasion of host cuticle by entomopathogenic fungi are consistent with the involvement of both enzymes and mechanical pressure. Insect cuticle comprises between 60–70% protein; therefore, it is perhaps not surprising that recent work has implicated proteases in particular in the penetration process (for review, see Charnley 2003).

Once the fungus breaks through the cuticle and underlying epidermis, it may grow profusely in the blood, in which case insect death is probably the result of starvation or physiological/biochemical disruption brought about by the fungus. Alternatively, insecticidal secondary metabolites may contribute to the demise of the insect and, in this case, extensive growth of the fungus may occur only on the cadaver of the host (Charnley 2003). For many fungi, the reality is probably somewhere between these two extremes. The absence of fungal proteases, so prominent during cuticle penetration, is a marked feature of this stage of mycosis (Wang et al. 2005). Few studies have examined the effect of fungal infection on host physiology/behaviour. This is unfortunate because, from the point of view of crop protection, sub-lethal or prelethal effects of mycosis may be just as useful as the death of the host. Detrimental effects of mycosis on food consumption, egg laying and flight behaviour have been recorded (Nnakumusana 1985; Seyoum et al. 1995).

The life cycle is completed when the fungus sporulates on the cadaver of the host. Under appropriate conditions, particularly high relative humidity (RH), the fungus will break out through the body wall of the insect, producing aerial spores. This may allow horizontal or vertical transmission of the dis-

ease within the insect population. Resting spores (chlamydospores) produced within the dead insect may enable the fungus to survive for long periods under adverse conditions (Samson et al. 1988). Additionally, in the locust pathogen *M. anisopliae* var. *acridum*, conidia may be produced in internal air spaces as the cadaver dries out under low humidity (Prior, personal communication).

## B. Host Response to Fungal Invasion

Host response has been extensively reviewed (Vilcinskas and Gotz 1999; Gillespie et al. 2000). The cuticle is not only the first but also the major barrier to host invasion. Structural features such as sclerotisation impede penetration, while enzyme inhibitors and tyrosinases, which generate antimicrobial melanins, are frontline defences against weak pathogens. Blood-borne defences seem to have little impact on virulent fungal pathogens. Phagocytosis by individual blood cells and cooperative behaviour between haemocyte subpopulations viz. encapsulation and granuloma formation are often not recorded after the initial incursion. This has been attributed to a failure of the insect's non-self recognition system, in some cases brought about by toxic fungal metabolites, in others due to the removal of immunogenic components from fungal cell walls (or even the walls themselves) in the blood of infected insects (see Charnley 2003). The important contribution of antifungal peptides in defence against non-adapted pathogens is also recognised (Kim and Kim 2005).

## IV. Epizootiology of Fungal Diseases in Insects

Although epizootics of insect fungal diseases are comparatively common, our understanding of population-level interactions between entomopathogenic fungi and their hosts is based only on a restricted number of studies (Carruthers and Soper 1987; Goettel et al. 2005). Natural disease development and spread are affected by the characteristics of the host and pathogen populations, the environment, and the impact of human activities (particularly in agroecosystems). Properties of the pathogen population which are important include virulence, dispersal, and survival in the host's environment, inoculum density

and spatial distribution. Host population factors which need to be considered are susceptibility, density, movement and spatial distribution. Abiotic environmental factors such as temperature, moisture and sunlight may determine whether infection can occur. Germination and sporulation are particularly dependent on moisture. Temperature may also be limiting for disease, particularly when short generation time for the host is favoured by a temperature which is above or below the optimum for the pathogen. Possible complex interactions between virulent and avirulent isolates were suggested by the outcome of an experimental dual infection in the leaf-cutting ant *Acromyrmex echinatior* (Hughes and Boomsma 2004). The normally avirulent *Aspergillus flavus* dominated when inoculated with *M. anisopliae* – probably the outcome of immunosuppression achieved by *Metarhizium* toxins.

Some of the more detailed epizootiology studies of insect pathogenic fungi come from agroecosystems. Examples include *Nomuraea rileyi* infection of *Anticarsia gematalis* on soybean (Ignoffo 1981) and *Entomophthora muscae* on the onion fly *Delia antiqua* (Carruthers and Haynes 1986). Forests are more diverse stable habitats than agroecosystems; consequently, the insect-pathogen interactions may be more complex. The spruce budworm *Choristoneura fumiferana* is a major defoliator in balsam and spruce trees in eastern North America. Among a range of pathogens which attack this insect, the fungi *Erynia radicans* and *Entomophaga aulicae* produce the highest mortality (Perry and Whitfield 1984). Disease incidence depends on insect age and position in the tree canopy; the role of abiotic factors is not completely understood. Humid, tropical forests have a rich, varied entomopathogenic mycoflora (Evans 1982), including in particular *Cordyceps* spp., and it has been suggested that these fungi have a significant role in the regulation of insect populations because of the stable microclimates in such habitats.

Rangelands are more stable than agricultural systems but more uniform in structure than forest ecosystems. Grasshoppers are often the dominant phytophagous insects in such habitats. *Entomophaga grylli* mycoses cause high mortality among populations of *Camnula pellucida* and *Melanoplus bivittatus* in western North America (Pickford and Riegert 1963).

Soil is a complex habitat which harbours a large fauna and flora. *Metarhizium anisopliae* is one of the most frequent mycopathogens of soil

insects in temperate regions, particularly of beetles (Keller and Zimmermann 1989). Epizootics have been found on wireworms (*Agriotes* spp.) and larvae of *Amphimallon solstitialis*. *M. anisopliae*, *B. bassiana* and *Paecilomyces* sp. are commonly isolated from temperate soils. The ubiquity in particular of the former two species must reflect their broad host ranges. *Beauveria brongniartii* is primarily a pathogen of cockchafers, *Melolontha* spp., and other Scarabidae. Investigations of the population dynamics of cockchafers in eastern Switzerland showed that *B. brongniartii* is the main regulating factor (Zimmermann 1992). Soil can also function as a reservoir for fungi which generally infect insects on aerial parts of plants. It has been shown experimentally that spores of the lepidopteran pathogen *N. rileyi* adhere to leaves of plant seedlings as they emerge through the soil (Ignoffo et al. 1977). Fungi may persist in soil as mycelium within mummified cadavers, conidia, resting spores (e.g. Entomophthorales) or pseudosclerotia. Temperature, pH, water and organic content can affect fungal survival (Keller and Zimmermann 1989).

Aquatic ecosystems present different problems to entomopathogenic fungi. In comparison with terrestrial habitats, fluctuations in temperature and sunlight may be less important whereas fluctuations in pH, salinity, currents and dissolved solutes may affect pathogen persistence. Although some pathogens may infect aquatic insects using specialist-shaped spores or motile zoospores to aid host location, e.g. *Lagenidium giganteum*, others confine themselves to the aerial adults, thereby avoiding the problems presented by the aquatic environment (Lacey and Undeen 1986).

## V. Pest Control

### A. Approaches to the Use of Insect Pathogenic Fungi for Pest Control

Several strategies have been adopted for the use of insect pathogenic fungi in pest control. The most cost effective is permanent introduction (also known as 'classical control'). This involves the establishment of a disease in a population where it does not occur normally, in order to obtain long-term or permanent suppression of the pest. Although there is a history of this approach, there are comparatively few examples where detailed population studies have determined the

effectiveness of introductions. Milner et al. (1982) introduced an Israeli isolate of *Zoophthora radicans* into alfalfa fields in Australia in an attempt to control an accidentally introduced pest, the spotted alfalfa aphid *Therioaphis trifolii maculata*. The fungus became established and spread from the release site in subsequent seasons, causing high percentage mortality among aphid populations. *Entomophaga maimaiga* was introduced from Japan into eastern North America in 1910–1911 to control the gypsy moth *Lymantria dispar*. The fungus did not appear to have become established. However, in 1989 and 1990, it caused extensive mortality among gypsy moth in 10 states in the USA (Hajek et al. 1995). It is possible that it was present but unrecognised for many years, as symptoms of mycosed gypsy moths resemble those of moths infected with a baculovirus. Resting spores of the fungus have been used successfully to introduce the fungus into new areas.

Promoting natural fungal epizootics by adopting appropriate cultural and crop protection practices is an alternative way of harnessing entomopathogenic fungi for pest control. A classic example is the development of early harvesting strategies for alfalfa to maximise development and spread of natural infections of *Zoophthora* sp. among alfalfa weevils (*Hypera postica*) (Brown and Nordin 1986). Steinkraus et al. (1995) showed that natural infections of *Neozygites fresneii* (Entomophthorales) could reduce or even eliminate the need for chemical control of aphids on cotton. They were able to predict epizootics a week in advance and advise farmers to refrain from chemical sprays at crucial times.

Epizootics of fungal pathogens on crop pests often occur too late to be of economic value. Application of an additional inoculum can accelerate the process. When this results in secondary spread of disease, the process is termed 'inoculative augmentation', otherwise the strategy is called 'inundative augmentation'; in reality, these are the two extremes of a continuum. Augmentation now also encompasses the application of a fungus in a situation where mycosis may not naturally occur. Mass production for inundative control is known as the 'microbial pesticide' or 'mycoinsecticide' approach (Tanada and Kaya 1993). The development of mycoinsecticides has received the most attention in recent times and is focussed on here.

Mycoinsecticides may perform inconsistently for a variety of reasons but particularly due to unfavourable environmental conditions (see

below). Therefore, strategies have been developed to increase efficiency and accelerate kill, e.g. by combining fungi with sub/low lethal doses of chemical pesticides. This approach is based on the assumption that, weakened by another stressor, the insect will succumb more readily to mycosis. In China, combinations of *B. bassiana* and certain insecticides have been recommended for application against crop and forest pests (Feng et al. 1994). Most experimental studies with mixtures suggest additive effects of the ingredients. However, synergy occurred between imidacloprid and *B. bassiana* against the Colorado beetle (Furlong and Groden 2001) and the caterpillar *Spilarctia obliqua* (Purwar and Sachan 2006). The same insecticide was antagonistic with *B. bassiana* against *Bemisia argentifolii* (James and Elzen 2001). *B. bassiana* and *Bacillus thuringiensis* toxins were synergistic against the Colorado beetle (Wraight and Ramos 2005). An alternative is to use the two treatments sequentially, rather than in combination. Early season use of *B. bassiana* followed by insecticides gave good control of beetle and caterpillar pests on crucifer crops (Vandenberg et al. 1998).

The alternative to the use of a chemical stressor is to combine entomopathogenic fungi with other microbial pathogens (see Zimmermann 1994). Though there are few examples where this strategy has been tried, it is given credence by observations of mixed infection in field-collected insects. Laboratory and glasshouse trials established the efficacy of a combination of *M. anisopliae* and entomopathogenic nematodes against the beetle *Hoplia philanthus* (Ansari et al. 2004). In Columbia, there is a product called Micobiol Completo, which is a mixture of *B. bassiana*, *M. anisopliae*, *N. rileyi*, *P. fumosoroseus* and the bacterium *B. thuringiensis* at  $1 \times 10^9$  spores of each pathogen per g of product, for control of larvae and adult Lepidoptera, Coleoptera, Hemiptera and Diptera, and mites (Alves et al. 2003). However, it seems unlikely that combinations will be used widely, as this strategy complicates application procedures as well as increasing the costs of pest control and initial development (particularly registration).

Limitation of fungal isolates to particular climatic conditions could theoretically be remedied by co-formulation of isolates, from the same or different species, with different ecological tolerances. This receives some support from the literature, e.g. Inglis et al. (1997). Interestingly, rarely is synergy observed and often one isolate dominates over the other (Wang et al. 2002; Rao et al. 2006).

## B. Current Use

### 1. Status of Mycoinsecticides

Crop protection is still dominated by chemical pesticides. Sales of biopesticides, at ca. US\$600 million, account for only 2% of the global crop protection market (Anon 2005). Mycoinsecticides accounted for a small fraction of the biopesticide market, the major share being taken by products based on the toxicogenic bacterium *Bacillus thuringiensis* (Bt). The controversial but successful introduction of crops, e.g. cotton, maize, potato and rice, genetically engineered to produce Bt toxin heralds a new era for insect control (James 2005).

Other than for transgenic crops, biopesticide production is dominated by small to medium-sized companies (SMEs). Large multinational agrochemical companies who, encouragingly, invested in biological forms of pest control in the 1980s have

since pulled out; an exception is the current involvement of some Japanese companies, e.g. Certis is the biopesticide arm of Mitsui & Co Ltd. However, it is clear that concerns over insecticide resistance, the impact of synthetic chemicals in environmentally sensitive areas, cost of registering chemicals for high-value but specialist crops, and the growing interest in 'organic' food ensure a market for biological forms of pest control including products based on entomopathogenic fungi.

Any attempt to produce a definitive list of recommendable commercial producers is hindered by the difficulty in obtaining realistic information, rather than company hype. A particular problem is gauging the size of an operation. The companies and products detailed in Table 10.1. appear to be the most important in the countries in which they originate. Taken as a whole, the list indicates a considerable level of interest and involvement in mycoinsecticide use.



**Fig. 10.2.** a Vertalec, a product based on *Lecanicillium longisporum*, targeted against glasshouse aphids (see Table 10.1.). b Initial brochure of the multi-donor funded LUBILOSA project on developing *Metarhizium anisopliae* for locusts and grasshopper control. c Logo of the product, based on *Metarhizium anisopliae* var. *acridum*, developed for locust control by LUBILOSA (see Table 10.1.). d Mycotrol, a product based on *Beauveria bassiana*, for control of a number of pests. Wettable powder formulation and a plate culture of the fungus are also shown (see Table 10.1.). E Botanigard, a product based on *Beauveria bassiana*, for control of a number of pests (see Table 10.1.).

Table 10.1. Commercial production of mycoinsecticides<sup>a</sup>

Country of the company	Product	Company/*registration other than home country	Target	Active ingredient/ production	Formulation and shelf life	Label crops
Austria	Melocont® Pilzgerste	Kwizda Agro GesmbH	Cockchafers	<i>Beauveria brongniartii</i>	Barley kernels, 7.5 × 10 <sup>9</sup> spores g <sup>-1</sup> , 12 months at 2 °C	Grassland, forest, horticulture crops
Australia	BioCane™	Becker Underwood Pty Ltd. (www.beckerunderwood.com)	Greyback cane grub	<i>Metarhizium anisopliae</i>	Granules, on grain, 2 × 10 <sup>9</sup> g <sup>-1</sup> , 6 months at 5–10 °C	Sugarcane
	BioGreen™	ditto	Red-headed cockchafer	<i>Metarhizium anisopliae</i>	Granules	Pasture and turf
	Green Guard™	ditto	Australian plague locust, wingless grasshoppers	<i>M. anisopliae</i> var. <i>acridum</i> , aerial conidia, solid state	Ultra-low volume, 12 months under recommended storage	Agricultural areas, pastures, crops, forage crops
Belgium	PreFeRal WG	Biopest N.V. (www.biobest.be), *Finland, France, Luxembourg, The Netherlands, Norway, Poland, Sweden	Greenhouse whitefly (all stages)	<i>Paecilomyces fumosoroseus</i>	Water-dispersible granule, 2 × 10 <sup>9</sup> g <sup>-1</sup> , 6 months at 2–6 °C	Ornamentals, cucumber and tomatoes
Bolivia	Probiomet	Probioma (www.probioma.org.bo)	Caterpillars	<i>M. anisopliae</i>		Range including ornamentals, rice, fruit trees
	Probiovert	ditto	Whitefly, scales	<i>Verticillium lecanii</i>		Range including vegetables, fruit trees, coffee plants
	Probiobass	ditto	Variety of beetle pests	<i>Beauveria bassiana</i>		Range including vegetables, fruit trees, coffee
Brazil	Metarhizium PM	Biocerto (www.biocerto.com.br)	Range of larval pests	<i>M. anisopliae</i>	Rice granules	Sugarcane, pasture
	Boveril wp	Itaforte (www.itafortebioproductos.com.br)	Whitefly	<i>B. bassiana</i>		Sugarcane, coffee
	Metarril wp	ditto	Sucking insects	<i>M. anisopliae</i>		
	Vertirril WP	ditto	Whitefly, aphids	<i>V. lecanii</i>		
China	Kilocat™	Chongqing Chongda Bio-Tech Development Co. Ltd. (www.ccct.com)	Locusts	<i>M. anisopliae</i> var. <i>acridum</i>	5 × 10 <sup>10</sup> g <sup>-1</sup> , oil formulation	Pasture and field crops
	Tianli	Nature and Man Group (www.jxtianren.com)		<i>Dendrolimus punctatus</i>		Pine forests
Columbia	Bassaniil	Edafon (www.controlbiologico.com)		Various beetle pests	<i>B. bassiana</i>	3 months, at 4–10 °C
	Metabiol	ditto		Various beetle pests	<i>M. anisopliae</i>	3 months, at 20 °C
	Vertilec	ditto	Whitefly, scales	<i>V. lecanii</i>	3 months, at 20 °C	Tomatoes

Table 10.1. (continued)

Country of the company	Product	Company*registration other than home country	Target	Active ingredient/ production	Formulation and shelf life	Label crops
Bassiana	Semicol Ltd. (www.semicol.com.co) ditto	Various, including whitefly Various, including whitefly	<i>B. bassiana</i> <i>B. brongniartii</i>			
Cebiopest	ditto ditto	Various, including whitefly	<i>B. brongniartii</i>			
Anisafer Vercani	ditto	Various, including whitefly	<i>M. anisopliae</i> <i>V. lecanii</i>			
Cuba	Bibisav-2	Inisav (www.inisav.cu)				
France	Betel	Betel Reunion SA (Head Company Arysta Lifescience), *Reunion	Sugarcane beetle	<i>B. brongniartii</i>	3 months, at 10-20 °C, 1 × 10 <sup>9</sup> conidia g <sup>-1</sup> , bait composition	Sugarcane
Guatemala	Met-92	Agricola el Sol (www.agricolaelsol.com) ditto	Various insects	<i>M. anisopliae</i>	WP, 3 months at 20-25 °C	Grass, sugarcane, banana, coffee
Teraboveria			Various insects	<i>B. bassiana</i>	WP, 3 months at 20-25 °C	Grass, sugarcane, banana, coffee
India	ZeroOK Bio-Catch	ditto T. Stanes & Co (www.tstanes.com/products.html) ditto	Cockroaches Sucking insects	<i>M. anisopliae</i> <i>V. lecanii</i>	Contains mineral talc WP, 1 × 10 <sup>8</sup> g <sup>-1</sup> , 8 months at 20-25 °C	Buildings
Bio-magic			Beetles	<i>M. anisopliae</i>	ditto	Ornamentals, field vegetables, turf
Priority	ditto		Mites	<i>P. fumosoroseus</i>	ditto, 8 months at 20-25 °C	Variety including cereals, vegetables, fruit crops, greenhouses
Multiplex Baba	Multiplex (www.multiplexgroup.com)		Sucking insects on vegetables and ornamentals	<i>B. bassiana</i>	Liquid and powder	ditto Variety including vegetables, ornamentals, indoor and outdoor nurseries
Multiplex Mycomite	ditto		Red spider mite	<i>P. fumosoroseus</i>	ditto	Coffee and tea plantations
Multiplex Metarhizium Meallkil	ditto		Beetle pests and termites	<i>M. anisopliae</i>	WP	
Pacer	Agri-Life (www.sompphyto.com) ditto		Mealbugs and scale insects	<i>V. lecanii</i>	WP, 1 × 10 <sup>8</sup> g <sup>-1</sup> , 6 months under recommended storage	
			termites	<i>M. anisopliae</i>	WP, 8 × 10 <sup>8</sup> g <sup>-1</sup> , 12 months	Buildings, interiors, agricultural fields, orchards

Table 10.1. (continued)

Country of the company	Product	Company*registration other than home country	Target	Active ingredient/ production	Formulation and shelf life	Label crops
	Racer	ditto	Caterpillars on a variety of crops	<i>B. bassiana</i>	$1 \times 10^8 \text{ g}^{-1}$ , 6 months under recommended storage	Rice, cotton, vegetables, chillies, oil seeds, pulses, tea, cardamom
Biovert Rich	Plantrich (www.plantrich.com)	Sucking insects including aphids, and whitefly	<i>Verticillium</i> sp.	$2 \times 10^9 \text{ g}^{-1}$	Variety including spices, citrus and horticulture crops	
Pacihit Rich	ditto	Thrips, whitefly	<i>Paecilomyces</i> sp.	$2 \times 10^9 \text{ ml}^{-1}$	Variety including spices, banana, coconut, arecanut	
Bioguard Rich	ditto	Variety of caterpillars	<i>B. bassiana</i>	$2 \times 10^9 \text{ ml}^{-1}$	Variety including sugarcane, tea, coffee, spices	
Biomet Rich	ditto	Variety of insects	<i>M. anisopliae</i>	$2 \times 10^9 \text{ ml}^{-1}$ , liquid formulation	Variety including sugarcane, tea, coffee, spices	
Indonesia	Bevaria	Bio Brahma Nusantara, Jakarta, Barat	<i>B. bassiana</i>			
	Biometeor	ditto				
	NirAma	ditto				
	Beavaria brong	Agrifutur s.r.l	Cockchafers			
Italy	Biolisa Kamikiri	Nitto Denko	Cerambycid beetles in citrus orchards	<i>B. brongniartii</i>	Non-woven fibre bands, impregnated conidia	Variety including grassland, horticulture, fruit
Mexico	Bea-Sin	Plant Health Care (www.phcmexico.com.mx)	Various insects	<i>B. bassiana</i>		
The Netherlands	Meta-Sin Mycotal	ditto Koppert B.V. (www.koppert.nl), *Finland, Italy, Norway, Switzerland, Turkey, UK (Denmark Pending)	Various insects	<i>M. anisopliae</i> <i>Lecanicillium</i> <i>muscarium</i>	Wettable powder, $1 \times 10^{10} \text{ g}^{-1}$ , 6 months under recommended storage	Various, including glasshouse vegetables and ornamentals
	Vertalec (Fig. 10.2a)	ditto, *Finland, Japan, Norway, Switzerland, UK	Aphids	<i>L. longisporum</i>	Blastospores, WP, $1 \times 10^9 \text{ g}^{-1}$ , 6 months at 2–6 °C	Glasshouse crops
New Zealand	Vertikil	Crop Solutions Ltd. (parent company Millenium Microbes) (www.millenniummicrobes.com)	Thrips, whitefly	Two strains of <i>L. muscarium</i>	WP, $1 \times 10^{10} \text{ g}^{-1}$ , applied in water with some vegetable oil	Vegetables, flowers under glass.
						Outdoor crops include kiwifruit and cucurbits

Table 10.1. (continued)

Country of the company	Product	Company*registration other than home country	Target	Active ingredient/ production	Formulation and shelf life	Label crops
	Vertiblast	ditto	Aphids, potato Psyllid Sucking insects, particularly thrips Red spider mites and aphids	<i>L. longisporum</i> , blastospores liquid ditto <i>B. bassiana</i>	Wettable powder, $1 \times 10^9 \text{ g}^{-1}$ , WP, $1 \times 10^{10} \text{ g}^{-1}$ , ditto	Outdoors
South Africa	Bb Plus	Biological Control Products SA (Pty) Limited (BCP) (www.biococontrol.co.za)	Banana weevil	<i>B. bassiana</i>	WP, $2 \times 10^{10} \text{ g}^{-1}$ , 9 months at $4^\circ\text{C}$	Protected crops
	Bb Weevil	ditto, *throughout Africa	Desert locust	<i>M. anisopliae</i> var. <i>acridum</i>	Dusting powder, $2 \times 10^{10} \text{ g}^{-1}$ , 9 months at $4^\circ\text{C}$ Oil formulation, $5 \times 10^{10} \text{ g}^{-1}$ , 1 year at $4^\circ\text{C}$ or 6 months in oil formulation	Bananas Unspecified
	Green Muscle™	ditto, *throughout Africa	Cockchafer	<i>B. brongniartii</i>	Conidia on barley kernels, >1 year at $2^\circ\text{C}$	Meadow turf
Switzerland	Engerlingspilz	Andermatt BioControl AG, Grossdietwil (www.biococontrol.ch)	A wide variety of soft-bodied insects	<i>B. bassiana</i>	$2 \times 10^{11} \text{ g}^{-1}$ , 18 months at $27^\circ\text{C}$	A wide variety of greenhouse and field crops
USA	BotaniGard® ES (Fig. 10.2e), BotaniGard® 22WP Mycotrol O (Fig. 10.2d)	Laverlam Int'l Corporation (www.bioworksbiococontrol.com), *Europe ditto, *Denmark, Italy, Sweden Spain, Switzerland	Sucking insects, including whitefly, aphids	<i>B. bassiana</i>	ES, $2 \times 10^{10} \text{ g}^{-1}$ , 18 months at $27^\circ\text{C}$	Organic agriculture
	Naturalis™ L	Troy Biosciences Inc. TM (*OECD Greece, Italy, Mexico, Spain, Switzerland)	Various, including whitefly, weevils, caterpillars		Flowable formulation, $2.3 \times 10^7 \text{ ml}^{-1}$	Various: glasshouse and outdoors
	Naturalis™ HG	ditto	Various, including leaf-feeding caterpillars, Colorado potato beetles		Liquid formulation, $2.3 \times 10^7 \text{ ml}^{-1}$	Various: vegetables, ornamentals, lawns
Venezuela	Meta-Ven	San Pedro Agrobiológicos (www.agrobiologicos.com.ve/)	Various caterpillars and bugs	<i>M. anisopliae</i> + <i>B. bassiana</i>	WP	
	Bassi	ditto	Sucking insects and weevils	<i>B. bassiana</i> + <i>P. fumosoroseus</i>	WP	

<sup>a</sup> WP=wettable powder, ES=emulsifiable concentrate, *Verticillium*=has not been renamed *Lecanicillium* unless the identity of the species is certain. This table was compiled from a search of the worldwide web, information direct from companies and additional information supplied by CPL Business Consultants

Products are based on a restricted number of species, primarily *M. anisopliae*, *B. bassiana*, *B. brongniartii*, *P. fumosoroseus*, *Lecanicillium longisporum*, *L. muscarium*. Some companies have retained the name *V. lecanii*. The fungi in question would probably now be classified as *L. longisporum* or *L. muscarium*. However, unless the identity is certain, the original names have been retained in Table 10.1. Most production is based on solid or di-phasic systems where conidia are concerned. Blastospores are produced by liquid fermentation, e.g. Vertalec (Fig. 10.2a) and Vertiblast. There are some products which have more than one active ingredient, either two isolates of the same fungus (Vertikil) or several different species (Meta-Ven and Bassi).

There is little reliable information on the situation in Russia. This is unfortunate because Boverin, a product based on *B. bassiana* and used against the Colorado beetle *Leptinotarsa decemlineata* and the codling moth *Cydia pomonella*, used to be an oft-quoted success story. There may be over 100 companies/organisations producing microbial pesticides in India. The ones cited in Table 10.1. appear to be the most significant. Elsewhere, in Asia AppliedChem in Thailand make Metazan (*M. anisopliae*) and Buverin (*B. bassiana*).

Throughout South America, particularly in Brazil, Peru, Columbia, Cuba, Mexico and Argentina and, to a much lesser extent, in Ecuador, Uruguay, Panama, Paraguay, Dominican Republic, Guatemala, Honduras, Nicaragua, Costa Rica and Trinidad and Tobago, small to medium commercial producers, research stations and universities are involved with producing mycoinsecticides (Alves et al. 2003; Ray Quinlan, personal communication). These are largely based on *M. anisopliae* and *B. bassiana* but there is some use of *P. fumosoroseus*, *V. lecanii*, *Nomuraea rileyi*, *Sporothrix insectorum*, *E. virulenta*. Another oft-quoted product, 'Metaquino', used in Brazil for the control of spittle bugs, *Mahanarva posticata*, on sugarcane is produced by a local authority/NGO project.

There are more than 60 workshops/cottage-style producers of unregistered mycoinsecticides in China. New regulations require that producers of biopesticides must not only register their products but also have a product license and a company license to produce pesticides (Yuxian Xia, personal communication). Registration requirements for mycoinsecticides are the same as for chemical pesticides. At the moment, only the two companies detailed in Table 10.1. have satisfied the new regulatory requirements. Entomopathogenic fungi

have been used more widely on forest pests than on crop pests (Feng 2003). The registered products are aimed at pests in these two ecosystems.

The most influential mycoinsecticide development project was the multidonor LUBILOSA programme (Fig. 10.2b). Initiated in 1989, it involved research institutes from the UK, The Netherlands and the republics of Benin and Niger. The aim was to develop fungi as biocontrol agents for grasshoppers and locusts. Twelve years later this programme, which involved 40 scientists at a cost of US\$17 million, resulted in a commercial product, Green Muscle™ (Fig. 10.2c). It gives 70–90% control of grasshoppers and locusts as soon as 5–15 days after application but, under less favourable conditions, at 14–20 days. It is recommended for control of these pests by FAO (Bateman 1997; Jenkins et al. 1998). Lessons in formulation and application, in particular, learned from LUBILOSA have influenced much subsequent research and development, e.g. Green Guard™ for control of locusts in Australia (see Table 10.1.).

## 2. Constraints on Efficiency

A need for high humidity for disease initiation and spread has often been considered the major constraint on the use of fungi for insect control, though this may not be as important with some preparations as previously thought. Furthermore, RH in microenvironments, e.g. on a leaf surface due to transpiration, may be higher than that measured by equipment on a larger scale (Vidal et al. 2003). The inclusion of moisture-retaining substances in aqueous formulations and the use of oil-based formulations (see below) may help to overcome the requirement for a high environmental RH. Ability of the disease to spread within a population is not necessarily an issue with an efficiently applied virulent mycoinsecticide; however, one key advantage over chemical pesticides is lost if an epizootic or, at least, vertical inter-generational spread does not occur. Development of mycosis is affected by extremes of temperature (<15 °C and >32 °C) but often the fungus is only delayed. In tropical regions, more equitable night temperatures may provide an opportunity for a fungus to initiate disease. However, insects such as locusts, which have the ability to regulate their body temperature behaviourally, exploit this fungal weakness; maintenance of a high set point (behavioural fever) prevents mycosis and limits mycoinsecticide efficiency in the field (Blanford et al. 1998). Fungi are sensitive to UV.

Experimentally, it has been possible to alleviate this problem by the addition of protectants to the formulation (Hunt et al. 1995). In practice, the ingredients may be too costly, though newer oil formulations themselves provide some UV protection (Alves et al. 1998) and field persistence is often longer than anticipated from UV studies.

Mycoinsecticides should be compatible with other crop protection measures. Several studies have shown that fungicides, herbicides and insecticides can prevent germination and/or mycelial growth of entomopathogenic fungi in vitro (e.g. Moorhouse et al. 1992). However, pest control by fungi is often not affected by chemical pesticides, as long as there is a ca. 7-day gap between the two applications (Anderson and Roberts 1983; Moorhouse et al. 1992).

Fungal pathogens act generally in a density-dependent fashion against their hosts and have relatively slow kill; thus, they are not good candidates for pest control in crops with low damage thresholds. Specificity is often perceived to be an advantage of microbial pesticides generally. However, specificity can be a problem when there is a pest complex and no single pathogen can give control (Powell and Jutsum 1993; Ravensberg 1994).

Plants have evolved chemical and physical defences against pathogens and herbivorous insects. However, these attributes can also influence susceptibility of insects to their own pathogens. Whilst some of the defensive phytochemicals may be sufficiently stressful to cause an increase in the susceptibility of generalist feeders to pathogens, some adapted insects can sequester these chemicals and may thereby acquire some protection from their own pathogens (Gallardo et al. 1990). Interestingly, the entomopathogen *Neozygites tanajoae* produced more conidia when exposed to volatiles from leaves damaged by the cassava mite, *Mononychellus tanajoae*, than to clean air (Hountondji et al. 2005). Pea aphids were more susceptible to the Entomophthoralean fungus *Pandora neoaphidis* on pea cultivars with reduced wax blooms, because spores adhered more effectively to leaf surfaces with less wax (Duetting et al. 2003).

### 3. Integration in Pest Management Schemes

Although mycoinsecticides can provide stand-alone pest control, they may be regarded as one weapon in an armoury of techniques employed in integrated pest management schemes (Dent 2000). A virulent pathogen could have indirect

detrimental effects on existing natural control, e.g. by reducing the availability of hosts for parasitic insects. However, use of fungi can help maintain biocontrol on protected crops in the Northern Europe mid-season when arthropod natural enemies falter due to adverse environmental conditions, and also help with pest clearance at the end of the season (Jacobson et al. 2001).

## C. Development of a Mycoinsecticide

### 1. Isolate Selection

There are some 700 species of entomopathogenic fungi known from 85 genera. However, comparatively few have been investigated as potential mycoinsecticides. Natural epizootics caused in particular by fungi in the order Entomophthorales occur frequently in natural and agricultural terrestrial ecosystems (Samson et al. 1988). This has led to a number of attempts to use Entomophthoralean fungi, e.g. for pest control of aphids, with varying degrees of success. Problems have included the inability to culture certain species in vitro and the fact that the most stable spore form is the resting spore, which is not infective and not produced by the most pathogenic isolates (Latgé 1986). The genera *Cordyceps* and *Torubiella* also contain some virulent but obligate insect pathogens. As a consequence, most development work has focused on certain Ascomycota, particularly *M. anisopliae*, *B. bassiana*, *N. rileyi*, *Aschersonia aleyrodis* and *L. longisporum*, and Oomycota, e.g. *Lagenidium giganteum*, which are more readily cultured in vitro.

Isolates are often selected on the basis of laboratory bioassay using cultured insects under optimal conditions. However, lead isolates need to be checked in a commercial setting. Immersion of test insects in a conidial suspension as part of a bioassay represents a temporary exposure to high inoculum. Prolonged exposure of insects, e.g. in soil, may provide a lethal dose from a sub-lethal bioassay concentration through the accumulation of spores with time (Ferron 1985). Laboratory assays do not allow for avoidance of the pathogen biologically, ecologically or behaviourally.

There is no consensus about whether isolates originating from the target host (homologous isolates) or isolates from other hosts (heterologous isolates) of an established pathogen, or an exotic fungus (a species which is not present in the geographical area of application), are likely to provide the most suitable candidate for a myco-

secticide. One aspect of the debate is whether an adapted pathogen (homologous isolate) evolves towards a balanced relationship with its host which precludes high virulence. There is evidence from work on human viral and bacterial pathogens that this may not always be the case (Ewald 1993); similarly, in a screen for fungal pathogens of the desert locust, *Schistocerca gregaria*, the majority of isolates of *Metarhizium* spp. with high virulence came from this or related Acridids (Prior 1992). However, in other studies heterologous isolates have proved the most virulent (e.g. see Vestergaard et al. 1995). Since there are concerns over the possible non-target effects of exotic fungi (e.g. see Lockwood 1993), it may be wise to search and screen isolates from the country where the mycoinsecticide is to be deployed, in order to facilitate commercial registration (Prior 1992). Passage of the key isolate through the target host may select for increased virulence (Quesada-Moraga and Vey 2003).

Studies have shown correlations between virulence and attributes such as spore size, speed of germination and attachment to cuticle (*P. fumosoroseus* against the diamond-back moth, Altare et al. 1999), and toxin production (*B. bassiana* against locusts, Quesada-Moraga et al. 2006). Thus, a rational approach to strain selection has been advocated. Barranco-Florido et al. (2002) selected *V. lecanii* isolates based on better growth associated with low water activity, and high chitinase and protease activities as well as CO<sub>2</sub> production (measure of growth). However, the reality seems to be that such studies may only go some way to explaining why a particular isolate is virulent, rather than providing a basis for a priori selection.

The relevance of isolate virulence to pest control is clear. However, high sporulation, stability during bulk storage, and epizootic potential may be equally important. Selection for ability to operate over a range of abiotic conditions, including soil chemistry, light, pH, likely to be encountered in practice while having little impact on natural enemies and other non-targets is a 'biorational approach' which has clear advantages (Yeo et al. 2003). Since natural habitats have highly variable temperatures, incorporation of cycling temperature regimes into selection programmes is an insightful development (Lecuona et al. 2005).

## 2. Production and Formulation

Large-scale in vivo cultivation of a fungus in laboratory-reared or field-derived insects is usu-

ally employed only for obligate pathogens which do not grow readily outside their hosts. This may be a viable method of inoculum production for a programme of 'introduction' (Soper and Ward 1981). However, it is unlikely to be economic for large-scale mycoinsecticide use. The method adopted for in vitro cultivation must take into account that:

1. The inoculum produced must have optimum virulence and retain viability over an extended period in storage and after application in the field.
2. Serial in vitro transfer can lead to loss of virulence (Brownbridge et al. 2001). Use of single spore isolates can help to alleviate this problem and ensure uniformity of the product. However, genetic change through chromosome transformation, transposable elements, cytoplasmically transmitted genetic elements and the parasexual cycle may promote drift (Couteaudier et al. 1994) and loss or reduction of pathogenicity factors (Shah and Butt 2005). Storage of the original culture under liquid nitrogen and periodic passage of the fungus through the host may be required to maintain virulence of a product.
3. The culture medium should balance the needs of cost effectiveness in terms of yield per unit outlay with the production of a highly virulent, stable inoculum.
4. The production system may affect the propagule type and virulence, which may impact on killing power, shelflife, environmental stability, and the formulation and application strategies.
5. The production system may have to be scaled up to produce cost-effective treatment on thousands or even millions of hectares (Lisansky and Hall 1983; Bradley et al. 1992; Jenkins and Goettel 1995).
6. Conditions for maximum productivity may not be the best for producing good-quality spores for field application (Tarocco et al. 2005).

Three types of production system have been employed: submerged (liquid) fermentation, surface cultivation, and diphasic fermentation (see, e.g. review by Deshpande (1999)).

*Submerged liquid fermentation* would be the preferred option because existing large-scale deep-tank fermentation equipment could be used, and the process is most easily controlled and can be much faster than other methods. The major drawback is that dimorphic fungi like *M. anisopliae*, *L. longisporum*, *Paecilomyces farinosus* and

*B. brongniartii* typically produce blastospores, rather than true conidia in liquid culture. In vitro-produced blastospores are similar to the in vivo yeast phase which enables many fungi to develop and spread quickly in the haemolymph of the host. However, the wall structure of blastospores is often similar to that of the mycelium and, being unpigmented, in vitro-produced blastospores are often unstable with limited shelf life and field stability. Yields of spores from submerged cultures are not as good as from aerial production systems (see below), e.g.  $5 \times 10^9 \text{ ml}^{-1}$  of *V. lecanii* (Latgé et al. 1986) and  $1.1 \times 10^9 \text{ ml}^{-1}$  *Paecilomyces fumosoroseus* (Jackson et al. 2003). In fact,  $5 \times 10^{12} \text{ ml}^{-1}$  produced in a short time frame (45 h) may be required for commercial viability (Bradley et al. 1992). Despite constraints on their use, blastospores form the basis of some commercial formulations, e.g. Vertalec (Fig. 10.2a) produced by Koppert, and Vertiblast produced by Crop Solutions Ltd (see Table 10.1.). Furthermore, blastospores are more virulent than conidia of *P. fumosoroseus* against the silverleaf whitefly (Jackson et al. 2003). Certain strains of *B. bassiana* (Feng et al. 1994), *M. anisopliae* var. *acridum* (formerly *M. flavoviride*) (Jenkins and Prior 1993; Leland et al. 2005) and *Hirsutella thompsonii* (Van Winkelhoff and McCoy 1984) will conidiate in liquid culture in appropriate media, and yields of  $1.5 \times 10^9 \text{ ml}^{-1}$  have been achieved with *M. anisopliae* var. *acridum* – being hydrophilic, however, these spores will require different formulation to the hydrophobic aerial conidia. Resting spores but not infective spores of *Entomophthora* spp. are formed in liquid culture (Latgé 1986), as are the sexually derived oospores of *Lagenidium giganteum* (Kerwin et al. 1986).

Several methods have been developed for producing mycelia in submerged cultures. Mycelium is applied in the field where it will sporulate, producing infective conidia. In the 'Marcescent process', first developed for Entomophthorales (McCabe and Soper 1985) and then adapted for Ascomycota (Rombach et al. 1988), mycelium is dried in a sugar desiccation process, milled and then stored at low temperature prior to use. Fluid bed-dried mycelial granules of *M. anisopliae* with a shelf life of at least a year at  $4^\circ\text{C}$  can provide good long-term control of the vine weevil *O. sulcatus* when incorporated into compost (Stenzył et al. 1992). Dried mycelia fragments have been successfully field tested (Rombach et al. 1987) and a product, BIO1020, based on dried mycelial granules, has been developed by Bayer AG in Germany (Sten-

zel et al. 1992). However, none of these mycelial preparations have been developed into commercial products.

In *surface cultivation*, the use of solid substrate is the most common method of production (see Krishna 2005), though pH, temperature, nutrient status, and aeration may be more difficult to control than in submerged liquid culture (Feng et al. 2000). Large-scale production has been carried out on agricultural, brewing or other wastes, e.g. Dalla Santa et al. (2005), though such media can be too variable and of low immediate metabolic availability. The high surface-area-to-volume ratio of small cereal grains such as sorghum and rice leads to better nutrient absorption, gas exchange and heat transfer. Aeration can be improved by the use of a rotating drum; however, premature germination and reduced yield can occur when conidia are dislodged from conidiophores (Lisansky and Hall 1983). The size of the initial inoculum may influence spore yield, and procedures need to be optimised to minimise hyphal growth and maximise sporulation. Kang et al. (2005) used a packed-bed bioreactor with rice and straw to produce *B. bassiana* for control of the diamond-back moth. The yield was  $4.9 \times 10^8$  conidia per g without support and  $1.1\text{--}1.2 \times 10^{10}$  conidia per g on polypropylene foam. New designs for solid-state fermentation bioreactors have been developed experimentally, including that of Ye et al. (2006). The latter comprised upright multitrayed conidiation chambers which produce  $2.4 \times 10^{12}$  *Beauveria* conidia per kg rice. Mycotech Corporation (now owned by Laverlam International) used a computer-controlled, forced-aeration solid-state fermenter to produce *B. bassiana* at a rate of  $3 \times 10^{13}$  per kg of starting material in 1 l of fermenter space (Wraight et al. 2001). Given the capital outlay with sealed, automated bioreactors, such systems are only cost effective with high output; otherwise, simpler labour-intensive systems are employed (Jenkins et al. 1998), n.b. the cottage-style industries which have proliferated in certain parts of the world.

In *diphasic fermentation*, in most cases solid-state fermentation is preceded by liquid fermentation in order to provide a mycelial inoculum. Fungus is grown in fermentation tanks to the end of log phase. The resulting mycelium is then applied to either a nutrient or non-nutrient surface. The latter allows fine tuning of the nutrients provided to promote optimum spore production. A large surface area to volume is important (Jenkins et al. 1998).

Solid, absorbent non-nutrient surfaces such as clay granules, vermiculite, sponge or clothe are used. Grain and particularly white rice are commonly used as nutrient support surfaces. The second-stage fermentation of *B. bassiana* as Boverin has been based on the use of shallow layers of sterilised liquid media in polypropylene plastic bags inoculated with mycelium (Bradley et al. 1992). Such an approach does not appear to be in current use. Mycotech have developed a two-step solid-state fermentation method for producing conidia of *B. bassiana*. In this system, inoculum is produced in liquid fermenters and then placed in bioreactors (see last section) where it is absorbed onto a starch-based solid substrate.

Harvesting the fungus from liquid culture is usually a matter of centrifugation, then rapid controlled drying to prevent bacterial growth, though excessive temperatures can reduce viability. Following solid-substrate cultivation, spores can be washed off the substrate or dried in situ to a suitable moisture content, then milled. Water content has an important bearing on conidial storage characteristics and temperature tolerance; increasing the level of desiccation can increase temperature tolerance of *M. anisopliae* and *M. anisopliae* var. *acridum* conidia (Moore et al. 1995). Dry harvesting may be achieved using apparatus like the cyclone MycoHarvester (DropData at International Application Research Centre, Imperial College <http://www.dropdata.net/mycoharvester/>, and ACIS <http://www.acis.co.uk/index.htm>).

The fungus needs to be formulated to help stabilise the product during storage and to facilitate delivery to the insect target in the field (Burges 1998; Wraight et al. 2001). An 18-month storage period is ideal for economic use. Mechanical harvesting and dry storage of unformulated conidia can prolong viability (71%, 12 months, 4 °C, *B. bassiana*; Chen et al. 1990, quoted in Feng et al. 1994). Blastospores are more difficult to formulate because of their instability. Freeze-drying has been used (3 months, *B. bassiana*, Belova 1978). Lactose- and sucrose-enhanced desiccation tolerance of *P. fumosoroseus* blastospores during freeze-drying while whole milk in a starch-oil formulation allowed storage at -20 °C for a year without loss of viability (Jackson et al. 2006). Spray-dried blastospores of *M. anisopliae* var. *acridum* were 68% viable after 1 year at 20 °C (Stephan and Zimmermann 1998). *M. anisopliae* var. *acridum* (= *flavoviride*) conidia harvested in the light petroleum fraction oil Edelex or groundnut veg-

etable oil, then diluted with Shellsol K, deodorised kerosene (Edelex) or an antioxidant (groundnut oil), retained 60% viability after storage at 17 °C for 30 months, as long as they were dried by the addition of non-indicating silica gel to the formulation (Moore et al. 1995). In dry or wettable powder formulations, the main ingredient may dilute the 'active ingredient' to a concentration which can be handled more easily. Phyllosilicates (clays) are the most commonly used, as they are relatively inert and cheap. They can promote conidial viability over extended periods (Ward 1984). Coating of ingredients may include stickers, humectants, UV protectants, an emulsifier for water-based spraying of hydrophobic spores and nutrients, though inclusion of nutrients may be a natural consequence of the production process. Either way, nutrients in the formulation may allow saprophytic growth and sporulation on foliage, increasing inoculum potential, and enhancing the chances of secondary pickup and vertical infection, e.g. aphid formulation Vertalec® of *L. longisporum* (Fig. 10.2a).

Many of the currently available mycoinsecticides comprise conidia in wettable powders which can be delivered in water using simple hydraulic sprayers. The development of oil-based formulations in the 1990s was a significant development. Vegetable (e.g. sunflower, soybean), mineral and paraffinic oils (e.g. Shellsol K, Ondina) are compatible with hydrophobic conidia of *Beauveria* and *Metarrhizium*, can be applied from ULV sprayers and do not evaporate (Bateman 1997; Burges 1998). Emulsifiable adjuvant oils (EAO) (paraffin- or vegetable oil-based) allow conidia and oil to be mixed in water, thereby reducing the amount of oil which has to be used (Polar et al. 2005). An adjuvant of emulsified vegetable oil ('Addit') increases the efficiency of the whitefly formulation Mycotal® of *L. muscarium* at low RH. Plastic-lined foil (trilaminate) bags, with small packets of silica gel to ensure dry conditions, allowed storage of *M. anisopliae* var. *acridum* for over a year at 30 °C, though rapid rehydration of these dry spores is lethal (Jenkins et al. 1998). Dry powder formulations retained 70% viability after 7 years at 5 °C (Dave Moore, personal communication).

Use of mycelial preparations presents different problems for formulation. Pure dry mycelium treated with maltose or sucrose produced more conidia after storage (Pereira and Roberts 1990). Bayer's BIO1020, consisting of mycelial granules, was stored vacuum packed after fluid-bed drying

and retained the ability to form conidia after rehydration following 12-month storage at 4 °C. Incorporation of mycelium into alginate pellets, with or without additional nutrient sources, has been tried successfully with *B. bassiana* (Pereira and Roberts 1991), providing enhanced shelf life and environmental stability particularly against solar radiation after application. Cornstarch or cornstarch oil formulation also enhanced conidial production by mycelium after several months storage (Pereira and Roberts 1991).

### 3. Application

The timing of application may be important. Suitable weather conditions (high humidity, equitable temperature) may occur during late evening/early morning. The pest should be at the most susceptible stage (the most juvenile stages are often the most susceptible and easiest to control). The method of application depends on the nature of the inoculum and the niche of the pest insect.

Conidia or blastospores of Ascomycota such as *B. bassiana*, *M. anisopliae* and *L. longisporum* can be suspended in a liquid or mixed with a powder carrier and sprayed with conventional machinery used for the application of synthetic chemical insecticides. High-volume hydraulic sprayers are used to apply *L. longisporum* spores in water against aphids on chrysanthemums in glasshouses. Secondary spore pickup, rather than just direct hit, is critical to success, which makes sedentary insects like *Aphis gossypii* more difficult to control. Repeat low-dose treatments may be helpful (Helyer and Wardlow 1987), though regular use of large volumes of water on a crop may promote plant fungal disease. Koppert recommend 2–3 applications of Mycotal 7 days apart to control whiteflies on cucumber. Improved targeting against aphids, which prefer abaxial leaf surfaces, can be provided by electrostatic sprayers. The equipment imparts a charge to droplets, thereby increasing abaxial deposition and aphid control (Sopp et al. 1989). A more recent study was less encouraging of this approach (Saito 2005).

Mist blowers and helicopters have been used successfully to apply *B. brongniartii* to swarming adults of the cockchafer *Melolontha melolontha* in Switzerland ( $2 \times 10^{14}$  spores ha<sup>-1</sup>). Ultra-low-volume (ULV) application of water- or mineral oil-based formulations on the ground or by aircraft has been used on >0.8 million hectares in China for the control of various forest and crop insects

(Ying 1992). Bateman et al. (1993) have shown in laboratory experiments that mineral or vegetable oil formulations of *M. anisopliae* and *M. anisopliae* var. *acridum* were more virulent than water-based formulations against the desert locust, *Schistocerca gregaria*, and decreased reliance on high environmental RH. These formulations in ULV sprays have been used successfully in field trials against the brown locust *Locusta pardalina* in South Africa (Bateman et al. 1994), the variegated grasshopper *Zonocerus variegatus* in West Africa (Lomer et al. 1993) and Australian plague locust (Hunter et al. 2001). ULV oil locust formulations of *M. anisopliae* need to be sprayed as droplets of 40–120 µm, with 500–10,000 spores per drop at a concentration of  $5 \times 10^{12}$  spores per litre and rate of 1 l per ha (Bateman and Chapple 2001). Only a few droplets are needed to produce a dose which kills in 2–3 weeks. Diseased insects are usually so slow that they are taken by predators before death from mycosis (Dave Moore, personal communication).

Soil-borne pests can be treated either prophylactically or curatively with fungal spores. Prior incorporation of conidia of *M. anisopliae* into compost can give yearlong protection of *Impatiens wallerana* against the vine weevil (Moorhouse et al. 1993). The success of drenches of aqueous spore suspensions is influenced by the depth to which spores percolate, the volume of the drench, adsorption to soil particles, and movement of the insect which will facilitate uptake of a lethal dose. Direct drilling of *M. anisopliae* conidia using existing (crop sowing) machinery to a depth of 20–25 mm in pasture gave long-term control of the redheaded cockchafer *Adorphorus couloni* in trials in Tasmania (Rath 1992).

A novel method of pest control was suggested by the observation that *B. bassiana* exists as an endophyte in certain genotypes of maize (Vakili 1990). Bing and Lewis (1991) prepared a granular formulation of *B. bassiana* by spraying a suspension of *B. bassiana* ( $1.1 \times 10^8$  conidia g<sup>-1</sup>) onto corn grits in a rotating drum of a Gustafon minimixer. Handheld inoculators were used to apply 0.4 g of the granules to whorls of plants ( $10^7 \times 4.55$  conidia plant<sup>-1</sup>  $2.5 \times 10^{12}$  conidia ha<sup>-1</sup>). Conidia of *B. bassiana* applied in this way moved within the plants and provided season-long control of the European cornborer, *Ostrinia nubilalis*. No further progress has been made with this system, though establishing *Beauveria* as an endophyte in cotton expressing a *Bt* toxin gene implies the possibility of

synergy between these forms of pest control (Lewis et al. 2001).

Baits and traps provide ways of bringing the insects to a source of inoculum, rather than the reverse. Application of conidia of *B. bassiana* to bran has given some success in trials against range-land grasshoppers in Canada (Johnson and Goettel 1993). Sex pheromone has been used to attract male diamond-back moths *Plutella xylostella* to traps where they are infected with *Zoophthora radicans*. Fungus is carried by contaminated moths to susceptible larvae, thereby initiating or enhancing an epizootic (Furlong et al. 1995). Traps laced with conidia of *M. anisopliae* were commercially available in the USA for cockroach control. Such autodissemination methods are target specific, reduce the amount of fungus required, and protect it while it is in the trap (Shah and Pell 2003).

Experimental infection of aphids with *L. longisporum* is enhanced in the presence of aphid alarm pheromone or a sub-lethal dose of the insecticide imidacloprid, which increase movement and the likelihood of acquiring a lethal dose of spores from a leaf (Roditakis et al. 2000).

Preparations of dried mycelial pellets which need to be rehydrated prior to the production of infective conidia are particularly suitable for application to soil (Stenzl et al. 1992). Direct drilling of cereal grains with sporulating fungus is also used (see Table 10.1.).

#### 4. Safety

It is sometimes assumed a priori that microbial pesticides must be considerably safer to humans and their environment than synthetic chemical insecticides. However, quite correctly, registration procedures in most countries require that mycoinsecticides, like chemical insecticides, are safety tested. Risk assessment frameworks are still evolving. Current registration protocols are often based on those devised for synthetic chemical insecticides and are not appropriate for insect pathogens (Hokkanen et al. 2003a). Risk assessments need to address the following: define the ecological context in which the mycoinsecticide will be operating and choose the most appropriate non-target species to test; determine host specificity; assess the ability of the fungus to disperse from the area of treatment; assess the ability of the fungus to become established; determine direct effects of the fungus on non-target species; determine the indirect effects on non-targets (including the possibility

of competitive displacement); check specifically for mammalian allergenicity; finally, collate the information into a 'risk assessment' (Goettel and Jaronski 1997). The possible side effects of entomopathogenic fungi may be summarised as infections, toxicosis and allergies in non-target animals or humans. However, more subtle effects may occur, such as competitive displacement of native entomopathogens if non-indigenous isolates are used, and depletion of other natural enemies because of the decline in host population. Among non-target arthropods, mycoses of honeybees, bumblebees, parasitoids and predatory beetles have been shown in laboratory tests and field experiments (Hokkanen et al. 2003b; Vestergaard et al. 2003). The absence of natural epizootics of candidate fungi among pollinators suggests that the risks are low, while several studies have shown the compatibility of fungi with parasitoids and predators in integrated control programmes. For example, Jaronski et al. (1998) found a low impact on natural enemies of 'above label' doses of *B. bassiana* applied against whitefly on cotton. Interestingly, a number of experimental studies have shown avoidance behaviour by predators (Meyling and Pell 2006) and parasites (Lord 2001) when faced with conidia from insect pathogens. Some studies have shown mortality of non-target invertebrates in the field. However, they are lower than those found in laboratory tests and can be minimised with appropriate application methods, doses and timing (Vestergaard et al. 2003). The key point appears to be that laboratory assays with fungi like *B. bassiana*, *B. brongniartii*, *M. anisopliae* and *P. fumosoroseus* can result in mortality among beneficials which is largely absent in field applications for ecological or behavioural reasons or absence of stress. Judicious choice of isolates will also help; Butt et al. (1998) showed that honeybees could be used to vector *M. anisopliae* to cause infection of pollen beetles without adverse effect.

A further aspect of risk evaluation is the need to monitor the fate and impact of an introduced fungus (Bidochka 2001). This requires techniques to be able to identify a genotype in an environment containing related organisms (Goettel and Jaronski 1997). A variety of approaches have been taken to produce suitable molecular markers (Bidochka 2001). RFLP analysis, allozymes and cloned DNA probes enabled differentiation between two closely related Entomophthoralean pathogens, *E. aulicae* and *E. maimaiga*, in populations of the gypsy moth in eastern USA. The dominance of the introduced

*E. maimaiga* during epizootics was elegantly shown (see Bidochka 2001). A single, molecular signature would be much more efficient (see, e.g. Castrillo et al. 2003); the approach taken by Hu and St Leger (2002) involved tracking an isolate of *Metarhizium* expressing a green fluorescent protein gene (see next section).

A particular safety problem occurs in areas of China and India where the silkworm, *Bombyx mori*, is reared in large numbers in the silk industry. Beauveriosis caused by native isolates of *B. bassiana* is potentially a natural, severe problem and thus mycoinsecticides are kept away from areas where the industry is prevalent.

Very occasionally, vertebrate infections have been reported with entomopathogenic fungi, though none have been associated with their use as pest control agents. None of the entomopathogenic fungi currently in use or under consideration are invasively pathogenic to humans. However, immunocompromised individuals are open to opportunistic infections which very occasionally have included entomopathogens (see, e.g. Revankar et al. 1999), though none of the fungi under development can grow efficiently at 37 °C.

Many Hypocreales produce toxic secondary metabolites in vitro which have detrimental effects on whole animals and cultured cells. The extent to which these chemicals contribute to the disease process is not known, though several studies have implicated cyclic peptides in the pathogenesis of *B. bassiana* and *M. anisopliae* (see Charnley 2003). The amount of the cyclic peptide, destruxin, produced by *M. anisopliae* in the inoculum or during mycosis in an insect is very small and, thus, environmentally damaging levels are unlikely to occur (Strasser et al. 2000). Furthermore, studies suggest that known toxins such as destruxins do not enter the food chain or contaminate the water supply following field application (Vey et al. 2001). Several groups have been investigating the best way to determine the toxicity of mycotoxins during safety assessments (Skrobek et al. 2006), though risk of exposure appears to be more important to those charged with regulating products (Dave Chandler, personal communication). Choosing an isolate which produces no or little toxin would minimise this risk. Quality control during production is also essential to ensure there is no contamination from toxicogenic spoilage fungi such as *Aspergillus flavus*.

Hypersensitive reactions to fungal antigens derived from hyphae, spores or from metabolites are

perhaps the most likely health hazards to humans. Experiments with mice, rats and guinea pigs suggest that the main route of sensitisation is the respiratory system, and people involved with large-scale production are most at risk (e.g. Ward et al. 2000).

## 5. Registration

The cost, complexity and between-country inconsistency of registration are major constraints on the development and use of biopesticides. However, pragmatism is becoming the order of the day. Most registration schemes for biopesticides have followed the chemical pesticide model in requiring studies on efficacy, toxicity, and impact against non-target and beneficial species (Thomas et al. 2000). However, recognition of the problems of small companies, the additional environmental benefit of the use of biopesticides, and the intrinsically benign nature of biocontrol agents have resulted in the introduction of reduced registration requirements. Within the European Union, a recent development of Objective 3 of the Directive 91/414/EEC 'Plant Protection Products Regulations' advocates 'reducing the levels of harmful active substances by substituting the most dangerous with safer (including non-chemical) alternatives' (NAT/156 2003). In addition, the thematic strategy on the sustainable use of pesticides by the EC Commission gives clear encouragement for biopesticide development.

An interesting development in the EU is the introduction of tailored requirements for biopesticide registration, rather than the use of a simplified version of those required for synthetic chemicals. Thus, 2001/36/EC, for example, asks for the life cycle of the microorganism and its infectiveness, relationships to known human and animal pathogens, stability and ability to produce toxins (Hamer 2003). On the back of the EU initiative, UK PSD launched a new system in 2006 which has halved the registration charge for a biopesticide (PSD 2006). The US EPA has a tiered approach to the registration process. If the results of the first tier tests indicate no adverse effects, then data from the other tiers are not required. Furthermore, there is no need for proof of efficacy (Cole 2004). The reduction in data requirements has led to significant cost savings, and biopesticides are now often registered in less than 1 year, compared to an average of over 3 years for conventional insecticides (Redbond 2004).

## VI. Future Developments

### A. Potential Targets

Entomopathogenic fungi have potential for control of some but not all insect pests, and it is important to identify appropriate targets for mycoinsecticide development. Fungi, in contrast to bacteria and viruses, invade their hosts by actively penetrating the exoskeleton (cuticle). Therefore, fungi are particularly important natural pathogens of sucking insects such as aphids, whitefly, thrips and leafhoppers, since the feeding strategy of these insects tends to preclude acquisition of pathogens which are infectious per os. Larval and adult beetles are frequently hosts to fungal infections but appear to have comparatively few bacterial and viral pathogens. Thus, fungi are often the pathogens of choice for bug and beetle pests (Samson et al. 1988).

Certain ecological niches lend themselves particularly well to the deployment of mycoinsecticides. The habitats in question have in common that chemical control is difficult or inappropriate (concerns over human or environmental health) and the environments are conducive to fungal infection (e.g. high RH). The overwhelming interest has been in exploring the potential for *M. anisopliae* and *B. bassiana*.

Some examples of recent investigations are work on thrips (Ekesi and Maniania 2003), the Sunn Pest (*Eurygaster integriceps*, a pentatomid bug), a major insect pest of wheat and barley in West and Central Asia (Parker et al. 2003), and the rice water weevil, a pest of rice in North and South America (Chen et al. 2005). However, failure of research in the 1980s, on fungal control of the brown plant hopper *Nilaparvata lugens*, the major rice pest in SE Asia (see, e.g. Aguda et al. 1987), to result in a commercial product is a reminder of the difficulty of treating a large-acreage, low-value crop economically with a biopesticide. Of insects which target animals and humans, rather than crops, work on tsetse flies (Maniania et al. 2003) and mosquitoes (Blanford et al. 2005) can be highlighted. Interestingly, the *B. bassiana* isolate used against mosquitoes was not the most virulent. Instead, one which formed the basis of an existing agricultural mycoinsecticide was employed, as it was thought that this would make registration easier. Parasitic mites (Smith et al. 2000), ticks (Samish et al. 2004), blowflies (Wright et al. 2004), reduviid bugs such as *Triatoma* (Lazzarini et al. 2006) and the bee parasite *Varroa destructor* (Shaw

et al. 2002) have all been investigated as potential targets for mycoinsecticides.

Of beetle pests, the Asian longhorned beetle, *Anoplophora glabripennis*, which is already used to control *B. brongniartii* in its native Japan (see Table 10.1.), is being considered for treating introduced populations in the USA and Canada. Studies on stored product pests (Throne and Lord 2004), fire ants (Brinkman and Gardner 2004), mound-building termites (Milner 2003) and Brassica root flies (*Delia radicum* and *Delia floralis*) (Eilenberg and Meadow 2003) show the variety of potential targets for mycoinsecticides. A *Metarrhizium*-based product aimed at cockroaches and developed in the USA was never effective enough to become a commercial reality.

### B. Constraints on the Commercial Use of Entomopathogenic Fungi

Development of mycoinsecticides would have occurred more quickly with greater investment from multinational agrochemical companies. The commercial view of insect pathogens has been that they are too specific, too expensive, difficult to formulate, too erratic, have a short shelf life and are difficult to patent (Lisansky 1999; Butt et al. 2001). This has led on the whole to the development of microbial insecticides by small to medium-sized companies for niche markets, viz. where chemicals do not work well (through resistance or the withdrawal of registration, for environmental reasons, of effective products, e.g. protected crops), or are environmentally unacceptable and have been banned (e.g. forests in North America). In addition, these niches have environments which promote activity of mycoinsecticides, viz. protection from temperature extremes, UV and desiccation, and crops which can sustain some damage without economic loss. The successful development of fungi for use against locusts and grasshoppers (the product Green Muscle; Fig. 10.2c), which came from the LUBILOSA project (Fig. 10.2b; Bateman 1997), needed significant public investment. Green Muscle gave long-term control through low-level recycling with low impact on natural enemies in inhospitable terrain over large areas; by comparison, resurgence occurred in plots where organophosphate insecticides were used.

Implementation of re-registration schemes for existing chemical pesticides and government-backed use reduction schemes in a number of

countries, e.g. Denmark, has reduced the number of chemical products available and may open up more niche markets for microbials, including fungi. Growing public demand for food with low or no chemical residues increases the pressure for biological alternatives to synthetic pesticides. The cost of registration in the past has been a major constraint on the development of mycoinsecticides. However, as indicated in Sections V.C.5 and VI, there are grounds for optimism with new initiatives both in Europe and the USA in this domain.

The rate of application of a mycoinsecticide required to give adequate control is critical to commercial success. The rate is influenced by the sigmoidal dose-response of insects to doses of spores from entomopathogenic fungi. This results in the 'Allee effect', i.e. there is a threshold number of spores before an infection can be initiated (Devi and Rao 2006). Whatever the reason for this, it increases the rate required for effective pest control;  $10^{13}$  conidia  $\text{ha}^{-1}$  is the current bench mark (Bradley et al. 1992). Good control of the vine weevil on glasshouse ornamentals was achieved experimentally using an equivalent dose of  $1.6 \times 10^{14}$  conidia  $\text{ha}^{-1}$  (Moorhouse et al. 1993). The recommended rate for the use of *L. muscarium* as Mycotol against glasshouse whitefly is  $3 \times 10^{13}$  conidia  $\text{ha}^{-1}$  on cucumber. *B. bassiana* as Boverin was used at  $6 \times 10^{12}$ – $2.2 \times 10^{13}$  spores  $\text{ha}^{-1}$  against the Colorado beetle. Since Mycotol contains  $10^{10}$  conidia  $\text{g}^{-1}$  and Boverin contains  $6 \times 10^9$ – $1.2 \times 10^{10}$  conidia  $\text{g}^{-1}$ , these rates represent as much as 10 kg or more of product per hectare (Bradley et al. 1992). Improvements in application technology, e.g. ULV spraying of oil formulation, have resulted in much lower rates being used. Only  $100 \text{ g ha}^{-1}$  is recommended for the locust mycoinsecticide Green Muscle (Fig. 10.2c), and as little as  $25 \text{ g ha}^{-1}$  can be effective (Dave Moore, personal communication).

### C. Strain Improvement

As our understanding of the epizootiology of disease and the biochemical basis of pathogenicity/virulence of entomopathogenic fungi improves, and techniques are developed for their genetic manipulation, it will be possible to devise strategies for strain improvement. Characteristics which could be addressed include: enhanced shelf life and environmental stability (e.g. UV resistance, temperature tolerance), improved sporulation during mass production, ability to initiate infection at low

humidity, expansion of the host range, accelerated kill (reduced  $\text{LT}_{50}$ ), and increased killing power (reduced  $\text{LD}_{50}$ ).

*Metarrhizium* spp. and *Beauveria* spp. are facultatively saprophytic, with soil-dwelling (rhizosphere) and pathogenic stages. Thus, epizootic potential may be improved by engineering traits which increase saprophytic potential. To this end, Wang et al. (2005) used ESTs and cDNA microarrays to explore gene expression during growth on a plant root exudate. Intriguingly, the transcriptome in this medium (nutrient poor) was similar to that on SDA broth (nutrient rich) but very different to that on either host (*Manduca sexta*) cuticle or haemolymph.

Culture conditions can influence the characteristics of fungal spores and can be manipulated to increase mycoinsecticide efficiency. Blastospores of *B. bassiana* from nitrogen-limited cultures had higher concentrations of carbohydrate and lipid and were significantly more virulent (lower  $\text{LT}_{50}$ ) towards the rice green leafhopper than were blastospores from carbon-limited cultures (Lane et al. 1991). Growth of *B. bassiana*, *M. anisopliae* and *P. farinosus* on agar-based media with low water activity or with a high concentration of glycerol encouraged accumulation of polyols in conidia which were more pathogenic at lower RH than those produced on control media (Hallsworth and Magan 1994, 1995).

Genetic modification of entomopathogenic fungi to improve efficiency of pest control is complicated by the fact that the leading candidates are largely Ascomycota with no easily manipulated sexual stage. The parasexual cycle and protoplast fusion have been used to cross isolates of *M. anisopliae* and *L. longisporum* (see review by Heale et al. 1989). However, rarely have the progeny had improved characteristics – indeed, the reverse can be the case. It has been suggested that disruption of clusters of pathogenicity genes is the cause. However, Riba et al. (1994) successfully crossed the non-entomopathogenic *B. sulfurescens*, which produces an entomotoxic glycoprotein, with an avirulent isolate of *B. bassiana*. Stable, partial diploid, hypervirulent, toxigenic recombinants ensued.

Strain selection and parasexual crossing may be the most effective method of obtaining environmental tolerance, as these traits are probably controlled polygenically. The alternative, direct genetic manipulation would provide enhanced targeting for single genes or gene clusters. Genetic en-

gineering needs the establishment of transformation and cloning systems, which have been achieved for *M. anisopliae*, *B. bassiana* and *P. fumosoroseus* (Bernier et al. 1989; St Leger et al. 1992b; Smithson et al. 1995; dos Reis et al. 2004; Lima et al. 2006).

The growing literature on mechanisms of pathogenesis and the development of techniques to genetically modify insect pathogenic fungi suggest strategies for rational strain improvement. It will be possible to increase isolate virulence and/or extend the host range by altering the timing and release of virulence factors, increase copy number of virulence genes, and introduce specificity genes from other isolates or toxin genes from other organisms (St. Leger and Screen 2001). Indeed, St Leger et al. (1996) have inserted extra copies of the *pr1* gene (encoding the protease PR1a) from *M. anisopliae* into the genome of *M. anisopliae* such that the gene was constitutively overexpressed in the tobacco hornworm, *Manduca sexta*. This resulted in the activation of the host's prophenoloxidase system. In comparison with insects infected with wild type, the combined effects of PR1 and the reaction products of phenoloxidase caused a 25% reduction in time to death and a 40% reduction in food consumption by insects infected with engineered fungus. Transforming *M. anisopliae* to express an insect-specific scorpion toxin has also enhanced virulence against mosquitoes and caterpillars (*M. sexta*) (St. Leger, personal communication). An additional strategy for the control of vector-borne diseases is to use fungi not only to control the insect but as a vehicle for introducing anti-parasite genes, e.g. *M. anisopliae* versus mosquitoes which transmit *Plasmodium*, the causative agent of malaria (Blanford et al. 2005).

Molecular biological studies on other entomopathogens such as baculoviruses, though much more advanced than those on fungi, have still not resulted in the commercial production and use of GM forms. This is despite the fact that baculoviruses expressing insect-specific toxins from mites and spiders have been shown in carefully regulated, small-scale field trials to be more effective than wild type and environmentally safe (Kamita et al. 2005). Any attempt to assess the likely environmental impact of a genetically engineered fungus is hampered by a basic lack of understanding of the population structure of naturally occurring fungal genotypes (Hajek et al. 2000). Hu and St Leger (2002) used a *M. anisopliae* isolate expressing green fluorescent protein (GFP) gene alone or with extra copies of a homologous

protease gene *pr1* in an EPA-approved field release into a plot of cabbage plants. This study showed: the usefulness of GFP for monitoring fungi; no evidence for transmission to non-target organisms; genetically modified organisms were stable for >1 year under field conditions; no displacement or depression of native culturable, fungal microflora and maintenance of the *Metarhizium* in the rhizosphere with decline in the non-rhizosphere soil. Before further use could be contemplated, however, detailed assessment of potential environmental risk of a candidate, genetically modified mycoinsecticide would be necessary along the lines of that advocated by Snow et al. (2005) to minimise the likelihood of negative ecological effects.

## VII. Conclusions

Mycoinsecticides have had comparatively little global impact on insect pest control to date. However, the pressing need for alternatives to chemical pesticides, and progress in research on epizootiology, mass production, formulation, application and mechanisms of pathogenesis and host defence suggest optimism for the future of mycoinsecticides. Furthermore, the drive to pesticide-free production in some sectors is a helpful development, e.g. the British Tomato Growers Association has a commitment to pesticide-free production in the next 10 years, which is re-enforced by retailer drive for zero-pesticide residues. The US EPA's introduction of comparative risk assessment for pesticide registration, as part of a policy to promote replacement of existing products with safer alternatives, has reduced conventional pesticide registrations and increased that of biologicals and 'low-risk chemicals' in the last 6 years. Despite these and other initiatives to encourage the development of biological alternatives to pesticides market (see Sect. V.C.5.), entry costs for SMEs for microbials are still onerous. In The Netherlands, there is a programme which subsidises up to half of the registration costs of biopesticides. Likewise, in the USA, the IR4 programme subsidises costs for agents designed for small-market use. There is a strong argument for more widespread use of public money to subsidise biopesticide development, as this is in both national and international interest. It is to be hoped that more governments will recognise and act in this way and thus promote actively the development and use of mycoinsecticides.

**Acknowledgements.** We would like to thank Ray St Leger for permission to quote from unpublished work, Ray Quinlan for up-to-date information on commercial-scale production of mycoinsecticides, and Dave Chandler and Dave Moore for constructive comments on an earlier version of the manuscript.

## References

- Aguda RM, Rombach MC, Im DJ, Shepard BM (1987) Suppression of population of the brown planthopper *Nilaparvata lugens* Stal Homoptera: Delphacidae in field cages by entomogenous fungi Deuteromycotina on rice in Korea. *J Appl Entomol* 104:167–172
- Altre JA, Vandenberg JD, Cantone FA (1999) Pathogenicity of *Paecilomyces fumosoroseus* isolates to diamondback moth, *Plutella xylostella*: Correlation with spore size, germination speed, and attachment to cuticle. *J Invertebr Pathol* 73:332–338
- Alves RT, Bateman RP, Prior C, Leather SR (1998) Effects of simulated solar radiation on conidial germination of *Metarrhizium anisopliae* in different formulations. *Crop Protect* 17:675–679
- Alves SB, Pereira RM, Lopes RB, Tamai MA (2003) Use of entomopathogenic fungi in Latin America. In: Upadhyay RK (ed) Advances in microbial control of insect pests. Kluwer, New York, pp 193–212
- Anderson TE, Roberts DW (1983) Compatibility of *Beauveria bassiana* isolates with insecticide formulations used in Colorado potato beetle (Coleoptera: Chrysomelidae) control. *J Econ Entomol* 76:1437–1441
- Anonymous (2005) Spotlight on biopesticides. *Crop Protection Monthly* 31 March Issue 184 (<http://www.crop-protection-monthly.co.uk>)
- Ansari MA, Tirry L, Moens M (2004) Interaction between *Metarrhizium anisopliae* CLO 53 and entomopathogenic nematodes for the control of *Hoplia philanthus*. *Biol Control* 31:172–180
- Barranco-Florido JE, Alatorre-Rosas R, Gutierrez-Rojas M, Viniegra-Gonzalez G, Saucedo-Castaneda G (2002) Criteria for the selection of strains of entomopathogenic fungi *Verticillium lecanii* for solid state cultivation. *Enzyme Microbial Technol* 30:910–915
- Bateman R (1997) The development of a mycoinsecticide for the control of locusts and grasshoppers. *Outlook Agric* 26:13–18
- Bateman R, Chapple A (2001) The spray application of mycopesticide formulations. In: Butt TM, Jackson C, Magan N (eds) Fungi as biocontrol agents. CABI, Wallingford, pp 289–311
- Bateman RP, Carey M, Moore D, Prior C (1993) The enhanced infectivity of *Metarrhizium flavoviride* in oil formulations to desert locusts at low humidities. *Ann Appl Biol* 122:145–152
- Bateman RP, Price RE, Muller EJ, Brown HD (1994) Controlling brown locust hopper bands in South Africa with a mycoinsecticide spray. In: Proc Brighton Crop Protection Conf, Pests and Diseases, vol 2. British Crop Protection Council, Farnham, pp 609–616
- Belova RN (1978) Development of the technology of boverin production by the submersion method. In: Ignoffo CM (ed) Proc 1st Joint US/USSR Conf Production, Selection and Standardisation of Entomopathogenic Fungi, US/USSR Joint Working Group Production of Substances by Microbiological Means. National Science Foundation, Washington, DC, pp 102–119
- Bernier L, Cooper RM, Charnley AK, Clarkson JM (1989) Transformation of the fungus *Metarrhizium anisopliae* to benomyl resistance. *FEMS Microbiol Lett* 60:261–266
- Bidochka MJ (2001) Monitoring the fate of biocontrol fungi. In: Butt TM, Jackson C, Magan N (eds) Fungi as biocontrol agents. CABI, Wallingford, pp 193–218
- Bing LA, Lewis LC (1991) Suppression of the European cornborer, *Ostrinia nubilalis* (Hubner) (Lepidoptera: Pyralidae) by endophytic *Beauveria bassiana* (Balsamo) Vuillemin. *Environ Entomol* 20:1207–1211
- Blanford S, Thomas MB, Langewald J (1998) Behavioural fever in the Senegalese grasshopper, *Oedaleus senegalensis*, and its implications for biological control using pathogens. *Ecol Entomol* 23:9–14
- Blanford S, Chan BHK, Jenkins N, Sim D, Turner RJ, Read AF, Thomas MB (2005) Fungal pathogen reduces potential for malaria transmission. *Science* 308:1638–1641
- Boucias DG, Pendland JC (1991) Attachment of mycopathogens to cuticle: the initial event of mycosis in arthropod hosts. In: Cole GT, Hoch HC (eds) The fungal spore and disease initiation in plants and animals. Plenum Press, New York, pp 101–128
- Bradley CA, Black WE, Kearns R, Wood P (1992) Role of production technology in mycoinsecticide development. In: Leatham GF (ed) Frontiers in industrial mycology. Chapman & Hall, New York, pp 160–173
- Brinkman MA, Gardner WA (2004) Red imported fire ant (Hymenoptera: Formicidae) control in nursery pots treated with *Beauveria bassiana* and bifenthrin. *J Entomol Sci* 39:175–187
- Brown GC, Nordin GL (1986) Evaluation of an early harvest approach for induction of *Erynia* epizootics in alfalfa weevil populations. *J Can Entomol Soc* 59:446–453
- Brownbridge M, Costa S, Jaronski ST (2001) Effects of in vitro passage of *Beauveria bassiana* on virulence to *Bemisia argentifolii*. *J Invertebr Pathol* 77:280–283
- Burges HD (1998) Formulation of microbial biopesticides: beneficial microorganisms, nematodes and seed treatments. Kluwer, Dordrecht
- Butt TM, Carreck NL, Ibrahim L, Williams IH (1998) Honeybee mediated infection of pollen beetle (*Meligethes aeneus* Fab.) by the insect-pathogenic fungus *Metarrhizium anisopliae*. *Biocontrol Sci Technol* 8:533–538
- Butt TM, Jackson C, Magan N (2001) Introduction – fungal biological control agents: progress, problems and potential. In: Butt TM, Jackson C, Magan N (eds) Fungi as biocontrol agents. CABI, Wallingford, pp 1–8
- Carruthers RI, Haynes DL (1986) Temperature, moisture and habitat effects on *Entomophthora muscae* (Entomophthorales, Entomophthoraceae) conidial germination and survival in the onion agroecosystem. *Environ Entomol* 15:1154–1160
- Carruthers RI, Soper RS (1987) Fungal diseases. In: Fuxa JR, Tanada Y (eds) Epizootiology of insects diseases. Wiley, New York, pp 357–416
- Castrillo LA, Vandenberg JD, Wraight SP (2003) Strain-specific detection of introduced *Beauveria bassiana* in agricultural fields by use of sequence-characterized amplified region markers. *J Invertebr Pathol* 82:75–83

- Charnley AK (2003) Fungal pathogens of insects: cuticle degrading enzymes and toxins. *Adv Bot Res* 40:241–321
- Chen CJ, Wu JW, Li ZZ, Wang ZX, Li YW, Chang SH, Yin XP, Dai LY, Tao L, Zhang YA, Tang J, Ding S, Ding GG, Gao XH, Tan YC (1990) Application of microbial pesticides in IPM (in Chinese). In: Chen CJ (ed) Integrated management of pine caterpillars in China. China Forestry Publishing House, Beijing, pp 214–308
- Chen H, Chen ZM, Zhou YS (2005) Rice water weevil (Coleoptera: Curculionidae) in mainland China: Invasion, spread and control. *Crop Protect* 24:695–702
- Clarkson JM, Charnley AK (1996) New insights into the mechanisms of fungal pathogenesis in insects. *Trends Microbiol* 4:197–203
- Cole D (2004) Biopesticides—the future? *Outlooks Pest Manage* 15:18–19
- Couetaudier Y, Maurer P, Viaud M, Riba G (1994) Genetic stability of fungi. In: Proc Int Colloquium Invertebrate Pathology and Microbial Control, Montpellier, France, vol 1, pp 343–349
- Dalla Santa HS, Dalla Santa OR, Brand D, Vandenberghe LPD, Soccol CR (2005) Spore production of *Beauveria bassiana* from agro-industrial residues. *Brazilian Arch Biol Technol* 48:51–60
- Dent D (2000) Integrated pest management. CABI, Wallingford
- Deshpande MV (1999) Mycopesticide production by fermentation: potential and challenges. *Crit Rev Microbiol* 25:229–243
- Devi KU, Rao CUM (2006) Allee effect in the infection dynamics of the entomopathogenic fungus *Beauveria bassiana* (Bals) Vuill. on the beetle, *Mylabris pustulata*. *Mycopathologia* 161:385–394
- Dos Reis MC, Fungaro MHP, Duarte RTD, Furlaneto L, Furlaneto MC (2004) *Agrobacterium tumefaciens*-mediated genetic transformation of the entomopathogenic fungus *Beauveria bassiana*. *J Microbiol Methods* 58:197–202
- Driver F, Milner RJ, Trueman JWH (2000) A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. *Mycol Res* 104:134–150
- Duetting PS, Ding HJ, Neufeld J, Eigenbrode SD (2003) Plant waxy bloom on peas affects infection of pea aphids by *Pandora neoaphidis*. *J Invertebr Pathol* 84:149–158
- Eilenberg J, Meadow R (2003) Fungi for biological control of Brassica root flies, *Delia radicum*, and *Delia floralis*. In: Upadhyay RK (ed) Advances in microbial control of insect pests. Kluwer, New York, pp 181–192
- Ekesi S, Manian NK (2003) *Metarhizium anisopliae*: an effective biological control agent for the management of thrips in horti- and floriculture in Africa. In: Upadhyay RK (ed) Advances in microbial control of insect pests. Kluwer, New York, pp 165–180
- Evans HC (1982) Entomogenous fungi in tropical forest ecosystems: an appraisal. *Ecol Entomol* 7:47–60
- Ewald PW (1993) The evolution of virulence. Scientific American, April, pp 56–62
- Feng M-G (2003) Microbial control of insect pests with entomopathogenic fungi in China: a decade's progress in research and utilization. In: Upadhyay RK (ed) Advances in microbial control of insect pests. Kluwer, New York, pp 213–234
- Feng MG, Poprawski TJ, Khachatourians GG (1994) Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: current status. *Biocontrol Sci Technol* 4:3–34
- Feng KC, Liu BL, Tzeng YM (2000) *Verticillium lecanii* spore production in solid-state and liquid-state fermentations. *Bioprocess Eng* 23:25–29
- Ferron P (1985) Fungal control. In: Kerkut GA, Gilbert LI (eds) Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol 12. Pergamon Press, Oxford, pp 313–346
- Freimoser FM, Screen S, Bagga S, Hu G, St. Leger RJ (2003a) Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. *Microbiology* 149:1–9
- Freimoser FM, Screen SE, Hu G, St. Leger RJ (2003b) EST analysis of genes expressed by the zygomycete pathogen *Conidiobolus coronatus* during growth on insect cuticle. *Microbiology* 149:239–247
- Furlong MJ, Groden E (2001) Evaluation of synergistic interactions between the Colorado potato beetle (Coleoptera: Chrysomelidae) pathogen *Beauveria bassiana* and the insecticides, imidacloprid, and cyromazine. *J Econ Entomol* 94:344–356
- Furlong MJ, Pell JK, Choo OP, Rahman SA (1995) Field and laboratory evaluation of a sex pheromone trap for the autodissemination of the fungal entomopathogen *Zoophthora radicans* (Entomophthorales) by the diamond-back moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae). *Bull Entomol Res* 85:331–337
- Gallardo F, Boethel DJ, Fuxa J, Richter A (1990) Susceptibility of *Heliothis zea* (Boddie) larvae to *Nomuraea rileyi* (Farlow) Samson effects of alpha-tomatine at the 3rd trophic level. *J Chem Ecol* 16:1751–1759
- Gillespie AT (1988) Use of fungi to control pests of agricultural importance. In: Burge MN (ed) Fungi in biological control systems. Manchester University Press, Manchester, pp 37–60
- Gillespie JP, Bailey AM, Cobb B, Vilcinskas A (2000) Fungi as elicitors of insect immune responses. *Arch Insect Biochem Physiol* 44:49–68
- Goettel MS, Jaronski ST (1997) Safety and registration of microbial agents for control of grasshoppers and locusts. *Memoirs Entomol Soc Can*, pp 83–99
- Goettel S, Eilenberg J, Glare T (2005) Entomopathogenic fungi and their role in regulation of insect populations. In: Gilbert LB, Iatrou K (eds) Comprehensive molecular insect science. Elsevier Pergamon, Oxford, pp 361–406
- Hajek AE, St Leger RJ (1994) Interactions between fungal pathogens and insect hosts. *Annu Rev Entomol* 39:293–322
- Hajek AE, Humber RA, Elkington JS (1995) Mysterious origin of *Entomophaga maimaiaga* in North America. *Am Entomol spring*, pp 31–42
- Hajek AE, Delalibera I, Mcmanus ML (2000) Introduction of exotic pathogens and documentation of their establishment and impact. In: Lacey LA, Kaya HK (eds) Field manual of techniques in invertebrate pathology. Kluwer, Dordrecht, pp 339–370
- Hallsworth JE, Magan N (1994) Improved biological control by changing polyols/trehalose in conidia of entomopathogens. In: Proc Brighton Crop Protection Conf, Pests and Diseases, vol 3. British Crop Protection Council, Farnham, pp 1091–1096

- Hallsworth JE, Magan N (1995) Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water activity. *Microbiology* 141:1109–1115
- Hamer A (2003) Registration of biopesticides in Europe and OECD countries. In: Wabule MN, Ngaruuya PN, Kimmins FK, Silversides PJ (eds) Proc Pest Control Products Board/Kenya Agri Research Institute/Department for International Development Crop Protection Programme Worksh, Najura, pp 111–117 (<http://www.cpp.uk.com/UPLOADS/publications/downloads/1prelimpages.pdf>)
- Heale JB, Isaac JE, Chandler D (1989) Prospects for strain improvement in entomopathogenic fungi. *Pesticide Sci* 26:79–92
- Helyer NL, Wardlow LR (1987) Aphid control on chrysanthemums using frequent, low dose applications of *Verticillium lecanii*. *WPRS Bull X*/2:62–65
- Hokkanen HMT, Bigler F, Burgio F, Van Lenteren JC, Thomas MB (2003a) Ecological risk assessment framework for biological control agents. In: Hokkanen HMT, Hajek AE (eds) Environmental impacts of microbial insecticides. Kluwer, Dordrecht, pp 1–14
- Hokkanen HMT, Zeng Q-Q, Menzler-Hokkanen I (2003b) Assessing the impacts of *Metarhizium* and *Beauveria* on bumblebees. In: Hokkanen HMT, Hajek AE (eds) Environmental impacts of microbial insecticides. Kluwer, Dordrecht, pp 63–72
- Holder DJ, Keyhani NO (2005) Adhesion of the entomopathogenic fungus *Beauveria (Cordyceps) bassiana* to substrata. *Appl Environ Microbiol* 71:5260–5266
- Hounchondji FCC, Sabelis MW, Hanna R, Janssen A (2005) Herbivore-induced plant volatiles trigger sporulation in entomopathogenic fungi: the case of *Neozygites tanajoae* infecting the cassava green mite. *J Chem Ecol* 31:1003–1021
- Hu G, St Leger RJ (2002) Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Appl Environ Microbiol* 68:6383–6387
- Hughes WOH, Boomsma JJ (2004) Let your enemy do the work: within-host interactions between two fungal parasites of leaf-cutting ants. *Proc R Soc Lond Series B-Biol Sci* 271:S104–S106
- Hunt TR, Moore D, Higgins PM, Prior C (1995) Effect of sunscreens, irradiance and resting periods on the germination of *Metarhizium flavoviride* conidia. *Entomophaga* 39:313–322
- Hunter DM, Milner RJ, Spurgin PA (2001) Aerial treatment of the Australian plague locust, *Chortoicetes terminifera* (Orthoptera: Acrididae) with *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes). *Bull Entomol Res* 91:93–99
- Ignoffo CM (1981) The fungus *Nomuraea rileyi* as a microbial insecticide. In: Burges HD (ed) Microbial control of pests and plant diseases 1970–1980. Academic Press, London, pp 513–538
- Ignoffo CM, Garcia C, Hostetter DL, Pinnel RE (1977) Laboratory studies of the entomopathogenic fungus *Nomuraea rileyi*: soil born contamination of soybean seedlings and dispersal of diseased larvae of *Trichoplusia ni*. *J Invertebr Pathol* 29:147–152
- Inglis GD, Johnson DL, Cheng KJ, Goettel MS (1997) Use of pathogen combinations to overcome the constraints of temperature on entomopathogenic hyphomycetes against grasshoppers. *Biol Control* 8:143–152
- Jackson MA, Cliquet S, Iten LB (2003) Media and fermentation processes for the rapid production of high concentrations of stable blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. *Biocontrol Sci Technol* 13:23–33
- Jackson MA, Erhan S, Poprawski TJ (2006) Influence of formulation additives on the desiccation tolerance and storage stability of blastospores of the entomopathogenic fungus *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes). *Biocontrol Sci Technol* 16:61–75
- Jacobson RJ, Chandler D, Fenlon J, Russel KM (2001) Compatibility of *Beauveria bassiana* (Balsamo) Vuillemin with *Amblyseius cucumeris* Oudemans (Acarina: Phytoseiidae) to control *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) on cucumber plants. *Biocontrol Sci Technol* 11:381–400
- James C (2005) Global Status of Commercialized Biotech/GM Crops: 2005. Ithaca, NY, International Service for the Acquisition of Agri-Biotech Applications Brief 34
- James RR, Elzen GW (2001) Antagonism between *Beauveria bassiana* and imidacloprid when combined for *Bemisia argentifolii* (Homoptera: Aleyrodidae) control. *J Econ Entomol* 94:357–361
- Jaronski ST, Lord J, Rosinska J, Bradley C, Hoelmer K, Simmons G, Osterlind R, Brown CRS (1998) Effect of *Beauveria bassiana*-based mycoinsecticide on beneficial insects under field conditions. In: Proc 1998 Brighton Conf Pests and Diseases, British Crop Protection Council, Farnham, vol 2, pp 651–657
- Jenkins NE, Goettel MS (1995) Methods for the mass production of microbial agents of grasshoppers and locusts. In: Goettel MS, Johnson DL (eds) Microbial control of grasshoppers and locusts. *Mem Entomol Soc Can* 171:37–48
- Jenkins NE, Prior C (1993) Growth and formulation of true conidia by *Metarhizium flavoviride* in a simple liquid medium. *Mycol Res* 7:1489–1494
- Jenkins NE, Heviego G, Langewald J, Cherry AJ, Lomer CJ (1998) Development of mass production technology for aerial conidia for use as mycopesticides. *Biocontrol News Information* 19:21N–31N
- Johnson DL, Goettel MS (1993) Reduction of grasshopper populations following field application of the fungus *Beauveria bassiana*. *Biocontrol Sci Technol* 3:165–175
- Kamita SG, Kang K-D, Hammock BD, Inceoglu AB (2005) Genetically modified baculoviruses for pest insect control. In: Gilbert LB, Iatrou K (eds) Comprehensive molecular insect science. Elsevier Pergamon, Oxford, pp 271–322
- Kang SW, Lee SH, Yoon CS, Kim SW (2005) Conidia production by *Beauveria bassiana* (for the biocontrol of a diamondback moth) during solid-state fermentation in a packed-bed bioreactor. *Biotechnol Lett* 27:135–139
- Keller S, Zimmermann G (1989) Mycopathogens of soil insects. In: Wilding N, Collins NM, Hammond PM, Webber JF (eds) Insect-fungus interactions. Academic Press, London, pp 239–270
- Kerwin JL, Simmons CA, Washino RK (1986) Osporogenesis by *Lagenidium giganteum* in liquid culture. *J Invertebr Pathol* 47:258–270

- Kim T, Kim YJ (2005) Overview of innate immunity in *Drosophila*. *J Biochem Mol Biol* 38:121–127
- Krishna C (2005) Solid-state fermentation systems – an overview. *Crit Rev Biotechnol* 25:1–30
- Lacey LA, Undeen AH (1986) Microbial control of blackflies and mosquitoes. *Annu Rev Entomol* 31:265–296
- Lane BS, Trinci AP, Gillespie AT (1991) Influence of cultural conditions on the virulence of conidia and blastospores of *Beauveria bassiana* to the green leafhopper, *Nephrotettix virescens*. *Mycol Res* 95:829–833
- Latgé J-P (1986) The Entomophthorales after the resting spore stage. In: Samson RA, Vlak JM, Peters D (eds) Fundamental and applied aspects of invertebrate pathology. Foundation IVth Int Colloquium Invertebrate Pathology, Wageningen, The Netherlands, pp 651–652
- Latgé J-P, Hall RA, Cabrera Cabrera RI, Kerwin JC (1986) Liquid fermentation of entomopathogenic fungi. In: Samson RA, Vlak JM, Peters D (eds) Fundamental and applied aspects of invertebrate pathology. Foundation IVth Int Colloquium Invertebrate Pathology, Wageningen, The Netherlands, pp 603–606
- Lazzarini GMJ, Rocha LFN, Luz C (2006) Impact of moisture on in vitro germination of *Metarhizium anisopliae* and *Beauveria bassiana* and their activity on *Triatoma infestans*. *Mycol Res* 110:485–492
- Lecuona RE, Rodriguez J, La Rossa FR (2005) Effect of constant and cyclical temperatures on the mortality of *Triatoma infestans* (Klug) (Hemiptera: Reduviidae) treated with *Beauveria bassiana* (Bals.) Vuill. (Hyphomycetes). *Neotrop Entomol* 34:675–679
- Leipe DD, Wainright PO, Gunderson JH, Porter D, Patterson DJ, Valois F, Himmerich S, Sogin M L (1994) The Stramenopiles from a molecular perspective – 16S-like ribosomal-RNA sequences from *Labyrinthuloides minuta* and *Cafeteria roenbergensis*. *Phycologia* 33:369–377
- Leland JE, Mullins DE, Vaughan LJ, Warren HL (2005) Effects of media composition on submerged culture spores of the entomopathogenic fungus, *Metarhizium anisopliae* var. *acridum*. Part 2. Effects of media osmolality on cell wall characteristics, carbohydrate concentrations, drying stability, and pathogenicity. *Biocontrol Sci Technol* 15:393–409
- Lewis LC, Bruck DJ, Gunnarson RD, Bidne KG (2001) Assessment of plant pathogenicity of endophytic *Beauveria bassiana* in *Bt* transgenic and non-transgenic corn. *Crop Sci* 41:1395–1400
- Lima IGP, Duarte RTD, Furlaneto L, Baroni CH, Funaro MHP, Furlaneto MC (2006) Transformation of the entomopathogenic fungus *Paecilomyces fumosoroseus* with *Agrobacterium tumefaciens*. *Lett Appl Microbiol* 42:631–636
- Lisansky SG (1999) Biopesticides: markets, technology, legislation and companies. CPL Scientific Information Services Ltd, Newbury
- Lisansky SG, Hall RA (1983) Fungal control of insects. In: Smith JE, Berry DR, Kristiansen B (eds) Filamentous Fungi vol IV. Edward Arnold, London, pp 325–345
- Lockwood JA (1993) Environmental issues involved in the biological control of rangeland grasshoppers (Orthoptera: Acrididae) with exotic agents. *Environ Entomol* 22:503–518
- Loher CJ, Bateman RP, Godonou I, Kpindu D, Shah PA, Paraíso A, Prior C (1993) Field infection of *Zonocerus variegatus* following application of an oil based formulation of *Metarhizium flavoviride*. *Biol Control Sci Technol* 3: 337–346
- Lord JC (2001) Response of the wasp *Cephalonomia tarsalis* (Hymenoptera: Bethylidae) to *Beauveria bassiana* (Hyphomycetes: Moniliales) as free conidia or infection in its host, the sawtoothed grain beetle, *Oryzaephilus surinamensis* (Coleoptera: Silvanidae). *Biol Control* 21:300–304
- Maniania NK, Laveissiere C, Odulaja A, Ekesi S, Herren HR (2003) Entomopathogenic fungi as potential biocontrol agents for tsetse flies. In: Upadhyay RK (ed) Advances in microbial control of insect pests. Kluwer, New York, pp 145–164
- McCabe D, Soper RS (1985) Preparation of an entomopathogenic fungal insect control agent. US Patent no 4,530,834, July 23, pp 1–4
- McCoy CW, Samson RA, Boucias DG (1988) Entomogenous fungi. In: Ignoffo CM (ed) CRC Handbook of Natural Pesticides vol V. Microbial insecticides, part A. Entomogenous protozoa and fungi. CRC Press, Boca Raton, FL, pp 151–236
- Meyling NV, Pell JK (2006) Detection and avoidance of an entomopathogenic fungus by a generalist insect predator. *Ecol Entomol* 31:162–171
- Milner RJ (2003) Application of biological control agents in mound building termites (Isoptera: Termitidae) – Experiences with *Metarhizium* in Australia. *Sociobiology* 41:419–428
- Milner RJ, Soper RS, Lutton GG (1982) Field release of an Israeli strain of the fungus *Zoophthora radicans* for the biological control of *Theroaphis trifolii* f. *maculata*. *J Austral Entomol Soc* 21:113–118
- Misra JK (1998) Trichomycetes – Fungi associated with arthropods: review and world literature. *Symbiosis* 24:179–219
- Moore D, Bateman RP, Carey M, Prior C (1995) Long-term storage of *Metarhizium flavoviride* conidia in oil formulations for the control of locusts and grasshoppers. *Biocontrol Sci Technol* 5:193–199
- Moorhouse ER, Gillespie AT, Sellers EK, Charnley AK (1992) Influence of fungicides and insecticides on the entomogenous fungus *Metarhizium anisopliae*, a pathogen of the vine weevil, *Otiorrhynchus sulcatus*. *Biocontrol Sci Technol* 2:49–58
- Moorhouse ER, Gillespie AT, Charnley AK (1993) Application of *Metarhizium anisopliae* (Metsch.) Sor. conidia to control *Otiorrhynchus sulcatus* (F.) (Coleoptera: Curculionidae) larvae on glasshouse pot plants. *Ann Appl Biol* 122:623–636
- Nat/156 (2003) Communication from the Commission to the council, the European Parliament and the European Economic and Social Committee: towards a thematic strategy on the sustainable use of pesticides. <http://ec.europa.eu/environment/ppps/pdf/finalopinion.pdf>
- Nnakamusana ES (1985) Laboratory infection of mosquito larvae by entomopathogenic fungi with particular reference to *Aspergillus parasiticus* and its effects on fecundity and longevity of mosquitoes exposed to conidial infections in larval stages. *Curr Sci* 54:1221–1228

- Parker BL, Skinner M, Costa SD, Gouli S, Reid W, El Bouhssini M (2003) Entomopathogenic fungi of *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae): collection and characterization for development. *Biol Control* 27:260–272
- Pereira RM, Roberts DW (1990) Dry mycelium preparations of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*. *J Invertebr Pathol* 56:39–46
- Pereira RM, Roberts DW (1991) alginate and cornstarch mycelial formulations of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*. *J Econ Entomol* 84:1657–1661
- Perry DF, Whitfield GH (1984) The interrelationships between microbial entomopathogens and insect hosts: a system study approach with particular reference to the Entomophthorales and the Eastern spruce budworm. In: Anderson JM, Rayner ADM, Walton D (eds) Animal-microbial interactions. Cambridge University Press, Cambridge, pp 307–331
- Pickford R, Riegert PW (1963) The fungus disease caused by *Entomophthora grylli* Fres and its effect on grasshopper populations in Saskatchewan in 1963. *Can Entomol* 96:1158–1166
- Polar P, Kairo MTK, Moore D, Pegram R, John SA (2005) Comparison of water, oils and emulsifiable adjuvant oils as formulating agents for *Metarhizium anisopliae* for use in control of *Boophilus microplus*. *Mycopathologia* 160:151–157
- Powell KA, Jutsum AR (1993) Technical and commercial aspects of biocontrol products. *Pesticide Sci* 37:315–321
- Prior C (1992) Discovery and characterization of fungal pathogens for locust and grasshopper control. In: Lomer CJ, Prior C (eds) Biological control of locusts and grasshoppers. CABI, Wallingford, pp 159–180
- PSD (2006) Launch of new biopesticide scheme. <http://www.pesticides.gov.uk/environment.asp?id=1846>
- Purwar JP, Sachan GC (2006) Synergistic effect of entomogenous fungi on some insecticides against Bihar hairy caterpillar *Spilarctia obliqua* (Lepidoptera: Arctiidae). *Microbiol Res* 161:38–42
- Quesada-Moraga E, Vey A (2003) Intra-specific variation in virulence and in vitro production of macromolecular toxins active against locust among *Beauveria bassiana* strains and effects of in vivo and in vitro passage on these factors. *Biocontrol Sci Technol* 13:323–340
- Quesada-Moraga E, Maranhao EAA, Valverde-Garcia P, Santiago-Alvarez C (2006) Selection of *Beauveria bassiana* isolates for control of the whiteflies *Bemisia tabaci* and *Trialeurodes vaporariorum* on the basis of their virulence, thermal requirements, and toxicogenic activity. *Biol Control* 36:274–287
- Rath AW (1992) *Metarhizium anisopliae* for control of the Tasmanian pasture scarab *Adoryphorus couloni*. In: Jackson TA, Glare TR (eds) Use of pathogens in scarab pest management. Intercept, Andover, pp 217–227
- Rao CUM, Devi KU, Khan P A (2006) Effect of combination treatment with entomopathogenic fungi *Beauveria bassiana* and *Nomuraea rileyi* (Hypocreales) on *Spodoptera litura* (Lepidoptera: Noctuidae). *Biocontrol Sci Technol* 16:221–232
- Ravensberg WJ (1994) Biological control of pests: current trends and future prospects. In: Proc Brighton Crop Protection Conf, Pests and Diseases. British Crop Protection Council, Farnham, pp 591–600
- Redbond M (2004) Biopesticides – the future? *Crop Protection Monthly* 170:16–18 (<http://www.crop-protection-monthly.co.uk>)
- Revankar SG, Sutton DA, Sanche SE, Rao JT, Zervos M, Dashti F, Rinaldi MG (1999) *Metarhizium anisopliae* as a cause of sinusitis in immunocompetent hosts. *J Clin Microbiol* 1:195–198
- Riba G, Couteaudier Y, Maurer P, Neuvéglise (1994) Molecular methods offer a new challenge for fungal bioinsecticides. In: Proc Int Colloquium Invertebrate Pathology and Microbial Control, Montpellier, France, vol 1, pp 16–22
- Roditakis E, Couzin ID, Balrow K, Franks NR, Charnley AK (2000) Improving secondary pick up of insect fungal pathogen conidia by manipulating host behaviour. *Ann Appl Biol* 137:329–335
- Rombach MC, Aguda RM, Roberts DW (1987) Biological control of the brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae) with dry mycelium applications of *Metarhizium anisopliae* (Deuteromycotina). *Philipp Entomol* 613–619
- Rombach MC, Aguda RM, Roberts DW (1988) Production of *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) in different liquid media and subsequent conidiation of dry mycelium. *Entomophaga* 33:315–324
- Saito T (2005) Preliminary experiments to control the silverleaf whitefly with electrostatic spraying of a mycoinsecticide. *Appl Entomol Zool* 40:289–292
- Samish M, Ginsberg H, Glazer I (2004) Biological control of ticks. *Parasitology* 129:S389–S403
- Samson RA, Evans HC, Latgé JP (1988) Atlas of entomopathogenic fungi. Springer, Berlin Heidelberg New York
- Seyoum E, Moore D, Charnley AK (1995) Reduction in flight activity and food consumption by the desert locust, *Schistocerca gregaria*, after infection with *Metarhizium flavoviride*. *Z angew Entomol* 118:310–315
- Shah FA, Butt TM (2005) Influence of nutrition on the production and physiology of sectors produced by the insect pathogenic fungus *Metarhizium anisopliae*. *FEMS Microbiol Lett* 250:201–207
- Shah PA, Pell JK (2003) Entomopathogenic fungi as biological control agents. *Appl Microbiol Biotechnol* 61:413–423
- Shaw KE, Davidson G, Clark SJ, Ball BV, Pell JK, Chandler D, Sunderland KD (2002) Laboratory bioassays to assess the pathogenicity of mitosporic fungi to *Varroa destructor* (Acari: Mesostigmata), an ectoparasitic mite of the honeybee, *Apis mellifera*. *Biol Control* 24:266–276
- Skrobek A, Boss D, Defago G, Butt TM, Maurhofer M (2006) Evaluation of different biological test systems to assess the toxicity of metabolites from fungal biocontrol agents. *Toxicol Lett* 161:43–52
- Smith KE, Wall R, French NP (2000) The use of entomopathogenic fungi for the control of parasitic mites, *Psoroptes* spp. *Vet Parasitol* 92:97–105

- Smithson SL, Paterson IC, Bailey AM, Screen SE, Cobb B, Hunt BA, Cooper RM, Charnley AK, Clarkson JM (1995) Cloning and characterisation of a gene encoding a cuticle-degrading protease from the insect pathogenic fungus *Metarhizium anisopliae*. *Gene* 166:161–165
- Snow AA, Andow DA, Gepts P, Hallerman EM, Power A, Tiedje JM, Wolfenbarger LL (2005) Genetically engineered organisms and the environment: current status and recommendations. *Ecol Appl* 15:377–404
- Soper RS, Ward MG (1981) Production, formulation and application of fungi for insect control. In: Papavizas GC (ed) Biological control in crop production. Beltsville Agricultural Research Centre Symp no 5. Allanheld and Osmun, Montclair, NJ, pp 161–180
- Sopp P, Gillespie AT, Palmer A (1989) Application of *Verticillium lecanii* by a low-volume electrostatic rotary atomiser and a high-volume hydraulic sprayer. *Entomophaga* 34:417–428
- Steinkraus DC, Hollingsworth RG, Slaymaker PH (1995) Prevalence of *Neozygites fresenii* (Entomophthorales, Neozygitiaceae) on cotton aphids (Homoptera, Aphididae) in Arkansas cotton. *Environ Entomol* 24:465–474
- Stenzel K, Hoelters J, Andersch W, Smit TAM (1992) BIO 1020: granular *Metarhizium*: a new product for biocontrol of soil pests. In: Proc Brighton Crop Protection Conf, Pests and Diseases, vol 3. British Crop Protection Council, Farnham, pp 363–368
- Stephan D, Zimmermann G (1998) Development of a spray-drying technique for submerged spores of entomopathogenic fungi. *Biocontrol Sci Technol* 8:3–11
- St. Leger R, Screen S (2001) Prospects for strain improvement. In: Butt TM, Jackson C, Magan N (eds) *Fungi as biocontrol agents*. CABI, Wallingford, pp 219–239
- St Leger RJ, May B, Allee LL, Frank DC, Staples RC, Roberts DW (1992a) Genetic differences in allozymes and in formation of infection structures among isolates of the entomopathogenic fungus *Metarhizium anisopliae*. *J Invertebr Pathol* 60:89–101
- St Leger RJ, Frank DC, Roberts DW, Staples RC (1992b) Molecular cloning and regulatory analysis of the cuticle-degrading protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. *Eur J Biochem* 204:991–1001
- St Leger RJ, Joshi L, Bidochka MJ, Roberts DW (1996) Construction of an improved mycoinsecticide overexpressing a toxic protease. *Proc Natl Acad Sci USA* 93:6349–6354
- Strasser H, Vey A, Butt TM (2000) Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium*, *Tolypocladium* and *Beauveria* species? *Biocontrol Sci Technol* 10:717–735
- Tanada Y, Kaya HK (1993) *Insect pathology*. Academic Press, San Diego, CA
- Tarocco F, Lecuona RE, Couto AS, Arcas JA (2005) Optimization of erythritol and glycerol accumulation in conidia of *Beauveria bassiana* by solid-state fermentation, using response surface methodology. *Appl Microbiol Biotechnol* 68:481–488
- Thomas M, Klass J, Blanford S (2000) The year of the locust. *Pesticide Outlook* 11:192–195
- Throne JE, Lord JC (2004) Control of sawtoothed grain beetles (Coleoptera: Silvanidae) in stored oats by using an entomopathogenic fungus in conjunction with seed resistance. *J Econ Entomol* 97:1765–1771
- Vakili NG (1990) Biocontrol of stalk rot in corn. In: Proc 44th Annu Corn and Sorghum Research Conf, 6–7 December 1989, Chicago, IL. American Seed Trade Association, vol 44, pp 87–105
- Vandenberg JD, Shelton AM, Wilsey WT, Ramos M (1998) Assessment of *Beauveria bassiana* sprays for control of diamond back moth (Lepidoptera: Plutellidae) on crucifers. *J Econ Entomol* 91:624–630
- Van Winkelhoff AJ, McCoy CW (1984) Conidiation of *Hirsutella thompsonii* var. *synnematosata* in submerged culture. *J Invertebr Pathol* 43:59–68
- Vestergaard S, Gillespie AT, Butt TM, Schreuter G, Eilenberg (1995) Pathogenicity of the *Frankliniella occidentalis*. *Biocontrol Sci Technol* 5:185–192
- Vestergaard S, Cherry A, Keller S, Goettel M (2003) Safety of Hyphomycete fungi as microbial control agents. In: Hokkanen HMT, Hajek AE (eds) *Environmental impacts of microbial insecticides*. Kluwer, Dordrecht, pp 35–62
- Vey A, Hoagland RE, Butt TM (2001) Toxic metabolites of fungal biocontrol agents. In: Butt TM, Jackson C, Magan N (eds) *Fungi as biocontrol agents*. CABI, Wallingford, pp 311–346
- Vidal C, Fargues J, Rougier M, Smits N (2003) Effect of air humidity on the infection potential of hyphomycetous fungi as mycoinsecticides for *Trialeurodes vaporarium*. *Biocontrol Sci Technol* 13:183–198
- Vilcinskas A, Gotz P (1999) Parasitic fungi and their interactions with the insect immune system. *Adv Parasitol* 43:267–313
- Wang CS, Leger RJS (2005) Developmental and transcriptional responses to host and nonhost cuticles by the specific locust pathogen *Metarhizium anisopliae* var. *acridum*. *Eukaryotic Cell* 4:937–947
- Wang CS, Li ZZ, Butt TM (2002) Molecular studies of co-formulated strains of the entomopathogenic fungus, *Beauveria bassiana*. *J Invertebr Pathol* 80:29–34
- Wang C, Hu G, St Leger RJ (2005) Differential gene expression by *Metarhizium anisopliae* growing in root exudate and host (*Manduca sexta*) cuticle or hemolymph reveals mechanisms of physiological adaptation. *Fungal Genet Biol* 42:704–718
- Ward MG (1984) Formulation of biological insecticides. In: Scher HB (ed) *Advances in pesticide formulation technology*. Proc ACS Symposium Series, American Chemical Society, Washington, DC, pp 175–184
- Ward MDW, Madison SL, Sailstad DM, Gavett SH, Selgrade MK (2000) Allergen-triggered airway hyper-responsiveness and lung pathology in mice sensitized with the biopesticide *Metarhizium anisopliae*. *Toxicology* 143:141–154
- Wraight SP, Ramos ME (2005) Synergistic interaction between *Beauveria bassiana* and *Bacillus thuringiensis tenebrionis* based biopesticides applied against field populations of Colorado potato beetle larvae. *J Invertebr Pathol* 90:139–150
- Wraight SP, Jackson MA, De Kock SL (2001) Product stabilization and formulation of fungal biocontrol agents. In: Butt TM, Jackson C, Magan N (eds) *Fungi as biocontrol agents*. CABI, Wallingford, pp 253–288

- Wright C, Brooks A, Wall R (2004) Toxicity of the entomopathogenic fungus, *Metarrhizium anisopliae* (Deuteromycotina: Hyphomycetes) to adult females of the blowfly *Lucilia sericata* (Diptera: Calliphoridae). Pest Manage Sci 60:639–644
- Ye SD, Ying SH, Chen C, Feng MG (2006) New solid-state fermentation chamber for bulk production of aerial conidia of fungal biocontrol agents on rice. Biotechnol Lett 28:799–804
- Yeo H, Pell JK, Alderson PG, Clark SJ, Pye BJ (2003) Laboratory evaluation of temperature effects on the germination and growth of entomopathogenic fungi and on their pathogenicity to two aphid species. Pest Manage Sci 59:156–165
- Ying FW (1992) Current situation of *Beauveria bassiana* for pine caterpillar and its prospect in China. In: Proc XIX Int Congr Entomology, Beijing, p 300
- Zimmermann (1992) Use of the fungus *Beauveria brongniartii* for control of the European cockchafer, *Melolontha melolontha* spp. in Europe. In: Jackson TA, Glare TR (eds) Use of pathogens in scarab pest management. Intercept, Andover, pp 199–208
- Zimmermann G (1994) Strategies for the utilization of entomopathogenic fungi. In: Proc VIth Int Colloquium Invertebrate Pathology and Microbial Control, Montpellier, France, vol 1, pp 67–73

---

# 11 Bacterial Weapons of Fungal Destruction: Phyllosphere-Targeted Biological Control of Plant Diseases, with Emphasis on Sclerotinia Stem Rot and Blackleg Diseases in Canola (*Brassica napus* L.)

W.G.D. FERNANDO<sup>1</sup>, R. RAMARATHNAM<sup>1</sup>, T. DE KIEVIT<sup>2</sup>

## CONTENTS

I. Introduction .....	189
II. Biological Control .....	190
III. Phyllosphere Biocontrol .....	191
A. Chemical and Physical Environment of the Leaf Surfaces .....	191
B. Bacterial Adaptations in the Phyllosphere .....	191
IV. Bacterial Phyllosphere Biocontrol of Plant Diseases, with Special Emphasis on Sclerotinia Stem Rot and Blackleg .....	191
A. Timing of Application .....	192
B. Mechanisms of Biocontrol .....	192
1. Antibiosis .....	192
2. Pre-Emptive Colonization of Target Site .....	194
3. Antifungal Volatiles .....	194
4. Induced Systemic Resistance .....	195
V. Conclusions .....	196
References .....	196

## I. Introduction

Canola, also known as oilseed rape, has worldwide production, with China, Canada, Australia and the European Union leading the production. In Canada, the 10-year average is 11.3 million acres harvested, making it the largest single producer of canola (Canola Council of Canada 2006). Developed in 1974, a rapeseed variety to qualify as canola has to fit the following definition: "an oil must contain less than 2% C22:1, and the solid component of the seed must contain less than 30 micromoles of any one or mixture of 3-butetyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butetyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate per gram of air dry, oil free solid" (Adolphe et al. 2002). Canola is currently more valuable than peanut, cottonseed and sunflower as a source of

vegetable oil (Sovero 1993). It is the third most important source of vegetable oil.

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, and blackleg, caused by *Leptosphaeria maculans* (Desm.) Ces & De Not (anamorph, *Phoma lingam* (Tode: Fr./Desm.)), are the two most economically important diseases of canola. Both diseases lead to significantly high yield loss in western Canada, with up to 5–100% (Manitoba Agriculture 2002) reported for stem rot and 50% for blackleg (Petrie et al. 1985). The primary inoculum of both diseases originates from overwintering structures. For stem rot, the overwintering sclerotia germinate carpogenically to produce apothecia (Willets and Wong 1980) which, in turn, produce ascospores, the primary inoculum which germinates and infects senescing petals. The infected petals, which act as a nutrient source for the germinating ascospore, fall onto the leaves where the fungus infects through mechanical pressure. The infection then moves from the leaves to the stem (Martens et al. 1994). In severe cases of infection, the sclerotia develop in the stem and are cycled back into the soil during harvest. For blackleg, ascospores are the primary and main source of infection. They are produced in pseudothecia in the infected stubble. Seedling infection is achieved by penetration of the cotyledons or young leaves through stomata or natural openings. The initial infection of the tissue is biotrophic, but most of the hyphal front becomes necrotrophic. The necrotrophic infection leads to the production of the asexual structures (pycnidia) in the dead tissue (Hammond et al. 1985; Hammond and Lewis 1987). During a rain event, the pycnidiospores are spread by splashing and, thus, are thought to infect other leaves and neighbouring plants. The colonization of the intercellular spaces follows the initial infection, which leads to the colonization of the xylem or the spaces between the xylem parenchyma and the cortex in the

<sup>1</sup> University of Manitoba, Department of Plant Science, 66 Dafoe Road, Winnipeg, Manitoba R3T 2N2, Canada

<sup>2</sup> University of Manitoba, Department of Microbiology, Winnipeg, Manitoba R3T 2N2, Canada

petiole. This intercellular growth is systemic, biotrophic and visually symptomless (Hammond et al. 1985). The fungus eventually invades and kills the cells of the stem cortex, resulting in a blackened canker which completely girdles the base of the stem. Thus, the disease is named "blackleg".

Control of sclerotinia stem rot by traditional methods has not been very effective. It is difficult to breed for resistance against *S. sclerotiorum*, since resistance is governed by multiple genes (Fuller et al. 1984). Control based on crop rotations is unrealistic due to the persistence of survival structures (sclerotia) in the soil for long periods and because *Sclerotinia* has such a wide host range (Nelson 1998). These factors necessitate the use of fungicides, which have been known to have adverse effects on non-target organisms (Rose 1995; Gilmour 2001; McGrath 2001). Several strategies based on, for example, crop rotation, stubble management, chemical control, sanitation and resistant cultivars have been advised for blackleg control (Guo et al. 2005). With the introduction of cultivars with major gene resistance to *L. maculans*, a high potential exists for increased prevalence of the aggressive isolates of *L. maculans*, or evolution of new virulent pathotypes of the pathogen (Mayerhofer et al. 1997). The report on the appearance of the more aggressive pathogenicity group 3 (PG3) and 4 (PG4) isolates in western Canada and the North Central USA, where PG2 is the predominant group, is a good example of this phenomenon (Fernando and Chen 2003; Chen and Fernando 2005; Bradley et al. 2005). With respect to chemical control with fungicides, the perceived health and environmental risks of using these chemicals has led to increased interest in alternative disease management strategies (Jacobsen and Backman 1993).

A clear understanding of the life cycle and mode of infection of both pathogens gives us an opportunity to design alternative disease control strategies, such as biological control, for the management of these pathogens and their diseases. For sclerotinia stem rot, as most inoculums are ascosporic, a few germinating sclerotia can lead to significant infection levels in the field (Davies 1986). Also, ascospores can travel long distances from neighbouring fields to infect petals (Venette 1998). Therefore, there is a need for research into the biocontrol of *S. sclerotiorum* on canola, specifically on limiting petal infection by ascospores. Foliar applications of biocontrol agents (BCA) are

important in the Sclerotinia/canola system, as the ascospores generally infect senescing petals at the flowering stage (Turkington and Morrall 1993). As this is a narrow window to protect the plant from infection, biological control may work well in controlling the germination of ascospores on petal surfaces. Similarly, the narrow susceptibility period of canola to blackleg favours the use of biocontrol as a viable disease control strategy. The disease is destructive to the canola crop only when the infection occurs early in plant development, from the cotyledon to the six-leaf growth stage (West et al. 2001). If the plant can be protected during this most highly susceptible period, then the disease could be managed to minimise yield loss. Therefore, biological control represents a promising, alternative control strategy which can be implemented in the integrated management of sclerotinia stem rot and blackleg diseases of canola.

## II. Biological Control

Baker and Cook, in 1974, defined biological control as "the reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonist". Bacteria have proved to be excellent sources of antagonists, owing to their multiple mechanisms of disease control. Earlier work on the biocontrol of plant diseases focused more on the root-colonizing rhizobacteria, with special emphasis on their ability to produce secondary metabolites, such as siderophores. Siderophores efficiently sequester iron and deprive the pathogen of this vital element, essential for metabolic functioning and the process of pathogenesis (Kloepper et al. 1980). Over the past two decades, numerous studies have established the role of other mechanisms such as antifungal antibiotics, enzymes and volatiles produced by bacteria in plant disease control (Weller et al. 1988; Whipps 1997). Other than their direct role in pathogen control, bacteria also produce metabolites which enhance plant growth (e.g. root growth), or trigger the induction of systemic resistance which acts in the form of immunization, thereby preventing plant disease (Van Loon et al. 1998). With the above-mentioned multiple mechanisms of disease control, bacteria serve as excellent antagonists.

### III. Phyllosphere Biocontrol

#### A. Chemical and Physical Environment of the Leaf Surfaces

There is limited knowledge on the chemical characteristics of the leaf surface which make it suitable for microbial growth. The unevenness of the thick waxy cuticle, along with the presence of veins and trichomes, affect the microclimate of the leaf surface. Factors such as rain, dew and leaf exudates result in the transport of nutrients from plant cells, and other microbes or pollutants which help bacteria to maintain their metabolic activity and population size (Suslow 2002). On the leaf surface, bacteria acquire their carbon and nitrogen sources mainly from the exudates, which are comprised of sugars such as sucrose, glucose and fructose. In addition, other organic acids, alcohols and amino acids are present on the leaves. The concentration of nutrients on the leaves is very low, ranging from 1–20 µg/leaf (Tukey 1971). Wilson and Lindow (1994a, b) have clearly shown that the growth of bacteria on the leaf surface is limited by the availability of carbon-containing nutrients under physical environmental conditions conducive for bacterial multiplication.

The microclimate of the leaf, under highly variable environmental conditions such as for humidity, temperature, leaf wetness and ultraviolet radiation, poses a major challenge for colonization by bacteria. Leaf surfaces are subjected to lack of free moisture and rapid fluctuations in relative humidity or temperature (Beattie and Lindow 1995, 1999). The ultraviolet radiation present in sunlight causes severe damage to bacterial cells (Sundin and Jacobs 1999). An additional challenge faced by these bacteria is the diurnal change from cool nights of ample moisture in the form of dew to higher temperatures and drier conditions which cause osmotic shock during the daytime.

#### B. Bacterial Adaptations in the Phyllosphere

Bacteria have evolved mechanisms to overcome these constraints and establish themselves under the harsh conditions present in the phyllosphere. Bacteria arrive through airborne, waterborne and vector-borne deposition from sources of plant origin such as tree buds, seeds and residues of previous crops (Manceau and Kasempour 2002). The bacteria multiply on the leaf surface, when free wa-

ter is available, and form aggregates which enhance their ability to survive when ecological conditions become less favourable on the surface. Aggregation occurs along the anticlinal walls of epidermal cells and around the trichomes, where leaching of nutrients is higher (Backman et al. 1997). Larger bacterial aggregates are present at the base of trichomes, probably due to their ability to retain water, and secrete mucilage and essential nutrients, such as sugars and amino acids (Simon 1997; Ascensao and Pais 1998). Bacteria increase their fitness in the phyllosphere by the expression of phenotypic features such as flagellar motility, extracellular polysaccharide production, production of biosurfactants, phytohormone production, and phytotoxins and siderophore production (Lindow 1991; Beattie and Lindow 1995, 1999). Bacteria also adopt an endophytic mode of life style when they colonize the inside of leaf tissues to escape the harsh conditions occurring on the leaf surface when the environment becomes dry (Whitesides and Spots 1991). Epiphytic bacteria adapt themselves to an endophytic mode of survival by multiplying in the apoplast and escaping plant defence systems (Kazempour 1998). Other than epiphytic bacteria, saprophytic bacteria develop mechanisms of tolerance to stress, such as pigmentation which is often correlated with UV tolerance, to maintain population levels high enough to survive in the phyllosphere (Wilson et al. 1999).

### IV. Bacterial Phyllosphere Biocontrol of Plant Diseases, with Special Emphasis on Sclerotinia Stem Rot and Blackleg

*Pseudomonas* spp. and *Bacillus* spp. mediate crop protection by exerting multiple mechanisms of inhibitory activity such as the production of extracellular enzymes (Dunlap et al. 1996; Pleban et al. 1997), competition (Lugtenberg et al. 1999), induced systemic resistance, ISR (Yan et al. 2002; Ryu et al. 2003), and antibiosis (Silo-Suh et al. 1994; Raaijmakers et al. 2002).

One of the most successful examples of phyllosphere biocontrol is the suppression of *Erwinia amylovora* by epiphytic bacteria, and the reduction of fire blight disease in apple and pear. *Pseudomonas fluorescens* A506 occupies the same sites on stigma as those colonized by *E. amylovora*,

and utilizes nutrients important for the growth of the pathogen (Wilson and Lindow 1993). On the other hand, *Pantoea agglomerans* produces antibiotics (Ishimaru et al. 1988; Vanneste et al. 1992; Kearns and Hale 1996; Wright and Beer 1996), in addition to other growth-limiting nutritional substrates and thereby competes for sites, suppressing *E. amylovora* on stigmas (Hattingh et al. 1986; Wilson et al. 1992). *Bacillus* has been one of the key genera used for the phyllosphere biocontrol of foliar diseases. *Bacillus* spp. form endospores, which are structures capable of surviving desiccation, heat, oxidizing agents and UV radiation (Setlow 1995). These characteristics offer ecological fitness and also enable long-term storage and relatively easy commercialization of *Bacillus*-based products. Control of bean rust by *Bacillus subtilis* (Baker et al. 1985), and control of *Alternaria* on tomato (Stevens et al. 1996) and *Cercospora* on peanut by *Bacillus cereus* (Kokalis-Burelle et al. 1992) are good examples.

Biological control of foliar pathogens at the phyllosphere has been less exploited, as is evident in the huge literature available on the biocontrol of root pathogens. Harsh conditions in the phyllosphere, which make for difficult establishment and survival of the biocontrol agent, is one of the main reasons for the lack of attempts on phyllosphere biocontrol. Yet, there are some host-disease systems in which phyllosphere biocontrol has been successfully used in disease control. The following section deals with the timing of application of the bacterial biocontrol agent (BCA), and the various mechanisms exerted by the bacterial BCA for the phyllosphere control of plant disease, such as antifungal antibiosis, efficient colonization, volatile production and induced systemic resistance. The section also lays emphasis on the biocontrol of sclerotinia stem rot and blackleg of canola by bacterial BCAs.

#### A. Timing of Application

A clear understanding of the pathogen life cycle, the growth stage at which the host is susceptible to infection by the pathogen, and the mechanism of host infection would help in the application of the BCA at the appropriate place and time. Also, if there is a narrow window of infection by a pathogen, this favours the use of BCAs as a control strategy, as the antagonist need only survive, establish its population and prevent pathogen infection dur-

ing that short critical period. The successful use of biocontrol relies on an effective delivery system and subsequent survival of bacteria in the infection court. Since the primary infection of *S. sclerotiorum* on canola occurs through ascosporic infection of senescing petals (Adams and Ayers 1979), biocontrol studies performed in our laboratory employ direct delivery of bacteria onto the petals, in the form of one or two spray applications during the blooming stage (Fernando et al. 2007). Results from two field trials conducted during 2003 and 2004 indicate that either one or two applications of bacterial strains suppressed stem rot under field conditions. Applications of *Pseudomonas chlororaphis* strain PA23 and *Bacillus amyloliquefaciens* strain BS6 twice at 30% and 50% bloom significantly reduced the percent canola stem rot incidence in both trials. The results are comparable to those obtained for the application of the fungicide Rovral-Flo, and are significantly different from those for the pathogen-inoculated control.

Similarly, bacterial application at the cotyledon stage, i.e. the stage most susceptible to pathogen infection, plays an important role in the prevention of blackleg infection (Ramarathnam 2006). This phenomenon was clearly established in our field study where *B. amyloliquefaciens* strain DFE16 applied at the cotyledon stage suppressed the disease as efficiently as the fungicide which was tested. Bacteria seem to prevent early infection of the cotyledon leaves, reducing the chances for systemic infection, and girdling and cankering of the stem. The need for BCA application at the cotyledon stage is further strengthened by our finding that the same bacteria applied only at the 3–4 leaf stage failed to suppress the disease, suggesting infection of the plant could have occurred prior to the application of the bacteria. The cotyledon stage of canola is a narrow window of infection potential and, therefore, it represents an opportunity to be exploited for biocontrol of blackleg.

#### B. Mechanisms of Biocontrol

##### 1. Antibiosis

Direct antagonism of the pathogen through antibiosis is one of the mechanisms by which disease-suppressive bacteria achieve disease control. Antibiosis is mediated through the production of a chemically heterogeneous group of organic, low-molecular weight compounds (Raaijmakers et al. 2002) which, at low concentrations, are

deleterious to the growth or metabolic activities of other microorganisms (Fravel 1988; Thomashow et al 1997). Restriction of growth, due either to limitation in nutrients or to high cell density, leads to the initiation of secondary metabolism and antibiotic production, which helps the organisms remain competitive in their environment (Vining 1990).

Stigma colonization by *E. amylovora* is the crucial initial step in the development of most fire blight infections in apple and pear trees. Suppression at this point of the disease process by antagonists of *E. amylovora*, such as *Pantoea agglomerans* (*Erwinia herbicola*) strain Eh1087, has resulted in significant success. The ability to produce a phenazine antibiotic, D-alanylgriseoluteic acid (AGA), helps the antagonistic strain to outcompete *E. amylovora* in the colonization of the stigma (Giddens et al. 2003). In competition experiments on the stigmas of apple flowers, *E. amylovora* was significantly less successful against the *P. agglomerans* AGA-producing wild type than against the AGA-non-producing mutant. Further, it was found that AGA production significantly enhanced the competitiveness of Eh1087 when it was applied at the same time or 24 h before the pathogen. This study clearly established the role of this antibiotic in the ecological fitness of the producing strain, and its role in disease control.

*Pseudomonas chlororaphis* strains PA23 (PA23) and DF190 (DF190) are strong antagonists of *S. sclerotiorum* and *L. maculans* respectively. The bacteria were identified as producers of the antifungal antibiotics phenazine and pyrrolnitrin (Ramarathnam and Fernando 2006; Zhang et al. 2006). Control of *Gaeumannomyces graminis* var. *tritici* in wheat by phenazine produced by *P. chlororaphis* PCL1391 (Chin-A-Woeng et al. 1998), and control of *Rhizoctonia solani* in cotton by pyrrolnitrin produced by *P. fluorescens* BL915 (Ligon et al. 2000) are well-known examples of an established role for these antibiotics in biocontrol. The production of phenazines and pyrrolnitrin corresponds to the antifungal activity of PA23 culture extracts in the inhibition of sclerotial and/or spore germination of several plant pathogens, including *Fusarium oxysporum*, *R. solani*, *Sclerotium rolfsii*, *Macrophomina phaseolina*, *Alternaria solani* and *Botryodiplodia theobromae* (Kavitha et al. 2005). Production of multiple antibiotics, with overlapping or different degrees of activity, may account for the suppression of specific or multiple plant pathogens (Raaijmakers et al. 2002). Characterization of a PA23Tn5 mutant, called PA23-63, revealed a Tn insertion in

*phzE*, which forms part of the phenazine biosynthetic cluster. Despite producing no phenazines, this strain exhibited wild-type levels of antifungal and biocontrol activity against *S. sclerotiorum* and *L. maculans* (Poritsanos 2005; Ramarathnam 2006). Our findings indicate that phenazine production is not essential for PA23 biocontrol of these two pathogens. Mutant PA23-63 produces pyrrolnitrin at levels equal to that of the wild type (Paulitz, personal communication). Therefore, we believe that pyrrolnitrin is mainly, but not exclusively, responsible for the antibiosis-mediated biocontrol of *S. sclerotiorum*, the stem rot pathogen (Poritsanos 2005), and *L. maculans*, the blackleg pathogen of canola (Ramarathnam 2006). The primary antifungal mechanism of pyrrolnitrin is the interference of the osmotic signal transduction pathway, and the secondary mechanism is thought to be inhibition of respiration, evident at a high dosage of the antibiotic in *N. crassa* and other fungi (Okada et al. 2005). This explains the antifungal activity of pyrrolnitrin over a wide range of basidiomycetes, deuteromycetes and ascomycetes (Ligon et al. 2000).

Another *Pseudomonas* BCA isolated by our laboratory, *Pseudomonas* sp. strain DF41 (DF41), demonstrated strong antagonistic activity against *S. sclerotiorum*. By means of Tn5 mutagenesis studies, a DF41 antifungal-deficient mutant was isolated harbouring a Tn insertion in a gene with 91% identity to the *syrB* gene of the *P. syringae* syringomycin biosynthetic cluster (C. Berry, W.G.D. Fernando, T.R. de Kievit, unpublished data). A Tn5 insertion in this gene completely abolishes antifungal activity, suggesting a molecule similar to syringomycin, probably a cyclic lipopeptide, may be responsible for the antibiosis in strain DF41.

*Bacillus* spp., especially *B. subtilis*, *B. cereus* and *B. amyloliquefaciens*, are reported to be effective for the control of plant diseases caused by soil-borne, foliar and post-harvest fungal pathogens (Silo-Suh et al. 1994; Raupach and Kloepffer 1998; Shoda 2000; Janisiewicz and Korsten 2002; Chiou and Wu 2003; Tjamos et al. 2004). *Bacillus cereus* strain DFE4 (DFE4), and *B. amyloliquefaciens* strains BS6 (BS6) and DFE16 (DFE16) exhibited agar-diffusible antifungal activity, and greenhouse and field suppression of sclerotina stem rot and blackleg of canola (Fernando et al. 2005; Ramarathnam 2006). All three *Bacillus* strains harbour biosynthetic genes for the lipopeptide antibiotics iturin A, bacillomycin D and surfactin. Moreover, cell extract analysis confirmed the production

of iturin A, bacillomycin D and surfactin by these BCAs (Ramarathnam 2006). Bacillomycin D has been reported to exhibit strong antifungal activity towards aflatoxin-producing fungi such as *Aspergillus flavus* (Moyné et al. 2001). Iturin A is known to exhibit strong antifungal activity and potential for biocontrol (Yoshida et al. 2002; Cho et al. 2003). The multiple antibiotics produced by these *Bacillus* spp. could possibly explain the broad spectrum of antifungal activity exhibited by these bacteria over a range of pathogens.

## 2. Pre-Emptive Colonization of Target Site

Competence of the BCA and synchronization of its activity, in both time and space, with the pathogen are key factors determining the efficiency of a BCA (Folman et al. 2003). Earlier studies by Bull et al. (1991) on the colonization of roots by *P. fluorescens* in the suppression of *G. graminis* var. *tritici*, and by Parke (1990) on the successful colonization of the pea spermosphere in the prevention of *Pythium* infection are good examples which stress the importance of colonization of BCAs at the target site as a prerequisite for suppression of plant pathogens. Pre-emptive stigma colonization by the antagonistic *P. agglomerans* strain Eh107 resulted in a population which was resilient to subsequent invasion by the pathogen. Similarly, the colonization of the stigma by the pathogen, prior to the antagonist, prevented the establishment of the antagonist. This clearly established the importance of pre-emptive colonization and utilization of the nutrients required by the pathogen (Giddens et al. 2003). This experiment suggested that niche exclusion has a dominant influence on the dynamics of bacterial populations on stigmas, and also in the suppression of the fire blight disease.

The time of inoculation of the BCA plays a very important role in the suppression of both sclerotinia stem rot and blackleg disease of canola. The application of the *Pseudomonas* sp. strain DF41 (Savchuk and Fernando 2004), strains PA23 or BS6 (N. Poritsanos, C. Selin, W.G.D. Fernando, S. Nakkeeran and T.R. de Kievit, unpublished data) 24 h prior to the pathogen, or co-inoculation with the pathogen were crucial in the suppression of stem rot disease under greenhouse conditions. Antagonistic bacteria, when applied 24 and 48 h after ascospore application, did not control the disease. The same phenomenon was also applicable in the blackleg suppression assays done on cotyledon plants of canola in the greenhouse.

The inoculation of the bacteria PA23, DF190 and DFE4, 24 and 48 h prior to the application of pycnidiospores of *L. maculans* on wounded canola cotyledons, led to significantly lower levels of disease, which was manifested in a low, resistant interaction phenotype (IP) ratings (<2). The correct timing of application of the BCA, suitable application strategy, and establishment of the BCA in the target area are the critical elements determining the success of a biocontrol strategy (Baker and Cook 1974; Weller et al. 1988; Campbell 1989). Early application provides the bacteria with ample time for the establishment of appropriate population size and successful colonization of the infection court of the pathogen. This, in turn, helps in preventing the germination of ascospores of *S. sclerotiorum* or pycnidiospores of *L. maculans*, and the penetration and establishment of the pathogen in the host.

## 3. Antifungal Volatiles

In addition to the phyllosphere inhibition of ascospores of *S. sclerotiorum* on petals, strain PA23 has been demonstrated to produce antifungal volatiles which are inhibitory to vegetative mycelium growth, germination of ascospores, and sclerotia formation of *S. sclerotiorum* (Fernando et al. 2005). Two compounds identified by GC-MS, nonanal and benzothiazole, completely inhibited mycelial growth or sclerotia formation under exposure to filter-disks soaked with these compounds (100 and 150 µl) in subdivided Petri plates. These observations suggest nonanal and benzothiazole may play a role in biological control. The antifungal activity of nonanal has been demonstrated earlier; this is one of the major constituents of the essential oil of *Hibiscus cannabinus*, responsible for the fungitoxic activity of the oil towards three species of *Colletotrichum* (Kobaisy et al. 2001). Also, nonanal produced by cotton leaves is responsible for the production of the unique aerial hyphae and decrease in aflatoxin production of *Aspergillus flavus* and *A. parasiticus* (Green-McDowell et al. 1999). Benzothiazole is a sulphur-based compound; many commercially used fungicides and soil fumigants are also sulphur-based. Sclerotial control would reduce apothecial formation, which would decimate ascospore production, the most important infection units in many crops, thereby reducing several diseases (Abawi and Grogan 1979). Hence, these bacteria may be good soil amendments for

the management of overwintering structures in soil. Pathogen-infested stubble and sclerotinia are substrates for the production of sexual and asexual spores which cause primary infection of the crop. Destruction of the overwintering structures would greatly limit primary inoculum production and the establishment of the disease in the crop.

#### 4. Induced Systemic Resistance

The efficiency of BCAs can be improved through elucidation of the mechanism(s) of their action. Plants may be protected against pathogens by way of endogenous defence mechanisms triggered in response to the attack of either an insect or pathogen (Heil 2001). Induced resistance by bacterial antagonists in several crops is associated with the enhancement of lignification, and stimulation of host-defence enzymes and synthesis of pathogenesis-related (PR) proteins (Hammerschmidt and Kuc 1995). Our results suggest that two applications of strain PA23 induced resistance against *S. sclerotiorum* infection (Fernando et al. 2007). An application of PA23 at both 30 and 50% bloom, followed by challenge inoculation with *Sclerotinia* ascospores, induced significantly higher chitinase and  $\beta$ -1,3-glucanase activity. In contrast, the activity was less in the healthy control, ascospore-inoculated control, and PA23 treatments. Accumulation of these hydrolytic enzymes reached the highest level 4 days after inoculation (DAI) for chitinase (2.5-fold increase over inoculated control), and 6 DAI for  $\beta$ -1,3-glucanase (threefold increase over inoculated control), followed by a slow decline thereafter. One chitinase isoform (34 kDa) was detected by western blotting using tobacco chitinase antiserum in PA23-treated plants with or without challenge inoculation by *S. sclerotiorum*. Expression was very low in the inoculated control. The PR proteins chitinase and  $\beta$ -1,3-glucanase both inhibit fungal pathogens (Mauch et al. 1988). The enhanced accumulation of PR proteins (chitinase and  $\beta$ -1,3-glucanase) and oxidative enzymes (peroxidases) in PA23-treated canola leaf tissues may contribute to the reduction of *Sclerotinia* infection in pathogen-inoculated plants. Since fungi have chitin and glucan as cell wall components (Sing et al. 1999), increased activity of chitinase and  $\beta$ -1,3-glucanase in canola plants exposed to biocontrol bacteria may prevent the establishment of pathogens. Similarly, phyllosphere inoculation studies have been carried out earlier for induced systemic resistance (ISR)

with *B. mycoides* strain Bac J for the control of *Cercospora* leaf spot in sugar beet (Bargabus et al. 2002), and *P. putida* WCS358r and *P. fluorescens* WCS374r for the control of bacterial wilt caused by *R. solanacearum* in *Eucalyptus urophylla* (Ran et al. 2005). Inoculation of *B. mycoides* strain Bac J induced the activity of chitinase,  $\beta$ -1,3-glucanase and peroxidase, and significantly reduced *Cercospora* leaf spot in sugar beet (Bargabus et al. 2002). Also, the bacteria were not isolated from the pathogen-inoculated leaves, which demonstrated the role of ISR in disease suppression. *B. subtilis* strain BacB formed cell aggregates which induced systemic resistance against *Cercospora* leaf spot, even though the cell number on the leaf reduced significantly (Collins et al. 2003). Disease control could not be attributed to either antibiosis or parasitism in this study.

Plant growth promoting rhizobacteria (PGPR) strains with ISR activity can be active against a wide range of pathogens (Raupach and Kloepffer 1998). Application of fluorescent pseudomonads strengthens the host cell wall structures and results in the restriction of pathogen invasion of host-plant tissue (Chen et al. 2000). Rhizobacteria-mediated ISR leads to enhanced sensitivity of the induced tissue to jasmonic acid (JA) and ethylene (ET), rather than increasing their production (Pozo et al. 2005). Conrath et al. (2002) term this phenomenon "priming", which is the enhanced capacity of induced tissues for rapid and effective activation of cellular defence response upon challenge by pathogen infection. This has been proved with analysis of local and systemic levels of JA and ET in plants expressing ISR, where induced resistance was not associated with detectable changes in their production (Pieterse et al. 2000). Priming has been well established towards disease reduction in tobacco, where cells showed faster and stronger lipid peroxidation and protein phosphorylation in response to fungal elicitors after preconditioning by MeJA treatment (Dubery et al. 2000).

In contrast to the ISR in mature canola elicited by strains PA23 and BS6, no such induction was observed in the suppression of blackleg in canola cotyledons. Lipopolysaccharides (LPS) of the bacterial cell wall and iron-chelating siderophores have clearly been shown to elicit systemic resistance in plants (Whipps 2001). In addition, antifungal antibiotics have also been reported to induce systemic resistance (SR). Audenaert et al. (2002) demonstrated that phenazine-1-carboxylic

acid and a siderophore, pyochelin, produced by *Pseudomonas aeruginosa* strain 7NSK2 both contributed to the ability of this isolate to induce SR in tomato against *Botrytis cinerea*. Among the lipopeptide antibiotics produced by *Bacillus* spp., treatment of potato tuber cells with purified fengycins resulted in the accumulation of some plant phenolics involved in, or derived from phenylpropanoid metabolism (Ongena et al. 2005). Production of siderophores and antifungal antibiotics by strains PA23 (Poritsanos 2005; Zhang et al. 2006), DF190, DFE4 and DFE16 (Ramarathnam 2006) have been established. These bacteria were tested for their ability to induce SR in canola cotyledons. Living bacterial cells and their culture broth extract were tested through split and local inoculation assays. Split inoculation determined the ability of the bacteria to induce SR, and the local inoculation tested for direct antifungal activity on the pycnidiospores of *L. maculans*. Both the bacteria and their culture broth extract failed to suppress the pycnidiospores of *L. maculans*, and the blackleg lesion on cotyledons in the split inoculations. The bacteria and the broth extracts suppressed the blackleg disease lesion on cotyledons when inoculated locally with the pathogen spores. This clearly indicated a lack of systemic induction by the bacteria and broth extracts, but localized inhibition of the pathogen. To further ascertain the mechanism involved in the localized suppression of the disease, the activity of PR enzymes was studied. None of the bacterial treatments (except for DF190-inducing  $\beta$ -1,3-glucanase) had significant induction of chitinase,  $\beta$ -1,3-glucanase or peroxidase (Ramarathnam 2006). The lack of localized PR-enzyme activity, but suppression of the pycnidiospores of *L. maculans*, suggest the potential role of direct antifungal activity of the bacterial cells.

## V. Conclusions

In the quest for safer, more environmentally friendly alternatives to pesticides, BCAs emerge as attractive candidates for the management of plant diseases. Thus far, natural bacterial isolates have shown inconsistent performance in the field. Poor field performance can be attributed in part to varying environmental conditions which, in turn, influences expression of essential biocontrol factors. Thus, if BCAs are ever to become realistic

alternatives to chemical pesticides, it is essential that we understand, at a molecular level, both biotic and abiotic factors regulating the expression of antifungal metabolites. By employing transcriptomics and proteomics, we can advance our understanding of gene/protein expression not only in the BCAs and fungal pathogens but also in the plant host during complex interactions in the phytosphere. This will provide us with a fuller picture of beneficial plant-microbe interactions. Defining factors which influence antibiosis, ISR, as well as predation and niche exclusion will ultimately lead to better-performing biocontrol products. Notably, this knowledge can be used to create genetically engineered strains exhibiting improved biocontrol capacity. It is expected that genetically modified strains will be the biocontrol products of the future, able to rival chemical fungicides in managing plant diseases.

## References

- Abawi GS, Grogan RG (1979) Epidemiology of plant diseases caused by *Sclerotinia* species. *Phytopathology* 69:899–904
- Adams PB, Ayers WA (1979) Ecology of *Sclerotinia* species. *Phytopathology* 69:896–899
- Adolphe D, Daun J, Fitzpatrick K, Gruener L, Macyk D, Mayko J, Peters L, Wilkins D (2002) Canola (<http://www.canola-council.org>, accessed 12 December 2002)
- Ascensao L, Pais MS (1998) The leaf capitate trichomes of *Leonotis leonurus*: histochemistry, ultrastructure and secretion. *Ann Bot* 81:263–71
- Audenaert VK, Pattery T, Cornelis P, Hofte M (2002) Induction of systemic resistance to *Botrytis cinerea* in tomato by *Pseudomonas aeruginosa* 7NSK2: role of salicylic acid, pyochelin, and pyocyanin. *Mol Plant Microbe Interact* 15:1147–1156
- Backman PA, Brannen PM, Mahaffee WF (1997) Bacteria for biological control of plant diseases. In: Rechcigl NA, Rechcigl JE (eds) Environmentally safe approaches to crop disease control. Lewis, New York, pp 95–109
- Baker KF, Cook RJ (1974) (eds) Biological control of plant pathogens. W.H. Freeman, San Francisco, CA
- Baker CJ, Stavely JR, Mock N (1985) Biocontrol of bean rust by *Bacillus subtilis* under field conditions. *Plant Disease* 69:770
- Bargabus RL, Zidack NK, Sherwood JW, Jacobsen BJ (2002) Characterization of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. *Physiol Mol Plant Pathol* 61:289–298
- Beattie GA, Lindow SE (1995) The secret life of bacterial colonist on leaf surfaces. *Annu Rev Phytopathol* 33:145–172

- Beattie GA, Lindow SE (1999) Bacterial colonization of leaves: a spectrum of strategies. *Phytopathology* 89:353–359
- Bradley C, Parks PS, Chen Y, Fernando WGD (2005) First report of pathogenicity groups 3 and 4 of *Leptosphaeria maculans* on canola in North Dakota. *Plant Disease* 89:776
- Bull CT, Weller DM, Thomashow LS (1991) Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* strain 2-79. *Phytopathology* 81:954–959
- Campbell R (1989) Biological control of microbial plant pathogens. Cambridge University Press, Cambridge
- Canola Council of Canada (2006) Crop production ([http://www.canola-council.org/cropproduction9/cropproduction9\\_1.html?gInitialPosX=10px&gInitialPosY=10px&gZoomValue=100](http://www.canola-council.org/cropproduction9/cropproduction9_1.html?gInitialPosX=10px&gInitialPosY=10px&gZoomValue=100), accessed 15 May 2006)
- Chen Y, Fernando WGD (2005) First report of canola blackleg caused by pathogenicity group 4 of *Leptosphaeria maculans* in Manitoba. *Plant Disease* 89:339
- Chen C, Belanger RR, Benhamou N, Paultz TC (2000) Defense enzymes induced in cucumber roots by treatment with plant growth promoting rhizobacteria. *Physiol Mol Plant Pathol* 56:13–23
- Chin-A-Woeng TFC, Bloomberg GV, van der Bij AJ, van der Drift KMGM, Schripsema J, Kroon B, Scheffer RJ, Keel C, Bakker PAHM, Tichy HV, de Bruijn FJ, Thomas-Oates JE, Lugtenberg BJJ (1998) Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Mol Plant Microbe Interact* 11:1069–1077
- Chiou AL, Wu WS (2003) Formulation of *Bacillus amyloliq-uefaciens* B190 for control of lily grey mould (*Botrytis elliptica*). *J Phytopathol* 151:13–18
- Cho SJ, Lee SK, Cha BJ, Kim YH, Shin KS (2003) Detection and characterization of the *Gloeosporium gloeosporioides* growth inhibitory compound iturin A from *Bacillus subtilis* strain KS03. *FEMS Microbiol Lett* 223:47–51
- Collins DP, Jacobsen BJ, Maxwell B (2003) Spatial and temporal population dynamics of a phyllosphere colonizing *Bacillus subtilis* biological control agent of sugar beet *Cercospora* leaf spot. *Biol Control* 26:224–232
- Conrath U, Pieterse CMJ, Mauch-Mani B (2002) Priming in plant pathogen interactions. *Trends Plant Sci* 7:210–216
- Davies J (1986) Diseases of oilseed rape. In: Scarsbrick DH, Daniels RW (eds) Oilseed rape. Mackays of Chantham, Kent, pp 195–236
- Dubery JA, Teodorczuk LG, Louw AE (2000) Early responses in methyl jasmonate-preconditioned cells towards pathogen derived elicitors. *Mol Cell Biol Res Comm* 3:105–110
- Dunlap C, Delaney J, Fenton A, Lohrke S, Moënne-Loccoz Y, O'Gara F (1996) The biotechnology and application of *Pseudomonas* inoculants in the biocontrol of phytopathogens. In: Stacey G, Mullin B, Gresshoff PM (eds) Biology of plant microbe interactions. International Society for Molecular Plant Microbe Interactions, St. Paul, MN, pp 441–448
- Fernando WGD, Chen Y (2003) First report on the presence of *Leptosphaeria maculans* pathogenicity group 3, causal agent of blackleg of canola in Manitoba. *Plant Disease* 87:1268
- Fernando WGD, Ramarathnam R, Krishnamoorthy AS, Savchuk S (2005) Identification and use of potential bacterial organic volatiles in biological control. *Soil Biol Biochem* 37:955–964
- Fernando WGD, Nakkeeran S, Zhang Y, Savchuk S (2007) Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Pseudomonas* and *Bacillus* species on canola petals. *Crop Protect* 26:100–107
- Folman LB, Postma J, van Veen JA (2003) Inability to find consistent bacterial biocontrol agents of *Pythium aphanidermatum* in cucumber using screens based on ecophysiological traits. *FEMS Microbiol Ecol* 45:72–87
- Fravel DR (1988) Role of antibiosis in the biocontrol of plant diseases. *Annu Rev Phytopathol* 26:75–91
- Fuller P, Coyne D, Steadman J (1984) Inheritance of resistance to white mold disease in a diallel cross of dry beans. *Crop Sci* 24:929–933
- Giddens SR, Houlston GJ, Mahanty HK (2003) The influence of antibiotic production and pre-emptive colonization on the population dynamics of *Pantoea agglomerans* (*Erwinia herbicola*) Eh1087 and *Erwinia amylovora* in planta. *Environ Microbiol* 5:1016–1021
- Gilmour G (2001) Canola growers losing Benlate fungicide. *Manitoba Cooperator* 58:1
- Green-McDowell D, Ingber MB, Wright MS, Zeringue HJ, Bhatnagar D, Cleveland TE (1999) The effect of selected cotton-leaf volatiles on the growth, development and aflatoxin production of *Aspergillus parasiticus*. *Toxicon* 37:883–893
- Guo XW, Fernando WGD, Entz MH (2005) Effects of crop rotation and tillage on the blackleg disease of canola. *Can J Plant Pathol* 27:53–57
- Hammerschmidt R, Kuc J (1995) Induced resistance to disease in plants. Kluwer Academic, Dordrecht
- Hammond KE, Lewis BG (1987) The establishment of systemic infection in leaves of oilseed rape by *Leptosphaeria maculans*. *Plant Pathol* 36:135–147
- Hammond KE, Lewis BG, Musa TM (1985) A systemic pathway in the infection of oilseed rape plants by *Leptosphaeria maculans*. *Plant Pathol* 34:557–565
- Hattingh MJ, Beer SV, Lawson EW (1986) Scanning electron microscopy of apple blossoms colonized by *Erwinia amylovora* and *Erwinia herbicola*. *Phytopathology* 76:900–904
- Heil M (2001) The ecological concept of induced systemic resistance (ISR). *Eur J Plant Pathol* 107:137–146
- Ishimaru CA, Klos EJ, Brubaker RR (1988) Multiple antibiotic production by *Erwinia herbicola*. *Phytopathology* 78:746–750
- Jacobsen BJ, Backman PA (1993) Biological and cultural disease controls: alternatives and supplements to chemicals in IPM systems. *Plant Disease* 77:311–315
- Janisiewicz WJ, Korsten L (2002) Biological control of post-harvest diseases of fruits. *Annu Rev Phytopathol* 40:411–441
- Kavitha K, Mathiyazhagan S, Sendhilvel A, Nakkeeran S, Chandrasekar G, Fernando WGD (2005) Broad spectrum action of phenazine against active and dormant structures of fungal pathogens and root knot nematode. *Arch Phytopathol Plant Protect* 38:69–76
- Kazempour MN (1998) Caractère épiphyte et endophyte de la colonisation de la tomate par *Pseudomonas syringae* et étude du rôle de composants au pouvoir pathogène dans la survie et la multiplication des bactéries in

- planta. Thèse Institut National d'Agronomie de Paris, Paris
- Kearns LP, Hale CN (1996) Partial characterization of an inhibitory strain of *Erwinia herbicola* with potential as a biocontrol agent of *Erwinia amylovora*, the fire blight pathogen. *J Agric Res* 62:745–753
- Kloepper JW, Leong J, Teintze M, Schroth MN (1980) *Pseudomonas* siderophores: a mechanism explaining disease suppressive soils. *Curr Microbiol* 4:317–320
- Kobaisy M, Tellez MR, Webber CL, Dayan FE, Schrader KK, Wedge DE (2001) Phytotoxic and fungitoxic activity of the essential oil of kenaf (*Hibiscus cannabinus* L.) leaves and its compositions. *J Agric Food Chem* 49:3768–3771
- Kokallis-Burelle N, Backman PA, Rodriguez-Kabana R, Ploper LD (1992) Biological control of early leafspot of peanut using *Bacillus cereus* and chitin as foliar amendments. *Boil Control* 2:321
- Ligon JM, Hill DS, Hammer PE, Torkewitz NR, Hofmann D, Kempf HJ, Van Pee KH (2000) Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. *Pest Manage Sci* 56:688–695
- Lindow SE (1991) Determinants of epiphytic fitness in bacteria. In: Hirano SS, Andrew J (eds) *Microbiology of the phyllosphere*. Springer, Berlin Heidelberg New York, pp 295–314
- Lugtenberg BJJ, Dekkers LC, Bansraj M, Bloemberg GV, Camacho M, Chin-A-Woeng TFC, van den Hondel C, Kravchenko L, Kuiper I, Lagopodi AL, Mulders I, Phoelich C, Ram A, Tikhonovich I, Tuinman S, Wijffelman C, Wijfjes A (1999) *Pseudomonas* genes and traits involved in tomato root colonization. In: De Wit PJGM, Bisseling T, Stiekema WJ (eds) 1999 IC-MPMI Congr Proc Biology of Plant-Microbe Interactions, vol 2. International Society for Molecular Plant-Microbe Interactions, St. Paul, MN, pp 324–330
- Manceau CR, Kasempour MN (2002) Endophytic versus epiphytic colonization of plants: what comes first? In: Lindow SE, Hecht-Poinar EI, Elliott VJ (eds) *Phyllosphere microbiology*. American Phytopathological Society, APS Press, St. Paul, MN, pp 115–123
- Manitoba Agriculture (2002) Pest management – Plant disease. *Sclerotinia* (<http://www.gov.mb.ca/agriculture/crops/diseases/facts07s00.html>, accessed 25 June 2004)
- Martens J, Seaman W, Atkinson G (1994) (eds) Diseases of field crops in Canada. Canadian Phytopathological Society, ON
- Mauch F, Mauch-Mani B, Boller T (1988) Antifungal hydro-lases in pea tissue. II. Inhibition of fungal growth by combinations of chitinases and two  $\beta$ -1,3 glucanases. *Plant Physiol* 87:936–942
- Mayerhofer R, Bansal VK, Thiagarajah MR, Stringam GR, Good AG (1997) Molecular mapping of resistance to *Leptosphaeria maculans* in Australian cultivars of *Brassica napus*. *Genome* 40:294–301
- McGrath MT (2001) Fungicide resistance in curcurbit powdery mildew: experiences and challenges. *Plant Disease* 85:236–245
- Moyné AL, Shelby R, Cleveland TE, Tuzun S (2001) Bacilomycin D: an iturin with antifungal activity against *Aspergillus flavus*. *J Appl Microbiol* 90:622–629
- Nelson B (1998) Biology of *Sclerotinia*. In: Proc 10th Int Sclerotinia Worksh, 21 January 1998, Fargo, ND. North Dakota State University Department of Plant Pathology, Fargo, ND, pp 1–5
- Okada A, Banno S, Ichiiishi A, Kimura M, Yamaguchi I, Fujimura M (2005) Pyrrolnitrin interferes with osmotic signal transduction in *Neurospora crassa*. *J Pesticide Sci* 30:378–383
- Ongena M, Jacques P, Touré Y, Destain J, Jabrane A, Thonart P (2005) Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of *Bacillus subtilis*. *Appl Microbiol Biotechnol* 69:29–38
- Parke JL (1990) Population dynamics of *Pseudomonas cepacia* in the pea spermosphere in relation to biocontrol of *Pythium*. *Phytopathology* 80:1307–1311
- Petrie GA, Mortensen K, Dueck J (1985) Blackleg and other diseases of rapeseed in Saskatchewan, 1978 to 1981. *Can Plant Disease Surv* 65:35–41
- Pieterse CMJ, Ton J, Parchmann S, Mueller MJ, Buchala AJ, Metraux JP, Van Loon LC (2000) Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. *Physiol Mol Plant Pathol* 57:123–134
- Pleban S, Chernin L, Chet I (1997) Chitinolytic enzymes of an endophytic strain of *Bacillus cereus*. *Lett Appl Microbiol* 25:284–288
- Poritsanos NJ (2005) Molecular mechanisms involved in the secondary metabolite production and biocontrol of *Pseudomonas chlororaphis* PA23. Masters Thesis, University of Manitoba
- Pozo MJ, van Loon LC, Pieterse CMJ (2005) Jasmonates – signals in plant-microbe interactions. *J Plant Growth Regul* 23:211–222
- Raaijmakers JM, Vlami M, de Souza JT (2002) Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek* 81:537–547
- Ramarathnam R (2006) Phyllosphere bacterial biological control of *Leptosphaeria maculans*, the blackleg pathogen of canola (*Brassica napus* L.): screening for potential antibiotic producers, investigation of the mechanism of control, biochemical detection of the antifungal compounds, and establishment of the role of antibiosis. PhD Thesis, University of Manitoba
- Ramarathnam R, Fernando WGD (2006) Preliminary phenotypic and molecular screening for potential bacterial biocontrol agents of *Leptosphaeria maculans*, the blackleg pathogen of canola. *Biocontrol Sci Technol* 16:567–582
- Ran LX, Li ZN, Wu GJ, van Loon LC, Bakker PAHM (2005) Induction of systemic resistance against bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp. *Eur J Plant Pathol* 113:59–70
- Raupach GS, Kloepper JW (1998) Mixtures of plant growth promoting rhizobacteria enhance biocontrol of multiple cucumber pathogens. *Phytopathology* 8:1158–1164
- Rose RI (1995) Ecological effects. In: Proc USDA IR-4/EPA Minor Use Biopesticide Worksh, 7–8 November 1995, Washington, DC, pp 50–54
- Ryu CM, Hu CH, Reddy MS, Kloepper JW (2003) Different signaling pathways of induced resistance by rhizobacteria in *Arabidopsis thaliana* against two pathovars of *Pseudomonas syringae*. *New Phytol* 160:413–420

- Savchuk S, Fernando WGD (2004) Effect of timing of application and population dynamics on the degree of biological control of *Sclerotinia sclerotiorum* by bacterial antagonists. *FEMS Microbiol Ecol* 49:379–388
- Setlow P (1995) Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. *Annu Rev Microbiol* 49:29–54
- Shoda M (2000) Bacterial control of plant diseases. *J Biosci Bioeng* 89:515–521
- Silo-Suh LA, Lethbridge BJ, Raffel SJ, He H, Clardy J, Handelsman J (1994) Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. *Appl Environ Microbiol* 60:2023–2030
- Simon LE (1997) Morphologie, ontogénie et valeur adaptative des surfaces glandulaires mucilagènes dans le genre *Chenopodium* L. (Chenopodiaceae). *C R Acad Sci Paris Sér III* 320:615–20
- Sing PP, Shin YC, Park CS, Chung YR (1999) Biological control of *Fusarium* wilts of cucumber by chitinolytic bacteria. *Phytopathology* 89:92–99
- Sovero M (1993) Rapeseed, a new oilseed crop for the United States. In: Janick J, Simon JE (eds) New crops. Wiley, New York (<http://www.hort.psu.edu/newcrop/proceedings1993/v2-302.html>, accessed 23 March 2001)
- Stevens C, Khan VA, Ploper LD, Backman PA, Rodriguez-Kabana R, Collins DJ, Brown JE, Wilson MA, Igwegbe ECK (1996) Reduction of tomato early blight by combining soil solarization and biological control strategies. In: Proc 26th National Agricultural Plastics Congr, American Society of Plasticulture, State College, PA, vol 24, pp 88
- Sundin GW, Jacobs JL (1999) Ultraviolet radiation (UVR) sensitivity analysis and UVR survival strategies of a bacterial community from the phyllosphere of field grown peanut (*Arachis hypogaea* L.). *Microbial Ecol* 38:27–38
- Suslow TV (2002) Production practices affecting the potential for persistent contamination of plants by microbial foodborne pathogens. In: Lindow SE, Hecht-Poinar EI, Elliott VJ (eds) Phyllosphere microbiology. American Phytopathological Society, APS Press, St. Paul, MN, pp 241–256
- Thomashow LS, Bonsall RF, Weller DM (1997) Antibiotic production by soil and rhizosphere microbes in situ. In: Hurst CJ, Knudsen GR, McInerney MJ, Stetzenbach LD, Walter MD (eds) Manual of environmental microbiology. ASM Press, Washington, DC, pp 493–499
- Tjamos EC, Tsitsigiannis DI, Tjamos SE, Antoniou PP, Katinakis P (2004) Selection and screening of endorhizosphere bacteria from solarized soils as biocontrol agents against *Verticillium dahliae* of solanaceous hosts. *Eur J Plant Pathol* 110:35–44
- Tukey HB (1971) Leaching of substances from plants. In: Preece TF, Dickinson CH (eds) Ecology of leaf surface microorganisms. Academic Press, London, pp 640
- Turkington TK, Morrall RAA (1993) Use of petal infestation to forecast sclerotinia stem rot of canola: the influence of inoculum variation over the flowering period and canopy density. *Phytopathology* 83:682–689
- Van Loon LC, Bakker PAHM, Pieterse CMJ (1998) Systemic resistance induced by rhizosphere bacteria. *Annu Rev Phytopathol* 36:453–483
- Vanneste JL, Yu J, Beer SV (1992) Role of antibiotic production by *Erwinia herbicola* Eh252 and *Pseudomonas fluorescens* A506 separately or in combination. *Acta Hortic* 411:351–353
- Venette J (1998) Sclerotinia spore formation, transport and infection. In: Proc Sclerotina Worksh, 21 January 1998, Fargo, ND
- Vining LC (1990) Functions of secondary metabolites. *Annu Rev Microbiol* 44:395–427
- Weller DM (1988) Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu Rev Phytopathol* 26:379–407
- Weller DM, Howie WJ, Cook RJ (1988) Relationship between in-vitro inhibition of *Gaeumannomyces graminis* var *tritici* and suppression of take-all of wheat by fluorescent pseudomonads. *Phytopathology* 78:1094–1100
- West JS, Kharbanda PD, Barbetti MJ, Fitt BDL (2001) Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathol* 50:10–27
- Whipps JM (1997) Development in the biological control of soil-borne plant pathogens. *Adv Bot Res* 26:1–134
- Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. *J Exp Bot* 52:487–511
- Whitesides SK, Spots RA (1991) Frequency, distribution and characterization of endophytic *Pseudomonas syringae* in pear trees. *Phytopathology* 81:453–457
- Willets HJ, Wong JAL (1980) The biology of *Sclerotinia sclerotiorum*, *S. trifolium*, and *S. minor* with emphasis on specific nomenclature. *Bot Rev* 46:101–165
- Wilson M, Lindow SE (1993) Interactions between the biological control agent *Pseudomonas fluorescens* strain A506 and *Erwinia amylovora* in pear blossoms. *Phytopathology* 83:117–123
- Wilson M, Lindow SE (1994a) Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. *Appl Environ Microbiol* 60:4468–4477
- Wilson M, Lindow SE (1994b) Ecological differentiation and coexistence between epiphytic *Ice<sup>+</sup>* *Pseudomonas syringae* strains and an *Ice<sup>-</sup>* biological control agent. *Appl Environ Microbiol* 60:3128–3137
- Wilson M, Epton HAS, Sigee DC (1992) Interactions between *Erwinia herbicola* and *Erwinia amylovora* on the stigma of hawthorn blossoms. *Phytopathology* 82:914–918
- Wilson M, Hirano SS, Lindow SE (1999) Location and survival of leaf-associated bacteria in relation to pathogenicity and potential for growth within the leaf. *Appl Environ Microbiol* 65:1435–1443
- Wright SAI, Beer SV (1996) The role of antibiotics in biological control of fire blight by *Erwinia herbicola* strain Eh318. *Acta Hortic* 411:309–311
- Yan Z, Reddy MS, Ryu CM, McInroy JA, Wilson M, Kloepfer JW (2002) Induced systemic protection against tomato late blight elicited by plant growth-promoting rhizobacteria. *Phytopathology* 92:1329–1333
- Yoshida S, Shirata A, Hiradate S (2002) Ecological characteristics and biological control of mulberry anthracnose. *Japan Agric Res Q* 36:89–95
- Zhang Y, Fernando WGD, de Kievit T, Berry C, Daayf F, Paulitz T (2006) Detection of antibiotic-related genes from bacterial biocontrol agents with polymerase chain reaction. *Can J Microbiol* 52:476–481

---

## 12 Effects of Animals Grazing on Fungi

T.P. McGONIGLE<sup>1</sup>

### CONTENTS

I. Introduction .....	201
II. The Fungivores in Soil and Litter Systems .....	202
A. General Considerations .....	202
B. A Survey of Relevant Groups .....	202
1. Acari .....	202
2. Collembola .....	203
3. Annelida .....	203
4. Nematoda .....	203
5. Others .....	203
III. Some Limitations to the Study of Fungivore Grazing .....	204
A. Gut Contents .....	204
B. Food Preference Tests .....	204
C. Enumeration of Hyphae .....	204
IV. Grazing and Community Structure .....	204
A. Changes in Species Richness and Community Diversity .....	204
1. Grazing on a Dominant Fungus .....	204
2. Grazing on a Fungus Which is not Dominant .....	205
B. Replacement of some Species by Others .....	206
C. Modifying Factors .....	206
1. Selectivity of Grazing .....	206
2. Intensity of Grazing .....	207
3. Responses of the Grazed Fungi .....	207
V. Conclusions from Laboratory Studies and Extrapolation to the Field .....	209
References .....	209

### I. Introduction

This review considers the impact which fungivore grazing can have on communities of fungi in soil and litter. The fungi are represented by large numbers of species (Christensen 1989) and considerable biomass (Kjøller and Struwe 1982) in soil and litter systems. Up to 75% of the soil fauna biomass resides in the fungivore trophic category (McGonigle 1995), and so grazing by soil fauna on fungal tissues is expected to play a significant role in shaping the fungal communities of field systems.

In order to assess the impact of grazing of fungi on community structure, it is useful initially to briefly consider fungi in relation to general principles of community ecology. The individuals within a species and at a given location comprise a single population. Discussion of the concept of the individual with respect to fungi has been made elsewhere (Rayner and Todd 1982). The indeterminate growth of many plants has led to the quantification of modules of plant growth, rather than of individuals (Harper 1977). Fungi can be treated similarly where recognizable structures such as sporophores are visible (Shaw 1985). However, fruit bodies may represent only less than 1% of the unseen but living vegetative fungal biomass (Frankland 1982). Communities are collections of populations which are present inside defined limits in space, and within specified taxonomic or functional groups (Begon et al. 2006). Communities can be described most simply in terms of species richness, which is the number of species present. Diversity refers to the relative abundance of each of the species being considered (Begon et al. 2006). For a given species richness, the diversity of the community will be determined by the relative abundances of the species present; high abundance of a small number of species equates to low diversity whereas greater evenness, or equitability of the species abundances, reflects higher diversity. Fungivore grazing can be expected to affect species richness by promoting the elimination or the introduction of fungal species. In addition, the grazing of fungi can be expected to affect relative abundance and community diversity.

Characterization of fungal communities has in some cases evaluated species richness, although this is in itself a mammoth undertaking (Swift 1976; Christensen 1981). Even prolonged isolation studies appear to be unable to complete the species list for a given sampling location; Christensen (1989) found that after more than one thousand isolates had already been taken from one location, each

<sup>1</sup> Department of Botany, Brandon University, 270-18th Street, Brandon, Manitoba R7A 6A9, Canada

increment of 100 additional isolates consistently yielded approximately 10 species new to the site. When isolation frequencies are used as a measure of relative fungal abundance, fungal communities follow a lognormal distribution (Lussenhop 1981); this is consistent with communities for other types of organisms in other kingdoms (Begon et al. 2006). In theory, the lognormal distribution reflects the way in which species abundances are the result of the interplay of a variety of independent factors (May 1975).

Swift (1976, 1982) considered the question of niche diversification among sympatric fungal species. Although many fungi show a surprisingly broad range of occurrence, there is some specialization for different resource types; these include various plant parts like leaves and twigs, and the different plant species they originate from. There is also the opportunity for specialization among the different microenvironments within resource types, such as between the vein and mesophyll of a leaf, and among the various carbon substrates therein.

Reviews of fungivore grazing have considered the processes of comminution of substrate, dispersal of fungi, and the direct impact of feeding itself on fungal community structure (Visser 1985) and nutrient cycling (Ingham 1992; McGonigle 1995). Comminution of substrate stimulates microbial activity and accelerates decomposition (Swift et al. 1979). The dispersal of fungi through the movements of grazing animals was clearly established in earlier studies (Brasier 1978; Wiggins and Curl 1979). Enhanced dispersal will encourage a community to fulfil the potential it intrinsically has for a poor or rich species compliment, playing an important role in the establishment of microbial communities. However, in this review it is the biological interactions which occur after dispersal, and which are involved in the determination of the fungal community structure, which are considered. The effect of the disruptive physical action of grazing animals on fungi, such as by the process of trampling, is not easy to distinguish from the effects resulting from ingestion of the fungal material. The breaking-up of fungal material by animal body movements has been suggested (Wicklow and Yocom 1982) to be one of the reasons why fungal communities change in response to grazing.

This review will initially outline the relevant features of the various types of invertebrate fungivores in soil and litter, and proceed to consider some limitations encountered in the study of graz-

ing on fungi. Examples will then be given to show the ways in which grazing can affect fungal communities through effects on species richness, community diversity, and replacement of some species by others. In the final section before the conclusions, various features of the fungivores and fungi which can modify the outcome of grazing will be discussed. Specifically, these modifying features are the selectivity and intensity of grazing, and the ability of the fungus being grazed to respond to that grazing.

## II. The Fungivores in Soil and Litter Systems

### A. General Considerations

The soil fauna is composed of a great diversity of species distributed among many phyla. Of particular importance with regard to fungivory are three phyla: the Annelida, the Arthropoda and the Nematoda. The Enchytraeidae within the Annelida display extensive fungivory, and gut passage in Lumbricidae also affects fungi. The arthropod classes Arachnida and Insecta contain the groups Acari and Collembola respectively, each of which has many fungivorous members. Among the nematodes, the Dorylaimida and Tylenchida contain an abundance of fungivores.

Using the terminology of Moore et al. (1988), animals feeding on hyphae can be divided into two categories: engulfing fungivores and fluid-feeding fungivores. These feeding modes are mostly consistent for fungivores within defined taxonomic boundaries.

### B. A Survey of Relevant Groups

#### 1. Acari

Mites are very abundant in soil and litter, and many of them consume fungi. Chitin-rich cell walls of engulfed hyphae pass through mite gut without being digested whereas the fungal disaccharide trehalose is utilized (Hubert et al. 2001). Soil mites comprise four major groups. Many members of the Cryptostigmata, or oribatid mites, are generalist feeders (Swift et al. 1979) and will ingest decaying plant material, fungal hyphae, and algae. However, there is some specialization within the group: members of the family Phthiracaridae feed only on plant residues and are able to digest cellulose

but not the fungal storage carbohydrate trehalose; by contrast, the Oppiidae and Eremaeidae specialize on fungi and cannot digest cellulose (Luxton 1972). Some Cryptostigmata readily feed on nematodes (Rickett 1980). The well-known accumulation of  $^{15}\text{N}$  in consumers relative to the materials they ingest has been applied to the question of niche diversification within the Cryptostigmata. Schneider et al (2004) determined that  $^{15}\text{N}/^{14}\text{N}$  ratios among species of oribatids below beech and oak indicated trophic-level separation within the oribatid community. Members of the Gamasina in the Mesostigmata are predatory, with different genera showing varying degrees of specialization in their choice of prey (Moore et al. 1988). The Astigmata show a diverse range of feeding activity: members of the family Acaridae feed by engulfing hyphae, as well as taking live nematodes and protozoa, whereas some members of the family Histiostomatidae specialize in the ingestion of a slurry of decaying residues mixed with microbes (Walter and Kaplan 1990). The Prostigmata have fluid-feeding fungivores in several families, e.g. Tydidae and Tarsonemidae; however, Prostigmata also contain families of predatory mites, such as the Bdellidae and Stigmaeidae (Moore et al. 1988). In forest systems, the Cryptostigmata are most abundant (Hogervost et al. 1993) whereas, in desert systems, the fungivorous Prostigmata are the more important group (Santos and Whitford 1981).

## 2. Collembola

The majority of Collembola are primarily fungivorous, and can be divided (Wallwork 1976) into two distinctive life forms as follows: those at the soil surface or in litter layers are usually large, pigmented individuals with well-developed eyes. The Collembola of deeper layers are more often small, weakly pigmented and with reduced eyes. Based on analysis of gut contents, the two life form groups show little difference in the extent to which they engage in fungal feeding (Takeda and Ichimura 1983). Collembola collected from the field often have no gut contents, which may be due to intermittent cessation of feeding caused by the moulting cycle of the animals (Christensen 1990).

## 3. Annelida

Gut contents of enchytraeids contain a mixture of fungal hyphae, plant residues and soil materials, with fungal material typically representing one

third of those gut contents (Dash et al. 1980). When offered fungal baits, the gut content of enchytraeids with fungal material can increase to between 50–70% of the total (Dash and Cragg 1972). The gut contents of some enchytraeid species can contain twice as much fungal material as would be expected on the basis of random ingestion of substrate (O'Connor 1967). Estimates of fungivory in enchytraeids range from 25% (Persson et al. 1980) to as much as 80% (Whitfield 1977). Feeding biology of the various enchytraeids needs further work before the role of this group in the functioning of soil systems is adequately appraised (Didden 1993). The burrowing earthworm *Lumbricus terrestris* L. feeds on surface litter, although fungi on the litter are a major component of their diet (Tiunov and Scheu 2000).

## 4. Nematoda

Based on their anterior morphology, free-living soil nematodes can be identified as fungivores, bacterivores, predators or omnivores (Twinn 1974). The diet of omnivorous nematodes can consist of algae, fungal spores, protozoa and other nematodes (Swift et al. 1979). In addition, there are also some free-living soil nematodes which feed on root epidermal cells and root hairs (Yeates et al. 1993). Fungivorous nematodes pierce hyphae with their stylets, and feed on the fluid protoplasm of the fungus using a pumping action (Freckman and Baldwin 1990). Empty hyphal walls remain behind. Under culture conditions, the impact of grazing by fungivorous nematodes can be severe, killing all aerial hyphae and reducing growth on agar relative to that seen in the absence of the nematodes (Shafer et al. 1981).

## 5. Others

Various other groups in the soil fauna also eat fungi. Among the larvae of dipterous flies, those in the family Sciaridae graze fungi in dung deposited on the soil surface (Wicklow and Yocom 1982), members of the Phoridae are mycelial feeders (Tibbles et al. 2005), and larvae of the families Chironomidae and Mycetophilidae are mainly fungivorous (Swift et al. 1979). Interestingly, mycetophilids are attracted to the zones of antagonism at the contact between fungal individuals (Boddy 1983). The isopod *Oniscus asellus* L. was able to reduce fungal standing crop to one third of that in control leaf-fungus microcosms (Hanlon and Anderson

1980). A selection of genera of soil amoebae are able to feed on hyphae (Bamforth 1988), but the impact of this on populations of soil fungi is unclear (Chakraborty et al. 1983).

### III. Some Limitations to the Study of Fungivore Grazing

Several approaches are used to investigate grazing, including direct observation, examination of gut contents, and monitoring through time the hyphae present. Food preference tests can be used to ascertain the palatability of different fungi to the faunal taxa of interest.

#### A. Gut Contents

Examination of gut contents can provide valuable information on dietary choices of fauna among the available foods. However, some food types more easily retain their structural integrity and are recognizable in the gut for a longer time. Walter (1987) found that nematodes observed to be consumed by a selection of mites, which are normally considered mycophagous, were not detectable in gut boluses because of the lack of sclerotization of the nematode body.

#### B. Food Preference Tests

Collembola have been seen during feeding evaluation tests to switch from feeding predominately on one palatable fungus to feeding days later almost exclusively on a simultaneously offered but different palatable fungus (Visser and Whittaker 1977). Mixed diet seems to be important for Collembola (Scheu and Simmerling 2004) and fungivorous nematodes (Ruess et al. 2000) to avoid over-consumption of toxic metabolites.

#### C. Enumeration of Hyphae

Measurement of fungal biomass through time presents difficulties, because estimates of the proportion of hyphal length which is active vary according to the staining method used (Schubert and Mazzitelli 1989; Hamel et al. 1990).

## IV. Grazing and Community Structure

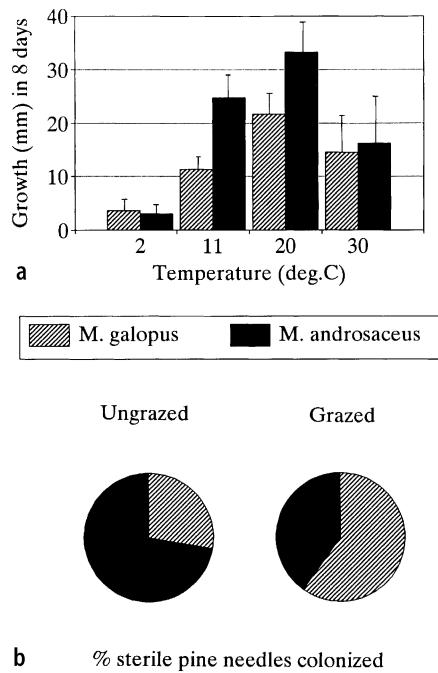
In various sections below, studies on grazing of plants are referred to in order to provide a theoretical framework in which we can consider grazing on fungi. Comparisons have been drawn previously between grazing in fungivore-fungus and herbivore-plant systems (Wicklow 1981; Visser 1985; Shaw 1992).

#### A. Changes in Species Richness and Community Diversity

##### 1. Grazing on a Dominant Fungus

Darwin (1859) reported that when a previously mown 90-cm by 120-cm turf plot was left to grow, the number of plant species within it was reduced from 20 to 11 because of the proliferation of some species. Thus, mowing can maintain increased species richness. Crawley (1983) argues that both mowing and grazing of herbaceous systems function in an essentially similar way, by the selective removal of more of some species than of others. Mammal grazing exclosures on grassland did not change species richness but increased proliferation of a few herb species, showing that greater diversity and more equitability among species had previously been maintained by grazing (Tansley and Adamson 1925). However, under very intense grazing which fell just short of uncovering bare soil, plant species richness itself was reduced (Tansley and Adamson 1925). These studies (Darwin 1859; Tansley and Adamson 1925) established that grazing can increase species richness or increase community diversity by the suppression of species which would otherwise be more dominating. Alternatively, when the intensity of grazing is sufficiently high, then species richness can be reduced.

When one fungus is more productive than another, selective grazing on the more abundant fungus can suppress what would otherwise have been the dominant member of the community. The outcome of grazing in this situation will be a community of greater diversity. An example from Newell (1984a, b) is the suppression of the otherwise dominant *Marasmius androsaceus* (L. ex Fr.) Fr. under grazing pressure from the collembolan *Onychiurus latus* Gisin, leading to more extensive development of the sympatric species *Mycena galopus* (Pers ex Fr.) Kummer (Fig. 12.1). In the study of Newell (1984a, b), two species of fungi were con-



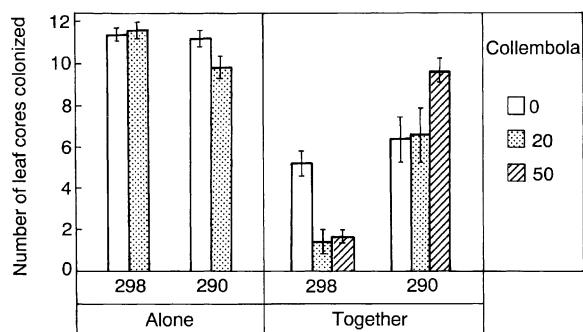
**Fig. 12.1.** **a** Growth of *Mycena galopus* and *Marasmius androsaceus* in culture as a function of temperature. *M. galopus* is the slower-growing species (data of Newell 1984a). **b** Effect of collembolan grazing on the percentage of pine needles colonized by *M. galopus* and *M. androsaceus* in microcosms. Grazing is seen to suppress *M. androsaceus* and allow greater proliferation of *M. galopus* (data of Newell 1984b)

sidered. However, the principles involved can be applied in theory to larger communities. Where increased representation of several otherwise suppressed species occurs at the expense of one dominant, the result will be greater equitability among species. An analogous effect may occur with the redistribution of nutrient resources among members of herbaceous communities through arbuscular mycorrhizal plant-to-plant connections (Grime et al. 1987).

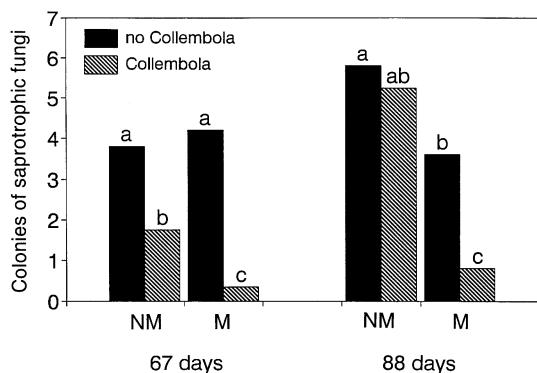
## 2. Grazing on a Fungus Which is not Dominant

Selective grazing on a fungus which is of equal aggressiveness compared to a neighbour can act to polarize the community, making the ungrazed fungus more abundant relative to the grazed neighbour. Fungivore selection of a fungus already showing relatively low production within the community will act to reinforce or further exaggerate the dominance of the ungrazed fungus. In both cases, the trend will be towards a community of reduced diversity. One example of selective grazing

on seemingly co-dominant fungi can be seen in Parkinson et al. (1979), where the collembolan *Onychiurus subtenuis* Folsom was introduced into aspen leaf microcosms which had been inoculated with two fungi isolated from an aspen litter system. When the two fungal isolates were kept in separate microcosms, grazing by Collembola had little impact (Fig. 12.2). However, when the fungi were grown together, grazing by the Collembola acted to polarize the system by the suppression of isolate sterile dark 298 and promotion of isolate basidiomycete 290 (Fig. 12.2). It was noted (Parkinson et al. 1979) that basidiomycete 290 was completely unpalatable to *O. subtenuis* in feeding choice tests. Ek et al. (1994) demonstrated that grazing by *O. armatus* Tullb. was able to suppress the development of saprotrophic fungi in the genus *Paecilomyces* much more strongly when *Pinus contorta* Dougl. ex Loud. seedlings in the microcosms were mycorrhizal with *Paxillus involutus* (Batsch) Fr., compared to when they were non-mycorrhizal (Fig. 12.3). This example underlines the importance of the interplay between any direct impact of grazing, and the interactions between the various fungi present. Deletion competition for resources in the grazed system shifted in favour of *Paxillus* and away from *Paecilomyces*, leading to almost complete suppression of the latter. However, in the non-mycorrhizal situation, grazing was only able to cause a delay in the development of *Paecilomyces* (Fig. 12.3).



**Fig. 12.2.** Effect of collembolan grazing on the numbers of leaf cores colonized by the isolates sterile dark 298 and basidiomycete 290. In one series, the fungi were inoculated onto leaf cores so as to keep the fungi in separate microcosms. In the other series, the leaf cores were inoculated with the fungi mixed in the same microcosms. Collembola were added at 0, 20 and 50 per microcosm. Grazing after 10 days is seen to promote the competitive success of basidiomycete 290 over sterile dark 298 (data of Parkinson et al. 1979)



**Fig. 12.3.** The number of sporulating colonies of saprotrophic fungi seen per microcosm after 67 and 88 days of development of *Pinus contorta*. Seedlings were either non-mycorrhizal (NM) or mycorrhizal (M) with *Paxillus involutus*, and they were with or without 50 Collembola added per microcosm. The majority of the saprotrophic fungi belonged to the genus *Paecilomyces*. Bars with different letters (a–c) are significantly ( $P < 0.05$ ) different (data of Ek et al. 1994)

### B. Replacement of some Species by Others

Selective grazing can speed the transition between successional stages. Lubchenco (1983) showed that the intertidal mollusc *Littorina littorea* L. preferentially grazed on early-successional-stage seaweed species, thereby making the change to later-stage seaweed species occur more swiftly. Klironomos et al. (1992) showed the action of grazing to promote the transition from early-stage

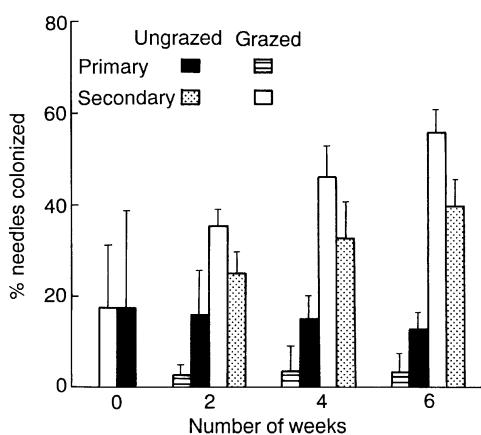
to later-stage successional species of fungi. Sterile *Picea abies* (L.) Karst. needles were inoculated with one of three primary saprotrophs from the genera *Cladosporium*, *Epicoccum* and *Phoma* (Klironomos et al. 1992), which typically invade senescent needles in the canopy. On falling to the floor, these primary saprotrophs are replaced by secondary saprotrophs from the community of soil fungi. Klironomos et al. (1992) added tagged needles colonized exclusively by primary saprotrophs to similar cultures of needles colonized only by secondary saprotrophs from the genera *Penicillium* and *Trichoderma*. Treatments with or without a population of the collembolan *Folsomia candida* Willem. were set up, and needles added were sampled over 6 weeks. Grazing by the Collembola on the primary saprotrophs, which were known from feeding choice tests to be preferred over the secondary saprotrophs, almost eliminated the primary saprotrophs and facilitated their replacement by the secondary saprotrophs (Fig. 12.4).

### C. Modifying Factors

Irrespective of the relative dominance of any grazed fungus, and of the position of the fungal community along a successional sequence, the impact of grazing on community structure will be influenced for the most part by three modifying influences: how selective the grazing is, how intense the grazing is, and the ability of the fungi to respond to grazing.

#### 1. Selectivity of Grazing

If given a choice, Collembola prefer to feed on saprotrophic fungi, rather than arbuscular mycorrhizal fungi (Klironomos and Kendrick 1996; Klironomos et al. 1999; Gange 2000) and rather than ectomycorrhizal fungi (Hiol Hiol et al. 1994). However, where grazing does occur on mycorrhizal fungi, mutualistic function can be impaired (McGonigle and Fitter 1988; Hiol Hiol et al. 1994; Schreiner and Bethlenfalvay 2003). Collembola also show preferences among pathogenic fungi (Curl et al. 1988) and ectomycorrhizal fungi (Shaw 1988) fungi. Collembola are attracted by the quality of fungal odours (Bengtsson et al. 1988; Hedlund et al. 1995). In contrast to Collembola, fungivorous nematodes seem to prefer mycorrhizal hyphae, rather than saprotrophic hyphae, if both types



**Fig. 12.4.** Effect of collembolan grazing in microcosms on the percentage of *Picea abies* needles colonized by primary saprotrophic fungi and secondary saprotrophic fungi through time. Grazing is seen to encourage the replacement of primary saprotrophic by the less palatable secondary saprotrophic (data of Klironomos et al. 1992)

are available (Giannakis and Sanders 1989; Ruess et al. 2000). Selectivity in feeding has also been shown for oribatid mites, several species of which prefer ectomycorrhizal and ericoid mycorrhizal hyphae to those of saprotrophs (Schneider et al. 2005). Earthworm impact on fungi seems also to be selective in terms both of the fungal species taken (Moody et al. 1995) and the survival of fungi during gut passage (Moody et al. 1996; Tiunov and Scheu 2000).

Microarthropods show a strong preference among available saprotrophic fungi, grazing particularly the dark pigmented forms (Kaneko et al. 1995; Varga et al. 2002). This preference for dark pigmented forms is pronounced for Collembola (Maraun et al. 2003), Oribatida (Schneider and Maraun 2005) and Astigmata (Hubert et al. 2004). Further, some preference is shown among species of dark pigmented fungi (Schneider and Maraun 2005). Selection of dark pigmented forms makes sense, given that these fungi form 30–60% of isolates from soil and are a high-quality food (Maraun et al. 2003).

Crawley (1983) explains that removal of species in proportion to their abundance will not be able to affect the structure of a community; for species richness or community diversity to be influenced requires selective removal of greater quantities of some species. It seems that grazing of fungi is almost always found to involve selective feeding, and it can therefore be expected to affect fungal community structure.

## 2. Intensity of Grazing

Generally, intense levels of grazing lead to elimination of some species. Wicklow and Yocom (1982) imposed a range of grazing intensities by varying the number of *Lycoriella mali* Fitch. dipteran fly larvae added to 2-g dry-mass-equivalent samples of rabbit dung. In the absence of grazing, 14 fungal species were recovered from each sample. However, the number fell to between six and nine species per sample when the grazing intensity was above 10 larvae  $\text{g}^{-1}$  dung (Fig. 12.5). This reduction in species richness was due to the loss of what appeared to be grazing-sensitive species. Over and above the impact of grazing seen in Fig. 12.5, some fungal species had a reduced frequency of occurrence in the more highly grazed microcosms, although other species were unaffected (Wicklow and Yocom 1982). Reduction of species richness in response to

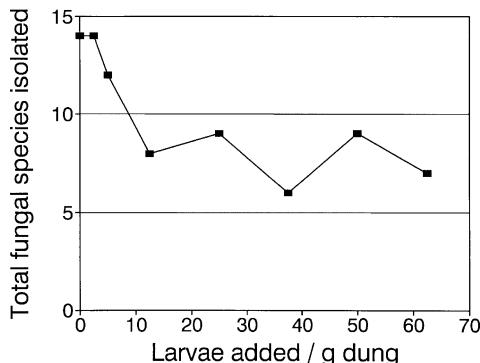


Fig. 12.5. Number of fungal species recovered from rabbit-dung microcosms in response to the number of fungivorous dipteran larvae added. Increasing grazing intensity reduces species richness, although no further consistent reduction is seen above 12.5 larvae  $\text{g}^{-1}$  dung (data of Wicklow and Yocom 1982)

high intensities of grazing have been seen in the fungi of aquatic systems (Bärlocher 1980).

## 3. Responses of the Grazed Fungi

Any negative impact of grazing on the vigour of fungi, through loss of biomass or damage to tissues, might be offset by compensatory growth produced in response to that grazing. Indeed, where senescent parts of the mycelium are removed and this, in turn, is able to permit re-growth from an otherwise functionally dormant hyphal system, grazing can have a positive, rather than a negative impact on the grazed fungus. Grazing of *Botrytis cinerea* Pers ex Fr. by *Folsomia candida* doubled the respiration of the fungus (Hanlon 1981), which was attributed to removal of senescent parts of the colony. Removal of old hyphae allowed new fungal growth where nutrient availability permitted (Hanlon 1981). A similar effect on respiration was shown for grazing of *Onychiurus quadriocellatus* Gisin on the fungi growing on Diplopod faecal pellets (van der Drift and Jansen 1977). One collembolan per pellet decreased fungal standing crop, yet increased fungal respiration by 10% over controls (van der Drift and Jansen 1977).

The impact of grazing on fungal biomass is nonlinear. Low grazing intensities can stimulate fungal production whereas higher levels reduce that production. Such responses for enchytraeid fungivores have been likened (Hedlund and Augustsson 1995) to the process of grazing optimization as described for mammalian herbivores (McNaughton 1979). A density of 0.1–0.4 Collem-

bola g<sup>-1</sup> soil appears to be a threshold, above which the quantity (Bakonyi et al. 2002) and function (McGonigle 1995) of arbuscular mycorrhizal fungi is restricted. The detrimental impact of higher Collembola densities on arbuscular mycorrhizae is severance of the hyphae (Klironomos and Ursic 1998). *Folsomia candida*, at a density of 0.25 animals g<sup>-1</sup> dry soil, increased hyphae of *Fusarium oxysporum* Schlect. to 4.8 mg<sup>-1</sup> dry soil (Moore 1988), from 3.2 mg<sup>-1</sup> dry soil in ungrazed controls. However, at a density of 1.0 g<sup>-1</sup> dry soil, these Collembola reduced the hyphae to 1.2 mg<sup>-1</sup> dry soil (Moore 1988).

Based on a modelling approach, Bengtsson et al. (1993) concluded that fragmentation of fungal thalli into patches, such as might be caused by grazing, would be expected to promote compensatory growth by the fungi. The effect of grazing on fungal respiration appears to consist of two temporal phases of fungal response. Grazing of *Onychiurus armatus* reduced respiration of *Mortierella isabellina* Oudem during the 2-day period in which the Collembola were in contact with the mycelium, but fungal respiration was stimulated relative to ungrazed controls in a subsequent 5-day grazing-free interval (Bengtsson and Rundgren 1983).

*Mortierella isabellina* adopts an alternate and faster-growing mode of more aerial hyphae in

response to grazing, possibly in an attempt by the fungus to escape the grazer (Hedlund et al. 1991). Morphological responses to grazing are also pronounced in wood-decomposing basidiomycete fungi (Fig. 12.6). Utilization of wood is limited mostly to white-rot basidiomycetes and xylariaceous ascomycetes, both able to degrade lignin, as well as brown-rot basidiomycetes able to utilize cell-wall polysaccharides in the presence of lignin; these constitute a relatively limited number of fungi providing a grazing resource of immense potential, given global wood production (Swift and Boddy 1984). Wood-decay fungi forming outgrowths from one wood-resource block to the next are particularly susceptible to grazing when the strength of the resource base is reduced in terms of time spent on the source block or in terms of a small size of the source block (Harold et al. 2005). Variability in grazing impact also arises because of different levels of grazing intensity and for different species of Collembola (Kampichler et al. 2004). However, a general pattern emerges of a response to grazing in the form of faster growth in some sections of the outgrowth mycelium, compared to arrested growth in other sections. This response is interpreted as a strategy adopted by the fungus to escape local grazing (Kampichler et al. 2004) and forage for additional wood resources to colonize (Tordoff et al. 2006).

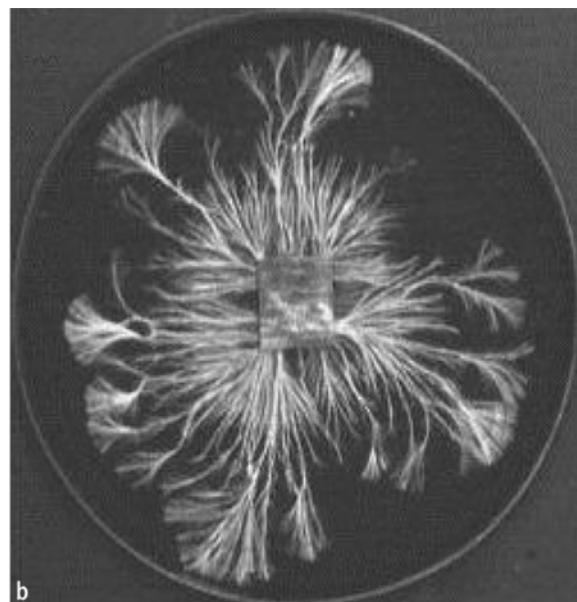
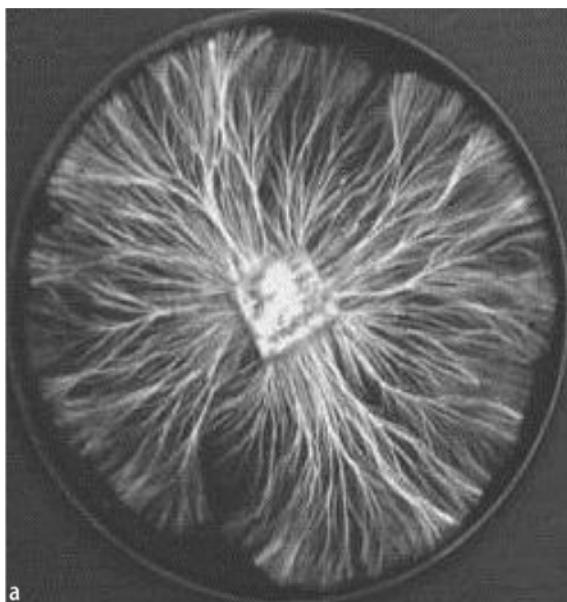


Fig. 12.6. Outgrowth from wood blocks of the wood-decay fungus *Hypholoma fasciculare* **a** without and **b** with grazing

by Collembola. Grazing leads to marginal fans connected to the wood resource by cords (data of Tordoff et al. 2006)

**Table 12.1.** A summary of responses noted by Riffle (1971) for fungi cultured individually and grazed by the nematode *Aphelenchoides*. The 43 fungi studied were subdivided by

Group	Fungal linear growth rate	Sub-culture viability	Nematode density developed	Number of fungal species	Example
I	Unchanged	Good	High	11	<i>Flammulina velutipes</i> (Curt. ex Fr.) Karst
II	Unchanged	Good	Low	12	<i>Suillus variegatus</i> (Fr.) O. Kuntze
III	Reduced	Reduced	High	5	<i>Amanita rubescens</i> ([Pers] Fr.) S. F. Grey
IV	Reduced	Lost	Low	7	<i>Boletus edulis</i> Bull. ex Fr.
V	Reduced	Lost	High	8	<i>Armillaria mellea</i> (Vahl ex Fr.) Kummer

Following introduction of low densities of the fungivorous nematode *Aphelenchoides* into agar cultures of various fungi, Riffle (1971) noted a variety of responses by both the fungus and grazer (Table 12.1). Fungal characteristics are clearly important in determining the outcome: Riffle (1971) noted that members of group I (Table 12.1) were fast growers of which the rate of mycelium production exceeded the rate of mycelium consumption by nematodes, while those in group IV were slow-growing fungi; group II were suspected of producing anti-feeding metabolites (Riffle 1971). The diversity of responses seen in Table 12.1 for fungal productivity and viability after imposing a standardized grazing regime emphasizes the complexity of ways in which grazing can modify fungal community structure. Depending on the type of fungus, grazing can be seen to have no effect or, alternatively, a strong negative effect on the grazed fungi. Simultaneously, for both the unaffected and grazing-damaged fungi, examples are to be seen where the nematode population is stimulated to reach high densities, and in other cases not. These different types of responses of the grazer population can, in turn, be expected to feed back in different ways to modify fungal community structure through different levels of subsequent grazing.

## V. Conclusions from Laboratory Studies and Extrapolation to the Field

Grazing of fungi has been shown under controlled conditions to have the capacity to change fungal community structure. Feeding on fungi which would be dominant in the absence of grazing will enhance diversity. Alternatively, grazing on co-dominant or subordinate fungi will act to polarize the fungal community, and diversity will

Riffle (1971) into five groups (I to V), according to the response of fungus and nematode to grazing

be reduced. Grazing can facilitate the transition from one successional stage of fungi to the next. The impact of grazing is modified by three factors: (i) grazing must be selective to impact community structure, (ii) high intensities of grazing lead to the loss of fungal species from the community and (iii) responses of the fungi to grazing can in some cases negate or exceed any negative impact of grazing.

There is an abundance of animals in soil and litter which eat fungi. As might be expected, fungivores are found to be particularly associated with mycelia in the field. For example, Cromack et al. (1988) reported the biomass per unit soil mass of Collembola, oribatid mites and nematodes to be 2.0, 1.5 and 1.2 times greater in ectomycorrhizal fungal mats than in adjacent non-mat soil respectively. Microcosms have shown grazing can modify saprotrophic fungal communities when isolated from active mycelium after removal of spores by washing (Tiunov and Scheu 2005). The potential clearly exists for grazing by fungivores to modify fungal communities extensively in the field.

## References

- Bakonyi G, Posta K, Kiss I, Fabian M, Nagy P, Nosek JN (2002) Density-dependent regulation of arbuscular mycorrhiza by collembola. *Soil Biol Biochem* 34:661–664
- Bamforth SS (1988) Interactions between Protozoa and other organisms. *Agric Ecosystems Environ* 24:229–234
- Bärlocher F (1980) Leaf-eating invertebrates as competitors of aquatic hyphomycetes. *Oecologia* 47:303–306
- Begon M, Townsend CR, Harper JL (2006) Ecology: from individuals to ecosystems, 4th edn. Blackwell, Malden
- Bengtsson G, Rundgren S (1983) Respiration and growth of a fungus, *Mortierella isabellina*, in response to grazing by *Onychiurus armatus* (Collembola). *Soil Biol Biochem* 15:469–473

- Bengtsson G, Erlandsson A, Rundgren S (1988) Fungal odour attracts soil Collembola. *Soil Biol Biochem* 20:25–30
- Bengtsson G, Hedlund K, Rundgren S (1993) Patchiness and compensatory growth in a fungus-Collembola system. *Oecologia* 93:296–302
- Boddy L (1983) Attraction of fungus gnats to zones of intraspecific antagonism on agar plates. *Trans Br Mycol Soc* 81:149–151
- Brasier CM (1978) Mites and reproduction in *Ceratocystis ulmi* and other fungi. *Trans Br Mycol Soc* 70:81–89
- Chakraborty S, Old KM, Warcup JH (1983) Amoebae from a take-all suppressive soil which feed on *Gaeumannomyces graminis tritici* and other soil fungi. *Soil Biol Biochem* 15:17–24
- Christensen M (1981) Species diversity and dominance in fungal communities. In: Wicklow DT Carroll GC (eds) *The fungal community*. Marcel Dekker, New York, pp 201–232
- Christensen M (1989) A view of fungal ecology. *Mycologia* 81:1–19
- Christensen K (1990) Insecta: Collembola. In: Dindal DL (ed) *Soil biology guide*. Wiley, New York, pp 965–995
- Crawley MJ (1983) *Herbivory*. Blackwell Scientific, Oxford
- Cromack K, Fischer BL, Moldenke AM, Entry JA, Ingham ER (1988) Interactions between soil animals and ectomycorrhizal fungal mats. *Agric Ecosystems Environ* 24:161–168
- Curl EA, Lartey R, Peterson CM (1988) Interactions between root pathogens and soil microarthropods. *Agric Ecosystems Environ* 24:249–261
- Darwin C (1859) *The origin of species*. John Murray, London
- Dash MC, Cragg JB (1972) Selection of microfungi by Enchytraeidae (Oligochaeta) and other members of the soil fauna. *Pedobiologia* 12:282–286
- Dash MC, Nanda B, Behera N (1980) Fungal feeding by Enchytraeidae (Oligochaeta) in a tropical woodland in Orissa, India. *Oikos* 34:202–205
- Didden WAM (1993) Ecology of terrestrial Enchytraeidae. *Pedobiologia* 37:2–29
- Ek H, Sjögren M, Arnebrant K, Söderström B (1994) Extramatrical mycelial growth, biomass allocation and nitrogen uptake in ectomycorrhizal systems in response to collembolan grazing. *Appl Soil Ecol* 1:155–169
- Frankland JC (1982) Biomass and nutrient cycling by decomposer basidiomycetes. In: Frankland JC, Hedger JN, Swift MJ (eds) *Decomposer basidiomycetes*. Cambridge University Press, Cambridge, pp 241–261
- Freckman DW, Baldwin JG (1990) Nematoda. In: Dindal DL (ed) *Soil biology guide*. Wiley, New York, pp 155–200
- Gange A (2000) Arbuscular mycorrhizal fungi, Collembola and plant growth. *Trends Ecol Evol* 15:369–372
- Giannakis N, Sanders FE (1989) Interactions between mycophagous nematodes, mycorrhizal and other soil fungi. *Agric Ecosystems Environ* 29:163–167
- Grime JP, Mackay JML, Hillier SH, Read DJ (1987) Floristic diversity in a model system using experimental microcosms. *Nature* 328:420–422
- Hamel C, Fyles H, Smith DL (1990) Measurement of development of endomycorrhizal mycelium using three different vital stains. *New Phytol* 115:297–302
- Hanlon RDG (1981) Influence of grazing by Collembola on the activity of senescent fungal colonies grown on media of different nutrient concentration. *Oikos* 36:362–367
- Hanlon RDG, Anderson JM (1980) Influence of macroarthropod feeding activities on microflora in decomposing oak leaves. *Soil Biol Biochem* 12:255–261
- Harold S, Tordoff GM, Jones TH, Boddy L (2005) Mycelial responses of *Hypholoma fasciculare* to collembola grazing: effect of inoculum age, nutrient status and resource quality. *Mycol Res* 109:927–935
- Harper JL (1977) *Population biology of plants*. Academic Press, London
- Hedlund K, Augustsson A (1995) Effects of enchytraeid grazing on fungal growth and respiration. *Soil Biol Biochem* 27:905–909
- Hedlund K, Boddy L, Preston CM (1991) Mycelial responses of the soil fungus, *Mortierella isabellina*, to grazing by *Onychiurus armatus* (Collembola). *Soil Biol Biochem* 23:361–366
- Hedlund K, Bengtsson G, Rundgren S (1995) Fungal odour discrimination in two sympatric species of fungivorous collembolans. *Funct Ecol* 9:869–875
- Hiol Hiol F, Dixon RK, Curl EA (1994) The feeding preference of mycophagous Collembola varies with the ectomycorrhizal symbiont. *Mycorrhiza* 5:99–103
- Hogervorst RF, Verhoef HA, Van Straalen NM (1993) Five-year trends in soil arthropod densities in pine forests with various levels of vitality. *Biol Fert Soils* 15:189–195
- Hubert J, Zilova M, Pekar S (2001) Feeding preferences and gut contents of three panphytophagous oribatid mites (Acaria: Oribatida). *Eur J Soil Biol* 37:197–208
- Hubert J, Jarosik V, Mourek J, Kubatova A, Zdarkova E (2004) Astigmatid mite growth and fungi preference (Acaria: Acaridida): comparisons in laboratory experiments. *Pedobiologia* 48:205–214
- Ingham RE (1992) Interactions between invertebrates and fungi: effects on nutrient availability. In: Carroll GC, Wicklow DT (eds) *The fungal community*, 2nd edn. Marcel Dekker, New York, pp 669–690
- Kampichler C, Rolschewski J, Donnelly DP, Boddy L (2004) Collembolan grazing affects the growth strategy of the cord-forming fungus *Hypholoma fasciculare*. *Soil Biol Biochem* 36:591–599
- Kaneko N, McLean MA, Parkinson D (1995) Grazing preference of *Onychiurus subtilis* (Collembola) and *Opiliella nova* (Oribatei) for fungal species inoculated on pine needles. *Pedobiologia* 39:538–546
- Kjøller A, Struwe S (1982) Microfungi in ecosystems: fungal occurrence and activity in litter and soil. *Oikos* 39:389–422
- Klironomos JN, Kendrick WB (1996) Palatability of microfungi to soil arthropods in relation to the functioning of arbuscular mycorrhizae. *Biol Fert Soils* 21:43–52
- Klironomos JN, Ursic M (1998) Density dependent grazing on the extraradical hyphal network of the arbuscular mycorrhizal fungus, *Glomus intraradices*, by the collembolan, *Folsomia candida*. *Biol Fert Soils* 26:250–253
- Klironomos JN, Widden P, Deslandes I (1992) Feeding preferences of the collembolan *Folsomia candida* in relation to microfungal successions on decaying litter. *Soil Biol Biochem* 24:685–692
- Klironomos JN, Bednarczuk EM, Neville J (1999) Reproductive significance of feeding on saprobic and arbuscular

- mycorrhizal fungi by the collembolan, *Folsomia candida*. *Funct Ecol* 13:756–761
- Lubchenco J (1983) *Littorina* and *Fucus*: effects of herbivores, substratum heterogeneity, and plant escapes during succession. *Ecology* 64:1116–1123
- Lussenhop J (1981) Analysis of microfungal component communities. In: Wicklow DT, Carroll GC (eds) *The fungal community*. Marcel Dekker, New York, pp 37–45
- Luxton M (1972) Studies on the oribatid mites of a Danish beech wood soil. I. Nutritional biology. *Pedobiologia* 12:434–463
- Maraun M, Martens H, Migge S, Theenhaus A, Scheu S (2003) Adding to the enigma of soil animal diversity: fungal feeders and saprophagous soil invertebrates prefer similar food substrates. *Eur J Soil Biol* 39:85–95
- May RM (1975) Patterns of species abundance and diversity. In: Cody ML, Diamond JM (eds) *Ecology and evolution of communities*. Harvard University Press, Cambridge, MA, pp 81–121
- McGonigle TP (1995) The significance of grazing on fungi in nutrient cycling. *Can J Bot suppl* 1:S1370–S1376
- McGonigle TP, Fitter AH (1988) Ecological consequences of arthropod grazing on VA mycorrhizal fungi. *Proc R Soc Edinb* 94B:25–32
- McNaughton SJ (1979) Grazing as an optimization process: grass-ungulate relationships in the Serengeti. *Am Nat* 113:691–703
- Moody SA, Briones MJI, Pearce TG, Dighton J (1995) Selective consumption of decomposing wheat straw by earthworms. *Soil Biol Biochem* 27:1209–1213
- Moody SA, Pearce TG, Dighton J (1996) Fate of some fungal spores associated with wheat straw decomposition on passage through the guts of *Lumbricus terrestris* and *Aporrectodea longa*. *Soil Biol Biochem* 28:533–537
- Moore JC (1988) The influence of microarthropods on symbiotic and non-symbiotic mutualism in detrital-based below-ground food webs. *Agric Ecosystems Environ* 24:147–159
- Moore JC, Walter DE, Hunt HW (1988) Arthropod regulation of micro- and mesobiota in below-ground detrital food webs. *Annu Rev Entomol* 33:419–439
- Newell K (1984a) Interaction between two decomposer basidiomycetes and a collembolan under sitka spruce: distribution, abundance and selective grazing. *Soil Biol Biochem* 16:227–233
- Newell K (1984b) Interaction between two decomposer basidiomycetes and a collembolan under sitka spruce: grazing and its potential effects on fungal distribution and litter decomposition. *Soil Biol Biochem* 16:235–239
- O'Connor FB (1967) The Enchytraeidae. In: Burges A, Raw F (eds) *Soil biology*. Academic Press, New York, pp 213–257
- Parkinson D, Visser S, Whittaker JB (1979) Effects of collembolan grazing on fungal colonization of leaf litter. *Soil Biol Biochem* 11:529–535
- Persson T, Bååth E, Clarholm M, Lundkvist H, Söderström BE, Sohlenius B (1980) Trophic structure, biomass dynamics and carbon metabolism of soil organisms in a scots pine forest. *Ecol Bull* 32:419–459
- Rayner ADM, Todd NK (1982) Population structure in wood-decomposing basidiomycetes. In: Frankland JC, Hedger JN, Swift MJ (eds) *Decomposer basidiomycetes, their biology and ecology*. Cambridge University Press, Cambridge, pp 307–333
- Riffle JW (1971) Effect of nematodes on root-inhabiting fungi. In: Hacskaylo E (ed) *Mycorrhizae*. United States Department of Agriculture Forest Service misc publ 1189. United States Government Printing Office, Washington, DC, pp 97–113
- Rockett CL (1980) Nematode predation by oribatid mites (Acari: Oribatida). *Int J Acarol* 6:219–224
- Ruess L, Garcia Zapata EJ, Dighton J (2000) Food preferences of a fungal-feeding *Aphelenchoides* species. *Nematology* 2:223–230
- Santos PF, Whitford WG (1981) The effects of microarthropods on litter decomposition in a Chihuahuan desert ecosystem. *Ecology* 62:654–663
- Scheu S, Simmerling F (2004) Growth and reproduction of fungal feeding Collembola as affected by fungal species, melanin and mixed diets. *Oecologia* 139:347–353
- Schneider K, Maraun M (2005) Feeding preferences among dark pigmented fungal taxa ("Dematiaceae") indicate limited trophic niche differentiation of oribatid mites (Oribatida, Acari). *Pedobiologia* 49:61–67
- Schneider K, Migge S, Norton RA, Scheu S, Langel R, Reineking A, Maraun M (2004) Trophic niche differentiation in soil microarthropods (Oribatida, Acari): evidence from stable isotope ratios ( $^{15}\text{N}/^{14}\text{N}$ ). *Soil Biol Biochem* 36:1769–1774
- Schneider K, Renker C, Maraun M (2005) Oribatid mite (Acari, Oribatida) feeding on ectomycorrhizal fungi. *Mycorrhiza* 16:67–72
- Schreiner RP, Bethlenfalvay GJ (2003) Crop residue and Collembola interact to determine the growth of mycorrhizal pea plants. *Biol Fert Soils* 39:1–8
- Schubert A, Mazzatelli M (1989) Enzymatic activities of VAM extraradical mycelium at different stages of development. *Agric Ecosystems Environ* 29:349–353
- Shafer SR, Rhodes LH, Riedel RM (1981) In vitro parasitism of endomycorrhizal fungi of ericaceous plants by the mycophagous nematode *Aphelenchoides bicaudatus*. *Mycologia* 73:141–149
- Shaw PJA (1985) Grazing preferences of *Onychiurus armatus* (Insecta: Collembola) for mycorrhizal and saprophytic fungi of pine plantations. In: Fitter AH, Atkinson D, Read DJ, Usher MB (eds) *Ecological interactions in soil*. Blackwell Scientific, Oxford, pp 333–337
- Shaw PJA (1988) A consistent hierarchy in the fungal feeding preferences of the Collembola *Onychiurus armatus*. *Pedobiologia* 31:179–187
- Shaw PJA (1992) Fungi, fungivores, and fungal food webs. In: Carroll GC, Wicklow DT (eds) *The fungal community*, 2nd edn. Marcel Dekker, New York, pp 295–310
- Swift MJ (1976) Species diversity and the structure of microbial communities in terrestrial habitats. In: Anderson JM, Macfadyen A (eds) *The role of terrestrial and aquatic organisms in decomposition processes*. Blackwell Scientific, Oxford, pp 185–222
- Swift MJ (1982) Basidiomycetes as components of forest ecosystems. In: Frankland JC, Hedger JN, Swift MJ (eds) *Decomposer basidiomycetes, their biology and ecology*. Cambridge University Press, Cambridge, pp 307–333

- Swift MJ, Boddy L (1984) Animal-microbial interactions in wood decomposition. In: Anderson M, Rayner ADM, Walton DWH (eds) *Invertebrate-microbial interactions*. Cambridge University Press, Cambridge, pp 89–131
- Swift MJ, Heal OW, Anderson JM (1979) Decomposition in terrestrial ecosystems. University of California Press, Berkeley, CA
- Takeda H, Ichimura T (1983) Feeding attributes of four species of Collembola in a pine forest soil. *Pedobiologia* 25:373–381
- Tansley AG, Adamson RS (1925) Studies on the vegetation of the English chalk. III. The chalk grasslands of the Hampshire-Sussex border. *J Ecol* 13:177–223
- Tibbles LL, Chandler D, Mead A, Jervis M, Boddy L (2005) Evaluation of the behavioural response of the flies *Megaselia halterata* and *Lycoriella castanescens* to different mushroom cultivation materials. *Entomol Exp Appl* 116:73–81
- Tiunov AV, Scheu S (2000) Microfungal communities in soil, litter and casts of *Lumbricus terrestris* L. (Lumbricidae): a laboratory experiment. *Appl Soil Ecol* 14:17–26
- Tiunov AV, Scheu S (2005) Arbuscular mycorrhiza and Collembola interact in affecting community composition of saprotrophic microfungi. *Oecologia* 142:636–642
- Tordoff GM, Boddy L, Jones TH (2006) Grazing by *Folsomia candida* (Collembola) differentially affects mycelial morphology of the cord-forming basidiomycetes *Hypoloma fasciculare*, *Phanerochaete velutina* and *Resinicium bicolor*. *Mycol Res* 110:335–345
- Twinn DC (1974) Nematodes. In: Dickinson CH, Pugh GJF (eds) *Biology of plant litter decomposition*. Academic Press, London, pp 421–465
- van der Drift J, Jansen E (1977) Grazing of springtails on hyphal mats and its influence on fungal growth and respiration. *Ecol Bull* 25:203–209
- Varga J, Naar Z, Dobolyi C (2002) Selective feeding of collembolan species *Tomocerus longicornis* (Mull.) and *Orchesella cincta* (L.) on moss inhabiting fungi. *Pedobiologia* 46:526–538
- Visser S (1985) Role of the soil invertebrates in determining the composition of soil microbial communities. In: Fitter AH, Atkinson D, Read DJ, Usher MB (eds) *Ecological interactions in soil*. Blackwell Scientific, Oxford, pp 297–317
- Visser S, Whittaker JB (1977) Feeding preferences for certain litter fungi by *Onychiurus subtilis* (Collembola). *Oikos* 29:320–325
- Wallwork JA (1976) The distribution and diversity of soil fauna. Academic Press, London
- Walter DE (1987) Trophic behaviour of "mycophagous" microarthropods. *Ecology* 68:226–229
- Walter DE, Kaplan DT (1990) Feeding observations on two astigmatic mites, *Schwiebea rocketti* (Acaridae) and *Histiostoma bakeri* (Histiostomatidae) associated with *Citrus* feeder roots. *Pedobiologia* 34:281–286
- Whitfield DWA (1977) Energy budgets and ecological efficiencies on Truelove Lowland. In: Bliss LC (ed) *Truelove Lowland, Devon Island, Canada: a high arctic ecosystem*. University of Alberta Press, Edmonton, pp 607–620
- Wicklow DT (1981) The coprophagous fungal community: a mycological system for examining ecological ideas. In: Wicklow DT, Carroll GC (eds) *The fungal community*. Marcel Dekker, New York, pp 47–76
- Wicklow DT, Yocom DH (1982) Effect of larval grazing by *Lycoriella mali* (Diptera: Sciaridae) on species abundance of coprophilous fungi. *Trans Br Mycol Soc* 78:29–32
- Wiggins EA, Curl EA (1979) Interactions of Collembola and microflora of cotton rhizosphere. *Phytopathology* 69:244–249
- Yeates GW, Bongers T, Goede RGM de, Freckman DW, Georgieva SS (1993) Feeding habits in soil nematode families and genera – an outline for soil ecologists. *J Nematol* 25:315–331

---

# 13 Fungal Endophytes

P. BAYMAN<sup>1</sup>

## CONTENTS

I. Introduction .....	213
II. Definitions and Interactions .....	214
III. How Endophytes are Studied .....	214
A. Microscopy in Planta .....	214
B. Isolation in Pure Culture .....	215
C. PCR-Based Methods .....	215
D. Biochemical Methods .....	215
IV. Clavicipitaceous Endophytes of Grasses .....	215
A. Systematics, Evolution and Life Cycles .....	216
1. Clavicipitaceae and Key Genera .....	216
2. Life Cycles and Evolution .....	216
3. Coevolution and Interspecific Hybridization .....	217
B. Secondary Metabolites and Their Effects .....	217
C. Effects of Mutualistic Grass Endophytes on Plant Hosts and Other Organisms .....	218
1. Effects on Hosts .....	218
2. Effects on Plant Communities .....	218
3. Effects of Endophytes on Insect Herbivores .....	218
4. Effects of Endophytes on Mammalian Herbivores .....	218
V. Endophytes of Maize .....	218
VI. Endophytes of Trees .....	219
A. Transmission and Sources of Inoculum .....	220
B. Specificity .....	220
1. Species Specificity .....	220
2. Organ and Tissue Specificity .....	220
3. Site Specificity and Spatial Variation .....	221
C. Effects of Tree Endophytes .....	221
1. Endophytes as Latent Pathogens or Misplaced Pathogens .....	221
2. Effects on Host Plants .....	222
3. Effects on Insect Herbivores .....	222
4. Effects on Plant Pathogens .....	222
VII. Endophytes of Tropical Plants .....	222
VIII. Endophytes of Nonvascular Plants and Lichens .....	223
IX. Uses in Agriculture and Biotechnology .....	223
A. Clavicipitaceous Fungi of Grasses .....	223
B. Other Endophytes .....	223
C. Bioprospecting .....	224
X. Conclusions .....	224
References .....	224

## I. Introduction

Fungi are important in mysterious ways and mysterious in important ways. Part of their charm is that much of their lives is hidden from our view – hidden by the microscopic size of their hyphae and by their substrata – yet they play key roles in nature, in agriculture, in biotechnology, and in the lives of many other organisms.

Much of the mystery and importance of the fungi can be found in the endophytes. They are (by definition) hidden in plant tissues, but they have important implications for communities, agriculture and biodiversity. In addition, certain endophytes are an excellent model system for studying interactions between organisms.

No plant is an island. Each plant is a community, including diverse types of microorganisms. One guild of plant-associated microorganisms, the endophytic fungi, is the topic of this chapter. All plants and plant-like protists have endophytes, including lichens and nonvascular plants (see Stone et al. 2000) and algae (Stanley 1992).

Some endophytes are hitchhikers with no noticeable effect on their plant host, but others have intimate relations with their hosts and profound effects on their function and survival. The importance of endophytes remained largely hidden until 1975, when Charles Bacon and colleagues (Bacon et al. 1975) demonstrated that endophytes of pasture grasses caused toxic syndromes in cattle. This stimulated research in many aspects of the biology of endophytes, both basic and applied.

This chapter reviews the principal guilds of endophytes, their relationships with their hosts, and their importance for other organisms and human affairs. It focuses on the guilds of endophytes that have been most extensively studied: clavicipitaceous endophytes of grasses and endophytes of trees. The chapter is intended to be illustrative, rather than comprehensive. Its

<sup>1</sup> Departamento de Biología, Universidad de Puerto Rico–Río Piedras, P.O. Box 23360, San Juan, PR 00931, USA

purpose is to stimulate young investigators to consider endophytes in their research, because many interesting endophytes and aspects of endophytes are probably still hidden.

## II. Definitions and Interactions

The term 'endophyte' was coined by de Bary in 1866 to describe fungi that colonize internal tissues of stems and leaves (cited from Wilson 1995). De Bary's definition has since been modified many times. Two widely accepted definitions follow:

"Endophytes colonize symptomlessly the living, internal tissues of their host, even though the endophyte may, after an incubation or latency period, cause disease" (Petrini 1991).

"Endophytes are fungi or bacteria which, for all or part of their life cycle ... cause unapparent and asymptomatic infections entirely within plant tissues ..." (Wilson 1995).

Both of these definitions (but not de Bary's) encompass endophytes of roots, which are ubiquitous (Schulz et al. 2006). Latent stages of infections by pathogens are included in these definitions, but mycorrhizal fungi are considered to be excluded because they are partly external and often symptomatic (Saikkonen et al. 1998). Unlike the definitions of mycorrhizae or pathogens, these definitions of endophytes are based primarily on location, rather than type of symbiotic interaction.

In contrast, symbioses (e.g. parasitism, competition, mutualism, etc.) are defined by which partners benefit from the interaction. The best-known endophytic fungi are mutualists: *Neotyphodium* species that infect cool-season grasses (see Sect. IV. below). They are of great economic importance, and a superb model system to study the biology of interactions. Because *Neotyphodium* is an example of mutualism, there is a widespread misconception (in the case of people who do not work with endophytes) or fantasy (in the case of those of us who do) that most other endophytes are mutualists as well. However, in most cases a mutualistic relationship has not been demonstrated.

Endophytic interactions form a continuum, and the interaction between a given microorganism and host may change over time (Saikkonen et al. 1998; Schulz and Boyle 2003). For example, a mutualistic endophyte may become pathogenic and vice versa, depending on the environment and

the condition of the host plant. For this reason, the different types of symbiosis best describe an interaction at a particular point in time, rather than a species of microorganism (for most endophytes). Even a mutualistic interaction may be viewed as a balanced antagonism, where each partner tries to maximize the benefit obtained from the other without loss of its own resources (Saikkonen et al. 1998; Schulz et al. 1999). To further complicate matters, whether an endophytic interaction appears mutualistic, parasitic or commensal may depend on the scale at which it is viewed (Carroll 1995; Wilson 2000). Of course, these problems do not mean that endophytes are less interesting or less worthy of study; rather, they mean that one should be cautious about categorizing them.

Like mycorrhizae, endophytism is an ancient form of symbiosis, perhaps dating to the first land plants or even before. However, endophytism has arisen so many times independently, involving different fungi and plants, that the idea of a single evolutionary history does not make sense; rather, there are many different histories. An exception is the evolution of endophytism in the Clavicipitaceae, discussed below.

## III. How Endophytes are Studied

Since endophytes are (by definition) internal and asymptomatic, their detection and identification can be difficult. Four sets of techniques are used: microscopy in planta, isolation in pure culture, DNA- (and RNA-) based methods, and biochemical methods. Results may depend on choice of technique; each has advantages and limitations, and each may bias results in favour of certain taxa.

### A. Microscopy in Planta

The definitive way to detect endophytes and determine infection frequency is by direct microscopic observation. Microscopy is also useful for locating endophytes in specific tissues. For example, *Rhabdocline parkeri* and *Phyllosticta abietis* are both endophytes of Douglas fir needles, but the former infects epidermal cells and the latter spongy mesophyll cells (Stone 1987, 1988). This difference in location may be important for their interaction with the plant or with other organisms. However, the standard culturing and molecular techniques cannot detect such differences.

Nevertheless, microscopy in planta has a disadvantage: identification of the fungi is very difficult because hyphae of many different taxa look alike. Detection with taxon-specific probes or antibodies allows identification of certain target organisms but not others.

### B. Isolation in Pure Culture

The great majority of studies of fungal endophytes are done with surface-sterilized plant pieces incubated on culture media. Plant pieces are usually surface-sterilized in ethanol and/or sodium hypochlorite, and incubated on a general purpose medium (e.g. potato dextrose agar or malt extract agar) amended with antibiotics. Methods of isolation have been reviewed extensively (Schulz et al. 1993; Bills 1996). These methods are simple, inexpensive, and allow detection of a wide range of fungi. However, the choice of surface-sterilization method and culture medium will bias the results in favour of certain organisms. Fungi that are obligate biotrophs and do not grow in culture media will be overlooked, and weedy fungi that grow quickly on agar will be overrepresented. Distinctions between epiphytes and endophytes depend on the assumption that surface sterilization kills the former and not the latter. These limitations have stimulated the use of molecular techniques to identify endophytes.

Sampling strategy has a great impact on which endophytes will grow out of leaf pieces on agar. In general, using small leaf pieces reveals greater endophyte richness than when using large leaf pieces. Because leaf-to-leaf variation is considerable, several leaves should be sampled. Many studies on endophyte diversity are not rigorous in sampling strategy, which often makes it difficult to compare results between studies. These sampling issues have been discussed at length (Carroll 1995; Lodge et al. 1996; Wilson 2000; Gamboa et al. 2002; Bayman 2006).

### C. PCR-Based Methods

PCR-based methods are increasingly being used to detect and identify endophytic fungi. A variety of techniques are available, including DNA sequencing, RFLPs and variations, DGGE and RAPDS. In many such studies, fungi are first isolated in pure culture and DNA is then extracted. This approach is excellent for identification, but it cannot detect

unculturable fungi or escape the biases inherent in culturing.

To date, relatively few studies on endophytes have used direct amplification (also called environmental PCR). This approach can serve to detect fungi that do not grow in culture. But how many endophytic fungi are unculturable? A study in loblolly pine compared culturing vs. direct amplification, and identified fungi by DNA sequencing (Arnold et al. 2007). Direct amplification detected more species than culturing, but culturing detected more orders of fungi. Also, more species of Sordariomycetes were detected by culturing than by direct amplification. These results are surprising: in epiphytic bacteria (and many other guilds of bacteria), direct amplification reveals more diversity at all levels (Yang et al. 2001).

Direct amplification of endophytes from plant tissues will probably lead to the discovery of new, high-level clades of fungi. However, PCR-based methods and culturing are complimentary, as the loblolly pine study suggests. To maximize diversity of fungi identified and economy of materials, endophyte diversity studies should include both direct amplification and culturing (Bayman 2006). Even as large-scale sequencing and metagenomics investigations become more common and more revealing, much of the currently hidden diversity in fungi may be revealed by culturing.

### D. Biochemical Methods

Ergosterol content can be used to detect and quantify fungal infection of plants, as can fatty acids and other biochemical markers. To date, these have been used mostly for clavicipitaceous endophytes of grasses. Immunoblot assays and other assays based on monoclonal antibodies provide convenient and specific tools for detection of *Neotyphodium* in grass stems (Richardson and Logendra 1997; Clay et al. 2005; Koh et al. 2006). However, such tools are expensive to develop, and are applicable only to fungi for which monoclonal antibodies have been developed.

## IV. Clavicipitaceous Endophytes of Grasses

Clavicipitaceous fungi (that is, in the Family Clavicipitaceae) in grasses were first described over 100 years ago (see Wilson 1996). However, the

importance of these endophytes was not realized until 1975, when Charles Bacon et al. demonstrated their association with toxicity syndromes in cattle. In the 30+ years since Bacon's discovery, the story of grass-endophyte relationships has been pursued from many different perspectives, several of which are discussed below.

For graduate students and new investigators, there are several advantages of working in a field of study this young: the literature is recent, and in journals that are still available; the major players are still active as of this writing, and one can talk with them at meetings; and many interesting symbioses, problems and potential applications have yet to be studied. For example, *Neotyphodium* endophytes were recently found in wild wheat species in Turkey (Marshall et al. 1999).

## A. Systematics, Evolution and Life Cycles

### 1. Clavicipitaceae and Key Genera

The Clavicipitaceae (Ascomycota) are characterized by perithecia produced in stromata, ascii with apical thickenings, and filamentous, multicellular ascospores. They are biotrophic parasites of insects, fungi, and monocotyledonous plants. The most conspicuous genera are *Claviceps* and *Cordyceps*, pathogens of cereals and insects respectively. The stromata of both genera are used in medicine. *Claviceps* has influenced human history many times (Matossian 1989; Pendell 2005). The Clavicipitaceae of interest as endophytes are about 30 species of *Balansia*, *Myriogenospora*, *Atkinsiella*, *Balansiopsis*, *Epichloë* and *Neotyphodium* (Clay 1988). *Neotyphodium* species (formerly placed in *Acremonium*) are anamorphs of *Epichloë*.

*Balansia* includes epiphytes and endophytes of grasses and sedges in the Americas and Asia. The endophytic species of *Balansia* are found on warm-season grasses with C<sub>4</sub> photosynthesis in the Americas, and appear to have evolved from epiphytes (White et al. 2000; see below). Hyphae are epicuticular in epiphytes and intercellular in endophytes. They are easy to culture on a variety of standard media, though many require vitamins (Bacon 1985). The switch to endophytism may have conferred several advantages, including easier absorption of nutrients from the host, protection from the environment, and protection from surface-feeding insects and parasitic fungi (White et al. 2000). Studies on the evolution of endophytism in this group are very interesting, partly because they integrate

molecular phylogeny, morphology, life history, host range and secondary metabolism into a single story (White 1994; Clay and Schardl 2002).

*Epichloë* and *Neotyphodium* infect cool-season grasses native to Europe and North America with C<sub>3</sub> photosynthesis. Stromata of *Epichloë* always form around a grass inflorescence and adjoining leaf sheath; the blade of the leaf emerges from the stroma (White et al. 2000). Hyphae are mostly intercellular. The stromata produce conidia (which function as spermatia) and receptive hyphae; the conidia are transferred between stromata by flies. The mating system is heterothallic, at least in *E. typhina* (White and Bultman 1987). However, some of these fungi have lost the ability to make the perfect (*Epichloë*) stage, as described below.

## 2. Life Cycles and Evolution

The relationship between Clavicipitaceae and grasses is believed to be 40 million years old (Clay et al. 2005). The presumed ancestral condition in Clavicipitaceae is seen in *Claviceps*, which infects and replaces grass ovaries and has both intercellular and intracellular hyphae (Bacon and White 2000). It does not infect vegetative organs and produces disease symptoms rapidly, and is therefore not considered endophytic.

A more derived relationship with the host is seen in *Myriogenospora*, *Echinodothis*, *Atkinsiella* and some *Balansia* species. These fungi are mostly epiphytic and epicuticular, producing stromata on stems, leaves or inflorescences. If these fungi infect meristems, they may perennialize. Some of these fungi inhibit sexual reproduction in the host plant, by physically preventing the development of the inflorescence and perhaps by other means as well. Preventing seed set in the host may leave more resources available for their own sexual reproduction (Bacon and White 2000). The more derived species are endophytic and intercellular, showing epiphytic growth only when they emerge to produce stromata.

The most derived group of species is entirely endophytic and intercellular. They cause systemic infections and are transmitted vegetatively and vertically through the seeds, and usually produce no spores. They produce the *Neotyphodium* (= *Acremonium*) stage in culture but do not produce stromata or perithecia. Compared to some of the pathogenic species, the situation is reversed here: the grass can reproduce sexually but the fungus cannot. These species are mutualists.

Finally, some species are inconsistent, sometimes allowing the host to set seed and sometimes replacing inflorescences with their own stromata. These species are of great evolutionary interest, because they may represent transitional forms between pathogenic species that reproduce sexually and are horizontally transmitted, and the mutualistic species that reproduce asexually and are vertically transmitted (Schardl et al. 1994, 1997; Schardl and Clay 1997).

Most of the following discussion focuses on the systemic, endophytic and asexual species of *Neotyphodium*. They are the best studied, because they infect economically important grasses and because they are an excellent model of mutualistic interactions and coevolution.

The mutualistic *Neotyphodium* species are so intimately associated with their hosts that it is conceivable they may eventually lose their independent identity and become organelles: they are vertically transmitted through the hosts' seeds. They are never found in nature as free-living organisms (although they grow well in culture). They have lost the capacity for sexual reproduction, which limits their ability to adapt to new environments. And, they are widespread (although endophyte-free plants are found in populations of infected grasses).

### 3. Coevolution and Interspecific Hybridization

Gene genealogies of *Epichloë* and several tribes in the Pooideae are concordant, suggesting the fungi and their hosts may have coevolved (Schardl et al. 1997). Further evidence of coevolution comes from experiments of cross-inoculation of endophytes. Most *Epichloë* and *Neotyphodium* isolates produce no noticeable defence response when inoculated onto their natural hosts. Inoculation onto non-host grasses, however, often produces a response, including death of endophyte cells or host tissues (Lane et al. 2000). The absence of such responses on the natural hosts may be the result of coevolution.

*Epichloë* and *Neotyphodium* comprise a number of biological species, each isolated from the others by intersterility and often by differences in host range and geographical distribution (Schardl et al. 1997; Schardl and Wilkinson 2000). However, a hybrid origin has been postulated for several species. This is interesting and surprising because some of these species lack a sexual stage, and because interspecific hybridization is relatively rare in fungi (Burnett 2003).

The initial evidence of hybridization came from isozyme studies, in which most *Neotyphodium* isolates had single bands but others had double bands (Leuchtmann and Clay 1990). Further evidence came from phylogenetic trees based on  $\beta$ -tubulin sequences, in which some isolates contained two copies that belonged to different branches of the tree, suggesting a hybrid origin (Schardl et al. 1994; Tsai et al. 1994).

The most interesting case is *N. coenophialum*, a ubiquitous and important endophyte of tall fescue (*Festuca arundinaceae*) (Schardl and Wilkinson 2000). Tall fescue itself is a (polyploid) hybrid of *F. pratensis* (which has its own endophyte, *N. uncinatum*) and *F. glaucescens*. When the hybrid tall fescue first arose, it kept the *N. uncinatum* endophyte of *F. pratensis*. *N. uncinatum* later hybridized in separate events with two *Epichloë* species to produce *N. coenophialum*. This story is complex, but it is the simplest explanation for the multiple  $\beta$ -tubulin sequences in *N. coenophialum* and is compatible with host phylogeny.

These fungal hybridization events presumably occurred in host plants infected with multiple species of endophytes, at least some of which were asexual species. This suggests that the hybrids may have come from parasexual (or somatic) recombination events. In turn, hybridization may have eliminated the possibility of future sexual reproduction, due to meiotic irregularities.

The previous (1997) edition of *The Mycota* volume IV includes an interesting chapter on the evolution of mutualism in clavicipitaceous endophytes (Shardl and Clay 1997).

### B. Secondary Metabolites and Their Effects

The Clavicipitaceae are famous for alkaloid production; the most flamboyant are the ergot alkaloids of *Claviceps purpurea* used in medicine and the production of LSD. The alkaloids produced by endophytic species are less scandalous but of enormous ecological and agricultural interest.

In terms of chemical ecology, grasses and endophytes are a perfect match: grasses make relatively few secondary metabolites. Secondary metabolites are important in plant defences against herbivores and pathogens, so the grasses are relatively undefended compared to other plant families. Their clavicipitaceous endophytes provide chemical defences (Clay and Schardl 2002).

Four types of alkaloids are produced. Two appear to be most effective against mammalian herbi-

vores and two against insects (see Bush et al. 1997; Lane et al. 2000):

1. Ergot alkaloids, especially the lysergic acid amide derivative ergovaline: they are common in tall fescue, perennial ryegrass and other grasses. Concentrations are usually <5 ppm (= µg/g) but cases of 2000 ppm have been documented. A related group of compounds, the clavine alkaloids, lack the amino acid side chain of ergovaline. Ergot alkaloids are found in about 50% of grass species with mutualist endophytes.
2. Lolitrem and other indole-diterpenoids, especially lolitrem B; these are found in ryegrass, fescue and other grasses. Tremorgenic neurotoxins, called lolitrem, cause tremors in mammals and appear to be the causal agent of 'ryegrass staggers' of sheep. Lolitrem B has other toxic effects in mammals and insects. At the cellular level, it interferes with membrane potential and neurotransmitters. Lolitrem are found in about 10% of grass species with mutualist endophytes.
3. Loline (or pyrrolizidine) alkaloids: these are small molecules composed of two rings, a cyclic ether and an amino side chain. They are produced by *N. lolii* and *N. coenophialum*, and are found in *Festuca* and *Lolium* grasses at concentrations as high as 10,000 ppm. They are feeding deterrents in insects, and may be allelopathic. Effects on grazing mammals are less clear. They are found in about 35% of grass species with mutualist endophytes.
4. Peramine, a pyrrolopyrazine alkaloid: like the lolines, it is an insect-feeding deterrent. Peramine is found in >50% of grass species with mutualist endophytes.

### C. Effects of Mutualistic Grass Endophytes on Plant Hosts and Other Organisms

The mutualistic endophytes lend themselves to experiments that are simple and elegant in design. Endophyte-free plants are easy to produce, so experiments can compare plants of the same cultivar with and without infection.

#### 1. Effects on Hosts

The frequency of endophyte infection often increases in grass populations over time. For instance, old populations tend to have higher in-

fection rates than recently established populations (see Clay 1988). This suggests that the endophytes confer an adaptive advantage on their hosts, even though they grow at the expense of host metabolism. Endophyte infection increases growth rate of perennial ryegrass and tall fescue (see Clay 1988). The increase is significant for many growth parameters: shoot production, seed production, number of tillers, etc. *Neotyphodium* endophytes also increase drought tolerance in grasses, by means of osmoregulation and stomatal regulation (see Bacon and Hill 1996). These mechanisms have allowed perennial ryegrass to colonize large areas of the south-eastern United States that would otherwise be too hot and dry. Production of plant hormones may be part of the mechanism of action.

The species that abort inflorescences in their hosts obviously limit host sexual reproduction, but they also stimulate vegetative growth (see Clay 1988). Similarly, infection by *Balansia cyperi* increased dry weight and number of tubers in purple nutsedge (*Cyperus rotundus*) (but decreased the size of tubers; Stovall and Clay 1988). Since purple nutsedge is one of the world's worst weeds and uses tubers for vegetative reproduction, the presence of the endophyte may have serious consequences for agriculture.

#### 2. Effects on Plant Communities

Both experimental studies and field surveys show that the mutualistic *Neotyphodium* endophytes confer competitive advantages on their hosts. In plots seeded with infected and endophyte-free tall fescue, plant species richness was significantly lower for infected plots (Clay and Holah 1999). The main mechanism for these competitive advantages is herbivore deterrence (see below), but they may also reflect allelopathic effects of alkaloids (Clay et al. 1993; Bush et al. 1997).

Endophytes also affect decomposition rates of grasses: endophyte-infected tall fescue decomposed more slowly than endophyte-free plants (Lemons et al. 2005). The presence of endophytes also altered the composition of detritivore communities, particularly *Collembola*.

#### 3. Effects of Endophytes on Insect Herbivores

Effects of clavicipitaceous fungi on various herbivores have been demonstrated. The most notorious case is *Claviceps purpurea* on rye, where the herbivores are unsuspecting humans.

Several studies have found that endophyte-infected grasses significantly reduce survivorship and growth in insects, e.g. fall armyworm (Clay et al. 1993). However, endophytes and alkaloids can have different effects on closely related insect species. A single insect species may have very different responses to different species of endophyte-infected grasses. Many insects are relatively unaffected by ergot alkaloids at concentrations found in plants (Clay et al. 1993; see Richardson 2000). In contrast, peramine is much more toxic than ergot alkaloids to the Argentine stem weevil, a major pest of perennial ryegrass (see Bush et al. 1997).

#### 4. Effects of Endophytes on Mammalian Herbivores

In the 20th century, millions of hectares in the south-eastern United States were converted from native vegetation to tall fescue, a European grass that is productive and nutritious for livestock (see Bush et al. 1997). However, livestock grazing on tall fescue occasionally develop a variety of toxic syndromes, which are correlated with the presence of endophytes (Bacon et al. 1975, 1977). A similar relationship was discovered in New Zealand in sheep grazing on endophyte-infected perennial ryegrass (Latch et al. 1984). The ergot alkaloids are implicated in many such syndromes, and have diverse effects: they can cause weight loss, elevated body temperature, vasoconstriction, and reduced milk production in livestock (see Bush et al. 1997). Ergot alkaloids also reduce prolactin and melatonin levels in blood, have an affinity for dopamine receptors, and stimulate cAMP production.

Reproductive effects are also well known. Consuming endophyte-infected forage led to reduced conception, increased spontaneous abortions, and altered gestation times in cattle, sheep and horses (see Richardson 2000). Reproductive effects on rodents have also been found. Effects on sperm production and testosterone levels have also been shown.

Endophyte alkaloids are feeding deterrents for grazers. Since alkaloids tend to have a bitter taste, it is possible that herbivores can detect the presence of alkaloids directly. Cattle reduce forage consumption when presented with endophyte-infected grasses.

Lolitremes are implicated in neuroreceptor binding and inhibition of membrane channels. Although lolines are thought to be more toxic to insects than to mammals, they are immunosuppressive in mice and may cause vasoconstriction.

The list of toxicity syndromes in grazing animals caused by clavicipitaceous fungi is expected to keep growing.

#### V. Endophytes of Maize

Maize has an intimate and interesting relationship with endophytes. *Fusarium moniliforme* (= *Gibberella fujikuroi* or *G. moniliformis*) causes serious diseases of maize including seed rot, stalk rot, root rot, and kernel or ear rot (see Bacon and Hinton 1996). *F. moniliforme* produces five groups of mycotoxins, including fumonisins. Some of these mycotoxins are cytotoxic, mutagenic, immunosuppressive and estrogenic in animal studies, and have been implicated in human oesophageal cancer (see Bacon and Hinton 1996).

However, the same isolates of *F. moniliforme* that cause disease in some maize lines are endophytes in others. While the pathogenic *F. moniliforme* is horizontally transmitted, the endophytic *F. moniliforme* is vertically transmitted through the seed. This is potentially dangerous because infection is very common: *Fusarium* can almost always be isolated from maize seeds, infections are usually asymptomatic, and fumonisins can often be detected in seeds (John Puhalla, personal communication; see Kuldau and Yates 2000).

*Fusarium moniliforme* and maize are sometimes mutualists. Endophytic infection by *F. moniliforme* can protect against infection by *F. graminearum* (= *G. zae*), often a more aggressive pathogen (van Wyck et al. 1988), and against infection by other pathogens as well (see Kuldau and Yates 2000).

All these aspects of the *F. moniliforme*-maize interaction are reminiscent of the clavicipitaceous endophyte-grass interaction. *Fusarium* mycotoxins are a more direct threat to human health than the clavicipitaceous alkaloids because of the importance of maize in the human diet. However, the ecology of the interaction has not been studied as extensively. *Fusarium* endophytes are found in a wide range of other plants as well (Kuldau and Yates 2000).

#### VI. Endophytes of Trees

Most studies on endophytes of trees have focused on fungal ecology and systematics (Carroll 1995). Questions, techniques, levels of taxonomic resolu-

tion and sample size vary greatly among these studies, so it can be difficult to compare results among studies. However, some patterns have emerged.

#### A. Transmission and Sources of Inoculum

Transmission of endophytes in trees is mostly horizontal. In some plants, endophytes can be isolated from seeds but the infection is not passed on to the seedling (Wilson and Carroll 1994; Bayman et al. 1998). Most endophytes are probably transmitted as wind-, water- or animal-borne spores. In most cases, it is not clear where and when they sporulate, how they overwinter, or how infection occurs. In this sense, the endophytic habit is a paradox (Bayman 2006). To propagate and disperse to new hosts horizontally, most endophytes must (strictly speaking) become something other than an endophyte: either sporulate on the surface, sporulate on dead tissue, or be exposed by damage to the host organ (mechanically or by an animal vector).

However, a few cases of dispersal are well documented. For example, *Rhabdocline parkeri*, an endophyte of Douglas fir needles, produces conidia on midge galls on needles, and produces conidia and ascospores on fallen needles (Stone 1987; Carroll 1995). New needles are infected by rainwater containing spores. Similarly, *Discula quercina*, a common endophyte of Oregon white oak, sporulates on fallen leaves and probably on the bark of its host (Wilson and Carroll 1994). Rainwater collected below trees contained ascospores, and endophyte-free trees inoculated with the rainwater developed *D. quercina* infections. Filtered rainwater without spores (as a control) did not cause infection.

Newly emerged leaves are usually endophyte-free, though there are exceptions (Rodrigues 1994). Infection levels often increase with leaf age, which is consistent with horizontal transmission. Seasonal changes in infection frequency have been found in some endophytes but not in others (see Wilson 2000). Infection frequency is often correlated with rainfall. Insect wounds can be infection sites for some endophytes but not for all (Faeth and Hammon 1997).

#### B. Specificity

##### 1. Species Specificity

How specific are fungal endophytes for host plant species and vice versa? There is a wide range of interactions, from highly specific to generalist.

Most plants have diverse endophyte floras, including a few dominant taxa and many rare taxa – a typical log-normal distribution (Lodge et al. 1996). Some of the dominant taxa are often found only in one or a few host species, and can be considered host-specific. Examples include *Discula umbrinella* and *Hypoxyylon fragiforme* in European beech, *Lophiodermium piceae* in spruce, and *Phyllosticta multicorticulata* in balsam fir (see Petrini 1996); others are mentioned elsewhere in this chapter. Many other taxa can be isolated from a wide range of hosts, and some are usually weedy fungi that can be isolated from many other types of substrata as well. These weedy fungi (e.g. *Aspergillus*, *Penicillium* and *Cladosporium*) are often assumed to be accidental endophytes, for whom most endophytic infections are dead ends.

When endophyte communities of different tree species at the same site are compared directly, they usually differ – for example, of pine and beech, oak and willow (Petrini and Fisher 1988, 1990). Among species of pine, endophyte communities were more similar in related species than in more distantly related species (Hata and Futai 1996). However, in tropical trees the picture is not as clear (see below).

When a single fungal species infects various host species, it may comprise host-specific races (or cryptic species). For example, although *D. umbrinella* was isolated from chestnut, oak and beech, conidia of isolates from beech were capable of infecting only beech leaves (Toti et al. 1992).

On the plant side, intraspecific variation may be reflected in differences in endophyte floras. In hybrid cottonwood trees (*Populus fremontii* x *P. angustifolia*), frequency of twig endophytes was correlated with contribution of *P. fremontii* to the genome (Bailey et al. 2005). Endophyte frequency was negatively correlated with tannin concentration in twigs, which is lower in *P. fremontii* than in *P. angustifolia*. Endophyte genotype and host genotype were correlated in *Venturia ditricha* infections of birch (Ahlholm et al. 2002). These examples suggest that plant evolution can affect relationships with endophytes.

##### 2. Organ and Tissue Specificity

In *Gynoxis oleifolia*, a tropical tree, several endophytes were specific to roots, bark or leaves (Fisher et al. 1995). In some conifers and rainforest trees, fungal communities in leaf blades were different from those in petioles (Carroll et al. 1977; Petrini

1996; Lodge et al. 1996). Differences were also seen in different parts of palm leaves (Rodrigues 1994).

Different endophytes may lead separate lives within a single leaf blade. *Rhabdocline parkeri* and *Phyllosticta* sp. both infect Douglas fir needles, but the former grows in epidermal and hypodermal cells and the latter grows between mesophyll cells (Stone 1987, 1988). This shows that a leaf is a heterogeneous and complex environment for organisms the size of fungi, and may have implications for how endophytes interact. Unfortunately, few other studies have mapped endophytes at the level of specific tissues.

Endophyte and epiphyte communities of single leaves may differ. Of the five most frequent genera in coffee leaves, *Guignardia*, *Xylaria* and *Colletotrichum* were significantly more frequent as endophytes, while *Botryosphaeria* and *Pestalotia* were significantly more frequent as epiphytes (Santamaría and Bayman 2005). These differences are remarkable, considering that the two communities live less than a millimetre apart. Since most endophytes presumably arrive at the surface of the leaf, the epiphyte community probably plays a role in determining which new arrivals are successful at colonizing.

### 3. Site Specificity and Spatial Variation

Endophytes of a single host species may vary considerably from site to site. Environmental conditions, especially rainfall, influence infection frequency and community composition (Rodrigues 1994; see Carroll 1995, Wilson 2000 for reviews). On a local scale, differences among endophyte communities of a single host species may increase with distance, as was shown for cacao trees in Panama: similarity of endophyte communities among leaves decreased significantly as distance between trees increased (Arnold et al. 2001; Arnold and Herre 2003).

Individual leaves on a single tree may differ in endophyte community composition (Lodge et al. 1996; Gamboa and Bayman 2001). If the endophytes affect tree performance, then this variability may complicate other studies that assume uniformity within a single tree. This variability can be explained by the island theory of biogeography and a recent offshoot, the neutral theory of biodiversity (Hubbell 2001). If each leaf is viewed as an island, its endophyte community size and composition will be determined by its size, age and distance from source communities (Wilson 2000; Bayman 2006).

Assuming the source community is much larger and richer than the community of a single leaf, it is not surprising that individual leaves will vary.

Island biogeography has been used to explain distribution of epiphytic microorganisms on leaves, but not endophytes (Kinkel et al. 1989; Andrews and Harris 2000). Both epiphytes and endophytes would be good model systems for these questions because sample sizes can be very large. Spatial variation within and between tree populations, trees and leaves has been reviewed by Carroll (1995) and Wilson (2000).

## C. Effects of Tree Endophytes

### 1. Endophytes as Latent Pathogens or Misplaced Pathogens

The distinction between endophytes and pathogens is often blurred. Endophytes may be latent or opportunistic pathogens. Once established in plant tissues, they can remain quiescent until a change in the environment or a decline in host defences allows them to become pathogenic. Others may be pathogens that have colonized the wrong host and are thus unable to cause disease.

Endophytes and pathogens of western white pine have been compared using a phylogenetic approach (Ganley et al. 2004). Two thousand endophytes were identified by sequencing the nuclear ribosomal ITS and BLAST searches. It was found that 90% of endophytes belonged to a single family, *Rhytismataceae*, which also includes three major pathogens of western white pine. However, none of the endophytes were clearly conspecific with the pathogens. Rather, most were closely related to pathogens of other species of pines. This suggests that either they are specialized as endophytes (e.g. pathogens that have lost the ability to cause disease) or they are pathogens that have infected a non-host species and are therefore unable to cause disease. The authors found the first explanation more likely, based on host and endophyte phylogeography.

There is experimental evidence that an endophyte may be derived from a pathogen by loss of pathogenicity. A nonpathogenic *Colletotrichum* mutant capable of infecting plants was derived from a pathogen in the laboratory (Redman et al. 1999). However, many endophytes are still capable of producing phytotoxic secondary metabolites. In fact, endophytes produced more such compounds in vitro than pathogens of the same hosts (barley and larch) (Schulz et al. 1999). The authors

suggested that these endophytes were still capable of causing disease, but that the plant hosts were able to limit their growth.

## 2. Effects on Host Plants

Effects of endophytes are harder to demonstrate in trees than in herbaceous plants that can be more easily manipulated. For example, it is harder to generate endophyte-free leaves of trees to use as controls. However, effects of endophytes on leaves have been demonstrated. For example, endophytes of gorse and oak leaves promoted senescence (Fisher et al. 1986; Wilson 1993). Also, abscised oak leaves had lower levels of the endophyte *Ophiognomonia cryptica* than leaves still attached to the tree (Faeth and Hammon 1997). Endophytes probably influence coloration of senescent leaves as well.

Effects of endophytes on photosynthesis have been demonstrated, but are not always significant. For example, *Colletotrichum musae* in banana decreased photochemical capacity compared to endophyte-free plants (Rodrigues Costa Pinto et al. 2000).

Endophytes may help host plants survive heat and drought. In the herb *Dichanthelium lanuginosum*, plants with a *Curvularia* endophyte survived high soil temperature and water stress better than endophyte-free plants (Redman et al. 2002). This plant lives in areas where soil temperatures can reach 57 °C, so the presence of the endophyte may increase plant fitness, as with *Neotyphodium* in grasses (see Sect. IV.). Such symbioses are of increasing importance, since they might help plants adapt to global climate change (Rodriguez et al. 2004).

## 3. Effects on Insect Herbivores

Many studies have searched for effects of endophytes on insect herbivores of tree leaves. (In contrast, very few have looked at other types of herbivores.) Results have been interesting but inconsistent; few studies have conclusively demonstrated a negative effect on insects (see Carroll 1995). A survey of the literature on effects of tree leaf endophytes on insect herbivores found no consensus: of correlative studies, about equal numbers of studies found a negative correlation between endophytes and insects, a positive correlation, or no correlation (Wilson 2000). Of experimental studies, some found endophytes had negative effects on insects, inhibiting growth, survivorship or oviposition, but

others found no effect. Given the diverse nature of endophytes, the heterogeneity of endophyte–plant interactions, and the technical difficulties of these studies, such mixed results are not surprising.

## 4. Effects on Plant Pathogens

A protective effect of endophytes against plant pathogens has been demonstrated in cacao (Arnold et al. 2003). Endophyte-free seedlings were inoculated with a mixture of seven common endophytes of cacao. These seedlings and control seedlings were then inoculated with *Phytophthora infestans*, a serious pathogen of cacao. Endophyte-containing leaves had significantly smaller lesions and lower mortality than did control leaves. The difference was more dramatic in old leaves than young leaves, presumably because young leaves tend to contain more chemical defences against pathogens.

## VII. Endophytes of Tropical Plants

It is thought that endophytes of tropical plants are an important component of global fungal biodiversity (Hawksworth and Rossman 1997; Frolich and Hyde 1999). Why? First, the tropics are rich in undescribed plant species. Second, the ratio of plant-associated fungal species to plant species has been estimated at 6:1 in Britain. (Britain is used as a point of reference because its floras are well sampled.) If this ratio holds for the tropics, it would imply a vast number of undescribed endophyte species.

However, it is not clear what the fungus:plant ratio is. The plant species richness in the tropics may select against highly specific plant-associated microorganisms (May 1988), in which case the ratio would be lower than 6:1. This controversy has stimulated research on biodiversity and host specificity of tropical endophytes. Results are more mixed than for temperate trees: while some studies have found evidence of host specificity (Arnold et al. 2001), others studies have not (e.g. Bayman et al. 1997; Cannon and Simmons 2002).

Taxonomic studies support the view that many of the ‘missing fungi’ are endophytes, especially in tropical plants. Many new species of endophytes and saprophytes of palms have been described by Kevin Hyde and coworkers in Hong Kong (Hyde 2001). Based on the number of fungi they could identify from a single palm tree,

they argue that the 6:1 ratio of fungal species to plant species is too low. They propose a 33:1 ratio, which would greatly increase the extrapolated total number of fungal species (Frölich and Hyde 1999).

Comparisons of endophyte communities in neighbouring plant species usually show quantitative, rather than qualitative differences. For this reason, it may be more accurate to speak of endophyte host preferences, rather than host specificity (Lodge 1997), at least for tropical plants.

Specificity aside, the most frequent endophytes in the tropics often differ from those of temperate plants. *Xylaria* and its anamorphs have been isolated as endophytes in a wide range of tropical plants, including important crops, but in temperate areas they are saprotrophs and wood-rotters rather than endophytes (Rodrigues and Petrini 1997; Rogers 2000; Bayman 2006). *Xylaria* spp. are of special interest because they produce several types of bioactive secondary metabolites. Like many tropical endophytes, *Xylaria* is difficult to fruit in culture, making identification difficult. Studies on tropical endophytes often group sterile fungi together in morphospecies, on the basis of morphology in culture (Arnold et al. 2000; Gamboa and Bayman 2001). However, a comparison of morphospecies vs. DNA sequencing showed that morphospecies data are a fairly accurate way to estimate the number of species in a sample (Arnold et al. 2000). Other genera such as *Guignardia* fit the general pattern described here for *Xylaria*.

## VIII. Endophytes of Nonvascular Plants and Lichens

Endophyte floras of lichens are as rich as those of leaves of vascular plants, if not richer (Miadlikowska et al. 2005). Over 500 morphospecies were isolated from 17 lichen thalli (Petrini et al. 1990). Mosses and liverworts also harbour endophytes, including *Xylaria* (Davis et al. 2003). Endophytes of marine algae are common but largely unexplored (Stanley 1992). Some endophytes of marine algae are conspecific with terrestrial fungi, and it is not clear if they are accidental endophytes or adapted to the marine, endophytic habit. In the case of *Acremonium* isolated from the brown alga *Fucus serratus*, endophytic isolates formed a clade distinct from terrestrial isolates, suggesting that they are not accidental (Zuccaro et al. 2004).

## IX. Uses in Agriculture and Biotechnology

Endophytes have many potential uses in agriculture and biotechnology. This section focuses on three uses: manipulation of clavicipitaceous fungi of grasses for purposes of biocontrol; manipulation of other endophytes for biocontrol; and natural products discovery or bioprospecting.

### A. Clavicipitaceous Fungi of Grasses

Endophyte-infected turfgrasses are commercially available; their increased resistance to insects decreases the need for insecticides. For pasture grasses, the ideal biocontrol agent would produce alkaloids toxic to insects (loline and peramines) but not those toxic to mammals (ergot alkaloids and lolitrems; see Sect. IV.B. above). A *Neotyphodium* mutant unable to make ergovaline (a major ergot alkaloid) has been produced by knockout of a peptide synthetase gene (Panaccione et al. 2001). Such designer endophytes would have many advantages as biocontrol agents: they are stable, are not transmitted horizontally to non-target hosts, and are transmitted vertically to progeny (Clay 1989). Artificial inoculation of fungi into non-host plants is another way of producing endophyte-host combinations with desirable properties, though it tends to be hit-or-miss (Clay 1988). Clavicipitaceous endophytes have also been suggested as surrogates for genetic transformation of plants, since it is sometimes easier to transform the endophyte than the host (Clay 1988).

### B. Other Endophytes

Bacterial endophytes have been exploited more extensively than fungal endophytes for control of plant diseases (Azevedo et al. 2000). For example, *Bacillus subtilis* is effective for controlling mycotoxin production by *Fusarium* in corn, and its use has been patented (Bacon et al. 2001). *Curtobacterium flaccumfaciens* shows promise for control of citrus variegated chlorosis (Lacava et al. 2004), and genetically modified bacteria can be introduced into plants, allowing for inoculation with improved strains.

Nonetheless, inoculation with endophytic fungi can reduce the frequency and severity of disease, compared to endophyte-free plants. This technique has demonstrated a protective function

for endophytes against brown rust of wheat (*Puccinia triticina*, Dingle and McGee 2003), tan spot of wheat (*Pyrenophora tritici-repentis*, Istifadah and McGee 2006), and *Phytophthora infestans* on cacao (Arnold et al. 2003; see Sect. VI.C.4. above). The mechanisms of protection are not clear but could include antagonism between the endophyte and pathogen, parasitism of the pathogen by the endophyte, competition for nutrients, or induction of nonspecific host defences. Manipulation of endophytes and endophyte populations has great promise for control of plant diseases.

### C. Bioprospecting

Endophytes are a rich source of new natural products (Tan and Zou 2001). The best-known example is the production of taxol by a previously unknown endophyte of the Pacific yew, *Taxomyces andreae* (Stierle et al. 1993). Other fungal endophytes were later shown to produce taxol as well (Strobel and Daisy 2003). Until this discovery, the yew was the only organism known to produce taxol. These discoveries were exciting because at that time yew bark was the only known source of taxol, an important anti-cancer drug. Taxol is present in bark at such low concentrations that many trees had to be felled to meet demand. An organic semi-synthesis has replaced both bark and endophytes as the commercial source of taxol. However, there are still many undescribed endophytes capable of producing many new natural compounds. Recently isolated secondary metabolites from fungal endophytes are being explored as insecticides, antitumor drugs, antioxidants and immunosuppressants (Strobel and Daisy 2003).

### X. Conclusions

Many researchers who work with plants are completely unaware of endophytes, but endophytes may affect their results. Plant molecular systematists occasionally amplify and sequence endophyte genes, rather than plant genes – for example, in pines (Liston and Alvarez-Buylla 1995), bamboos (Zhang et al. 1997) and spruces (Camacho et al. 1997). In pines, phylogenetic trees based on the contaminant sequences reflected the predicted phylogeny of the pines (possibly as a result of coevolution), so contamination went undetected for some time. In other cases, sequences deposited

as plant sequences in databases have turned out to be fungal. Furthermore, variation in endophytes and their metabolites could be confounding factors in studies of plant breeding, plant physiology, plant ecology, and animal nutrition.

Endophytes embody the mystery and importance of the fungi. They are a taxonomically diverse group that have little in common, except a hidden way of life. They pose interesting questions on many levels, from natural products chemistry to community ecology, and lead us to address many fundamental questions about relationships.

**Acknowledgements.** I thank Miguel Angel Gamboa for contributions to this chapter.

### References

- Ahlholm JU, Helander M, Henriksson J, Metzler M, Saikkonen K (2002) Environmental conditions and host genotype direct genetic diversity of *Venturia ditricha*, a fungal endophyte of birch trees. *Evolution* 56:1566–1573
- Andrews JH, Harris RF (2000) The ecology and biogeography of microorganisms on plant surfaces. *Annu Rev Phytopathol* 38:145–180
- Arnold AE, Herre EA (2003) Canopy cover and leafage affect colonization by tropical fungal endophytes: ecological pattern and process in *Theobroma cacao* (Malvaceae). *Mycologia* 95:388–398
- Arnold AE, Maynard Z, Gilbert G, Coley PD, Kursar TA (2000) Are tropical fungal endophytes hyperdiverse? *Ecol Lett* 3:267–274
- Arnold AE, Maynard Z, Gilbert G (2001) Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. *Mycol Res* 105:1502–1507
- Arnold AE, Mejia L, Kyllo D, Rojas E, Maynard Z, Herre EA (2003) Fungal endophytes limit pathogen damage in a tropical tree. *Proc Natl Acad Sci USA* 100:15649–15654
- Arnold AE, Henk DA, Eells RL, Lutzoni F, Vilgalys R (2007) Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. *Mycologia* (in press)
- Azevedo JL, Maccheroni W Jr, Pereira JO, Araújo WL (2000) Endophytic microorganisms: a review on insect control and recent advances on tropical plants. *Electron J Biotechnol* <http://www.ejbiotechnology.info/content/vol3/issue1/full/4/index.html>
- Bacon CW (1985) A chemically defined medium for the growth and synthesis of ergot alkaloids by species of *Balansia*. *Mycologia* 77:418–423
- Bacon CW, Hill NS (1996) Symptomless grass endophytes: products of coevolutionary symbioses and their role in the ecological adaptations of infected grasses. In: Redlin SC, Carris LM (eds) *Endophytic fungi in grasses and woody plants*. APS Press, St Paul, MN, pp 155–178
- Bacon CW, Hinton DM (1996) Symptomless endophytic colonization of maize by *Fusarium moniliforme*. *Can J Bot* 74:1195–1202

- Bacon CW, White JF Jr (2000) Physiological adaptations in the evolution of endophytism in the Clavicipitaceae. In: Bacon CW, White JF Jr (eds) *Microbial endophytes*. Marcel Dekker, New York, pp 237–261
- Bacon CW, Porter JK, Robbins JD (1975) Toxicity and occurrence of *Balansia* on grasses from toxic fescue pastures. *Appl Environ Microbiol* 29:553–556
- Bacon CW, Porter JK, Robbins JD, Luttrell ES (1977) *Epichloë typhina* from toxic tall fescue grasses. *Appl Environ Microbiol* 34:576–581
- Bacon CW, Yates IE, Hinton DM, Meredith F (2001) Biological control of *Fusarium moniliforme* in maize. *Environ Health Perspect* 109:325–332
- Bailey JK, Deckert R, Schweitzer JA, Rehill BJ, Lindroth RL, Gehring C, Whitham TG (2005) Host-plant genetics affect hidden ecological players: links among *Populus*, condensed tannins and fungal endophyte infection. *Can J Bot* 83:356–362
- Bayman P (2006) Diversity, scale and variation of endophytic fungi in leaves of tropical plants. In: Bailey MJ, Lilley AK, Timms-Wilson TM, Spencer-Phillips PTN (eds) *Microbial ecology of aerial plant surfaces*. CABI, Wallingford, pp 37–50
- Bayman P, Lebrón LL, Tremblay RL, Lodge DJ (1997) Fungal endophytes in roots and leaves of *Lepanthes* (Orchidaceae). *New Phytol* 135:143–149
- Bayman P, Angulo-Sandoval P, Báez-Ortiz Z, Lodge DJ (1998) Distribution and dispersal of *Xylaria* endophytes in two tree species in Puerto Rico. *Mycol Res* 102:944–948
- Bills GF (1996) Isolation and analysis of endophytic fungal communities from woody plants. In: Redlin SC, Carris LM (eds) *Endophytic fungi in grasses and woody plants*. APS Press, St Paul, MN, pp 31–65
- Burnett J (2003) *Fungal populations and species*. Oxford University Press, Oxford
- Bush LP, Wilkinson HH, Schardl CL (1997) Bioprotective alkaloids of grass-fungal endophyte symbioses. *Plant Physiol* 114:1–7
- Camacho FJ, Gernandt DS, Liston A, Stone JK, Klein AS (1997) Endophytic fungal DNA, the source of contamination in spruce needle DNA. *Mol Ecol* 6:983–987
- Cannon PF, Simmons CM (2002) Diversity and host preference of leaf endophytic fungi in the Iwokrama Forest Reserve, Guyana. *Mycologia* 94:210–220
- Carroll G (1995) Forest endophytes: pattern and process. *Can J Bot* 73:S1316–S1324
- Carroll FE, Muller E, Sutton BC (1977) Preliminary studies on the incidence of needle endophytes in some European conifers. *Sydotia* 29:87–103
- Clay K (1988) Fungal endophytes of grasses: a defensive mutualism between plants and fungi. *Ecology* 69:10–16
- Clay K (1989) Clavicipitaceous endophytes of grasses: their potential as biocontrol agents. *Mycol Res* 92:1–12
- Clay K, Holah J (1999) Fungal endophyte symbiosis and plant diversity in successional fields. *Science* 285:1742–1744
- Clay K, Schardl C (2002) Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *Am Nat* 160:S99–S127
- Clay K, Marks S, Cheplick GP (1993) Effects of insect herbivory and fungal endophyte infection on competitive interactions among grasses. *Ecology* 74:1767–1777
- Clay K, Holah J, Rudgers JA (2005) Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition. *Proc Natl Acad Sci USA* 102:12465–12470
- Davis EC, Franklin JB, Shaw AJ, Vilgalys R (2003) Endophytic *Xylaria* (Xylariaceae) among liverworts and angiosperms: phylogenetics, distribution, and symbiosis. *Am J Bot* 90:1661–1667
- Dingle J, McGee PA (2003) Some endophytic fungi reduce the density of pustules of *Puccinia recondita* f. sp. *tritici* in wheat. *Mycol Res* 107:310–316
- Faeth SH (2002) Are endophytic fungi defensive plant mutualists? *Oikos* 98:25–36
- Faeth SH, Hammon KE (1997) Fungal endophytes in oak trees: long-term patterns of abundance and associations with leafminers. *Ecology* 78:810–819
- Fisher PJ, Anson AE, Petrini O (1986) Fungal endophytes in *Ulex europaeus* and *Ulex gallii*. *Trans Br Mycol Soc* 86:153–156
- Fisher PJ, Petrini LE, Sutton BC, Petrini O (1995) A study of fungal endophytes in leaves, stems and roots of *Gynoxis oleifolia* Muchler (Compositae) from Ecuador. *Nova Hedwigia* 60:589–594
- Frölich J, Hyde KD (1999) Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic? *Biodivers Conserv* 8:977–1004
- Gamboa MA, Bayman P (2001) Communities of endophytic fungi in leaves of a tropical timber tree (*Guarea guidonia*: Meliaceae). *Biotropica* 33:352–360
- Gamboa MA, Laureano S, Bayman P (2002) Does size matter? Estimating endophytic fungal diversity in leaf fragments. *Mycopathologia* 156:41–45
- Ganley RJ, Brunsfeld SJ, Newcombe G (2004) A community of unknown, endophytic fungi in western white pine. *Proc Natl Acad Sci USA* 101:10107–10112
- Hata K, Futai K (1996) Variation in fungal endophyte populations in needles of the genus *Pinus*. *Can J Bot* 74:103–114
- Hawksworth DL, Rossman AY (1997) Where are all the undescribed fungi? *Phytopathology* 87:888–891
- Hubbell SP (2001) *The unified neutral theory of biodiversity and biogeography*. Princeton University Press, Princeton, NJ
- Hyde KD (2001) Where are the missing fungi? Does Hong Kong have any answers? *Mycol Res* 105:1514–1518
- Istifadah N, McGee PA (2006) Endophytic *Chaetomium globosum* reduces development of tan spot in wheat caused by *Pyrenophora tritici-repentis*. *Australas Plant Pathol* 35:411–418
- Kinkel LL, Andrews JH, Nordheim EV (1989) Fungal immigration dynamics and community development on apple leaves. *Microbial Ecol* 18:45–58
- Koh S, Vicari M, Ball JP, Rakocevic T, Zaheer S, Hik DS, Bazyly DR (2006) Rapid detection of fungal endophytes in grasses for large-scale studies. *Funct Ecol* 20:736–741
- Kulda GA, Yates IE (2000) Evidence for *Fusarium* endophytes in cultivated and wild plants. In: Bacon CW, White JF Jr (eds) *Microbial endophytes*. Marcel Dekker, New York, pp 85–117
- Lacava PT, Araújo WL, Marcon J, Maccheroni W Jr, Azevedo JL (2004) Interaction between endophytic bacteria from citrus plants and the phytopathogenic bacteria *Xylella fastidiosa*, causal agent of citrus-variegated chlorosis. *Lett Appl Microbiol* 39:55–59

- Lane GA, Christensen MJ, Miles CO (2000) Coevolution of fungal endophytes with grasses: the significance of secondary metabolites. In: Bacon CW, White JF Jr (eds) *Microbial endophytes*. Marcel Dekker, New York, pp 341–388
- Latch GCM, Christensen MJ, Samuels GJ (1984) Five endophytes of *Lolium* and *Festuca* in New Zealand. *Mycotaxon* 20:535–550
- Lemons A, Clay K, Rudgers JA (2005) Connecting plant-microbial interactions above and belowground: a fungal endophyte affects decomposition. *Oecologia* 145:595–604
- Leuchtmann A, Clay K (1990) Isozyme variation in the *Acremonium/Epichloë* fungal endophyte complex. *Phytopathology* 80:1133–1139
- Liston A, Alvarez-Buylla E (1995) Internal transcribed spacer sequences of conifers: “There is a fungus among us”. *Inoculum* 46:26 [abstract]
- Lodge DJ (1997) Factors related to diversity of decomposer fungi in tropical forests. *Biodivers Conserv* 6:681–688
- Lodge DJ, Fisher PJ, Sutton BC (1996) Endophytic fungi of *Manilkara bidentata* leaves in Puerto Rico. *Mycologia* 88:733–738
- Marshall D, Tunali B, Nelson LR (1999) Occurrence of fungal endophytes in species of wild *Triticum*. *Crop Sci* 39:1507–1512
- Matossian MK (1989) Poisons of the past: molds, epidemics, and history. Yale University Press, London
- May RM (1988) How many species are there on Earth? *Science* 241:14419
- Miadlikowska J, Arnold AE, Higgins KL, Sarvate S, Gugger P, Way A, Hofstetter V, Lutzoni F (2005) Endolichenic fungi: random inhabitants or symbiotic partners? *Inoculum* 56:40 [abstract]
- Panaccione DG, Johnson RD, Wang J, Young CA, Damrongkool P, Scott B, Schardl CL (2001) Elimination of ergovaline from a grass-*Neotyphodium* endophyte symbiosis by genetic modification of the endophyte. *Proc Natl Acad Sci USA* 98:12820–12825
- Pendell D (2005) *Pharmako/gnosis: plant teachers and the poison path*. Mercury House, San Francisco, CA
- Petrini O (1991) Fungal endophytes of tree leaves. In: Andrews J, Hirano SS (eds) *Microbial ecology of leaves*. Springer, New York, pp 179–197
- Petrini O (1996) Ecological and physiological aspects of host-specificity in endophytic fungi. In: Redlin SC, Carris LM (eds) *Endophytic fungi in grasses and woody plants*. APS Press, St Paul, MN, pp 87–100
- Petrini O, Fisher PJ (1988) A comparative study of fungal endophytes in xylem and whole stems of *Pinus sylvestris* and *Fagus sylvatica*. *Trans Br Mycol Soc* 91:233–238
- Petrini O, Fisher PJ (1990) Occurrence of fungal endophytes in twigs of *Salix fragilis* and *Quercus robur*. *Mycol Res* 94:1077–1080
- Petrini O, Hake U, Dreyfuss MM (1990) An analysis of fungal communities from fruticose lichens. *Mycologia* 82:444–451
- Redman RS, Ranson JC, Rodriguez RJ (1999) Conversion of the pathogenic fungus *Colletotrichum magna* to a non-pathogenic, endophytic mutualist by gene disruption. *Mol Plant Microbe Interact* 12:969–975
- Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, Henson JM (2002) Thermotolerance conferred to plant host and fungal endophyte during mutualistic symbiosis. *Science* 298:1581
- Richardson MD (2000) Alkaloids of endophyte-infected grasses: defence chemicals or biological anomalies? In: Bacon CW, White JF Jr (eds) *Microbial endophytes*. Marcel Dekker, New York, pp 323–340
- Richardson MD, Logendra S (1997) Ergosterol as an indicator of endophyte biomass in grass seeds. *J Agric Food Chem* 45:3903–3907
- Rodrigues KF (1994) The foliar fungal endophytes of the Amazonian palm *Euterpe oleracea*. *Mycologia* 86:376–385
- Rodrigues KE, Petrini O (1997) Biodiversity of endophytic fungi in tropical regions. In: Hyde KD (ed) *Biodiversity of tropical microfungi*. Hong Kong University Press, Hong Kong, pp 57–69
- Rodrigues Costa Pinto LS, Azevedo JL, Pereira JO, Carneiro Vieira ML, Labate CA (2000) Symptomless infection of banana and maize by endophytic fungi impairs photosynthetic efficiency. *New Phytol* 147:609–615
- Rodriguez RJ, Redman RS, Henson JM (2004) The role of fungal symbioses in the adaptation of plants to high stress environments. *Mitigation Adaptation Strategies Global Change* 9:261–272
- Rogers JD (2000) Thoughts and musings on tropical Xylariaceae. *Mycol Res* 104:1412–1420
- Saikonen K, Faeth SH, Helander M, Sullivan TJ (1998) Fungal endophytes: a continuum of interactions with host plants. *Annu Rev Ecol Syst* 29:319–343
- Santamaría J, Bayman P (2005) Fungal epiphytes and endophytes of coffee leaves (*Coffea arabica*). *Microbial Ecol* 50:1–8
- Schardl CL, Clay K (1997) Evolution of mutualistic endophytes from plant pathogens. In: Carroll GC, Tudzynski P (eds) *The Mycota vol V part B. Plant relationships*. Springer, Berlin Heidelberg New York, pp 221–238
- Schardl CL, Wilkinson HH (2000) Hybridization and cospeciation hypotheses for the evolution of grass endophytes. In: Bacon CW, White JF Jr (eds) *Microbial endophytes*. Marcel Dekker, New York, pp 63–84
- Schardl CL, Leuchtmann A, Tsai HF, Collett MA, Watt DM, Scott DB (1994) Origin of a fungal symbiont of perennial ryegrass by interspecific hybridization of a mutualist with the ryegrass choke pathogen, *Epichloë typhina*. *Genetics* 136:1307–1317
- Schardl CL, Leuchtmann A, Chung KR, Penny D, Siegel MR (1997) Coevolution by common descent of fungal symbionts (*Epichloë* spp.) and grass hosts. *Mol Biol Evol* 14:133–143
- Schulz B, Boyle C (2003) The endophytic continuum. *Mycol Res* 109:661–686
- Schulz B, Wanke U, Draeger S, Aust HJ (1993) Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilisation methods. *Mycol Res* 97:1447–1450
- Schulz B, Rommert AK, Dammann U, Aust HJ, Strack D (1999) The endophyte-host interaction: a balanced antagonism? *Mycol Res* 103:275–283
- Schulz B, Boyle C, Draeger S, Römmert AK, Krohn K (2002) Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycol Res* 106:996–1004

- Schulz B, Boyle C, Sieber TN (eds) (2006) Microbial root endophytes. Springer, Berlin Heidelberg New York
- Stanley SJ (1992) Observations on the seasonal occurrence of marine endophytic and parasitic fungi. Can J Bot 70:2089–2096
- Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreaeae*, an endophytic fungus of Pacific yew. Science 260:214
- Stone JK (1987) Initiation and development of latent infections by *Rhabdochline parkeri* on Douglas-fir. Can J Bot 65:2614–2621
- Stone JK (1988) Fine structure of latent infections by *Rhabdochline parkeri* on Douglas-fir, with observations on uninfected epidermal cells. Can J Bot 66:45–54
- Stone JK, Bacon CW, White JF Jr (2000) An overview of endophytic microbes: endophytism defined. In: Bacon CW, White JF Jr (eds) Microbial endophytes. Marcel Dekker, New York, pp 3–29
- Stovall ME, Clay K (1988) The effect of the fungus, *Balansia cyperi* Edg., on growth and reproduction of purple nutgrass, *Cyperus rotundus* L. New Phytol 109:351–359
- Strobel G, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 67:491–502
- Tan RX, Zou WX (2001) Endophytes: a rich source of functional metabolites. Nat Prod Rep 18:448–459
- Toti L, Viret O, Chapela IH, Petrini O (1992) Differential attachment by conidia of the endophyte, *Discula umbrinella* (Berk & Br.) Morelet, to host and non-host surfaces. New Phytol 121:469–475
- Tsai H, Liu J, Staben C, Christensen MJ, Latch GCM, Siegel MR, Schardl CL (1994) Evolutionary diversification of fungal endophytes of tall fescue grass by hybridization with *Epichloë* species. Proc Natl Acad Sci USA 91:2542–2546
- van Wyk PS, Scholtz DJ, Marasas WFO (1988) Protection of maize seedlings by *Fusarium moniliforme* against infection by *Fusarium graminearum* in the soil. Plant Soil 107:251–257
- White JF Jr (1994) Taxonomic relationships among the members of the Balansieae (Clavicipitales). In: Bacon CW, White JF Jr (eds) Biotechnology of endophytic fungi of grasses. CRC Press, Boca Raton, FL, pp 3–20
- White JF Jr, Bultman TL (1987) Endophyte-host associations in forage grasses. VIII. Heterothallism in *Epichloë typhina*. Am J Bot 74:1716–1721
- White JF Jr, Reddy PV, Bacon CW (2000) Biotrophic endophytes of grasses: a systematic appraisal. In: Bacon CW, White JF Jr (eds) Microbial endophytes. Marcel Dekker, New York, pp 49–62
- Wilson D (1993) Fungal endophytes: out of sight but should not be out of mind. Oikos 68:379–384
- Wilson D (1995) Endophyte – the evolution of a term, and clarification of its use and definition. Oikos 73:274–276
- Wilson AD (1996) Resources and testing of endophyte-infected germplasm in National Grass Repository collections. In: Redlin SC, Carris LM (eds) Endophytic fungi in grasses and woody plants. APS Press, St Paul, MN, pp 179–195
- Wilson D (2000) Ecology of woody plant endophytes. In: Bacon CW, White JF Jr (eds) Microbial endophytes. Marcel Dekker, New York, pp 389–420
- Wilson D, Carroll GC (1994) Infection studies of *Discula quercina*, an endophyte of *Quercus garryana*. Mycologia 86:635–647
- Yang CH, Crowley DE, Borneman J, Keen NT (2001) Microbial phyllosphere populations are more complex than previously realized. Proc Natl Acad Sci USA 98:3889–3894
- Zhang W, Wendel JF, Clark LG (1997) Bamboozled again! Inadvertent isolation of fungal rDNA sequences from bamboos (Poaceae: Bambusoideae). Mol Phylogenet Evol 8:205–217
- Zuccaro A, Summerbell RC, Gams W, Schroers HJ, Mitchell JI (2004) A new *Acremonium* species associated with *Fucus* spp., and its affinity with a phylogenetically distinct marine *Emericellopsis* clade. Stud Mycol 50:283–297

---

# 14 Mycorrhizal Fungi: Their Habitats and Nutritional Strategies

M. GIRLANDA<sup>1</sup>, S. PEROTTO<sup>1</sup>, P. BONFANTE<sup>1</sup>

## CONTENTS

I. Introduction .....	229
II. Mycorrhizal Fungi Interact with both Soil and Plants .....	230
A. Fungal Diversity in Plant–Fungal Interactions .....	230
B. Fungal Diversity in Soil–Fungal Interactions .....	233
III. Nutritional Strategies of Mycorrhizal Fungi at the Soil–Root Interface .....	235
A. Mineral Nutrients .....	235
1. Utilisation of Phosphorus .....	235
a) AM Fungi .....	235
b) ECM Fungi .....	236
2. N Acquisition .....	237
a) ECM Fungi .....	237
b) AM Fungi .....	239
B. Carbon .....	239
1. Uptake of Plant-Derived C at the Symbiotic Interface by AM and ECM Fungi .....	240
2. Fate of Plant-Derived C in AM and ECM Mycelia .....	241
IV. At the Interface Between Several Host Plants: Common Mycelial Networks (CMNs), a Unifying Phenomenon in ECM and AM Fungi .....	242
A. Evidence for the Occurrence and Function of CMNs .....	242
B. What may be the Ecological Significance for the Host Plants? .....	243
1. Interactions Between Autotrophic Plants .....	243
2. Interactions Between Autotrophic and Heterotrophic Plants .....	244
C. ... and what Ecological Significance for the Mycorrhizal Fungi? .....	246
V. Conclusions .....	246
References .....	246

## I. Introduction

Mycorrhizal fungi are specialised root symbionts, engaging in intimate association with a great diversity of plants (Smith and Read 1997). The best

understood function of such fungi in the symbiosis is improvement of plant mineral nutrient acquisition, in exchange for some photosynthate, resulting in positive host growth responses. However, this symbiosis has a multifunctional character (Newsham et al. 1995) because mycorrhizal fungi may perform many other significant roles, including protection of the plant from biotic and abiotic stress, for instance, by altering host environmental tolerances to water deficit or pollutants, or reducing susceptibility to soil-borne pathogens.

Historically, the variety of mycorrhizal associations established between plants and fungi has been placed into seven categories (ectomycorrhiza, arbuscular endomycorrhiza, ericoid endomycorrhiza, orchid endomycorrhiza, ectendomycorrhiza, arbutoid mycorrhiza, monopodial mycorrhiza) based primarily on structural characteristics of the symbiotic interfaces and the taxonomic identity of the symbionts (Smith and Read 1997). Classification based on functional criteria has also been proposed, distinguishing ‘balanced’ and ‘exploitative’ mycorrhizal associations (Brundrett 2002, 2004). Although the association is generally assumed to be mutualistic, with bilateral nutrient exchange between the plant and fungal partners, host responses ranging from positive to negative may in fact be observed, with mycorrhizal fungi sometimes functioning as commensals, necrotrophs or antagonists of host or non-host plants, their roles varying during the lifespan of the association. Conversely, exploitation of the fungal partner by the host plant without any apparent benefit in return also occurs in peculiar mycorrhizal associations. Mycorrhizal outcomes are indeed conditional, depending upon complex interactions between environmental, developmental and genotypic factors, and the association is therefore best outlined as occupying a wider range on the symbiotic continuum, including commensalism and antagonism (Johnson et al. 1997; Brundrett 2002, 2004; Egger and Hibbett 2004).

<sup>1</sup> Department of Plant Biology, University of Torino, Viale PA Mattei 25, Torino 10125, Italy

The first periods of research on mycorrhiza have been dominated by a cataloguing, reductionist approach (Read 2002) that has allowed a basic framework to be established, the functioning of single pairs of symbionts in terms of nutrient exchange. Traditionally perceived as being restricted to the root niche, mycorrhizal fungi thrive in fact at the interface between two distinct habitats, a biotic (host roots) and abiotic (soil) habitat. Although already in 1973 the ‘mycorrhizosphere’ concept (the soil volume under the influence of mycorrhizal roots, extending beyond the area directly accessible to roots; Rambelli 1973) had recognized the key role of mycorrhizal fungi in linking plants to soil, only later has consideration of these fungi shifted from an almost exclusive focus on their relationship with the plant host to the realization of their environmental roles. Thus, greater attention has been paid to the functioning and importance of the extraradical mycelium of mycorrhizal fungi and its interactions with the biotic and abiotic environment and, due to innovative combinations of methods (for instance, root-free compartments coupled with isotope probing), the last decade has yielded valuable insights on the activities of this dynamic and functionally diverse component of the symbiosis (Leake et al. 2004a). At the same time, progress in molecular identification and characterization of fungal symbionts has revealed a much more diverse and complex picture of the multifunctional nature of mycorrhizal fungi. Martin et al. (2007) have reviewed the perspectives opened by environmental genomics, which combine community/population structure and function studies applying genomics. The fungal and plant genes, regulation of their expression, and biochemical pathways for nutrient exchange between symbiotic partners are now coming under intense study (see, e.g. Graham and Miller 2005), and will eventually be used to define the ecological nutritional role of the fungi. Another promising tool is stable isotope measurements, which rely on calculating ratios of heavy to light isotopes of key biological elements (e.g.  $^{13}\text{C}$ : $^{12}\text{C}$ ,  $^{15}\text{N}$ : $^{14}\text{N}$ ). This method has been increasingly applied to explore fungal functioning (Hobbie 2005). The study of natural abundance of stable isotopes in fungal structures, fungal-derived compounds and the environment, as well as the use of stable isotope tracers (the fate of which is followed into different ecosystem components, including fungi; cf. stable isotope probing; see, e.g. Dumont and Murrell 2005) will help us to link fungal identity to function, and to improve our un-

derstanding of fundamental ecosystem processes driven by fungi (Hobbie 2005).

Since these topics have been the objects of many recent reviews, the aim of this chapter is to shortly summarize the most relevant aspects of mycorrhizal fungi as a “tie that binds” (Read 1997) the host plant to the biotic and abiotic environment, with a focus on the two main types of mycorrhizal symbionts, i.e. arbuscular endomycorrhizal (AM) and ectomycorrhizal (ECM) fungi.

## II. Mycorrhizal Fungi Interact with both Soil and Plants

### A. Fungal Diversity in Plant–Fungal Interactions

The mycorrhizal habit has arisen multiple times in fungal evolution. Based on evidence from paleobotanical studies and the analysis of rDNA-based phylogenies, the most ancient mycorrhizal fungi are those forming arbuscular mycorrhizas. Their earliest fossil record dates back to the Ordovician, 460 million years ago (Redecker et al. 2000a), but molecular clock-based inferences estimate their origin at much earlier (Tehler et al. 2000; Berbee and Taylor 2001; Schüßler et al. 2001a, b), possibly as far back as between 1200 and 1400 Ma ago (Heckman et al. 2001). Such an ancient origin anticipates emergence of vascular terrestrial plants, and the thallus of bryophyte-like precursors had associations resembling modern arbuscular mycorrhizas even before roots evolved (reviewed in Brundrett 2002). Thus, the hypothesis has been put forward that symbiosis was a key factor in the colonization of land by plants (Taylor et al. 1995; Phipps and Taylor 1996). Modern arbuscular mycorrhizal (AM) fungi comprise a number of ancient lineages (Redecker et al. 2000b; Schüßler et al. 2001a; Schüßler 2002), which based on morphological, biochemical and ecological traits, can unequivocally be separated from all other major fungal groups. Based on 18S rDNA sequences, AM fungi appear to form a monophyletic (although weakly supported) group, which was given phylum status (Glomeromycota; Schüßler et al. 2001b). The Glomeromycota possibly diverged from the same common ancestor as the dikaryomycetes (Asco- and Basidiomycota; Gehrig et al. 1996; Tehler et al. 2000; Schüßler et al. 2001b). Information from different genomic loci, which is being increasingly

collected (see, e.g. Helgason et al. 2003; Corradi et al. 2004; Da Silva et al. 2006), is needed to provide further support to the phylum, and to clarify evolutionary patterns within the group. Information deriving from multigene trees will also improve our understanding of species boundaries in AM fungi. Based on a traditional, morphological species concept, the Glomeromycota are viewed as a species-poor group, with about 150 species recognized by conventional spore-based morphology (Morton and Benny 1990). Since these few morphospecies colonize about two-thirds of modern plants (Smith and Read 1997), arbuscular mycorrhizas appear to be not only the most ubiquitous and abundant terrestrial symbiosis but also nonspecific, with most AM fungi readily colonizing almost any susceptible plant species (Molina et al. 1992). The few AM fungi used in laboratory experiments indeed exhibit broad host spectra. However, several recent reports on the occurrence of natural root-colonising AM fungi, identified by their rDNA sequences in field-collected roots (Helgason et al. 1998, 2002; Husband et al. 2002; Vandenkoornhuyse et al. 2002, 2003; Öpik et al. 2003; Rosendahl and Stukenbrock 2004; Johnson et al. 2004; Scheublin et al. 2004; Öpik et al. 2006), have revealed challenging AM fungal richness and specificity (Sanders 2002, 2003, 2004). Whereas some plant species enter into specialised relationships with only a few AM fungi, others appear to associate with as many as 20 different AM fungi, thus hosting very diverse AM fungal communities that include currently unknown taxa. Possible relationships between plant responsiveness to mycorrhiza and their selectivity towards specific AM fungi remain to be elucidated (van der Heijden 2002), but it has been suggested that the AM fungal genetic diversity observed may be, at least partially, accounted for by diversification in functional traits, e.g. efficiency in functions such as mineral nutrient acquisition, soil binding, protection from pathogens, or sensitivity to abiotic and biotic environmental factors (Fitter 2005). AM fungal morphospecies or sequence groups have indeed been shown to be functionally diverse (Hart and Reader 2002a, b; Munkvold et al. 2004; van der Heijden et al. 2004; Avio et al. 2006; Koch et al. 2006).

On the other hand, the possibility that some of the sequence diversity found in these studies may be within – rather than among – individuals cannot be ruled out (Pawlowska and Taylor 2004; Rosendahl and Stukenbrock 2004). Uncommonly

polymorphic sequences of rDNA genes have been found within single AM fungal spores, which harbour hundreds of nuclei (Lloyd-MacGilp et al. 1996; Pawlowska 2005), in contrast with the situation found in other organisms, where homogenization of rDNA repeats is carried out through concerted evolution, a recombination-driven process (Dover 1982). No evidence for sexual reproduction is available either in ancestral or in modern AM fungi, leading to the suggestion that they may represent one of the oldest groups of clonally reproducing eukaryotic organisms on Earth (Judson and Normark 1996). The high intrasporal variability found in these fungi for normally conserved genes has provoked intense controversy over their genetic structure, and the processes governing it (reviewed in Pawlowska 2005). While one possible explanation involves distribution of the intrasporal rDNA variation among different, therefore genetically distinct nuclei (heterokaryosis; Kuhn et al. 2001; Bever and Wang 2005; Hijri and Sanders 2005), an alternative hypothesis envisages such variation as being contained in each individual nucleus (homokaryosis), with polyploid genome organization to accommodate intranuclear rDNA polymorphism and to buffer these apparently asexual organisms against the effects of accumulating mutations (Pawlowska and Taylor 2004, 2005). The reproductive mode (clonal vs. recombining) of AM fungi in nature remains elusive (Pawlowska 2005), and we still know too little about the basic genetics and genome organization of AM fungi to resolve these different scenarios. Approaches such as multiplex and global PCR amplification for individual spores (Gadkar and Rillig 2005; Stukenbrock and Rosendahl 2005), and genome sequencing of representative AM fungi such as *Glomus intraradices* (Martin et al. 2004) will hopefully provide new clues to widen our understanding of reproduction and genetics in these fungi.

In contrast with the situation of arbuscular mycorrhiza, established exclusively by a specific fungal group, the ectomycorrhizal (ECM), symbiosis involves a diverse range of fungi (a large number of homobasidiomycetes, some ascomycetes, and a handful of zygomycetes belonging in the genus *Endogone*; Smith and Read 1997). Unlike AM fungi, ECM fungi show conventional sexual mechanisms, with possibly a few exceptions such as the apparently sterile, widespread ascomycete *Cenococcum geophilum* (LoBuglio et al. 1996; Shinohara et al. 1999).

Most likely, the ECM symbiosis emerged long after the arbuscular association. Whereas divergence between Endogonales and Glomerales (the AM fungi) would have occurred at least about 600 Ma ago, well before any evidence of vascular plants, Asco- and Basidiomycota would have diverged from one another in the Palaeozoic, at least 500 Ma ago (Berbee and Taylor 2001). Although the only true ECM fossils are from recent Middle Eocene materials (LePage et al. 1997), it is likely that most ECM fungi (basidiomycetes) went through rapid diversification during the period of angiosperm radiation in the Cretaceous and in the Eocene-Oligocene transition (Bruns et al. 1998; Bruns and Shefferson 2004), perhaps assisting plant migration from the Tropics to the poorer temperate regions (Smith and Read 1997). The ectomycorrhizal assemblage is therefore clearly polyphyletic, since a common ECM precursor for all of these fungal groups should have evolved earlier than the Glomeromycota, and prior to land plant evolution (Bruns and Shefferson 2004). ECM ascomycetes belong in at least four distinct lineages (LoBuglio et al. 1996; Percudani et al. 1999), and several studies have provided evidence that the ECM habit has developed convergently in multiple lineages of homobasidiomycetes, evolving repeatedly from saprotrophic ancestors (Bruns et al. 1998; Hibbett et al. 2000; Moncalvo et al. 2000). Multiple reversion to a free-living, saprotrophic lifestyle has also been postulated, consistently with theoretical predictions that mutualism is inherently unstable (Hibbett et al. 2000). It is, however, unclear what ecological advantages, in terms of acquisition of open niches, reversion to saprotrophism would confer, since saprotrophic basidiomycetes had been well established prior to the evolution of the ECM habit (Bruns and Shefferson 2004). Whatever the directions of the evolutionary patterns, the polyphyletic origins of ECM fungi suggest considerable functional diversity in these fungi (Brundrett 2002), which is indeed indicated by physiological studies (e.g. Abuzinadah and Read 1986; Coleman et al. 1989; Arnebrant 1994; Keller 1996; Dickie et al. 1998; Read and Perez-Moreno 2003). Convergent evolution is likely to have occurred also on the plant side, since the ECM trait is present in unrelated groups such as the Pinaceae and at least 12 independent groups of angiosperms (Bruns and Shefferson 2004). Although specialists occur in both fungi and plants, the majority of ECM fungi and plants are capable of association with

multiple partners (e.g. Trappe 1962; Molina and Trappe 1982; Molina et al. 1992; Horton and Bruns 1998; Horton et al. 1999; Cullings et al. 2000). Such capability sets the stage for the establishment of impressively diverse ECM fungal communities, which are being described belowground by means of molecular identification (e.g. Dahlberg 2001; Horton and Bruns 2001; Taylor 2002; Chen and Cairney 2002; Dickie et al. 2002; Landeweert et al. 2003; Tedersoo et al. 2003; Anderson and Cairney 2004; Izzo et al. 2005; Koide et al. 2005; Saari et al. 2005; Genney et al. 2006; Toljander et al. 2006). Not only is sequence-based identification helping to dissect ECM community structure but it is also increasingly broadening the set of known ECM taxa even in well-investigated environments – such as temperate forests – where it has revealed unsuspected ECM capability in ascomycetous (see, e.g. Vrålstad et al. 2000; Tedersoo et al. 2006) and (hetero)basidiomycetous (Selosse et al. 2002a; Urban et al. 2003; Bidartondo et al. 2003) fungi.

An unexpected finding from these studies (sometimes coupled to resynthesis experiments) has been the recognition of both ecto- and endomycorrhizal competence in the same fungus. Simultaneous formation of ectomycorrhiza in tree hosts and ectoendomycorrhiza in plants of the subfamilies Arbutoideae and Monotropoideae (family Ericaceae) has long been known (Kamien-ski 1881; Björkman 1960; Smith and Read 1997), and often arbutoid and monotropoid ectoendomycorrhizae are, in fact, not considered as a distinct mycorrhizal category (Egger and Fortin 1988; Yu et al. 2001; Brundrett 2004). By contrast, fungi able to establish ericoid (i.e. *Cadophora* (syn. *Phialophora*) *finlandia*, a member of the so-called *Hymenoscyphus ericae* aggregate; Vrålstad et al. 2002) or orchid (i.e. *Sebacina* and *Tulasnella* species) endomycorrhiza have only recently been proven to be also able to develop typical ectomycorrhiza in trees (Selosse et al. 2002a, b; Taylor et al. 2002; Bidartondo et al. 2003, 2004; Urban et al. 2003; Villarreal-Ruiz et al. 2004; Weiss et al. 2004). This triangular relationship suggests that these fungi are part of a common guild (Vrålstad 2004), setting the intriguing scenario of mycobiont-mediated interplant interactions (see below). It also reveals fungal phenotypic plasticity in the production of distinct interfaces within different hosts, and suggests a decisive role of the host plant in determining the ecto- or endomycorrhizal character of the association. Symbiotic interfaces are complex compartments involved in nutrient

exchange between partners, and likely result from specific morphogenetic processes involving temporally and spatially controlled activity of genes and proteins. A fungus capable of developing such different interfaces as those characterizing ecto- vs. endomycorrhizae (cf. fungal hyphae remaining external to plant cell walls or breaching cell walls respectively, but remaining separated from the cell cytoplasm by a plant-derived membrane and an interfacial matrix; Peterson and Massicotte 2004) represents an interesting model to study the development of the structurally different ecto- and endomycorrhizal organs.

Recent molecular ecology investigations have also revealed a continuum between mycorrhizal fungi and other nonpathogenic root associates, the wide and diverse group of non-mycorrhizal root endophytes mostly referred to as DSE (dark septate endophytes) or DSM (dark sterile mycelia; Schulz and Boyle 2006). These conidial or sterile septate fungal endophytes, with known or likely affinities with distinct ascomycetous lineages, grow asymptotically in the roots either in the presence or absence of ecto- or endomycorrhizal mycobionts (Jumpponen and Trappe 1998; Sieber and Grünig 2006; Girlanda et al. 2006a). They may be as abundant and consistent as mycorrhizal fungi (Jumpponen and Trappe 1998; Girlanda et al. 2002; Mandyam and Jumpponen 2005) and, by virtue of such a constant association with roots, they can be qualified as true root symbionts (Schulz and Boyle 2006). Their peculiar structural interaction with roots, achieved by hyphae growing inter- and intracellularly in the root cortex and microsclerotia (Schulz 2006; Schulz and Boyle 2006), differs from the specialised interfaces that are diagnostic of mycorrhizae (Brundrett 2006). The fungal partner likely profits from a stable nutrient source and some protection from abiotic stresses and, although DSE exhibit variable life history strategies, several examples have been reported of nutritional and non-nutritional benefits to the host plant, thus indicating a possible mutualistic character of the association (Mandyam and Jumpponen 2005; Schulz 2006; Schulz and Boyle 2006). Fungi with endophytic behaviour in some plants are capable of forming typical mycorrhizal structures in other hosts (Bergero et al. 2000, 2003; Cairney 2006; Schulz 2006; Bayman and Otero 2006; Rice and Currah 2006; Girlanda et al. 2006a); on the other hand, classical mycorrhizal fungi may have endophytic phases in non-host plants, non-mycorrhizal plants, or plants with dual - AM

and ECM – mycorrhizal associations (Brundrett 2006), thus prompting contrasting interpretations of root endophytism. One view (Brundrett 2002, 2004, 2006) contrasts mycorrhizal and endophytic associations, and proposes the latter as possible evolutionary precursors of the former, hence postulating a continuum of association types, starting with endophytic occupation of roots by fungi and concluding with mycorrhizal associations with synchronised nutrient transfer. An alternative view includes root endophytes in a broader concept of symbiotic mycorrhizal associations, spanning from mutualism to parasitism (Johnson et al. 1997), and considers them as nonconventional mycorrhizal symbionts (Jumpponen 2001; Mandyam and Jumpponen 2005). Data available on DSE functions thus far are admittedly scant, and we still know little to correctly appreciate the degree of ecological and functional overlap between root endophytes and mycorrhizal symbionts. Whatever such a relationship, the possibility of multiple (endophytic and mycorrhizal) interaction for the same root coloniser reinforces the idea of plant control over the character of root associations (Girlanda et al. 2006a).

## B. Fungal Diversity in Soil-Fungal Interactions

The broad diversity of mycorrhizal fungi is mirrored by their interactions with the soil environment, engaged by the extraradical mycorrhizal mycelium (ERMM). Progress in our understanding of crucial processes such as transport of plant-derived carbon, production of extracellular enzymes, mineralization of nitrogen and phosphorus, uptake and transport of nutrients, weathering of minerals, soil aggregation, and interactions with other soil organisms has long been hindered by difficulties in observing and studying the dynamic and functionally diverse mycelial systems, extending beyond the colonised root into the opaque soil matrix without disturbing and destroying them. Only in the past decade have studies started to focus on the extent and functioning of ERMM in the field, and previously intractable aspects of biomass, structure and function of ERMM have been addressed with estimation of biochemical markers (e.g. ergosterol and specific phospholipid fatty acids as a signature for fungal membranes), and observations carried out in either mycorrhizal root-organ cultures (Fortin et al. 2002) or thin-layer soil microcosms

coupled with radioactive and stable isotope tracers (these methods, and the insights gained from their application, have been critically covered in several recent reviews, e.g. Olsson et al. 2002; Treseder 2004; Leake et al. 2004a; Cairney 2005; Rillig and Mumey 2006). Of particular importance has been the use of mesh barriers to provide root-free compartments into which mycorrhizal mycelium can grow, which have allowed us to distinguish the biomass and effects of ERMM from those of roots both in laboratory and, increasingly, in field experiments. Mesh bags buried in soil have, for instance, been used to estimate ERMM biomass in field settings and, although the difficulty in distinguishing ECM from saprotrophic mycelia remains a major problem with such systems, discrimination between the contributions of the two fungal components has been based on either analysis of the carbon isotopic composition of mycelia extracted from the mesh bags, comparison with 'closed' cores (i.e. PVC pipes that prevented the in-growth of mycorrhizal fungi), or the use of acid-washed sand to allow preferential development of mycorrhizal mycelia (see, e.g. Wallander et al. 2001; Höglberg and Höglberg 2002; Nilsson and Wallander 2003; Querejeta et al. 2003; Hendricks et al. 2006). Mesh-walled soil-filled cores, either left undisturbed or rotated to sever the hyphal connections with roots, have also been used to quantify in situ nutrient uptake by the ERMM (Schweiger et al. 1999; Schweiger and Jakobsen 2000; Johnson et al. 2001, 2002a, b), and transfer of C from the host plant.

Emerging from such experimental studies is the dynamic and functionally diverse nature of ERMM, its great contribution to soil biomass, carbon and nutrient fluxes, and global C, P and N cycles. ERMM, which may comprise up to 85% of the total mycorrhizal fungal biomass (Colpaert et al. 1992; Wallander et al. 2001), often constitutes 20–30% of total soil microbial biomass (e.g. Miller and Kling 2000; Olsson and Wilhemsson 2000; Höglberg and Höglberg 2002). By exceeding tens of metres per gram of soil (e.g. Miller et al. 1995; Ek 1997), it also provides extensive conduits for C and nutrient fluxes through the soil.

ERMM morphology and growth patterns as well as nutrient capturing mechanisms (see also Sect. III.A.) indicate different foraging strategies for AM and ECM fungi (Olsson et al. 2002). AM fungi form a uniformly distributed mycelium in soil, growing as aseptate thick-walled runner hyphae associated with lateral absorbing thin-walled

hyphae. In the patchy soil environment, AM hyphal proliferation has been observed near potential host roots as well as in response to several types of organic materials, although organic substrates are not generally exploited as a C source by the AM external mycelium (Green et al. 1999; Ravnskov et al. 1999). By contrast, the ECM usually form denser hyphal fronts than AM fungi, and they respond to both organic material and inorganic nutrients by increased growth. They exhibit the widest and most active set of enzymes for foraging on complex organic material, and also seem to be more active in the exudation of organic acids. Significant morphological differentiation is often observed in ECM species that produce the most extensive ERMM, with the formation of hydrophobic hyphal aggregates (hyphal cords or rhizomorphs) involved in long-distance transport, and hydrophilic distal mycelium involved in the uptake of soluble nutrients. Olsson et al. (2002) hypothesized that such differences between AM and ECM ERMM may reflect distinct evolutionary strategies, aimed at optimal search for potential new host roots in AM fungi, and at optimised nutrient capture in competition with other mycelia and translocation into the host roots in ECM fungi, possibly as a result of differences in size and persistence of energy supply by the host species.

Consideration of the functional importance of ERMM has also stimulated a new approach to the characterization of the diversity of natural mycorrhizal fungal communities. Some ECM produce little mycelium, apart from the mycorrhizal mantle, whereas others produce extensive external mycelial fans and strands. These differences most likely reflect distinct functional patterns of exploration and nutrient exploitation (Agerer 2001). Because they are relatively easy to culture, and amenable to laboratory manipulation, ECM fungi producing fairly extensive ERMM (such as *Suillus*, *Paxillus*, *Pisolithus*, *Rhizopogon* species) have been most popular for use in the microcosm studies on which so many of our notions of ECM functioning are based (Read and Perez-Moreno 2003; Leake et al. 2004a). Such species do not appear to dominate in the field, as judged by DNA-based characterization of ECM root tips (see, e.g. Horton and Bruns 2001), but they may be nevertheless functionally very important in carbon and nutrient cycles. Only recently have DNA-based identification methods been applied to ECM mycorrhizal mycelium in soil, and ERMM-based diversity data compared to the picture obtained from molecular identification

of mycorrhizal roots (e.g. Chen and Cairney 2002; Dickie et al. 2002; Landeweert et al. 2003; Wallander et al. 2003; Hunt et al. 2004; Koide et al. 2005; Kjöller 2006; Genney et al. 2006). These studies are opening a new window on the diversity and spatial distribution of ECM fungi in soil, having revealed, for instance, foraging at different spatial scales by different species, segregation of some mycorrhizas and corresponding ERMM in different soil layers, and unexpected high frequency of ERMM of smooth-type mycorrhizae (Genney et al. 2006). Just as the molecular identification of ECM roots has allowed us to shift from an 'aboveground' view based on surveys of sporocarps, which mostly reflects fruiting patterns, to a 'belowground' view providing information on the outcome of competition among the fungi for host roots (Horton and Bruns 2001), the 'mycelial' perspective on the ECM fungal community will be invaluable to establish functional differences between mycorrhizal fungi in the field (Leake et al. 2004a). This perspective appears to be more appropriate if we are primarily concerned with functions of mycelia such as water or nutrient uptake, or competition for physical space (Koide et al. 2005). The need to relate fungal diversity in the bulk soil to that in the plant roots clearly exists also in the case of AM fungi, and mycelium-based studies are eagerly awaited for these mycorrhizal symbionts (Johnson et al. 2005a).

### III. Nutritional Strategies of Mycorrhizal Fungi at the Soil–Root Interface

The extraradical mycorrhizal mycelium (ERMM) performs water and nutrient capture from the soil and is an important sink for host carbon. Within the root, carbohydrates and mineral nutrients are then transferred across interfaces that are bordered by plant and fungal plasma membranes (Smith and Smith 1990). Although the molecular mechanisms are not clearly understood, such bidirectional transfer is generally recognized as passive efflux into the symbiotic interfaces and then active uptake by the receiver organism (Smith and Read 1997).

Whereas details of molecular mechanisms for carbon and P exchange between AM symbionts have emerged rapidly from genetic studies on nutrient transport and metabolic pathways (Harri-

son 1999), dissection of host–fungal interactions for ECM fungi has been more problematic (Martin et al. 2001).

#### A. Mineral Nutrients

The success of mycorrhizal fungi in time and space mostly relies on the nutritional benefits they confer to their plant hosts: they take up phosphate (Pi) and other macronutrients as well as microelements and water from the soil, and transfer them to the plant. Despite the fact that mycorrhizal fungi play a crucial role in N, P and C cycling in ecosystems, their detailed function in nutrient dynamics is still unknown. Consistent with their wide genetic variability, mycorrhizal fungi differ in their functional abilities, thus offering distinct advantages to the host plant. Some fungi are particularly effective in scavenging organic N, and associate with plants for which acquisition of N is crucial (Peter et al. 2001); others are more effective at P uptake and transport. An important goal is therefore to develop approaches (i.e. the identification of  $^{13}\text{C}$  and  $^{15}\text{N}$  isotopic signatures) by which the functional abilities of the symbiotic fungal communities are assessed directly in the field.

##### 1. Utilisation of Phosphorus

Phosphate is an essential nutrient for all living cells, and plants have developed different strategies to ensure and enhance Pi acquisition (i.e. by modifying root architecture and extension to explore larger portions of soil, and by secreting organic acids or phosphatases that allow the release of bound Pi; Marschner 1995). The alternative adopted by most land plants is to use the Pi "catering service", as Requena (2006) has defined Pi uptake guaranteed by the symbiotic association with mycorrhizal fungi.

###### a) AM Fungi

Orthophosphate uptake is greatly enhanced during AM association (Smith and Gianinazzi-Pearson 1988). Current explanations for this increased efficiency are that (i) AM fungal mycelium explores the soil more efficiently than the root itself, and spreads beyond the phosphate depletion zone, (ii) it takes up phosphate from the soil and transfers it along the hyphae, and (iii) it delivers the nutrient to the plant root cells. Mycorrhizal plants can therefore acquire Pi either directly from the

soil through plant-specific phosphate transporters (PT) or through uptake and transport systems of the fungal symbiont. Both systems can work simultaneously but – at least for some plants – there is a preferential uptake via fungal hyphae. Recent data demonstrate how this fungus-mediated uptake requires the coordination of gene expression in both symbionts, such as up-/down-regulation of the high-affinity PT genes in both partners, and up-regulation of secreted acid phosphatase genes by the host (Ezawa et al. 2005).

In the extraradical mycelium, Pi is absorbed by active PTs operating at the fungus–soil interface (Harrison and Van Buuren 1995; Maldonado-Mendoza et al. 2001; Benedetto et al. 2005), and accumulates in the vacuoles of extraradical hyphae as inorganic polyphosphate (polyP), a linear polymer of Pi linked by high-energy bonds (Ezawa et al. 2004). Its synthesis and accumulation in AM hyphae occur rapidly but the molecular and biochemical bases of such processes are not yet known. Since polyP chains are accumulated in acidic compartments such as the vacuoles, they are supposedly transferred by means of a motile tubular vacuolar network (Uetake et al. 2002) in the intraradical compartment; Pi ions resulting from polyP hydrolysis are assumed to be released by membrane passive carriers into the periarbuscular space (Ezawa et al. 2002). Mycorrhiza-specific PTs possibly responsible for plant Pi uptake in arbuscule-containing cells have recently been characterized in many plants: potato, tomato, rice, maize, barley and *Medicago truncatula* (Rausch et al. 2001; Harrison et al. 2002; Paszkowski et al. 2002; Glassop et al. 2005; Nagy et al. 2005). The importance of such mycorrhiza-inducible PTs is indicated by the presence of multiple PTs in Solanaceae, suggesting a gene duplication event at least in these plants (Nagy et al. 2005). A combination of genetic, physiological and molecular approaches demonstrates how the mycorrhiza-inducible LePT3 and LePT4 are expressed also in mutant plants (*rmc*) where the block to fungal colonisation is not complete (Poulsen et al. 2005), suggesting that these PTs can be considered reliable markers for a functional Pi uptake. Fungal PT genes have been so far isolated exclusively from *Glomus* species (*G. versiforme*, *G. intraradices* and *G. mosseae*), and encode for high-affinity proton-coupled transporters (Harrison and Van Buuren 1995; Maldonado-Mendoza et al. 2001; Benedetto et al. 2005). As expected, they are predominantly detected in the extraradical mycelium, even though *G. mosseae* PT, differ-

ently from *G. intraradices* PT, shows a significant expression also in the mycorrhizal roots.

Taken as a whole, these data provide a rather complete picture of the molecular mechanisms that operate in order to guarantee the fungus-to-plant phosphate transfer. However, many questions, mostly concerning the way in which the fungus accumulates polyP and then releases it, as well as the cost paid by the plant for such symbiotic route, remain unanswered (Requena 2006).

### b) ECM Fungi

In the case of ectomycorrhiza, the host plants rely heavily on the fungus for Pi and N uptake because ECM fungi may be able to take up complex or immobilised forms of these nutrients that the root cannot absorb. Due to the production of extracellular acid and alkaline phosphomono- and phosphodi-esterases, phosphatases and phytases, ECM fungi can solubilize insoluble forms of Pi, such as Al and Ca phosphates and inositol hexaphosphates (Cumming and Weinstein 1990; Lapeyrie et al. 1991; Leake and Read 1997; Nilsson and Wallander 2003). The ability of some ECM fungi to mobilise Pi directly from minerals, through excretion of organic chelators such as organic acids (mainly citric and oxalic acids), has also become increasingly apparent (e.g. Wallander 2000a, b; van Breemen et al. 2000; Landeweert et al. 2001; Wallander et al. 2002). Weathering of rock substrates through physical and chemical mechanisms enables ectomycorrhizal plants to utilize essential nutrients from insoluble mineral sources, and plays a fundamental role in mineralogical processes, including pedogenesis and biogeochemical cycling of nutrients in forest systems (Burford et al. 2003). Once dissolved, Pi is taken up by ECM hyphae and then translocated to the host roots, its absorption and efflux being likely regulated by intracellular Pi and inorganic polyphosphates (polyP) pools. Although several low- and high-affinity Pi transporters have been identified in the genome sequence of *Laccaria bicolor* (F. Martin, unpublished data) and other symbiotic fungi, the molecular processes controlling Pi uptake in ECM fungi remain unknown.

Many ECM basidiomycetes also appear to have retained some of the saprophytic abilities of their decomposer relatives and, thereby, can access a range of organic sources of N and P from the soil, including partially decayed tree litter, pollen and nematodes (Read and Perez-Moreno

2003). Based on the efficiency of nutrient recovery from such detrital materials by ECM tree seedlings in soil microcosms, and estimates of the annual production of these nutrient sources in boreal forests, it has been suggested that as much as 15% of P and 12% of N supplied to trees in these forest ecosystems may come only from nutrient uptake from these sources by the ECM ERMM (Read and Perez-Moreno 2003).

## 2. N Acquisition

In addition to their function in P nutrition, AM and especially ECM fungi play a pivotal role in the uptake of N by plants.

While mycorrhiza research has emphasized the role of symbiosis in facilitating capture of mineral nutrients in ionic form, attention has shifted since the mid-1980s to analysing the mycorrhizal fungal abilities to release N and P from detrital materials of microbial and plant origins, which are the primary sources of these elements in terrestrial ecosystems (Read and Perez-Moreno 2003). The results obtained over the last decade have provided much support to the hypothesis (Read 1991) that, on a global scale, mycorrhizal fungi may be making significant contributions to ecosystem nutrient cycling (Read and Perez-Moreno 2003). In the forest ecosystems of Eurasia and North America, soil acidity, a high C:N ratio in litter material, and seasons marked by low temperatures and surface drought restrict nitrification and ammonification processes, and hence N availability to plants (Attewell and Adams 1993; Francis and Read 1994; Smith and Read 1997; Perez-Moreno and Read 2001a, b). The amount of free N in soil water is also limited by the tight association of ammonium to humic substances (Yu et al. 2002). In such environments, where accumulation of nutrient-poor recalcitrant litter and paucity of mobile N may threaten the fitness of autotrophs, selection has favoured associations with ECM fungal symbionts that are physiologically equipped – due to their well-developed saprotrophic capabilities – to facilitate capture of these elements from their locally predominant organic sources (Read 1991; Read and Perez-Moreno 2003; Read et al. 2004). The significance of AM colonisation in plant N acquisition, an aspect formerly overlooked (Smith and Read 1997), is now becoming increasingly clear in both agricultural and natural ecosystems (Johansen et al. 1996; Hodge et al. 2001). However, nitrogen available to both AM and ECM plants should not be

regarded as a single pool open to free competition, co-occurrence of ECM and AM fungi being allowed by exploitation of different niches in the same ecosystem.

### a) ECM Fungi

**Exploitation of organic N** ECM fungi have biochemical and physiological attributes that make them highly efficient in scavenging organic sources of N and P in surface soil horizons (Read et al. 2004). Being localised mostly in the upper, organically enriched soil horizon, the ECM ERMM is indeed ideally placed for nutrient acquisition from organic pools (Smith and Read 1997). ECM fungi may use soluble amino acids, and some of these fungi have highly developed proteolytic capabilities enabling them to directly access macromolecular N (Abuzinadah and Read 1989). Absorption of soluble or protein-derived amino acids by ECM fungi is mediated by high-affinity amino-acid uptake systems. Functional complementation of a yeast strain deficient in amino-acid transporters has allowed identification of amino-acid transporters from *A. muscaria* (Nehls et al. 1999) and *H. cylindrosporum* (Chalot et al. 2002; Wipf et al. 2002), which appear to be involved in both uptake of amino acids from the soil solution and retention of amino acids under N-deprivation conditions. While amino acids are readily assimilated by most ECM fungi and used as a N source, the fate of the carbon contained in these materials remains uncertain. Negligible transfer of glycine-derived  $^{13}\text{C}$  from roots to shoots in either mycorrhizal or non-mycorrhizal plants was found when seedlings of *Pinus sylvestris* were fed with double-labelled ( $^{15}\text{N}$  and  $^{13}\text{C}$ ) glycine as their sole N source, in contrast with the considerable quantities of  $^{15}\text{N}$  observed in the mycorrhizal tips, roots and shoots (Taylor et al. 2004). Recently, di- and tripeptide transporters were also isolated by yeast functional complementation using a *Hebeloma cylindrosporum* cDNA library, and were shown to mediate dipeptide uptake (Benjdia et al. 2006).

Whereas model proteins have been used to determine the potential of ECM fungi and their plant associates to mobilise N from polymeric macromolecules, investigations have subsequently focused on the use of detrital material of plant and animal origin (e.g. tree litter and nematode necromass), which presumably represent the major potential sources of N and P in the field (Read and Perez-Moreno 2003; Read et al. 2004). The ECM

ERMM efficiently colonise nutrient patches in the soil of thin-layer microcosms, and convert such material into more readily usable forms (reviewed in Leake et al. 2004a; Read et al. 2004). Many ECM fungi have been shown to deploy a broad spectrum of 'saprotrophic' enzyme activities, and to be directly involved both in the mobilisation of N and P from the organic polymers in which they are sequestered and in the degradation of the polymers themselves, thus challenging the conventional view that mycorrhizal fungi are fundamentally different from saprotrophs and lack the ability to directly participate in decomposition processes (Leake et al. 2004a). Such a situation sets the stage for intense competition, especially for the more labile N and P sources, between mycorrhizal and saprotrophic fungi in forest soils, in particular other large fungi such as the wood decomposers. Soil microcosm studies (reviewed in Leake et al. 2002, 2004a; Read et al. 2004; Cairney 2005) have revealed that mycelial systems of ECM and saprotrophic wood-decomposer fungi can be antagonistic to each other, both in growth and functioning. This antagonism can lead to significant transfer of nutrients between the two trophic groups upon interaction (Lindahl et al. 1999). Although symbiotic fungi may be favoured over saprotrophs by the host's continuous provision of carbon compounds, competition for lignocellulose seems unlikely, since actual ligninolytic capabilities are mostly modest in ECM fungi (Chen et al. 2001). Interactions between mycorrhizal fungi and saprotrophs may also be beneficial for nutrient acquisition. For instance, nutrient recovery by the ECM fungus *Pisolithus tinctorius*, from highly recalcitrant protein-tannin complexes that most ECM fungi tested to date have little or no enzymatic capacity to hydrolyse, is facilitated by pre-treatment of the complex by saprotrophs (Wu et al. 2003).

Through their participation in the removal of N and P from organic polymers, ECM fungi inevitably increase the C:N and C:P ratios of the residual materials and thus will contribute to C retention in soil, potentially strongly affecting the carbon source-sink relationships upon which global climate systems ultimately depend (Read and Perez-Moreno 2003).

#### **Scavenging of inorganic nitrogen ( $\text{NO}_3^-$ and $\text{NH}_4^+$ )**

AM and ECM mycelia are also effective scavengers of inorganic forms of N, either  $\text{NH}_4^+$  or  $\text{NO}_3^-$ .

ECM mycelia are particularly effective in the uptake of ammonium. Phyllosilicate weathering

has been demonstrated for *Pisolithus*, which gains access to  $\text{NH}_4^+$  and  $\text{Ca}_2^+$  ions trapped in between the vermiculite layer in pots probably by means of soluble exudates (Paris et al. 1995). Rapid and effective uptake of inorganic N by ECM has also been demonstrated in forest soils, as indicated by the lower  $\text{NH}_4^+$  and  $\text{NO}_3^-$  levels in a conifer forest with active mycorrhizal networks, compared to plots with plastic tubes inserted to exclude ECM hyphae (Nilsson and Wallander 2003).

The molecular bases of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  uptake have been investigated, and transporters and assimilating enzymes have been characterized in ECM fungi such as *Pisolithus*, *Laccaria* and *Tuber* (Jargeat et al. 2003; Javelle et al. 2003).  $\text{NH}_4^+$  absorbed by ERMM, or derived from  $\text{NO}_3^-$  reduction, may be rapidly assimilated into amino acids, mostly glutamine, and is subsequently incorporated into mycelial proteins or translocated to the host, glutamine being regarded also as the main translocation form in ECM mycelia (Martin and Botton 1993). In *Tuber*, the glutamine synthase gene is highly expressed both during fruitbody ripening (Lacourt et al. 2002) and N starvation (Montanini et al. 2003). Nitrate and high-affinity ammonium transporters are differentially expressed at least 1.5-fold in response to N deprivation (Montanini et al. 2006).

Incorporation of mineral N into amino acids entails a significant C cost for the fungus, as demonstrated by respiration increases observed following this nutrient uptake (e.g. Ek 1997). Recent studies in ECM and AM mycorrhiza have highlighted the potential for direct transfer of ammonia from fungal to plant cells at the symbiotic interface (Chalot et al. 2006). Together with the expression of putative ammonium exporter genes in the ectomycorrhizal fungus *Amanita muscaria*, expression analysis of a high-affinity ammonium importer from *Populus tremula* × *tremuloides* (*PttAMT1.2*) has revealed that *PttAMT1.2* expression is root-specific, is affected by N nutrition, and strongly increases in a N-independent manner upon ectomycorrhiza formation, thus suggesting that ammonium could act as a direct N source delivered by the fungus in ECM symbiosis (Selle et al. 2005). A close relationship could exist between C availability and the form of N transferred from an ectomycorrhizal fungus to the host plant: under C availability, the large flux of C compounds towards the fungal compartment would ensure the assimilation of inorganic N and the further release of organic nitrogen by the fungus whereas, under C depletion, the synthe-

sis of organic N might be strongly down-regulated, and ammonium would be transferred to the plant (Chalot et al. 2006).

### b) AM Fungi

Although Pi acquisition has received most attention, nitrogen is also an important element the uptake of which is improved during root colonisation by AM fungi. AM mycelia take up and assimilate  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and amino acids but the impact of such metabolisms on plant nutrition is still unknown (Smith and Read 1997).  $\text{NH}_4^+$  directly absorbed by ERMM mycelia, or derived from  $\text{NO}_3^-$  reduction, is rapidly assimilated through the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle found in ectomycorrhizal fungi (Martin et al. 2007). By measuring mRNA levels for key enzymes, such as the putative nicotinamide adenine dinucleotide (NAD)-dependent glutamate dehydrogenase (GDH) gene, Govindarajulu et al. (2005) found support that inorganic N assimilation also operates in the extraradical mycelium of AM fungi via the GS/GOGAT pathway. The mechanisms involved in N transport along the fungal hyphae, and then the delivery to the plant are still unclear. Very recently, Jin et al. (2005) supplied  $^{15}\text{N}$ - and/or  $^{13}\text{C}$ -labelled substrates to *Glomus intraradices* on Ri T-DNA-transformed carrot roots growing in two-compartment Petri dishes. They investigated the levels and labelling of free amino acids in the extra- and intraradical mycelium in mycorrhizal roots by gas chromatography/mass spectrometry and high-performance liquid chromatography, and demonstrated that arginine was the predominant free amino acid in the AM ERMM. The amino acid represents the major form of stored N, which is then transported to the intraradical mycelium. However,  $\text{NH}_4^+$  is the most likely form of N transferred to host cells following its generation from arginine breakdown (Jin et al. 2005).

Hodge et al. (2001) demonstrated that AMs also acquire nitrogen directly from organic material, a feature that – for a long time – has been considered characteristic of ECM fungi. There is clearly a need to understand the mechanisms involved in such organic N mobilisation by AM fungi, and to detect the molecular basis of such events. A putative amino-acid permease has been recently detected in *Glomus mosseae* (Cappellazzo et al., personal communication), its expression being exclusively located in the extraradical hyphae and N depen-

dent. As a further step, suppressive subtractive hybridization (SSH) and reverse Northern dot blot were performed on extraradical structures of *Glomus intraradices* grown on carrot hairy roots in order to isolate genes responsive to low versus high organic N concentrations. Genes involved in defence and signalling transduction pathways have been identified to be activated following a 48-h treatment with a 2  $\mu\text{M}$  amino-acid pool. This novel set of data indicates that *G. intraradices* extraradical structures perceive organic N limitation in the surrounding environment, leading to a specific response at transcriptional level, and supports the role of N as a signalling molecule in AM fungi (Cappellazzo et al. 2007).

## B. Carbon

In order to compete with other organisms in the patchy and changing soil environment, mycorrhizal fungi rely on a continuous supply of organic C from the plant. Whereas saprotrophic soil microorganisms are typically C limited – their C sources are spatially and temporally heterogeneous – mycorrhizal fungi gain direct access to a plant carbohydrate supply that is unparalleled amongst soil microbial populations in terms of both quality and quantity.

AM fungi receive all of their carbohydrates from the host plant. By contrast, many ECM fungi likely acquire C both via host photosynthesis and assimilation following the degradation of soil carbon polymers. As mentioned above, ECM fungi show saprotrophic capabilities that allow them to attack structural polymers in the organic detritus (reviewed in Leake et al. 2002; Read and Perez-Moreno 2003; Read et al. 2004; Lindahl et al. 2005), thus possibly reducing the amount of C needed from the host plant.

Quantitative estimates of the amount of C allocated to AM and ECM fungi by plants (reviewed in Leake et al. 2004a) range between about 2–20% of net fixation for AM and 7–30% for ECM fungi, although large variations may occur depending on individual plant–fungus combinations, amount of fungal biomass, and environmental conditions (Jakobsen 1999; Lerat et al. 2003a, b). Fungal C drain represents a cost of the symbiosis, but experiments with AM fungi indicate that the actual charge of mycorrhizal fungi to their host plants may be negligible because mycorrhizal colonisation can increase the rate of photosynthesis, alleviate shoot N and P

limitation, and cause a substantial increase in leaf area, a response that maximizes the area available for CO<sub>2</sub> assimilation (Wright et al. 1998a; Graham 2000; Jakobsen et al. 2002; Simard et al. 2002; Read and Perez-Moreno 2003). The fungus-mediated increase in photosynthesis does not appear to be an indirect result of enhanced mineral nutrition in mycorrhizal plants, and does not result in increased host biomass production (Wright et al. 1998a; Staddon et al. 1999; Jifon et al. 2002; Miller et al. 2002). Thus, it has been hypothesized that up-regulation of photosynthesis may be due to the carbohydrate demand by the mycorrhizal fungus (Wright et al. 1998b; Miller et al. 2002): mycorrhizal colonisation of the root, by increasing its sink strength, would stimulate the process of C assimilation.

In vivo NMR, coupled with <sup>13</sup>C labelling, radiorespirometry and transcriptome profiling studies, are beginning to unravel some details of C transport processes at the molecular level in mycorrhizal symbioses (Pfeffer et al. 1999, 2001; Bago et al. 2000, 2002; Lammers et al. 2001; Nehls et al. 2001; Ferrol et al. 2002a; Jakobsen et al. 2002; Simard et al. 2002). In both AM and ECM mycorrhiza, the primary transport carbohydrate in plants, sucrose, can be used as a C source by fungi only provided it is hydrolyzed by cell wall-bound invertases, with fungal absorption of glucose preceding that of fructose (Nehls et al. 2001; Simard et al. 2002).

### 1. Uptake of Plant-Derived C at the Symbiotic Interface by AM and ECM Fungi

Transfer of C from plant to fungus in an established mycorrhiza may be regulated by factors associated with either symbiont (Jakobsen et al. 2002).

In AM fungi, *in vitro* <sup>13</sup>C-NMR studies have shown that intraradical hyphae, but not external hyphae, import exogenously supplied hexoses, mainly in the form of glucose (Soliaman and Saito 1995; Shachar-Hill et al. 1995). While there is good evidence that arbuscules are the site of fungus-to-plant P transfer, the location of C transfer from plant to fungus remains unclear. The arbuscule could be the site of both hexose and phosphate transfer, and up-regulation of genes involved in sucrose hydrolysis (e.g. sucrose synthases and soluble acid invertase) has been taken as evidence that photoassimilate is directed towards arbuscule-containing cells (Blee and Anderson 1998; Ravnskov et al. 2003). However, there is as yet no evidence for a fungal hexose trans-

porter expressed on the arbuscular membrane (Smith et al. 2001). The plasma membranes of the arbuscular branches indeed lack ATPase staining, which might exclude these as a site for active sugar uptake by the fungus (which requires a proton gradient generated by an H<sup>+</sup>-ATPase). By contrast, the intercellular interfaces show high levels of ATPase activity, thus qualifying as the principal site of fungal hexose uptake (Gianinazzi-Pearson et al. 1991, 2000). Five ATPases have been identified from *G. mosseae* and two from *G. intraradices* (Ferrol et al. 2000, 2002b). Expression of one of these genes occurs in the intraradical phase of *G. intraradices*, indicating active transport processes in these hyphae, but the specific location of these transcripts remains to be determined. An alternative possibility involves passive uptake mediated by specific sugar carrier(s) in the fungal membrane, with the sugars concentration gradient between the fungus and the cortical apoplast being maintained by rapid conversion, in the fungus, to translocation and storage compounds (Bago et al. 2000; Jakobsen et al. 2002). The same mechanisms could operate not only in arbuscules but also in intercellular hyphae or coils.

In ECM, fungal uptake of hexoses has been suggested to take place at the plant-fungus interface (Hartig net) because the ECM represents a major carbohydrate sink. The mechanisms rely on the fungal conversion of glucose and fructose to fungal metabolites, thereby maintaining a concentration gradient from the plant to the ECM fungus, and allowing for continued fungal uptake of plant-derived hexoses (Simard et al. 2002). The poplar monosaccharide transporter gene *PttMST3.1* has been shown to be up-regulated by mycorrhiza formation, suggesting that root cells are able to compete with fungal hyphae for hexoses from the common apoplast during symbiosis (Grunze et al. 2004). Degenerate PCR primers from ESTs have been used to isolate a number of candidate genes for ECM fungal transport processes. In *Hebeloma cylindrosporum*, screening of genomic and cDNA libraries has revealed 38 tags corresponding to genes encoding carriers or channels that may play a role in nutrient uptake from the soil solution, from the host plant apoplast, or in transport towards the host plant (Chalot et al. 2002). So far, only one hexose transporter system (*AmMst1*) has been identified from an ECM fungus, *A. muscaria* (Nehls et al. 2000, 2001; Wiese et al. 2000). Increased monosaccharide concentrations at the fungus/plant interface have

been found to up-regulate expression of monosaccharide transporters in the fungus (Rieger et al. 1992; Nehls et al. 1998, 2001), resulting in enhanced photoassimilate sequestration by the fungus that will trigger additional photoassimilate supply by the host to the fungus, ultimately increasing rates of photosynthesis (reviewed in Simard et al. 2002). The results suggest active transport across fungal membranes, but the form of hexose transferred as well as the function and localization of transport proteins in mycorrhizal tissues are poorly known. Although it is assumed that most movement of C from root to fungus is by way of sugar transport (Smith and Read 1997), there is evidence of bidirectional movement of amino acids between symbionts (Lewis 1976), with carbon in amino acids usually moving from fungus to root by way of the glutamine/glutamate shuttle (see Sect. III.A.2.), but sometimes also moving as glutamine from root to fungus (Lewis 1976).

The identification of novel fungal transport genes in combination with the development of transformation techniques for mycorrhizal fungi (see, e.g. Harrier et al. 2002; Pardo et al. 2002; Marmeisse et al. 2004; Grimaldi et al. 2005; Kemppainen et al. 2005; Rodriguez-Tovar et al. 2005; Muller et al. 2006) will greatly advance our understanding of how carbon and nutrient release and transport events at the apoplastic interface are coordinated and controlled by the fungus and host.

## 2. Fate of Plant-Derived C in AM and ECM Mycelia

Once within the fungal mycelium, plant-derived C meets several fates. In both AM and ECM symbioses, sugars are used for translocation and storage synthesis, for the production of the large extraradical mycelium, and for support of the respiratory demands.

Knowledge of the nature of the substances transported from the intraradical to the extraradical mycelium has advanced considerably. What differentiates AM fungal C metabolism from ECM metabolism is the accumulation of lipids as the primary storage reserve. Lipids constitute by far the largest C pool in AM fungi, and are synthesised in the intraradical structures before being translocated to the external mycelium (Bago et al. 2000, 2002). A recent report confirms that fatty acid synthase activity of AM fungi is expressed exclusively in the intraradical mycelium (Trépanier et al. 2005). In AM intraradical mycelium, hexose

acquired from the root is converted into trehalose and then glycogen (Bago et al. 2000). Glycolysis, the TCA cycle, and the pentose phosphate pathways are functioning in the intraradical hyphae (Pfeffer et al. 1999), which synthesize and store large amounts of triacylglycerides (TAGS; Pfeffer et al. 1999; Bago et al. 2002). Since the extraradical mycelium is unable to take up exogenous hexose (Pfeffer et al. 1999), and no storage lipid production occurs in it, the carbohydrate requirement is likely met via translocation of TAGS from intraradical hyphae, and their conversion via the glyoxylate cycle (Bago et al. 2002). Glycogen is the other main C compound exported into the extraradical mycelium (Bago et al. 2003). Translocation of carbon compounds from intraradical to extraradical hyphae appears to occur in tubular vacuoles along the entire length of the hyphae (Ashford and Allaway 2002). The incorporation of mineral N into amino acids by the fungus, and their transfer to the plant would return some of the C skeletons originally supplied by the plant. These postulated metabolic and transfer pathways match those that are known to occur in ECM mycelium (Martin et al. 1998).

The amounts of C allocated to the extraradical AM and ECM mycelia by plants have been determined in some studies (reviewed in Leake et al. 2004a). Microcosm and field experiments with either AM (Johnson et al. 2002a, b) or ECM (Ek 1997; Bidartondo et al. 2001; Leake et al. 2001; Wu et al. 2002) plants have indicated that C allocation to the external fungal network may reach approx. 30% of net C fixation. Such experiments are corroborated by field studies showing that daily variations in the  $\delta^{13}\text{C}$  isotopic signature of recently fixed C in trees is closely followed, within a few days, by the isotope signature of root and rhizosphere respiration, which must include the external mycorrhizal mycelium (Ekblad and Högberg 2001).

Pulse labelling with  $^{13}\text{CO}_2$  combined with the use of root-excluding hyphal compartments (reviewed in Leake et al. 2004a) has indicated that there are two distinct fates for C within the extraradical mycorrhizal mycelium. A major part has short residence time in the hyphae, being rapidly translocated throughout the network and quickly respired. The fast turnover portion will also include some C that is transferred back to host plants (e.g. as amino acids generated from the hyphal uptake of inorganic N). Some of the C enters more stable, longer-term pools, for example, as structural components of the mycelium and hyphal walls. In

addition to their contribution to biomass, AM and ECM hyphae may produce metabolites, either secreted into the environment or contained in the hyphal walls (and then secondarily arriving in the soil via hyphal turnover and decomposition), such as the AM-secreted protein glomalin; accumulating in the soil, these have been implicated as an important mechanism in soil aggregation, and contribute a substantial amount of the more stable soil organic C (Rillig and Mumme 2006).

Given the considerable portion of soil microbial biomass accounted for by the mycorrhizal extraradical mycelium (see Sect. II.B.), this fungal network provides a major pathway for C movement from plants to soil, with an outstanding impact on C cycling at the ecosystem scale (Rillig 2004). Such a contribution is beginning to be recognized in quantitative terms (Leake et al. 2004a). In addition, through the extraradical mycorrhizal mycelium, carbon can also access other plants in the environment, thus setting the stage for the possibility for a reversal of C flux between plants and fungi.

#### **IV. At the Interface Between Several Host Plants: Common Mycelial Networks (CMNs), a Unifying Phenomenon in ECM and AM Fungi**

##### **A. Evidence for the Occurrence and Function of CMNs**

A breakthrough in mycorrhizal ecology has been the discovery that individual mycelia of either ECM or AM fungi can interlink different host plants, thus establishing common mycelial networks (CMNs, often referred to as the ‘wood-wide web’ in the case of ECM mycelia) that connect plants, belonging to the same or different species, and providing potential pathways for interplant transport of mineral nutrients and C (Simard et al. 2002; Simard and Durall 2004; Taylor 2006; Selosse et al. 2006).

Although occurrence of such networks had been postulated as early as 1881 (Kamienski 1881, 1882), only careful laboratory observations studies since 1969 (reviewed by Simard and Durall 2004) provided physical and functional evidence of hyphal interplant linkages. Transparent rhizoboxes (thin-layer soil microcosms) have been instrumental to visualize plant-to-plant mycelial

connections and to apply isotope probing and imaging (cf. the same techniques used to assess the transport of C and nutrients, growth, and spatial and temporal foraging activities of extraradical mycorrhizal mycelia; see Sect. II.B.). These techniques have demonstrated the concurrent existence of interplant mycorrhizal networks and transfer of elements from one root to another (e.g. McKendrick et al. 2000a; Wu et al. 2001).

In the field, direct visualization of CMNs is hampered by the opaque nature and structural complexity of soil as well as by intermingling of mycorrhizal and non-mycorrhizal mycelia. Sharing of mycorrhizal fungal species by different host plants growing together in the field, a feature of both ECM and AM symbioses (see Sect. II.A.), provides potential for the formation of common mycelial networks in plant communities (Kennedy et al. 2003), although only sharing of the same fungal individual (genotype, or genet) may allow establishment of CMNs. Many ECM fungi spread vegetatively below ground from root to root, and a single genet can colonise large areas. Genotyping using either basidiome and rhizomorphic tissue, or DNA extracted directly from soil (reviewed by Leake et al. 2004a; Cairney 2005), has indeed revealed that although genet size varies considerably between species and, for a given species, is strongly affected by factors such as disturbance and forest age, individual genotypes of some ECM taxa may span up to tens of metres, presumably by progressively infecting root tips, thus providing high potential for the formation on CMNs. Only recently, however, was the capability of a single fungal ECM genotype to colonise multiple host trees conclusively demonstrated by using microsatellite markers on ECM root tips to identify the genotypes of both the fungal symbiont and host tree (Lian et al. 2006). Similarly, data referred to Mediterranean dark septate endophytes (see Sect. II.A.) have indicated that the same DSE genotype may be shared between an ericoid and an ectomycorrhizal host (Bergero et al. 2000), setting the stage for a role of DSE in interactions between ecto- and endomycorrhizal plants (Girlanda et al. 2006a). Since it is likely that, in diverse plant communities, virtually all mycorrhiza-compatible plants will join common mycelial network but that not all plant species will share the same fungal partners (species or individuals of the same genet), complex overlapping host-fungus species interactions probably occur (Leake et al. 2004a). Formation of larger mycelia from the fusion of individual ones offers another mecha-

nism potentially enabling the formation of hyphal interplant linkages. The capability of anastomosis between self or genetically closely related hyphae (as determined by vegetative incompatibility systems; see, e.g. Esser 2006) has long been known for septate mycelia, and it has recently been shown to allow networks originating from plants of different species, genera and families to become interconnected (Giovannetti et al. 2004). Although burial of membranes on which the hyphae grow may reveal much about mycelial branching and hyphal anastomosis patterns (Balaz and Vosatka 2001), the actual extent of anastomosis formation in the field remains undetermined, as does the spread of individual AM mycelia in natural settings. Genet size studies also cannot ascertain whether these mycelia are actually continuous or have fragmented as ramets, i.e. spatially discontinuous mycelial units of the same genotype, a functionally important aspect because it will define, at least in part, the spatial limits within which the mycelia can effect movements of C and minerals through soil (Cairney 2005).

Evidence of a functional role of CMNs in nutrient transport and exchange derives from isotope labelling studies. These indicate that mineral resources such as N and, to a lesser extent, P move between plants via both AM and ECM CMNs (Simard et al. 2002; Tuffen et al. 2002; He et al. 2003, 2005, 2006). Carbon also moves within both ECM and AM CMNs, but actual net transport of C between interconnected plants remains controversial (Robinson and Fitter 1999; Simard et al. 2002; Simard and Durall 2004; Taylor 2006; Selosse et al. 2006). For AM, many laboratory studies have shown C movement from one mycorrhizal plant to another but none have demonstrated net transfer (Perry 1999), the labelled C remaining associated with the fungus. For instance, by using pulse labelling in a monoxenic culture system with *Ri* T-DNA-transformed carrot roots colonised by *Glomus intraradices* growing in subdivided Petri plates (to avoid CO<sub>2</sub> recycling and to eliminate label movement from one root system to another by diffusion), Pfeffer and colleagues (2004) found that carbon provided to a donor root as labelled glucose moved to recipient roots via a common AM fungal network, but remained in fungal compounds. Such data indicate that carbon taken up by the mycorrhizal fungus in association with one mycorrhizal root does not become nutritionally available to other roots. These findings on AM have raised serious doubts as to whether the detection of a carbon label in mycorrhizal roots represents a nutritionally meaningful transfer for

the plant or, indeed, any transfer at all (Fitter et al. 1998; Robinson and Fitter 1999; Wu et al. 2001; Pfeffer et al. 2004).

In contrast to the laboratory experiments, carbon exchange between plant species pairs sharing either ECM (Simard et al. 1997) and AM fungi (Lerat et al. 2002) has been demonstrated in field experiments, where labelled C pulsed to a donor plant was found in both the roots and shoots of the receiver plants. In these experiments, comparisons between mycorrhizal and non-mycorrhizal control plants (plants that did not tap into the CMN under examination) suggest that transfer occurred predominantly via the CMNs, as indicated by small or negligible transport to the incompatible control plants (Simard et al. 1997; Lerat et al. 2002; Simard and Durall 2004).

## B. What may be the Ecological Significance for the Host Plants?

### 1. Interactions Between Autotrophic Plants

The possibility of substantial interplant C transfer has led to the hypothesis that such transfers may influence interactions in plant communities, thus suggesting the need for a radical reappraisal of conventional concepts of competition in plant ecology (Leake et al. 2004a).

Field labelling experiments (Simard et al. 1997; Lerat et al. 2002) have shown that although bidirectional carbon transfer occurs between interconnected plants, a net C gain (up to 10%) can be found in one plant over that of its connected partner, thus confirming previous laboratory reports (Newman 1988; Miller and Allen 1992). Such a net flux has been suggested to be governed by a source–sink relationship, established, for example, by shading of one of the paired plants (Francis and Read 1984; Finlay and Read 1986; Simard et al. 1997). Similarly, one-way P transport has been shown to be regulated by fertilization of donor plants with phosphorus (e.g. Ritz and Newman 1986), and N has been observed to be transferred from N<sub>2</sub>-fixing mycorrhizal to non-N<sub>2</sub>-fixing mycorrhizal plants (He et al. 2003). Such unidirectional net transfers are likely to affect a range of interplant interactions, such as plant competition, plant diversity, plant community dynamics, and patterns of seedling establishment (Simard et al. 2002; Simard and Durall 2004; Taylor 2006; Selosse et al. 2006). A more even distribution of carbon among plants, as a result of belowground transfer, is likely to reduce dom-

inance and to allow co-occurrence of less aggressive species, thus contributing to maintain biodiversity, and therefore ecosystem productivity, stability and sustainability. Involvement in ecological succession has also been hypothesized (e.g. Horton et al. 1999), also in invasion by exotics outcompeting native species (e.g. Carey et al. 2004).

Resource sharing between adult high-canopy trees and understorey seedlings, which would counterbalance low light influx and inefficient photosynthesis by juveniles, has been proposed as a mechanism to explain the improved establishment frequently observed for ECM seedlings in close proximity to existing plants of the same and different species (Taylor 2006). Despite numerous studies on seedling establishment, no transfer of nutrients or C from nurse plants to seedlings has been demonstrated so far, and juvenile plants may actually benefit only indirectly from CMNs. For example, the costs to the seedling of supporting mycorrhizal fungi with carbon may be offset by the larger nurse plant. Even without carbon transfer, this situation would represent a clear benefit to seedlings, which would simply tap into a mycelial network already developed and supported by the surrounding vegetation (van der Heijden 2004; Simard and Durall 2004; Taylor 2006). This hypothesis can be corroborated by the observation that, based on natural variations in stable carbon isotope ratios ( $^{13}\text{C}$ ), overstorey trees appear to supply, partly or wholly, the nutrient-absorbing mycelia of their alleged competitors, the understorey trees (Högberg et al. 1999). Furthermore, CMNs could simply allow more rapid plant mycorrhizal colonisation, with benefits arising merely from increased mycorrhization, which may or may not require network connection (Simard and Durall 2004; Bever and Schultz 2005; Taylor 2006).

The extent to which interplant element transfers occur routinely between interlinked green plants in the field and over what scale, as well as their significance in plant ecology under field situations therefore remain contentious. Although there seems little doubt that there is considerable potential for their existence, the general significance of these networks in plant ecology remains unknown. Only fragmentary evidence is thus far available, experimental limitations (including the use of C isotope pulses, since the short application-to-harvest period of labels would not provide enough time for large amounts of carbon to move through fungi or for carbon to move from fungal tissues into plants; Carey et al. 2004) constrain the scope

of the conclusions reached and, in most cases, the importance of the observed CMN-mediated carbon transport for the total plant C budget has yet to be quantified. However, the outcome of any plant-fungus combination most often appears to be very context-dependent, and differences in CMN feedbacks among species or experimental conditions could be influenced by factors such as soil fertility, plant-fungus interactions (providing various carbon sink strengths), and the age of adult plants (Selosse et al. 2006). Other soil organisms, such as mycophagous collembolans and earthworms, may also significantly impact CMN-mediated transfers (Tuffen et al. 2002; Johnson et al. 2005b), thus providing another possible explanation for some of the contradictions reported between different nutrient transfer studies (Selosse et al. 2006). In autotrophic plant nutrition, therefore, the ecological relevance of CMN-derived C, at least under some circumstances (e.g. specific growth stages or environmental conditions), remains a fascinating possibility that cannot be entirely ruled out and, when coupled with differential effects of host plant-mycorrhizal fungus combinations, could contribute to our understanding of the influence of mycorrhizal fungi over plant competitive performances in mixed natural communities.

## 2. Interactions Between Autotrophic and Heterotrophic Plants

Whereas the evidence for nutritionally significant carbon movement from the fungus to the plant remains dubious in autotrophic plants communities, an undisputed example of interplant CMN-mediated net C transfer involves achlorophyllous plants. By lacking photosynthetic pigments, achlorophyllous plants behave as heterotrophs and deploy alternative strategies to acquire organic carbon for growth (Leake 1994). Some achlorophyllous species are direct epiparasites on photosynthetic plants whereas others acquire organic carbon through mycorrhizal association. Leake (1994) introduced the term "mycoheterotrophy" to describe this peculiar strategy, which relies on the ability of fungi to fetch organic compounds from the environment. This strategy arose repeatedly in angiosperm evolution, leading to over 400 mycoheterotrophic (MH) species in 87 genera derived from multiple independent lineages of green plants (Leake 1994). Recent studies on the identity and diversity of mycorrhizal fungi associated with MH plants belonging to distant

taxa have outlined common features and provided key information on their nutritional strategies (Taylor et al. 2002; Leake 2004; Bidartondo 2005). Most fungal partnerships of mycoheterotrophs are characterized by extreme specificity, contrasting with the generally broad specificity pattern of most mycorrhizal associations (see, e.g. Bidartondo and Bruns 2002; Taylor et al. 2002; Leake 2004). They involve ECM (Selosse et al. 2002a, b; Taylor et al. 2002) or AM fungi (Bidartondo et al. 2002) capable of forming simultaneous mycorrhiza on adjacent green plants that are the ultimate source of C for the mycoheterotroph. By taking carbon through their mycorrhizal partner, MH plants behave both as epiparasites on the green mycoheterotrophic (MH) plants that support the shared fungal partners with sugars (Leake 2004), and as "cheaters" towards the mycorrhizal fungus (Bidartondo 2005). The potential of mycorrhizal fungi for the formation of multiple interfaces with different neighbouring plants (see Sect. II.A.) is most likely the premise for the invasion of both ECM and AM by MH epiparasites (Leake 2004); in addition, the photosynthetic plant interacts only indirectly with the epiparasitic plant, and cannot select against the epiparasitic plant without selecting against a fungal mutualist (Bidartondo 2005). The significance of the unusually high specificity of MH plants, featuring exclusive associations with a single (or a narrow range of) fungal species even when surrounded by numerous potentially alternative fungal symbionts, is far from clear (Gardes 2002; Taylor et al. 2002; Leake 2004; Taylor 2004) but has been related to their strategy as cheating parasites, since parasitism tends to favour specificity by selection for resistance and evolutionary arms-races (Taylor and Bruns 1997; Taylor 2004).

Isotope-labelling experiments, coupled with morphological observations and biomass measurements in experimental microcosms with and without mycelial interconnections between autotrophs and mycoheterotrophs, have actually demonstrated C flow via a shared ECM mycelium from photosynthetic plants to either a *Monotropa*, MH orchid or liverwort (Björkman 1960; McKendrick et al. 2000a; Bidartondo et al. 2003). However, the physiological mechanism for the fungus-to-plant transfer remains unknown. Two distinct pathways for C transport from fungi to plants have been proposed: (i) the turnover of fungal biomass, particularly intracellular hyphae, an appealing option in cases such as the Orchidaceae where there is massive intracellular colonisation

followed by hyphal collapse; and (ii) the transfer of fungal compounds through ephemerally disrupted membranes at the fungal–plant interface, more likely in the Monotropoideae where there is minimal intracellular penetration (Bidartondo 2005). Whether digestion of intracellular hyphae by the plant may account for the entire amount of C transferred from the photosynthetic to MH plant, as well as for the rate of the process, remains uncertain. Carbon flux from AM fungi to MH plants also awaits demonstration (Bidartondo 2005).

Whatever the mechanism involved, MH plants exemplify both the ability of mycorrhizal networks to transport substantial quantities of C and nutrients and the potential importance of these networks for the control of species composition of natural communities (Leake et al. 2004a). Evidence has indeed been provided that in the absence of their critical fungal partners, MH plants fail to germinate and to establish (see, e.g. McKendrick et al. 2000b), and the distribution of a single mycorrhizal fungus can forcefully constrain the establishment and resulting distribution of a MH monotrope (Leake et al. 2004b).

Alongside plants that are obligately nonphotosynthetic throughout their lifetime, others exhibit an MH strategy during specific life stages. This is, for instance, the case of the early developmental stages of germination of most orchids, and the gametophyte stages of many ferns and lycopophytes, which can therefore be defined as 'initially MH' plants (Leake et al. 2004a). Some green orchids also exhibit prolonged stages of adult dormancy, consisting of periods of one or more years where no sprouts are produced and no photosynthesis occurs. A recent study on the genus *Cypripedium*, which exhibits this phenomenon, indicates that mycorrhizal specificity towards Tulasnellaceae is fairly high (Shefferson et al. 2005). Studies of natural isotope abundance have indicated that fully MH plants have distinctive stable isotope signatures (enrichment in both  $^{13}\text{C}$  and  $^{15}\text{N}$ ) relative to co-occurring autotrophic plants (Gebauer and Meyer 2003; Trudell et al. 2003), most similar to the signatures of ECM fungi. Such a distinctive heavy-isotope enrichment has also been found in some forest green-leaved orchids previously assumed to be autotrophs, thus indicating that these plants, although photosynthetic, depend at least in part upon fungal C (Gebauer and Meyer 2003). The term 'mixotrophy' has been proposed (Selosse et al. 2004) to indicate this dual (photosynthetic and mycoheterotrophic) strategy in the or-

chid genera *Cephalanthera*, *Epipactis* and *Limodorum*, which accompanies ectomycorrhizal association and, hence, connection to a CMN linking to surrounding trees (Bidartondo et al. 2004; Selosse et al. 2004; Julou et al. 2005; Girlanda et al. 2006b). Green forest orchids that grow in deeply shaded forest habitats likely experience reduced photosynthetic efficiency in a shaded environment. For *C. damasonium*, the amount of carbon obtained from fungi was estimated (by variation in stable isotope abundance) to span a quite wide range, from ca. 85% (Gebauer and Meyer 2003) to 30–50% (Bidartondo et al. 2004; Julou et al. 2005), suggesting a strong influence of the environment on the relative contribution of autotrophic photosynthesis and heterotrophism to the carbon metabolism of mixotrophic orchids. These findings parallel a variable extent of mycobiont specificity in green forest orchids (Bidartondo et al. 2004; McCormick et al. 2004; Julou et al. 2005), implying that the degree of specificity to a given fungal symbiont may mirror the degree of heterotrophy and nutritional dependency for carbon, possibly as a function of environmental conditions. By supplying mycobiont extraradical mycelium with  $^{13}\text{C}$ -labelled glycine and shoots with  $^{14}\text{CO}_2$ , bidirectional transfer of C was recently demonstrated between the green forest orchid *Goodyera repens* and its fungal symbiont *Ceratobasidium cornigerum* (Cameron et al. 2006).

Taken together, these findings suggest that whilst achlorophyllous plants are necessarily bound to heterotrophy, and mycoheterotrophy likely represents an extreme ‘cheating’ type of mycorrhiza (Bidartondo et al. 2002), a continuum of C transfer from fungi to plants possibly exists, with some chlorophyllous plants obtaining part of their carbon heterotrophically. Although evidence for interplant C transfer between fully autotrophic species is not that compelling as yet, the multiple evolutionary origins of mycoheterotrophy as well as the occurrence of mixotrophic plants occupying an intermediate position in a likely gradient between full autotrophy and full mycoheterotrophy suggest that plant-to-plant carbon transfer via mycorrhizal fungi may be a much more universal phenomenon than usually recognized (Leake et al. 2004a).

### C. ... and what Ecological Significance for the Mycorrhizal Fungi?

Within this framework, hardly anything is known about the significance of CMNs in fungal ecology, a situation that reflects our limited knowledge

of the biology of these microorganisms. Even in well-characterized situations such as those involving fully MH plants, the characteristics of fungi targeted by successful mycoheterotrophs remain a matter of speculation (Bidartondo 2005), as do possible benefits derived by the fungus from the association (e.g. see Bidartondo et al. 2000). The great majority of the work on CMNs has focused on the benefits and consequences for the aboveground plant community (Fitter et al. 1999), while the ‘myco-centric’ perspective on CMNs (i.e. an inverted mycorrhizosphere, or networks of fungi linked by shared trees) has been neglected in terrestrial ecology, largely biased towards a ‘phytocentric’ view (Selosse et al. 2006).

## V. Conclusions

In the last decade, application of modern techniques and innovative combinations of methodological approaches has yielded valuable new insights on the roles played by mycorrhizal fungi in natural communities, and the mechanisms underlying such fungal processes. A picture has emerged of greater than expected plasticity of these symbiotic organisms, which have therefore multiple potential of impacting their hosts from the molecular to the ecosystem level. By facing the challenge posed by natural complexity, mycorrhizal research has thus featured substantial advances towards ecological relevance (Read 2002). Due to their key position at the plant–soil interface, mycorrhizal fungi must be taken into account in the study of ecosystem impacts of multifactorial global changes including elevated atmospheric gas concentrations, increased atmospheric deposition of nutrients such as nitrogen, climatic variations, and invasions by exotic species (see, e.g. Rillig et al. 2002). Further efforts to place the mycorrhizal function within this broader context will be invaluable in predicting the future impacts of global environmental change on terrestrial ecosystems.

## References

- Abuzinadah RA, Read DJ (1986) The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. I. Utilization of peptides and proteins by ectomycorrhizal fungi. *New Phytol* 103:481–493
- Abuzinadah RA, Read DJ (1989) The roles of proteins in the nitrogen nutrition of ectomycorrhizal plants. 4. The utilization of peptides by birch (*Betula pendula* L.) infected with different mycorrhizal fungi. *New Phytol* 112:55

- Agerer R (2001) Exploration types of ectomycorrhizae – A proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. *Mycorrhiza* 11:107–114
- Anderson IC, Cairney JWG (2004) Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. *Environ Microbiol* 6(8):769–779
- Arnebrant K (1994) Nitrogen amendments reduce the growth of extramatrical ectomycorrhizal mycelium. *Mycorrhiza* 5:7–15
- Ashford AE, Allaway WG (2002) The role of the motile tubular vacuole system in mycorrhizal fungi. *Plant Soil* 244:177–187
- Attiwill PM, Adams MA (1993) Nutrient cycling in forests. *New Phytol* 124:561–582
- Avio L, Pellegrino E, Bonari E, Giovannetti M (2006) Functional diversity of arbuscular mycorrhizal fungal isolates in relation to extraradical mycelial networks. *New Phytol* 172:347–357
- Bago B, Pfeffer PE, Shachar-Hill Y (2000) Carbon metabolism and transport in arbuscular mycorrhizas. *Plant Physiol* 124:949–957
- Bago B, Zipfel W, Williams RM, Jun J, Arreola R, Lambers PJ, Pfeffer PE, Shachar-Hill Y (2002) Translocation and utilization of fungal storage lipid in the arbuscular mycorrhizal symbiosis. *Plant Physiol* 128:108–124
- Bago B, Pfeffer PE, Abubaker J, Jun J, Allen JW, Brouillette J, Douds DD, Lambers PJ, Shachar-Hill Y (2003) Carbon export from arbuscular mycorrhizal roots involves the translocation of carbohydrate as well as lipid. *Plant Physiol* 131(3):1496–1507
- Balaz M, Vosatka (2001) A novel inserted membrane technique for studies of mycorrhizal extraradical mycelium. *Mycorrhiza* 11:291
- Bayman P, Otero JT (2006) Microbial endophytes of orchid roots. In: Schulz BJE, Boyle CJC, Sieber TN (eds) *Microbial root endophytes*. Springer, Berlin Heidelberg New York, pp 153–178
- Benedetto A, Magurno F, Bonfante P, Lanfranco L (2005) Expression profiles of a phosphate transporter gene (*GmosPT*) from the endomycorrhizal fungus *Glomus mosseae*. *Mycorrhiza* 15(8):620–627
- Benjdia M, Rikirsch E, Muller T, Morel M, Corratge C, Zimmermann S, Chalot M, Frommer WB, Wipf D (2006) Peptide uptake in the ectomycorrhizal fungus *Hebeloma cylindrosporum*: characterization of two di- and tripeptide transporters (*HcPTR2A* and *B*). *New Phytol* 170(2):401–410
- Berbee ML, Taylor JW (2001) Fungal molecular evolution: gene trees and geologic time. In: McLaughlin DJ, McLaughlin EG, Lemke PA (eds) *The Mycota*, vol VII part B. Systematics and evolution. Springer, Berlin Heidelberg New York, pp 229–245
- Bergero R, Perotto S, Girlanda M, Vidano G, Luppi AM (2000) Ericoid mycorrhizal fungi are common root associates of a Mediterranean ectomycorrhizal plant (*Quercus ilex*). *Mol Ecol* 9:1639–1649
- Bergero R, Girlanda M, Bello F, Luppi AM, Perotto S (2003) Soil persistence and biodiversity of ericoid mycorrhizal fungi in the absence of the host plant in a Mediterranean ecosystem. *Mycorrhiza* 13:69–75
- Bever JD, Schultz PA (2005) Mechanisms of arbuscular mycorrhizal mediation of plant-plant interactions. In: Dighton J, White JF, Oudemans P (eds) *The fungal community. Its organization and role in the ecosystem*, 3rd edn. CRC Press, Taylor & Francis Group, Boca Raton, FL, pp 443–460
- Bever JD, Wang M (2005) Arbuscular mycorrhizal fungi – Hyphal fusion and multigenomic structure. *Nature* 433(7022):E3–E4
- Bidartondo MI (2005) The evolutionary ecology of myco-heterotrophy. *New Phytol* 167:335–352
- Bidartondo MI, Bruns TD (2002) Fine-level mycorrhizal specificity in the Monotropoideae (Ericaceae): specificity for fungal species groups. *Mol Ecol* 11:557–569
- Bidartondo MI, Kretzer AM, Pine EM, Bruns TD (2000) High root concentration and uneven ectomycorrhizal diversity near *Sarcodes sanguinea* (Ericaceae): a cheater that stimulates its victims? *Am J Bot* 87:1783–1788
- Bidartondo MI, Ek H, Wallander H, Soderstrom B (2001) Do nutrient additions alter carbon sink strength of ectomycorrhizal fungi? *New Phytol* 151(2):543–550
- Bidartondo MI, Redecker D, Hijri I, Wiemken A, Bruns TD, Dominguez L, Sérsic A, Leake JR, Read DJ (2002) Epiparasitic plants specialized on arbuscular mycorrhizal fungi. *Nature* 419:389–392
- Bidartondo MI, Bruns TD, Weiß M, Sérgio S, Read DJ (2003) Specialized cheating of the ectomycorrhizal symbiosis by an epiparasitic liverwort. *Proc R Soc Lond B* 270:835–842
- Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ (2004) Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proc R Soc Lond B* 271:1799–1806
- Björkman E (1960) *Monotropa hypopitys* L. – an epiparasite on tree roots. *Physiol Plantarum* 13:308–327
- Blee KA, Anderson AJ (1998) Regulation of arbuscule formation by carbon in the plant. *Plant J* 16:523–530
- Brundrett MC (2002) Coevolution of roots and mycorrhizas of land plants. *New Phytol* 154:275–304
- Brundrett MC (2004) Diversity and classification of mycorrhizal associations. *Biol Rev* 79:473–495
- Brundrett MC (2006) Understanding the roles of multi-functional mycorrhizal and endophytic fungi. In: Schulz BJE, Boyle CJC, Sieber TN (eds) *Microbial root endophytes*. Springer, Berlin Heidelberg New York, pp 281–298
- Bruns TD, Shefferson RP (2004) Evolutionary studies of ectomycorrhizal fungi: recent advances and future directions. *Can J Bot* 82(8):1122–1132
- Bruns TD, Szaro TM, Gardes M, Cullings KW, Pan JJ, Taylor DL, Horton TR, Kretzer A, Garbelotto M, Li Y (1998) A sequence database for the identification of ectomycorrhizal basidiomycetes by phylogenetic analysis. *Mol Ecol* 7:257–272
- Burford EP, Fomina M, Gadd GM (2003) Fungal involvement in bioweathering and biotransformation of rocks and minerals. *Mineral Mag* 67(6):1127–1155
- Cairney JWG (2005) Basidiomycete mycelia in forest soils: dimensions, dynamics and roles in nutrient distribution. *Mycol Res* 109:7–20

- Cairney JWG (2006) Mycorrhizal and endophytic fungi of epacrids (Ericaceae). In: Schulz BJE, Boyle CJC, Sieber TN (eds) Microbial root endophytes. Springer, Berlin Heidelberg New York, pp 247–260
- Cameron DD, Leake JR, Read DJ (2006) Mutualistic mycorrhiza in orchids: evidence from plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. *New Phytol* 171:405–416
- Cappellazzo G, Lanfranco L, Bonfante P (2007) A limiting source of organic nitrogen induces specific transcriptional responses in the extraradical structures of the endomycorrhizal fungus *Glomus intraradices*. *Curr Genet* 51(1):59–70
- Carey EV, Marilyn J, Marler MJ, Callaway RM (2004) Mycorrhizae transfer carbon from a native grass to an invasive weed: evidence from stable isotopes and physiology. *Plant Ecol* 172:133–141
- Chalot M, Javelle A, Blaudéz D, Lambilliotte R, Cooke R, Sentenac H, Wipf D, Botton B (2002) An update on nutrient transport processes in ectomycorrhizas. *Plant Soil* 244(1/2):165–175
- Chalot M, Blaudéz D, Brun A (2006) Ammonia: a candidate for nitrogen transfer at the mycorrhizal interface. *Trends Plant Sci* 11(6):263–266
- Chen DM, Cairney JWG (2002) Investigation of the influence of prescribed burning on ITS profiles of ectomycorrhizal and other soil fungi at three Australian sclerophyll forest sites. *Mycol Res* 106:532–540
- Chen DM, Taylor AFS, Burke RM, Cairney JWG (2001) Identification of genes for lignin peroxidases and manganese peroxidases in ectomycorrhizal fungi. *New Phytol* 152:151–158
- Coleman MD, Bledsoe CS, Lopushinsky W (1989) Pure culture response of ectomycorrhizal fungi to imposed water stress. *Can J Bot* 67:29–39
- Colpaert JV, van Assche JA, Luijten K (1992) The growth of extramatrical mycelium of ectomycorrhizal fungi and the growth response of *Pinus sylvestris* L. *New Phytol* 120:127–135
- Corradi N, Hijri M, Fumagalli L, Sanders IR (2004) Arbuscular mycorrhizal fungi (Glomeromycota) harbour ancient fungal tubulin genes that resemble those of the chytrids (Chytridiomycota). *Fungal Genet Biol* 41(11):1037–1045
- Cullings KW, Vogler DR, Parker VT, Finley SK (2000) Ectomycorrhizal specificity patterns in a mixed *Pinus contorta* and *Picea engelmannii* forest in Yellowstone National Park. *Appl Environ Microbiol* 66:4988–4991
- Cumming JR, Weinstein LH (1990) Utilization of AlPO<sub>4</sub> as a phosphorus source by ectomycorrhizal *Pinus rigida* Mill. seedlings. *New Phytol* 116:99–106
- Dahlberg A (2001) Community ecology of ectomycorrhizal fungi: an advancing interdisciplinary field. *New Phytol* 150:555–562
- Da Silva GA, Lumini E, Costa Maia L, Bonfante P, Bianciotto V (2006) Phylogenetic analysis of Glomeromycota by partial LSU rDNA sequences. *Mycorrhiza* 16(3):183–189
- Dickie IA, Koide RT, Stevens CM (1998) Tissue density and growth response of ectomycorrhizal fungi to nitrogen source and concentration. *Mycorrhiza* 8:145–148
- Dickie IA, Xu B, Koide RT (2002) Vertical niche differentiation of ectomycorrhizal hyphae in soil as shown by T-RFLP analysis. *New Phytol* 156(3):527–535
- Dover G (1982) Molecular drive: a cohesive mode of species evolution. *Nature* 299:111–117
- Dumont MG, Murrell JC (2005) Stable isotope probing – linking microbial identity to function. *Nature Rev Microbiol* 3(6):499–504
- Egger KN, Fortin JA (1988) Ectomycorrhizae: diversity and classification. In: Lalonde M, Piché Y (eds) Canadian workshop on mycorrhizae in forestry. C.R.B.F., Université Laval, Ste-Foy, Canada, pp 113–114
- Egger KN, Hibbett DS (2004) The evolutionary implications of exploitation in mycorrhizas. *Can J Bot* 82:1110–1121
- Ek H (1997) The influence of nitrogen fertilization on the carbon economy of *Paxillus involutus* in ectomycorrhizal association with *Betula pendula*. *New Phytol* 135:133–142
- Ekblad A, Höglberg P (2001) Natural abundance of C-13 in CO<sub>2</sub> respired from forest soils reveals speed of link between tree photosynthesis and root respiration. *Oecologia* 127:305–308
- Esser K (2006) Heterogenic incompatibility in fungi. In: Kües U, Fischer R (eds) The Mycota, vol I. Growth, differentiation and sexuality. Springer, Berlin Heidelberg New York, pp 141–166
- Ezawa T, Smith SE, Smith FA (2002) P metabolism and transport in AM fungi. *Plant Soil* 244(1/2):221–230
- Ezawa T, Cavagnaro TR, Smith SE, Smith FA, Ohtomo R (2004) Rapid accumulation of polyphosphate in extraradical hyphae of an arbuscular mycorrhizal fungus as revealed by histochemistry and a polyphosphate kinase/luciferase system. *New Phytol* 161:387–392
- Ezawa T, Hayatsu M, Saito M (2005) A new hypothesis on the strategy for acquisition of phosphorus in arbuscular mycorrhiza: up-regulation of secreted acid phosphatase gene in the host plant. *Mol Plant Microbe Interact* 18(10):1046–1053
- Ferrol N, Barea JM, Azcon-Aguilar C (2000) The plasma membrane H<sup>+</sup>-ATPase gene family in the arbuscular mycorrhizal fungus *Glomus mosseae*. *Curr Genet* 37:112–118
- Ferrol N, Barea JM, Azcon-Aguilar C (2002a) Mechanisms of nutrient transport across interfaces in arbuscular mycorrhizas. *Plant Soil* 244:231–237
- Ferrol N, Pozo MJ, Antelo M, Azcon-Aguilar C (2002b) Arbuscular mycorrhizal symbiosis regulates plasma membrane H<sup>+</sup>-ATPase gene expression in tomato plants. *J Exp Bot* 53:1683–1687
- Finlay RD, Read DJ (1986) The structure and function of the vegetative mycelium of ectomycorrhizal plants. I. Translocation of <sup>14</sup>C-labeled carbon between plants interconnected by a common mycelium. *New Phytol* 103:143–156
- Fitter AH (2005) Darkness visible: reflections on underground ecology. *J Ecol* 93:231–243
- Fitter AH, Graves JD, Watkins NK, Robinson D, Scrimgeour C (1998) Carbon transfer between plants and its control in networks of arbuscular mycorrhizas. *Funct Ecol* 12:406–412
- Fitter AH, Hodge A, Daniell TJ, Robinson D (1999) Resource sharing in plant-fungus communities: did the carbon move for you? *Trends Ecol Evol* 14:70–70
- Fortin JA, Becard G, Declerck S, Dalpé Y, St-Arnaud M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. *Can J Bot* 80:1–20

- Francis R, Read DJ (1984) Direct transfer of carbon between plants connected by vesicular-arbuscular mycorrhizal mycelium. *Nature* 307:53–56
- Francis R, Read DJ (1994) The contributions of mycorrhizal fungi to the determination of plant community structure. In: Robson AD, Abbott LK, Malajczuk N (eds) Management of mycorrhizas in agriculture, horticulture and forestry. Kluwer, Dordrecht, pp 11–25
- Gadkar V, Rillig MC (2005) Application of Phi 29 DNA polymerase mediated whole genome amplification on single spores of arbuscular mycorrhizal (AM) fungi. *FEMS Microbiol Lett* 242:65
- Gardes M (2002) An orchid-fungus marriage – physical promiscuity, conflict and cheating. *New Phytol* 154:4–7
- Gebauer G, Meyer M (2003) N-15 and C-13 natural abundance of autotrophic and myco-heterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. *New Phytol* 160:209–223
- Gehrig H, Schüßler A, Kluge M (1996) *Geosiphon pyriforme*, a fungus forming endocytobiosis with *Nostoc* (Cyanobacteria), is an ancestral member of the Glomales: evidence by SSU rRNA analysis. *J Mol Evol* 43:71–81
- Genney DR, Anderson IC, Alexander IJ (2006) Fine-scale distribution of pine ectomycorrhizas and their extramatrical mycelium. *New Phytol* 170:381–390
- Gianinazzi-Pearson V, Smith SE, Gianinazzi S, Smith FA (1991) Enzymatic studies on the metabolism of vesicular arbuscular mycorrhizas. 5. Is H<sup>+</sup>-ATPase a component of ATP-hydrolyzing enzyme activities in plant-fungus interfaces. *New Phytol* 117:61–74
- Gianinazzi-Pearson V, Arnould C, Oufattole M, Arango M, Gianinazzi S (2000) Differential activation of H<sup>+</sup>-ATPase genes by an arbuscular mycorrhizal fungus in root cells of transgenic tobacco. *Planta* 211:609–613
- Giovannetti M, Sbrana C, Avio L, Strani P (2004) Patterns of below-ground plant interconnections established by means of arbuscular mycorrhizal networks. *New Phytol* 164:75–181
- Girlanda M, Ghignone S, Luppi AM (2002) Diversity of root-associated fungi of two Mediterranean plants. *New Phytol* 155:481–498
- Girlanda M, Perotto S, Luppi AM (2006a) Molecular diversity and ecological roles of mycorrhiza-associated sterile fungal endophytes in Mediterranean ecosystems. In: Schulz BJE, Boyle CJC, Sieber TN (eds) Microbial root endophytes. Springer, Berlin Heidelberg New York, pp 207–226
- Girlanda M, Selosse MA, Cafasso D, Brilli F, Delfine S, Fabiani R, Ghignone S, Pinelli P, Segreto R, Loreto F, Cozzolino S, Perotto S (2006b) Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* (L.) Swartz is mirrored by specific association to ectomycorrhizal Russulaceae. *Mol Ecol* 15(2):491–504
- Glassop D, Smith SE, Smith FW (2005) Cereal phosphate transporters associated with the mycorrhizal pathway of phosphate uptake into roots. *Planta* 222:688–698
- Govindarajulu M, Pfeffer PE, Jin H, Abubaker J, Douds DD, Allen JW, Bucking H, Lammers PJ, Shachar-Hill Y (2005) Nitrogen transfer in the arbuscular mycorrhizal symbiosis. *Nature* 435:819–823
- Graham JH (2000) Assessing costs of arbuscular mycorrhizal symbiosis in agroecosystems. In: Podila GK, Douds DD (eds) Current advances in mycorrhizal research. APS Press, St. Paul, MN, pp 127–140
- Graham JH, Miller RM (2005) Mycorrhizas: gene to function. *Plant Soil* 274:79–100
- Green H, Larsen J, Olsson PA, Funck Jensen D, Jakobsen I (1999) Suppression of the biocontrol agent *Trichoderma harzianum* by the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices*. *Appl Environ Microbiol* 65:1428–1434
- Grimaldi B, de Raaf MA, Filetici P, Ottonello S, Ballario P (2005) *Agrobacterium*-mediated gene transfer and enhanced green fluorescent protein visualization in the mycorrhizal ascomycete *Tuber borchii*: a first step towards truffle genetics. *Curr Genet* 48(1):69–74
- Grunze N, Willmann M, Nehls U (2004) The impact of ectomycorrhiza formation on monosaccharide transporter gene expression in poplar roots. *New Phytol* 164(1):147–155
- Harrier LA, Millam S, Franken P (2002) Biolistic transformation of AM fungi: advances and applications. In: Giannuzzi S, Schuepp H, Barea JM, Haselwandter K (eds) Mycorrhiza technology: from genes to bioproducts – achievements and hurdles in arbuscular mycorrhizal research. Birkhäuser, Basel, pp S9–71
- Harrison MJ (1999) Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. *Annu Rev Plant Physiol Mol Biol* 50:361
- Harrison MJ, Van Buuren ML (1995) A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. *Nature* 378:626–629
- Harrison MJ, Dewbre GR, Liu J (2002) A phosphate transporter from *Medicago truncatula* involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. *Plant Cell* 14:2413–2429
- Hart MM, Reader RJ (2002a) Host plant benefit from association with arbuscular mycorrhizal fungi: variation due to differences in size of mycelium. *Biol Fert Soils* 36(5):357–366
- Hart MM, Reader RJ (2002b) Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. *New Phytol* 153(2):335–344
- He XH, Critchley C, Bledsoe C (2003) Nitrogen transfer within and between plants through common mycorrhizal networks (CMNs). *Crit Rev Plant Sci* 22(6):531–567
- He X, Critchley C, Ng H, Bledsoe C (2005) Nodulated N<sub>2</sub>-fixing *Casuarina cunninghamiana* is the sink for net N transfer from non-N<sub>2</sub>-fixing *Eucalyptus maculata* via an ectomycorrhizal fungus *Pisolithus* sp. using <sup>15</sup>NH<sub>4</sub><sup>+</sup> or <sup>15</sup>NO<sub>3</sub><sup>−</sup> supplied as ammonium nitrate. *New Phytol* 167:897–912
- He X, Bledsoe CS, Zasoski RJ, Southworth D, Horwath WR (2006) Rapid nitrogen transfer from ectomycorrhizal pines to adjacent ectomycorrhizal and arbuscular mycorrhizal plants in a California oak woodland. *New Phytol* 170:143–151
- Heckman DS, Geiser DM, Eidell BR, Stauffer RL, Kardos NL, Hedges SB (2001) Molecular evidence for the early colonization of land by fungi and plants. *Science* 293:1129–1133
- Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW (1998) Ploughing up the wood-wide web? *Nature* 394(6692):431–431

- Helgason T, Merryweather JW, Denison J, Wilson P, Young JPW, Fitter AH (2002) Selectivity and functional diversity in arbuscular mycorrhizas of co-occurring fungi and plants from a temperate deciduous woodland. *J Ecol* 90:371–384
- Helgason T, Watson IJ, Young JPW (2003) Phylogeny of the Glomerales and Diversisporales (Fungi: Glomeromycota) from actin and elongation factor 1-alpha sequences. *FEMS Microbiol Lett* 229(1):127–132
- Hendricks JJ, Mitchell RJ, Kuehn KA, Pecot SD, Sims SE (2006) Measuring external mycelia production of ectomycorrhizal fungi in the field: the soil matrix matters. *New Phytol* 171:179–186
- Hibbett DS, Gilbert LB, Donoghue MJ (2000) Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. *Nature* 407:506–508
- Hijri M, Sanders IR (2005) Low gene copy number shows that arbuscular mycorrhizal fungi inherit genetically different nuclei. *Nature* 433(7022):160–163
- Hobbie EA (2005) Using isotopic tracers to follow carbon and nitrogen cycling of fungi. In: Dighton J, White JE, Oudemans P (eds) *The fungal community. Its organization and role in the ecosystem*, 3rd edn. CRC Press, Taylor & Francis Group, Boca Raton, FL, pp 361–382
- Hodge A, Campbell CD, Fitter AH (2001) An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. *Nature* 413:297–299
- Högberg MN, Högberg P (2002) Extramatrical ectomycorrhizal mycelium contributes one-third of microbial biomass and produces, together with associated roots, half the dissolved organic carbon in a forest soil. *New Phytol* 154 (3):791–795
- Högberg P, Plamboeck AH, Taylor AFS, Fransson PMA (1999) Natural  $^{13}\text{C}$  abundance reveals trophic status of fungi and host-origin of carbon in mycorrhizal fungi in mixed forests. *Proc Natl Acad Sci USA* 96:8534–8539
- Horton TR, Bruns TD (1998) Multiple host fungi are the most frequent and abundant ectomycorrhizal types in a mixed stand of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) and bishop pine (*Pinus muricata* D. Don). *New Phytol* 139:331–339
- Horton TR, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Mol Ecol* 10:1855–1871
- Horton TR, Bruns TD, Parker VT (1999) Ectomycorrhizal fungi associated with *Arctostaphylos* contribute to *Pseudotsuga menziesii* establishment. *Can J Bot* 77:93–102
- Hunt J, Boddy L, Randerson PF (2004) An evaluation of 18S rDNA approaches for the study of fungal diversity in grassland soils. *Microb Ecol* 47(4):385–395
- Husband R, Herre EA, Turner SL, Gallery R, Young JPW (2002) Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in a tropical forest. *Mol Ecol* 11:2669–2678
- Izzo A, Agbowo J, Bruns TD (2005) Detection of plot level changes in ectomycorrhizal communities across years in an old-growth mixed-conifer forest. *New Phytol* 166:619–629
- Jakobsen I (1999) Transport of phosphorus and carbon in arbuscular mycorrhizas. In: Varma A, Hock B (eds) *Mycorrhiza: structure, function, molecular biology and biotechnology*, 2nd edn. Springer, Berlin Heidelberg New York, pp 305–332
- Jakobsen I, Smith SE, Smith FA (2002) Function and diversity of arbuscular mycorrhizae in carbon and mineral nutrition. In: van der Heijden MGA, Sanders IR (eds) *Mycorrhizal ecology*. Springer, Berlin Heidelberg New York, pp 75–92
- Jargeat P, Rekangalt D, Verner MC, Gay G, Debaud JC, Marmeisse R, Fraissinet-Tachet L (2003) Characterisation and expression analysis of a nitrate transporter and nitrite reductase genes, two members of a gene cluster for nitrate assimilation from the symbiotic basidiomycete *Hebeloma cylindrosporum*. *Curr Genet* 43:199–205
- Javelle A, Morel M, Rodriguez-Pastrana BR, Botton B, Andre B, Marini AM, Brun A, Chalot M (2003) High-affinity ammonium transporters and nitrogen sensing in mycorrhizas. *Trends Microbiol* 11:53
- Jifon J, Graham JH, Drouillard DL, Syvertsen JP (2002) Growth depression of mycorrhizal *Citrus* seedlings grown at high phosphorus supply is mitigated by elevated CO<sub>2</sub>. *New Phytol* 153:133–142
- Jin H, Pfeffer PE, Douds DD, Piotrowski E, Lammers PJ, Shachar-Hill Y (2005) The uptake, metabolism, transport and transfer of nitrogen in an arbuscular mycorrhizal symbiosis. *New Phytol* 168:687–696
- Johansen A, Finlay RD, Olsson PA (1996) Nitrogen metabolism of external hyphae of the arbuscular mycorrhizal fungus *Glomus intraradices*. *New Phytol* 133(4):705–712
- Johnson NC, Graham JH, Smith FA (1997) Functioning of mycorrhizal associations along the mutualism-parasitism continuum. *New Phytol* 135:575–585
- Johnson D, Leake JR, Read DJ (2001) Novel in-growth core system enables functional studies of grassland mycorrhizal mycelial networks. *New Phytol* 152:555–562
- Johnson D, Leake JR, Ostle N, Ineson P, Read DJ (2002a) In situ (CO<sub>2</sub>)-C-13 pulse-labelling of upland grassland demonstrates a rapid pathway of carbon flux from arbuscular mycorrhizal mycelia to the soil. *New Phytol* 153:327–334
- Johnson D, Leake JR, Read DJ (2002b) Transfer of recent photosynthate into mycorrhizal mycelium of an upland grassland: short-term respiratory losses and accumulation of C-14. *Soil Biol Biochem* 34:1521–1524
- Johnson D, Vandenkoornhuyse PJ, Leake JR, Gilbert L, Booth RE, Grime JP, Young JPW, Read DJ (2004) Plant communities affect arbuscular mycorrhizal fungal diversity and community composition in grassland microcosms. *New Phytol* 161:503–515
- Johnson D, Ijdo M, Genney DR, Anderson IC, Alexander IJ (2005a) How do plants regulate the function, community structure and diversity of mycorrhizal fungi? *J Exp Bot* 56(417):1751–1760
- Johnson D, Krsek M, Wellington EMH, Stott AW, Cole L, Bardgett RD, Read DJ, Leake JR (2005b) Soil invertebrates disrupt carbon flow through fungal networks. *Science* 309:1047
- Judson OP, Normark BB (1996) Ancient asexual scandals. *Trends Ecol Evol* 11:A41–A46
- Julou T, Burghardt B, Gebauer G, Berveiller D, Damesin C, Selosse MA (2005) Mixotrophy in orchids: insights from a comparative study of green individuals and

- nonphotosynthetic individuals of *Cephalanthera damasonium*. New Phytol 166(2):639–653
- Jumpponen A (2001) Dark septate endophytes – are they mycorrhizal? Mycorrhiza 11:207–211
- Jumpponen A, Trappe JM (1998) Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. New Phytol 140:295–310
- Kamienski F (1881) Die Vegetationsorgane der *Monotropa hypopitys* L. Bot Z 29:457–461
- Kamienski F (1882) Les organes végétatifs du *Monotropa hypopitys* L. Mémoires Soc Natl Sci Naturelles Mathématiques Cherbourg 24:5–40
- Keller G (1996) Utilization of inorganic and organic nitrogen sources by high-subalpine ectomycorrhizal fungi of *Pinus cembra* in pure culture. Mycol Res 100:989–998
- Kemppainen M, Circosta A, Tagu D, Martin F, Pardo AG (2005) Agrobacterium-mediated transformation of the ectomycorrhizal symbiont *Laccaria bicolor* S238N. Mycorrhiza 16(1):19–22
- Kennedy PG, Izzo AD, Bruns TD (2003) There is a high potential for the formation of common mycorrhizal networks between understorey and canopy trees in a mixed evergreen forest. J Ecol 91:1071–1080
- Kjöller R (2006) Disproportionate abundance between ectomycorrhizal root tips and their associated mycelia. FEMS Microbiol Ecol 58(2):214–224
- Koch AM, Croll D, Sanders IR (2006) Genetic variability in a population of arbuscular mycorrhizal fungi causes variation in plant growth. Ecol Lett 9(2):103–110
- Koide RT, Xu B, Sharda J (2005) Contrasting below-ground views of an ectomycorrhizal fungal community. New Phytol 166:251–262
- Kuhn G, Hijri M, Sanders IR (2001) Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi. Nature 414(6865):745–748
- Lacourt I, Duplessis S, Abba S, Bonfante P, Martin F (2002) Isolation and characterization of differentially expressed genes in the mycelium and fruit body of *Tuber borchii*. Appl Environ Microb 68(11):5788–5788
- Lammers PJ, Jun J, Abubaker J, Arreola R, Gopalan A, Bago B, Hernandez-Sebastia C, Allen JW, Douds DD, Pfeffer PE, Shachar-Hill Y (2001) The glyoxylate cycle in an arbuscular mycorrhizal fungus. Carbon flux and gene expression. Plant Physiol 127(3):1287–1298
- Landeweert R, Hoffland E, Finlay RD, Kuyper TW, van Breemen N (2001) Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. Trends Ecol Evol 16:248–254
- Landeweert R, Leeflang P, Kuyper TW, Hoffland E, Rosling A, Wernars K, Smit E (2003) Molecular identification of ectomycorrhizal mycelium in soil horizons. Appl Environ Microb 69:327–333
- Lapeyrie F, Ranger J, Vairelles D (1991) Phosphate-solubilizing activity of ectomycorrhizal fungi in vitro. Can J Bot 69:342
- Leake JR (1994) The biology of mycoheterotrophic ('saprophytic') plants. New Phytol 127:171–216
- Leake JR (2004) Myco-heterotroph/epiparasitic plant interactions with ectomycorrhizal and arbuscular mycorrhizal fungi. Curr Opin Plant Biol 7:422–428
- Leake JR, Read DJ (1997) Mycorrhizal fungi in terrestrial habitats. In: Wicklow DT, Söderström B (eds) The Mycota vol IV, 1st edn. Environmental and microbial relationships. Springer, Berlin Heidelberg New York, pp 281–301
- Leake JR, Donnelly DP, Saunders EM, Boddy L, Read DJ (2001) Rates and quantities of carbon flux to ectomycorrhizal mycelium following C-14 pulse labeling of *Pinus sylvestris* seedlings: effects of litter patches and interaction with a wood-decomposer fungus. Tree Physiol 21(2/3):71–82
- Leake JR, Donnelly DP, Boddy L (2002) Interactions between ecto-mycorrhizal fungi and saprotrophic fungi. In: van der Heijden MGA, Sanders IR (eds) Mycorrhizal ecology. Springer, Berlin Heidelberg New York, pp 345–372
- Leake JR, Johnson D, Donnelly DP, Muckle GE, Boddy L, Read DJ (2004a) Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. Can J Bot 82(8):1016–1045
- Leake JR, McKendrick SL, Bidartondo M, Read DJ (2004b) Symbiotic germination and development of the myco-heterotroph *Monotropa hypopitys* in nature and its requirement for locally distributed *Tricholoma* spp. New Phytol 163:405–423
- LePage BA, Currah RS, Stockey RA, Rothwell GW (1997) Fossil ectomycorrhizae from the middle Eocene. Am J Bot 84:410–412
- Lerat S, Gauci R, Catford JG, Vierheilig H, Piche Y, Lapointe L (2002) C-14 transfer between the spring ephemeral *Erythronium americanum* and sugar maple seedlings via arbuscular mycorrhizal fungi in natural stands. Oecologia 132:181–187
- Lerat S, Lapointe L, Gutjahr S, Piché Y, Vierheilig H (2003a) Carbon partitioning in a split-root system of arbuscular mycorrhizal plants is fungal and plant species dependent. New Phytol 157:589–595
- Lerat S, Lapointe L, Piché Y, Vierheilig H (2003b) Variable carbon-sink strength of different *Glomus mosseae* strains colonizing barley roots. Can J Bot 81:886–889
- Lewis DH (1976) Interchange of metabolites in biotrophic symbioses between angiosperms and fungi. In: Sutherland N (ed) Botany. Perspectives in Experimental Biology vol 2. Pergamon Press, Oxford, pp 207–219
- Lian C, Narimatsu M, Nara K, Hogetsu T (2006) *Tricholoma matsutake* in a natural *Pinus densiflora* forest: correspondence between above- and below-ground genets, association with multiple host trees and alteration of existing ectomycorrhizal communities. New Phytol 171:825–836
- Lindahl B, Stenlid J, Olsson S, Finlay R (1999) Translocation of P-32 between interacting mycelia of a wood-decomposing fungus and ectomycorrhizal fungi in microcosm systems. New Phytol 144(1):183–193
- Lindahl BD, Finlay RD, Cairney JWG (2005) Enzymatic activities of mycelia in mycorrhizal fungal communities. In: Dighton J, White JF, Oudemans P (eds) The fungal community. Its organization and role in the ecosystem, 3rd edn. CRC Press, Taylor & Francis Group, Boca Raton, FL, pp 331–348
- Lloyd-MacGill SA, Chambers SM, Dodd JC, Fitter AH, Walker C, Young JPW (1996) Diversity of the ribosomal internal transcribed spacers within and among isolates of *Glomus mosseae* and related mycorrhizal fungi. New Phytol 133:103–111

- LoBuglio KF, Berbee ML, Taylor JW (1996) Phylogenetic origins of the asexual mycorrhizal symbiont *Cenococcum geophilum* Fr. and other mycorrhizal fungi among Ascomycetes. *Mol Phylogen Evol* 6:287–294
- Maldonado-Mendoza IE, Dewbre GR, Harrison MJ (2001) A phosphate transporter gene from the extra-radical mycelium of an arbuscular mycorrhizal fungus *Glomus intraradices* is regulated in response to phosphate in the environment. *Mol Plant Microbe Interact* 14(10):1140–1148
- Mandyam K, Jumpponen A (2005) Seeking the elusive function of the root-colonising dark septate endophytic fungi. *Stud Mycol* 53:173–189
- Marmeisse R, Guidot A, Gay G, Lambilliotte R, Sentenac H, Combier JP, Melayah D, Fraissinet-Tachet L, Debaud JC (2004) *Hebeloma cylindrosporum* – a model species to study ectomycorrhizal symbiosis from gene to ecosystem. *New Phytol* 163(3):481–498
- Marshner H (1995) Mineral nutrition of higher plants, 2nd edn. Academic Press, London
- Martin F, Botton B (1993) Nitrogen metabolism of ectomycorrhizal fungi and ectomycorrhiza. *Adv Plant Pathol* 9:83
- Martin F, Boiffin V, Pfeffer PE (1998) Carbohydrate and amino acid metabolism in the *Eucalyptus globulus* X *Pisolithus tinctorius* ectomycorrhiza during glucose utilization. *Plant Physiol* 118(2):627–635
- Martin F, Duplessis S, Ditengou F, Lagrange H, Voilet C, Lapeyrie F (2001) Developmental cross talking in the ectomycorrhizal symbiosis: signals and communication genes. *New Phytol* 151:145–154
- Martin F, Tuskan GA, DiFazio SP, Lammers P, Newcombe G, Podila GK (2004) Symbiotic sequencing for the *Populus* mesocosm. *New Phytol* 161:330–335
- Martin F, Perotto S, Bonfante P (2007) Mycorrhizal fungi: a fungal community at the interface between soil and roots. In: Pinton R, Varanini Z, Nannipieri P (eds) The rhizosphere: biochemistry and organic substances at the soil-plant interface. Marcel Dekker, New York, (in press)
- McCormick MK, Whigham DF, O'Neill J (2004) Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytol* 163:425–438
- McKendrick SL, Leake JR, Read DJ (2000a) Symbiotic germination and development of myco-heterotrophic plants in nature: transfer of carbon from ectomycorrhizal *Salix repens* and *Betula pendula* to the orchid *Corallorrhiza trifida* through shared hyphal connections. *New Phytol* 145:539–548
- McKendrick SL, Leake JR, Taylor DL, Read DJ (2000b) Symbiotic germination and development of myco-heterotrophic plants in nature: ontogeny of *Corallorrhiza trifida* and characterization of its mycorrhizal fungi. *New Phytol* 145:523–537
- Miller SL, Allen EB (1992) Mycorrhizae, nutrient translocation, and interactions between plants. In: Allen MF (ed) Mycorrhizal functioning: an integrative plant-fungal process. Chapman and Hall, New York, pp 301–325
- Miller RM, Kling M (2000) The importance of integration and scale in the arbuscular mycorrhizal symbiosis. *Plant Soil* 226:295–309
- Miller RM, Reinhardt DR, Jastrow JD (1995) External production of vesicular-arbuscular mycorrhizal fungi in pasture and tallgrass prairie communities. *Oecologia* 103:17–23
- Miller RM, Miller S, Jastrow JD, Rivetta CB (2002) Mycorrhizal mediated feedbacks influence net carbon gain and nutrient uptake in *Andropogon gerardii* Vitman. *New Phytol* 155:149–162
- Molina R, Trappe JM (1982) Patterns of ectomycorrhizal host specificity and potential among Pacific Northwest conifers and fungi. *Forest Sci* 28:423–458
- Molina R, Massicotte H, Trappe JM (1992) Specificity phenomena in mycorrhizal symbiosis: community-ecological consequences, practical implications. In: Allen MF (ed) Mycorrhizal functioning: an integrated plant-fungal process. Chapman and Hall, London, pp 357–423
- Moncalvo JM, Lutzoni FM, Rehner SA, Johnson J, Vilgalys R (2000) Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *Syst Biol* 49(2):278–305
- Montanini B, Moretto N, Soragni E, Percudani R, Ottonello S (2002) A high-affinity ammonium transporter from the mycorrhizal ascomycete *Tuber borchii*. *Fungal Genet Biol* 36:22–34
- Montanini B, Bettini M, Marquez AJ, Balestrini R, Bonfante P, Ottonello S (2003) Distinctive properties and expression profiles of glutamine synthetase from a plant symbiotic fungus. *Biochem J* 373:357–368
- Montanini B, Gabella S, Abbà S, Peter M, Kohler A, Bonfante P, Chalot M, Martin F, Ottonello S (2006) Gene expression profiling of the nitrogen starvation stress response in the mycorrhizal ascomycete *Tuber borchii*. *Fungal Genet Biol* 43:630–641
- Morton JB, Benny GL (1990) Revised classification of arbuscular mycorrhizal fungi (Zygomycetes) – a new order, Glomales, 2 new suborders, Glomineae and Gigasporineae, and 2 new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. *Mycotaxon* 37:471–491
- Muller T, Benjdia M, Avolio M, Voigt B, Menzel D, Pardo A, Frommer WB, Wipf D (2006) Functional expression of the green fluorescent protein in the ectomycorrhizal model fungus *Hebeloma cylindrosporum*. *Mycorrhiza* 16(6):437–442
- Munkvold LKJ, Iler R, Vestberg M, Rosendahl S, Jakobsen I (2004) High functional diversity within species of arbuscular mycorrhizal fungi. *New Phytol* 164:357–364
- Nagy R, Karandashov V, Chague V, Kalinkevich K, Tamasloukht M, Xu G, Jakobsen I, Levy AA, Amrhein N, Bucher M (2005) The characterization of novel mycorrhiza-specific phosphate transporters from *Lycopersicon esculentum* and *Solanum tuberosum* uncovers functional redundancy in symbiotic phosphate transport in solanaceous species. *Plant J* 42:236–250
- Nehls U, Beguiristain T, Ditengou F, Lapeyrie F, Martin F (1998) The expression of a symbiosis-regulated gene in eucalypt roots is regulated by auxins and hypaphorine, the tryptophan betaine of the ectomycorrhizal basidiomycete *Pisolithus tinctorius*. *Planta* 207(2):296–302
- Nehls U, Ecke M, Hampp R (1999) Sugar- and nitrogen-dependent regulation of an *Amanita muscaria* phenylalanine ammonium lyase gene. *J Bacteriol* 181:1931–1933

- Nehls U, Wiese J, Hampp R (2000) Cloning of a *Picea abies* monosaccharide transporter gene and expression-analysis in plant tissues and ectomycorrhizas. *Trees* 14:334–338
- Nehls U, Mikolajewski S, Magel E, Hampp R (2001) Carbohydrate metabolism in ectomycorrhizas: gene expression, monosaccharide transport and metabolic control. *New Phytol* 150:533–541
- Newman EI (1988) Mycorrhizal links between plants: their functioning and ecological significance. *Adv Ecol Res* 18:243–270
- Newsham KK, Fitter AH, Merryweather JW (1995) Multifunctionality and biodiversity in arbuscular mycorrhiza. *Trees* 10:407–411
- Nilsson LO, Wallander H (2003) The production of external mycelium by ectomycorrhizal fungi in a Norway spruce forest was reduced in response to nitrogen fertilization. *New Phytol* 158:409–416
- Olsson PA, Wilhelmsson P (2000) The growth of external AM fungal mycelium in sand dunes and in experimental systems. *Plant Soil* 226(2):161–169
- Olsson PA, Jakobsen I, Wallander H (2002) Foraging and resource allocation strategies of mycorrhizal fungi in a patchy environment. In: van der Heijden MGA, Sanders IR (eds) *Mycorrhizal ecology*. Springer, Berlin Heidelberg New York, pp 93–116
- Öpik M, Moora M, Liira J, Köljalg U, Zobel M, Sen R (2003) Divergent arbuscular mycorrhizal fungal communities colonize roots of *Pulsatilla* spp. in boreal Scots pine forest and grassland soils. *New Phytol* 160:581–593
- Öpik M, Moora M, Liira J, Zobel M (2006) Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. *J Ecol* 94(4):778–790
- Pardo AG, Hanif M, Raudaskoski M, Gorfer M (2002) Genetic transformation of ectomycorrhizal fungi mediated by *Agrobacterium tumefaciens*. *Micol Res* 106:132–137
- Paris F, Bonnaud P, Ranger J, Robert M, Lapeyrie F (1995) Weathering of ammonium or calcium-saturated 2/1 phyllosilicates by ectomycorrhizal fungi in vitro. *Soil Biol Biochem* 27(10):1237–1244
- Paszkowski U, Kroken S, Roux C, Briggs SP (2002) Rice phosphate transporters include an evolutionarily divergent gene specifically activated in arbuscular mycorrhizal symbiosis. *Proc Natl Acad Sci USA* 99:13324–13329
- Pawlowska TE (2005) Genetic processes in arbuscular mycorrhizal fungi. *FEMS Microbiol Lett* 251:185–192
- Pawlowska TE, Taylor JW (2004) Organization of genetic variation in individuals of arbuscular mycorrhizal fungi. *Nature* 427:733–737
- Pawlowska TE, Taylor JW (2005) Arbuscular mycorrhizal fungi – Hyphal fusion and multigenomic structure – Reply. *Nature* 433(7022):E4–E4
- Percudani R, Trevisi A, Zambonelli A, Ottonello S (1999) Molecular phylogeny of truffles (Pezizales: Tuberaceae, Terfeziaceae) derived from nuclear rDNA sequence analysis. *Mol Phylogen Evol* 13:169–180
- Perez-Moreno J, Read DJ (2001a) Exploitation of pollen by mycorrhizal mycelial systems with special reference to nutrient recycling in boreal forests. *Proc R Soc Lond B Biol Sci* 268:1329–1335
- Perez-Moreno J, Read DJ (2001b) Transfer from soil nematodes to plants: a direct pathway provided by the mycorrhizal mycelial network. *Plant Cell Environ* 24:1219–1228
- Perry DA (1999) Reply from D.A. Perry. *Trends Ecol Evol* 14:70–71
- Peter M, Ayer F, Egli S (2001) Nitrogen addition in a Norway spruce stand altered macromycete sporocarp production and below-ground ectomycorrhizal species composition. *New Phytol* 149(2):311–325
- Peterson RL, Massicotte HB (2004) Exploring structural definitions of mycorrhizas, with emphasis on nutrient-exchange interfaces. *Can J Bot* 82(8):1074–1088
- Pfeffer PE, Douds D, Becard G, Shachar-Hill Y (1999) Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. *Plant Physiol* 120:587–598
- Pfeffer PE, Douds DD, Bücking H, Schwartz DP, Shachar-Hill Y (2004) The fungus does not transfer carbon to or between roots in an arbuscular mycorrhizal symbiosis. *New Phytol* 163:617–627
- Phipps CJ, Taylor TN (1996) Mixed arbuscular mycorrhizae from the Triassic of Antarctica. *Mycologia* 88:707–714
- Poulsen KH, Nagy R, Gao LL, Smith SE, Bucher M, Smith FA, Jakobsen I (2005) Physiological and molecular evidence for Pi uptake via the symbiotic pathway in a reduced mycorrhizal colonization mutant in tomato associated with a compatible fungus. *New Phytol* 168(2):445–453
- Querejeta JI, Egerton-Warburton LM, Allen MF (2003) Direct nocturnal water transfer from oaks to their mycorrhizal symbionts during severe soil drying. *Oecologia* 134:55–64
- Rambelli A (1973) The rhizosphere of mycorrhizae. In: Marks GC, Kozlowski TT (eds) *Ectomycorrhizae, their ecology and physiology*. Academic Press, New York, pp 299–349
- Rausch C, Daram P, Brunner S, Jansa J, Laloi M, Leggewie G, Amrhein N, Bucher M (2001) A phosphate transporter expressed in arbuscule-containing cells in potato. *Nature* 414(6862):462–466
- Ravnskov S, Larsen J, Olsson PA, Jakobsen I (1999) Effects of various organic compounds on growth and P uptake of an arbuscular mycorrhizal fungus. *New Phytol* 141:517–524
- Ravnskov S, Wu Y, Graham JH (2003) Arbuscular fungi differentially affect expression of genes in maize roots coding for sucrose synthases in maize roots. *New Phytol* 157:539–545
- Read DJ (1991) Mycorrhizas in ecosystems. *Experientia* 47:376–391
- Read DJ (1997) Mycorrhizal fungi – the ties that bind. *Nature* 388(6642):517–518
- Read DJ (2002) Towards ecological relevance – progress and pitfalls in the path towards an understanding of mycorrhizal functions in nature. In: van der Heijden MGA, Sanders IR (eds) *Mycorrhizal ecology*. Springer, Berlin Heidelberg New York, pp 3–29
- Read DJ, Perez-Moreno J (2003) Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? *New Phytol* 157:475–492
- Read DJ, Leake JR, Perez-Moreno J (2004) Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. *Can J Bot* 82(8):1243–1263
- Redecker D, Kodner R, Graham LE (2000a) Glomalean fungi from the Ordovician. *Science* 289:1920–1921

- Redecker D, Morton JB, Bruns TD (2000b) Ancestral lineages of arbuscular mycorrhizal fungi (Glomales). *Mol Phylogen Evol* 14:276–284
- Requena N (2006) Measuring quality of service: phosphate ‘à la carte’ by arbuscular mycorrhizal fungi. *New Phytol* 168:268–271
- Rice AV, Currah RS (2006) *Oidiodendron maius*: saprobe in sphagnum peat, mutualist in ericaceous roots? In: Schulz BJE, Boyle CJC, Sieber TN (eds) *Microbial root endophytes*. Springer, Berlin Heidelberg New York, pp 227–246
- Rieger A, Guttenberger M, Hampp R (1992) Soluble carbohydrates in mycorrhized and non-mycorrhized fine roots of spruce seedlings. *Z Naturforsch* 47(3/4):201–204
- Rillig MC (2004) Arbuscular mycorrhizae and terrestrial ecosystem processes. *Ecol Lett* 7:740–754
- Rillig MC, Mumey DL (2006) Mycorrhizas and soil structure. *New Phytol* 171:41–53
- Rillig M, Treseder KK, Allen MF (2002) Global change and mycorrhizal fungi. In: van der Heijden MGA, Sanders IR (eds) *Mycorrhizal ecology*. Springer, Berlin Heidelberg New York, pp 135–160
- Ritz K, Newman EI (1986) Nutrient transport between ryegrass plants differing in nutrient status. *Oecologia* 70:128–131
- Robinson D, Fitter A (1999) The magnitude and control of carbon transfer between plants linked by a common mycorrhizal network. *J Exp Bot* 50:9–13
- Rodriguez-Tovar AV, Ruiz-Medrano R, Herrera-Martinez A, Barrera-Figueroa BE, Hidalgo-Lara ME, Reyes-Marquez BE, Cabrera-Ponce JL, Valdes M, Xoconostle-Cazares B (2005) Stable genetic transformation of the ectomycorrhizal fungus *Pisolithus tinctorius*. *J Microbiol Methods* 63(1):45–54
- Rosendahl S, Stukenbrock EH (2004) Community structure of arbuscular mycorrhizae fungi in undisturbed vegetation revealed by analysis of LSU rDNA sequences. *Mol Ecol* 13:3179–3186
- Saari SK, Campbell CD, Russell J, Alexander IJ, Anderson IC (2005) Pine microsatellite markers allow roots and ectomycorrhizas to be linked to individual trees. *New Phytol* 165:295–304
- Sanders IR (2002) Specificity in the arbuscular mycorrhizal symbiosis. In: van der Heijden MGA, Sanders IR (eds) *Mycorrhizal ecology*. Springer, Berlin Heidelberg New York, pp 415–437
- Sanders IR (2003) Preference, specificity and cheating in the arbuscular mycorrhizal symbiosis. *Trends Plant Sci* 8(4):143–145
- Sanders IR (2004) Plant and arbuscular mycorrhizal fungal diversity – are we looking at the relevant levels of diversity and are we using the right techniques? *New Phytol* 164:413–418
- Scheublin TR, Ridgway KP, Young JPW, van der Heijden MGA (2004) Nonlegumes, legumes, and root nodules harbor different arbuscular mycorrhizal fungal communities. *Appl Environ Microbiol* 70(10):6240–6246
- Schulz BJE (2006) Mutualistic interactions with fungal root endophytes. In: Schulz BJE, Boyle CJC, Sieber TN (eds) *Microbial root endophytes*. Springer, Berlin Heidelberg New York, pp 261–280
- Schulz BJE, Boyle C (2006) What are endophytes? In: Schulz BJE, Boyle CJC, Sieber TN (eds) *Microbial root endophytes*. Springer, Berlin Heidelberg New York, pp 1–14
- Schüßler A (2002) Molecular phylogeny, taxonomy, and evolution of *Geosiphon pyriformis* and arbuscular mycorrhizal fungi. *Plant Soil* 244(1/2):75–83
- Schüßler A, Gehrig H, Schwarzott D, Walker C (2001a) Analysis of partial Glomales SSU rRNA gene sequences: implications for primer design and phylogeny. *Mycol Res* 105:5–15
- Schüßler A, Schwarzott D, Walker C (2001b) A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycol Res* 105:1413–1421
- Schweiger P, Jakobsen I (2000) Laboratory and field methods for measurement of hyphal uptake of nutrients in soil. *Plant Soil* 226:237–244
- Schweiger PF, Thingstrup I, Jakobsen I (1999) Comparison of two test systems for measuring plant phosphorus uptake via arbuscular mycorrhizal fungi. *Mycorrhiza* 8:207–213
- Selle A, Willmann M, Grunze N, Geßler A, Weiß M, Nehls U (2005) The high-affinity poplar ammonium importer PttAMT1.2 and its role in ectomycorrhizal symbiosis. *New Phytol* 168:697–706
- Selosse MA, Bauer R, Moyersoen B (2002a) Basal hymenomycetes belonging to the Sebacinaeae are ectomycorrhizal on temperate deciduous trees. *New Phytol* 155(1):183–195
- Selosse MA, Weiss M, Jany JL, Tillier A (2002b) Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) LCM Rich. & neighbouring tree ectomycorrhizae. *Mol Ecol* 11:1831–1844
- Selosse MA, Faccio A, Scappaticci G, Bonfante P (2004) Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (Neottiaeae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. *Microbial Ecol* 47:416–426
- Selosse MA, Richard F, He X, Simard SW (2006) Mycorrhizal networks: *des liaisons dangeureuses?* *Trends Ecol Evol* 21(11):621–628
- Shachar-Hill Y, Pfeifer PE, Douds D, Osman SF, Doner LW, Ratcliffe RG (1995) Partitioning of intermediate carbon metabolism in VAM colonized leek. *Plant Physiol* 108:7–15
- Shefferson RP, Weiss M, Kull T, Taylor DL (2005) High specificity generally characterizes mycorrhizal association in rare lady’s slipper orchids, genus *Cypripedium*. *Mol Ecol* 14:613–626
- Shinozaki ML, LoBuglio KF, Rogers SO (1999) Comparison of ribosomal DNA ITS regions among geographic isolates of *Cenococcum geophilum*. *Curr Genet* 35:527–535
- Sieber TN, Grünig CR (2006) Biodiversity of fungal root-endophyte communities and populations, in particular of the dark septate endophyte *Phialocephala fortinii* s.l. In: Schulz BJE, Boyle CJC, Sieber TN (eds) *Microbial root endophytes*. Springer, Berlin Heidelberg New York, pp 107–132
- Simard SW, Durall DM (2004) Mycorrhizal networks: a review of their extent, function, and importance. *Can J Bot* 82(8):1140–1165

- Simard SW, Perry DA, Jones MD, Myrold DD, Durall DM, Molina R (1997) Net transfer of carbon between ectomycorrhizal tree species in the field. *Nature* 388:579–582
- Simard SW, Durall D, Jones M (2002) Carbon and nutrient fluxes within and between mycorrhizal plants. In: van der Heijden MGA, Sanders IR (eds) *Mycorrhizal ecology*. Springer, Berlin Heidelberg New York, pp 33–74
- Smith SE, Gianinazzi-Pearson V (1988) Physiological interactions between symbionts in vesicular arbuscular mycorrhizal plants. *Annu Rev Plant Physiol Plant Mol Biol* 39:221
- Smith SE, Read DJ (1997) *Mycorrhizal symbiosis*, 2nd edn. Academic Press, London
- Smith SE, Smith FA (1990) Structure and function of the interfaces in biotrophic symbioses as they relate to nutrient transport. *New Phytol* 114:1–38
- Smith SE, Dickson S, Smith FA (2001) Nutrient transfer in arbuscular mycorrhizas: how are fungal and plant processes integrated? *Austr J Plant Physiol* 28:683–694
- Solaiman MDK, Saito M (1995) Use of sugars by intraradical hyphae of arbuscular mycorrhizal fungi revealed by radiorespirometry. *New Phytol* 136:533–538
- Staddon PL, Fitter AH, Robinson D (1999) Effects of mycorrhizal colonization and elevated atmospheric carbon dioxide on carbon fixation and below-ground carbon partitioning in *Plantago lanceolata*. *J Exp Bot* 50:853–860
- Stukenbrock EH, Rosendahl S (2005) Development and amplification of multiple co-dominant genetic markers from single spores of arbuscular mycorrhizal fungi by nested multiplex PCR. *Fungal Genet Biol* 42:73–80
- Taylor AFS (2002) Fungal diversity in ectomycorrhizal communities: sampling effort and species detection. *Plant Soil* 244:19–28
- Taylor DL (2004) Myco-heterotroph–fungus marriages – is fidelity over-rated? *New Phytol* 163:217–221
- Taylor AFS (2006) Common mycelial networks: life-lines and radical addictions. *New Phytol* 169:3–6
- Taylor DL, Bruns TD (1997) Independent, specialized invasions of ectomycorrhizal mutualism by two non photosynthetic orchids. *Proc Natl Acad Sci USA* 94:4510–4515
- Taylor TN, Remy W, Hass H, Kerp H (1995) Fossil arbuscular mycorrhizae from the early Devonian. *Mycologia* 87:560–573
- Taylor DL, Bruns TD, Leake JR, Read DJ (2002) Mycorrhizal specificity and function in myco-heterotrophic plants. In: van der Heijden MGA, Sanders IR (eds) *Mycorrhizal ecology*. Springer, Berlin Heidelberg New York, pp 375–413
- Taylor AFS, Gebauer G, Read DJ (2004) Uptake of nitrogen and carbon from double-labelled ( $^{15}\text{N}$  and  $^{13}\text{C}$ ) glycine by mycorrhizal pine seedlings. *New Phytol* 164:383–388
- Tedersoo L, Kõljalg U, Hallenberg N, Larsson K-H (2003) Fine scale distribution of ectomycorrhizal fungi and roots across substrate layers including coarse woody debris in a mixed forest. *New Phytol* 159:153–165
- Tedersoo L, Hansen K, Perry BA, Kjøller R (2006) Molecular and morphological diversity of pezizalean ectomycorrhiza. *New Phytol* 170:581–596
- Tehler A, Farris JS, Lipscomb DL, Källersjö M (2000) Phylogenetic analyses of the fungi based on large rDNA data sets. *Mycologia* 92:459–474
- Toljander JF, Eberhardt U, Toljander YK, Paul LR, Taylor AFS (2006) Species composition of an ectomycorrhizal fungal community along a local nutrient gradient in a boreal forest. *New Phytol* 170(4):873–883
- Trappe JM (1962) Fungus associates of ectotrophic mycorrhizae. *Bot Rev* 28:538–606
- Trépanier M, Bécard G, Peter Moutoglis P, Willemot C, Gagné S, Avis TJ, Rioux JA (2005) Dependence of arbuscular-mycorrhizal fungi on their plant host for palmitic acid synthesis. *Appl Environ Microbiol* 71(9):5341–5347
- Treseder KK (2004) A meta-analysis of mycorrhizal responses to nitrogen, phosphorus and atmospheric CO<sub>2</sub> in field studies. *New Phytol* 164:347–355
- Trudell SA, Rygiewicz PT, Edmonds RL (2003) Nitrogen and carbon stable isotope abundances support the myco-heterotrophic nature and host-specificity of certain achlorophyllous plants. *New Phytol* 160:391–401
- Tuffen F, Eason WR, Scuffen J (2002) The effect of earthworms and arbuscular mycorrhizal fungi on growth of and  $^{32}\text{P}$  transfer between *Allium porrum* plants. *Soil Biol Biochem* 34:1027–1036
- Uetake Y, Kojima T, Ezawa T, Saito M (2002) Extensive tubular vacuole system in an arbuscular mycorrhizal fungus, *Gigaspora margarita*. *New Phytol* 154:761–768
- Urban A, Weiß M, Bauer R (2003) Ectomycorrhizas involving sebacinoid mycobionts. *Mycol Res* 107(1):3–14
- van Breemen N, Lundstrom US, Jongmans AG (2000) Do plants drive podzolization via rock-eating mycorrhizal fungi? *Geoderma* 94(2/4):163–171
- Vandenkoornhuysse P, Husband R, Daniell TJ, Watson IJ, Duck M, Fitter AH, Young JPW (2002) Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. *Mol Ecol* 11:1555–1564
- Vandenkoornhuysse P, Ridgway KP, Watson IJ, Fitter AH, Young JPW (2003) Co-existing grass species have distinctive arbuscular mycorrhizal communities. *Mol Ecol* 12:3085–3095
- van der Heijden MGA (2002) Arbuscular mycorrhizal fungi as a determinant of plant diversity: in search for underlying mechanisms and general principles. In: van der Heijden MGA, Sanders IR (eds) *Mycorrhizal ecology*. Springer, Berlin Heidelberg New York, pp 243–266
- van der Heijden MGA (2004) Arbuscular mycorrhizal fungi as support systems for seedling establishment in grassland. *Ecol Lett* 7:293–303
- van der Heijden MGA, Scheublin TR, Brader A (2004) Taxonomic and functional diversity in arbuscular mycorrhizal fungi – is there any relationship? *New Phytol* 164(2):201–204
- Villarreal-Ruiz L, Anderson IC, Alexander IJ (2004) Interaction between an isolate from the *Hymenoscyphus ericae* aggregate and roots of *Pinus* and *Vaccinium*. *New Phytol* 164:183–192
- Vrålstad T (2004) Are ericoid and ectomycorrhizal fungi part of a common guild? *New Phytol* 164:7–10
- Vrålstad T, Fosseim T, Schumacher T (2000) *Piceirhiza bicolorata* – the ectomycorrhizal expression of the *Hymenoscyphus ericae* aggregate? *New Phytol* 145:549–563

- Vrålstad T, Myhre E, Schumacher T (2002) Molecular diversity and phylogenetic affinities of symbiotic root-associated ascomycetes of the Helotiales in burnt and metal polluted habitats. *New Phytol* 155:131–148
- Wallander H (2000a) Uptake of P from apatite by *Pinus sylvestris* seedlings colonised by different ectomycorrhizal fungi. *Plant Soil* 218(1/2):249–256
- Wallander H (2000b) Use of strontium isotopes and foliar K content to estimate weathering of biotite induced by pine seedlings colonised by ectomycorrhizal fungi from two different soils. *Plant Soil* 222(1/2):215–229
- Wallander H, Nilsson LO, Hagerberg D, Baath E (2001) Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field. *New Phytol* 151:753–760
- Wallander H, Johansson L, Pallon J (2002) PIXE analysis to estimate the elemental composition of ectomycorrhizal rhizomorphs grown in contact with different minerals in forest soil. *FEMS Microbiol Ecol* 39(2):147–156
- Wallander H, Mahmood S, Hagerberg D, Johansson L, Pallon J (2003) Elemental composition of ectomycorrhizal mycelia identified by PCR-RFLP analysis and grown in contact with apatite or wood ash in forest soil. *FEMS Microbiol Ecol* 44:57–65
- Weiss M, Selosse MA, Rexer KH, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. *Mycol Res* 108:1003–1010
- Wiese J, Kleber R, Hampp R, Nehls U (2000) Functional characterization of the *Amanita muscaria* monosaccharide transporter *AmMST1*. *Plant Biol* 2:1–5
- Wipf D, Benjdia M, Tegeder M, Frommer WB (2002) Characterization of a general amino acid permease from *Hebeloma cylindrosporum*. *FEBS Lett* 528:119–124
- Wright DP, Read DJ, Scholes JP (1998a) Mycorrhizal sink strength influences whole plant carbon balance of *Trifolium repens* L. *Plant Cell Environ* 21:881–891
- Wright DP, Scholes JD, Read DJ (1998b) Effects of VA mycorrhizal colonization on photosynthesis and biomass production of *Trifolium repens* L. *Plant Cell Environ* 21:209–216
- Wu B, Nara K, Hogetsu T (2001) Can <sup>14</sup>C-labeled photosynthetic products move between *Pinus densiflora* seedlings linked by ectomycorrhizal mycelia? *New Phytol* 149:137–146
- Wu BY, Nara K, Hogetsu T (2002) Spatiotemporal transfer of carbon-14-labelled photosynthate from ectomycorrhizal *Pinus densiflora* seedlings to extraradical mycelia. *Mycorrhiza* 12(2):83–88
- Wu TH, Sharda JN, Koide RT (2003) Exploring interactions between saprotrophic microbes and ectomycorrhizal fungi using a protein-tannin complex as an N source by red pine (*Pinus resinosa*). *New Phytol* 159(1):131–139
- Yu TEJ-C, Egger KN, Peterson RL (2001) Ectomycorrhizal associations – characteristics and functions. *Mycorrhiza* 11:167–177
- Yu Z, Zhang Q, Kraus TEC, Dahlgren RA, Anastasio C, Zassowski RJ (2002) Contribution of amino compounds to dissolved organic nitrogen in forest soils. *Biogeochemistry* 61:173–198

---

# 15 Applications of Fungal Ecology in the Search for New Bioactive Natural Products

J.B. GLOER<sup>1</sup>

## CONTENTS

I. Introduction .....	257
A. Fungal Natural Products: From Mycotoxins to Antibiotics .....	257
B. Chemical Ecology: A General Guide to Natural Product Discovery .....	259
II. Observations in Fungal Ecology Associated with the Production of Bioactive Metabolites .....	261
A. Competitive or Antagonistic Interactions .....	261
B. Resistance of Key Fungal Structures to Fungivory or Microbial Attack .....	265
1. <i>Claviceps</i> Ergot .....	265
2. Sclerotia of Other Fungi .....	266
3. Ascostromata .....	267
4. Other Fungal Structures .....	267
C. Fungi that May Confer Host Resistance to Herbivory or Disease .....	268
D. Fungi that Cause Diseases or Damage to Host Species .....	269
1. Plant Pathogens .....	269
2. Entomopathogenic and Nematophagous Fungi .....	270
3. Mycoparasitic and Fungicolous Fungi .....	270
III. Perspectives and Future Directions .....	274
References .....	276

## I. Introduction

### A. Fungal Natural Products:     From Mycotoxins to Antibiotics

Among the most fascinating and important properties of fungi is their ability to produce a tremendous variety of so-called secondary metabolites that display a broad range of biological activities (Demain et al. 2005). Fungi are widely known for the production of compounds that have a deservedly negative reputation due to their activities as carcinogens or mammalian toxins (mycotoxins). Such compounds include aflatoxins, ochratoxins, citroviridin, trichothecenes, fumonisins, and various indole-derived tremorgenics (Miller and Trenholm

1994; Cole and Schweikert 2003). In general, mycotoxins are not inherently more toxic than natural products from bacteria, plants, or other sources, but they are much more problematic because of their widespread occurrence as contaminants of food for humans and livestock, as well as indoor environments (Jarvis 2003; Jarvis and Miller 2005). Knowledge of mycotoxin chemistry is essential to efforts to monitor and reduce the levels of exposure to such compounds.

On the other hand, numerous important pharmaceuticals have also been discovered through studies of fungal chemistry (Masurekar 2005; Demain et al. 2005). This dichotomy is indicative of the diversity of bioactive compounds that fungi can produce. Despite a decrease in resources invested in industrial natural product discovery research in the USA over the last 25 years, natural products in general continue to be among the most important therapeutic agents and lead compounds in medicine (Cragg and Newman 2000; Newman et al. 2000, 2003). While the extent of their roles varies from one therapeutic area to another, natural products have been particularly important in the development of effective therapies for cancer, malaria, bacterial and fungal infections, and CNS and cardiovascular diseases (Newman et al. 2000). For example, over 60% of all drugs in clinical trials against cancer as of 2003 are either natural products, or are derived directly or indirectly from natural product leads (Cragg and Newman 2005). This is particularly impressive when one considers the many other intensively investigated approaches to the discovery of anticancer therapeutics. Natural products from various sources show both proven utility and promise in agriculture as well (Duke et al. 2003; Rimando and Duke 2006).

Fungi are particularly prolific sources of biologically active natural products (Peláez 2005). Many billions of dollars in annual sales of pharmaceuticals of various types can be traced to fungal natural product chemistry (Demain et al.

<sup>1</sup> Department of Chemistry, University of Iowa, Iowa City, IA 52242, USA

2005). Antibacterial agents such as penicillins and cephalosporins are perhaps the best known examples, but a variety of other compounds with distinctive pharmacological activities have also been discovered as fungal metabolites.

A textbook example is provided by mevinolin (=mevacor = lovastatin), the lead compound in the development of the so-called statin class of cholesterol-lowering drugs. Lovastatin was at one point a billion-dollar drug, and is still a useful product, though it is now off-patent. A slight structural modification of lovastatin afforded Zocor® (= simvastatin), an even more lucrative “blockbuster drug” product with over US\$5 billion/year in sales (until it also went off-patent in 2006). Other medically important compounds include cyclosporin, mycophenolic acid, and the ergot alkaloids. Many other fungal metabolites have been discovered as potential pharmaceuticals or leads thereto, with a wide range of pharmacologically relevant activities in mechanism-based and whole-organism assays (Caporale 1995; Masurekar 2005). A recent commercialized example is provided by Cancidas® (= caspofungin; Keating and Figgitt 2003), a member of the pneumocandin/echinocandin class of cyclic peptide fungal metabolites. Cancidas® was introduced by Merck in 2001 upon approval as a treatment for refractory invasive aspergillosis, and represented the first new class of systemic antifungal agents to enter the market in 25 years. This example also illustrates the intriguing fact that fungi can serve as sources of useful antifungal agents. In fact, a survey of the literature on bioactive fungal metabolites during the period from 1993–2001 (718 references) revealed that antifungal activity was the most common biological effect reported (Peláez 2005), although, as noted by the author, this is likely due at least in part to the modest resources needed for such assays. Fungi are effective in producing compounds with activity in assays relevant to other therapeutic areas as well, such as HIV (Singh et al. 2005). Fungal products also show considerable potential as natural agrochemicals (Gardner and McCoy 1992; Anke and Sterner 2002; Peláez 2005; Liu and Li 2005), with important examples including nodulisporic acids (insecticides/antiparasitics), strobilurins (fungicides), and various phytotoxins (herbicides).

The practical advantages of fungi as sources of useful natural products are well documented. Fungal metabolites are renewable resources, and methods for large-scale production of im-

portant fungal metabolites can be developed using established techniques. Modification of metabolite structure and dramatic improvements in metabolite production efficiency can be accomplished through strain mutation, medium variation, and optimization of culture conditions (Masurekar 2005). For example, manipulation of metabolite-producing cultures eventually resulted in a 6,000-fold improvement in penicillin production (Demain 1992), and a 900-fold improvement in compactin production (Chakravarti and Sahai 2004). Thus, although these steps are not trivial, promising metabolites isolated from fungi can be made available on a practical scale through application of existing technology. Of course, such compounds can also be used as models in the development of synthetic or semisynthetic derivatives or analogs that may have improved activity or other more desirable properties, as has been the case for penicillins, cephalosporins, and lovastatin (Demain et al. 2005). In addition, considerable advances are being made in development of techniques for exploiting the genetics of microbial biosynthesis (Khosla and Keasling 2003; Du et al. 2003; Keller et al. 2005; Wang et al. 2005). The discovery of fungi that express new biosynthetic pathways of interest can provide valuable new “raw material” for research in this area.

Of course, the odds against finding a truly useful agent are daunting. On average, it requires approximately 12 years and is estimated to cost over US\$800 million to develop and bring to market a new drug, and thousands of candidate compounds are dropped for every one that ultimately reaches the market (Anonymous 1995; DiMasi et al. 2003; Barton and Emanuel 2005). Even so, the potential payoff, and the track record of fungi as sources of useful compounds have fostered continued industrial interest in fungal natural products chemistry within many screening programs. In recent years, it has become increasingly difficult to find new bioactive natural products from microbial sources because of the extensive screening efforts that have already taken place. In fact, the need to derePLICATE cultures, i.e., to weed out well-known metabolites that are responsible for positive results in a bioassay (Harris 2005; Dinan 2005; Hansen et al. 2005), is a source of tremendous expense and frustration (Corley and Durley 1994), and is viewed by many as a significant negative feature of continued screening efforts. Part of this problem stems from long-term reliance on screening of sheer numbers

of actinomycetes and common fungi isolated mainly from soil samples as sources of bioactive metabolites, while other, less widely studied niche groups have been largely neglected. Inattention to the specific types of organisms chosen for screening and the habitats from which they are isolated, together with disinterest in fungi that are slow-growing, more difficult to isolate, and/or difficult to adapt to standard liquid fermentation protocols, combine to exacerbate the problem. Thus, many taxonomic and ecological groups of fungi have not been systematically explored for useful secondary metabolites, despite literature evidence that directly or indirectly indicates their potential in this area.

The fungi provide almost limitless potential for metabolic variation. Fungi rank second only to the insects in estimated species biodiversity. Conservative estimates suggest that there are likely to be over 1.5 million fungal species, of which only ca. 5% have been described (Hawksworth 1991, 2001). This is over five times the number of predicted plant species, and 50 times the estimated number of bacterial species (Hawksworth 1991). In recent years, an element of urgency has been conferred upon studies of the chemistry of certain fungi for the same reasons often cited to rationalize appeals for accelerated studies of plant chemistry, i.e., concerns about the loss of biodiversity (Balandrin et al. 1993). Many endangered plant and insect species are associated with specific fungal flora, and loss of those species would also result in a concomitant loss of fungal species.

The importance of seeking isolates for industrial screening programs from relatively unexplored niche groups or substrates has been recognized (Monaghan and Tkacz 1990; Miller 1991; Dreyfuss and Chapela 1994; Bills 1995; Caporale 1995), and a number of programs have made significant efforts to expand their scope in order to include such isolates. However, the primary objective of this chapter is to provide specific examples of how observations in fungal ecology can be linked to the search for bioactive natural products that may be useful in medicine or agriculture.

This chapter is an updated version of one that appeared in the previous edition in this series a decade ago (Gloer 1997). Examples from the literature will again be cited, with most taken from more recent literature. As before, emphasis will be placed on results from our own research program that demonstrate the potential value of this kind of approach as a complement to those typically

taken in random screening programs. Detailed chemistry results from our program that were described in the 1997 volume will not be repeated here, although overall summaries will be provided. Instead, descriptions of results from our laboratory will focus on highlights of explorations undertaken since the appearance of the earlier edition.

## B. Chemical Ecology: A General Guide to Natural Product Discovery

It is widely accepted that natural products play important roles in the ecology of many different types of organisms. It is also well known that studies of these types of ecological phenomena can lead to the discovery of novel natural products that have bioactivities of potential practical importance. Examples include compounds produced by plants that serve as chemical defenses against herbivores (Harborne 1987), and metabolites produced by marine invertebrates that deter attacks by predators (Harper et al. 2001). Many instances of the involvement of chemical agents in attack and defense are known among other groups as well, such as insects and amphibians (Daly 1998). By contrast, relatively little is known of the chemical ecology of microbial ecosystems. Principles of chemical ecology would suggest that slow-growing fungi inhabiting competitive niches, or those that produce long-lived physiological structures, would experience considerable evolutionary pressure to produce antagonistic secondary metabolites that could play roles (offensive or defensive) in their ecology. Even so, as noted above, many competitive niches have not been widely sampled, and slower-growing fungi are often overlooked or discarded by screening programs.

As suggested by a number of researchers, there is ample evidence to suspect that the search for bioactive agents from fungi may be aided by application of ecological rationale (Wicklow 1981; Monaghan and Tkacz 1990; Dreyfus and Chapela 1994; Caporale 1995; Gloer 1995a, b; Anke et al. 1995; Eckerman and Graham 2000). Fungi commonly thrive in competitive environments, and it is often hypothesized that some of their secondary metabolic capabilities may be influenced by selection pressures exerted by other organisms. However, the majority of known bioactive fungal natural products have been discovered through industrial programs that involve mass random screening of uncharacterized isolates using laboratory fermentation

conditions. Choices of isolates for chemical investigation are based strictly on bioassay results, as are metabolite isolation procedures. Most modern industrial programs utilize an extensive array of specialized, often proprietary biological assays for activity against specific molecular, cellular and/or whole-organism targets. Advances in assay miniaturization and robotics permit extremely high throughput in many of these screens. Because of the sheer numbers of samples needed to satisfy the capacity of these assays, such programs can rarely provide support for ecological or taxonomic investigations of the organisms being screened, let alone studies of the possible roles of their metabolites.

The most important fungal secondary metabolite discoveries in recent years have been due to the creative development and implementation of bioassays, rather than to chemical studies of rare or specially selected types of fungi. For example, once an assay for inhibitors of HMG-CoA reductase was selected and developed, many fungi were found that produced lovastatin and/or close analogs thereof (Chakravarti and Sahai 2004). Similarly, assays for 1,3- $\beta$ -D-glucan synthase inhibitors have proven to be effective in leading to discovery of various members of the powerful echinocandin class of antifungal agents (Barrett 2002; Wiederhold and Lewis 2003). Many other architecturally complex fungal metabolite-types with potent biological effects (e.g., trichothecenes, destruxins, cytochalasins, paspalinines, cyclosporin A, mycophenolic acid) have been found to occur as metabolites of disparate fungal taxa. On the other hand, a considerable number of bioactive metabolites have been reported only from a single species or a single isolate. The latter cases provide supporting arguments for studies targeted toward rare fungal taxa or relatively unexplored ecosystems. Secondary metabolite profiling of isolates being surveyed can be valuable in quickly recognizing known compounds and providing useful taxonomic information, and can also afford important clues about which species might be producing unknown metabolites (Frisvad 1989; Larsen et al. 2005). However, bioassay-guided fractionation remains the paradigm of natural product discovery in most laboratories.

Examples of fungal chemicals likely to be associated with ecological phenomena include toxic mushroom metabolites that are presumed to play roles in defending fruiting bodies from fungivores, phytotoxins from plant pathogenic fungi that play roles in disease processes, in-

secticidal metabolites responsible for the toxic effects of entomopathogenic fungi, and the ergot alkaloids, chemical defenses found in the ergot of *Claviceps* spp. and in certain grasses as metabolites of fungal endophytes. Chemical investigations of such sources could be viewed as "ecology-based" approaches leading to the discovery of fungal metabolites with somewhat predictable types of bioactivities. Studies of such phenomena are complementary to random screening because they incorporate rationale into the process of selecting fungi for chemical study. This kind of approach is particularly well suited to academic laboratories because resources for efficient mass screening on-site in an appropriately wide variety of assays are not typically available in academia. Realistically, throughput limitations in most academic laboratories, coupled with longer turnaround time for results and a somewhat more diverse (e.g., educational) mission, preclude true direct competitiveness with industrial in-house microbial chemistry programs. Ultimately, collaborations with industry are essential if compounds are to be fully evaluated. However, application of rationale at the beginning of the screening process limits the number of organisms that need to be investigated, reduces costs, facilitates dereplication, and permits evaluation (and subsequent result-based modification) of a hypothesis. Our own work has illustrated that such rationale can be very effective in leading to the isolation of new (and patentable) fungal metabolites with antifungal or antiinsectan activity, as well as other effects. Although we have not yet demonstrated through our own work that such an approach can lead to marketable products, application of ecological rationale in the search for novel bioactive fungal products may well result in findings with practical utility.

There are many elements of natural product science and efforts to apply ecological principles to the search for bioactive fungal metabolites that seem somewhat incongruous with typical approaches to commercial product development. Even if one assumes that some metabolites have evolved because they play a role in the life cycle of the producing species, the natural targets of these molecules are likely to be different from those of greatest interest in medicine. Such molecules are also unlikely to have built-in properties suitable for desirable pharmacokinetics. Furthermore, while focus in the search for new pharmaceutical or agrochemical agents is typically placed on a desire for discovery of extremely potent "magic-bullet"

compounds, natural functions that secondary metabolites might serve do not necessarily require high levels of potency or specificity, nor do they even require single entities. Synergistic effects occur commonly in nature, but can be difficult to sort out, and are much more complicated to take advantage of in pharmaceutical development. It could be argued that defensive or antagonistic effects exerted by cocktails of moderately active compounds with differing modes of action would be more effective against predator or competitor organisms because evolution of resistance to such mixtures would be more difficult. It is interesting to note that some recently marketed pharmaceutical products (including Vytorin®, a successor to Zocor® as a cholesterol-lowering drug) are actually mixtures of compounds that have different modes of action. Despite these incongruities, application of ecological considerations to the search for new bioactive natural products provides rationale for targeting certain organisms or types of organisms that reduces the level of randomness in the process, while leading to new information about potential natural roles of secondary metabolites, as well as new tools for ecological studies.

## II. Observations in Fungal Ecology Associated with the Production of Bioactive Metabolites

### A. Competitive or Antagonistic Interactions

Competitive and/or antagonistic interactions among fungal species constitute a general category of effects that are widely recognized to occur in nature (Shearer 1995), and also are among the most straightforward to attribute to secondary metabolites. Antagonism between species of naturally competing fungi has been reported in virtually every fungal ecosystem. Examples include coprophilous (Wicklow 1992a, b), carbonicolous (Wicklow and Hirschfield 1979a), lignicolous (Strunz et al. 1972; Boddy 2000), phylloplane (Fokkema 1976), rhizosphere (Carroll 1992), marine (Strongman et al. 1987), and freshwater aquatic fungi (Shearer and Zare-Maivan 1988). It has long been proposed that such interactions are important factors in determining the organization, composition, and pattern of succession within these ecosystems (Webster 1970; Wicklow 1981). The mechanism of antagonism often appears to

involve the production of a chemical agent (or agents) by one species that inhibits the growth of another, but many such reports have not been followed up by studies of the chemistry associated with these phenomena. Chemical studies of this nature have significant appeal, since the metabolites responsible for these effects are essentially natural antifungal agents. The need for new antifungal agents in both medicine and agriculture continues to grow. Although effective topical antifungal agents are relatively abundant, many types of topical fungal infections recur after cessation of treatment, and others (e.g., nail infections) are particularly difficult to treat effectively (Baker et al. 2005). More importantly, very few drugs are available that are therapeutically useful in the treatment of systemic fungal infections (Richardson and Marriott 1987; Balkovec 1998; Wong-Beringer and Kriengkauykit 2003). Fungal diseases have become increasingly common, and there are several risk groups of growing population (e.g., AIDS and chemotherapy patients) that are particularly susceptible to opportunistic fungal infections (Koltin 1990; Wong-Beringer and Kriengkauykit 2003). Moreover, resistance to existing antifungals has become problematic (Anderson et al. 2003). New agriculturally useful fungicides are also being continually sought. It can be argued that fungi are logical sources to explore in search of agents that regulate fungal growth, interact with important fungal receptors, or modulate the activities of key fungal enzymes. Indeed, several particularly promising antifungal leads have been isolated from fungal sources, including echinocandins, pneumocandins, sordarins, and papulacandins, and, as noted above, there are antifungal agents in use in both clinical and agricultural settings that are based on compounds originally obtained from fungal sources.

Our initial chemical studies of antagonistic interactions involved investigations of coprophilous (dung-colonizing) fungi (Malloch 1981). These fungi were targeted in part because of their well-documented successional patterns on natural substrates (i.e., replacement of early-occurring, fast-growing colonists by later, slower-growing competitors; Harper and Webster 1964; Webster 1970; Angel and Wicklow 1983), along with sporadic, but tantalizing reports of antagonism among such species that were presumed to be due to the production of antagonistic agents (Harper and Webster 1964; Ikediugwu and Webster 1970a, b; Singh and Webster 1973; Wicklow and Hirschfield

1979b). From an evolutionary perspective, it seems logical that slower-growing colonists might evolve mechanisms that help to eliminate fast-growing, nutrient-consuming competitors from the local substrate. Additional support for the concept of targeting coprophilous fungi was based on their taxonomic distinctiveness that is, in turn, associated in a significant way with their adaptations to this niche. Few coprophilous species had been previously studied from a chemical standpoint prior to our work, and it is, of course, appealing for a natural product chemist to explore a source that has not been previously investigated, since the probability of finding *new* agents would likely be higher under such circumstances.

Based on these and other considerations outlined in greater detail in the earlier volume (Gloer 1997), we targeted certain types of mid- to late-successional coprophilous fungi as potential sources of antifungal agents. In petri plate competition assays carried out in our laboratories, over 60% of the coprophilous isolates surveyed displayed inhibitory effects toward competitor fungi at a distance. The vast majority of these cultures represented genera and/or species for which no chemistry had been previously reported. Chemical

studies focusing on the antagonistic isolates were undertaken, and this work afforded a variety of new biologically active natural products, many of which displayed antifungal effects. Our early chemistry results in this area have been reviewed previously (Gloer 1997), but studies of coprophilous fungi in our laboratory have continued to afford new bioactive metabolites, and selected representatives encountered in more recent years are included in Fig. 15.1. Studies of *Sporormiella vexans* afforded a set of new aromatic metabolites called sporovexins (e.g., 1), along with a previously unreported preussomerin analog (2; Soman et al. 1999a). Preussomerins are intriguing and unusual structures that display a variety of biological effects, including antifungal activity. The first representatives of this class were originally reported by our group from a different coprophilous fungus (*Preussia isomera*; Weber and Gloer 1991). *Podospora communis* produced a series of at least eight novel polyketide-derived metabolites with furanone, cyclopentanoid, furanofuran, and furanocyclopentanoid structures (e.g., 3–5; Che et al. 2004a, 2005). The variety of structural types and different ring systems arising from an apparent common biogenetic precursor was particularly impressive in this in-

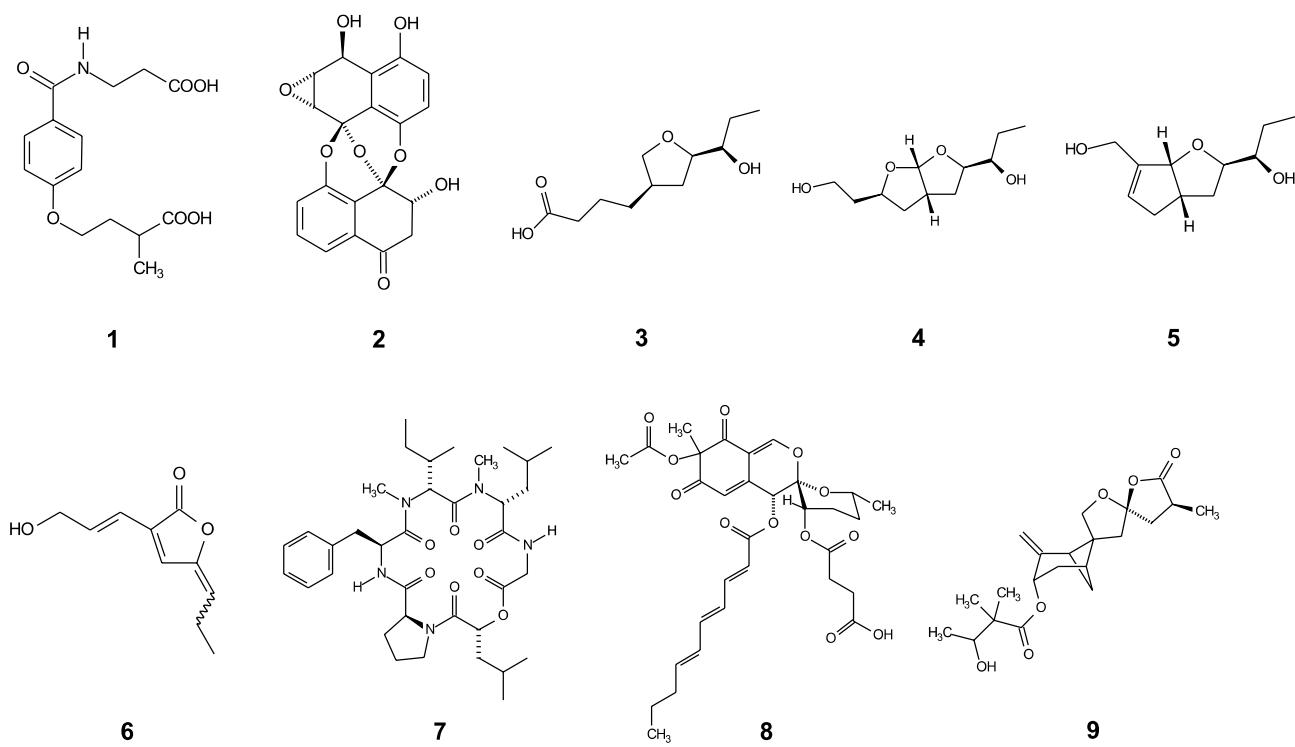


Fig. 15.1. Some representative new metabolites from coprophilous fungi (1–9)

stance. *Bombardioidea anartia* afforded a series of new anti-*Candida* furanones called bombardolides (e.g., 6), as mixtures of geometric isomers that could not be separated (Hein et al. 2001). Further examples include cyclic peptides (e.g., 7), additional polyketides (e.g., 8), and terpenoid-derived metabolites (e.g., 9; Che et al. 2001, 2002b).

Some of the new compounds encountered in this project showed potent activity against competitor fungi (MIC values < 5 µg/ml), and/or activity against more medically relevant organisms such as *Candida albicans* or *Aspergillus flavus*. Naturally, known compounds were also encountered, and some of the new metabolites are closely related to compounds that were previously known, but others contain some quite novel structural features. In many instances, these findings arose from studies of fungal species for which no prior chemistry had been described. Given the high incidence of antifungal activity and new chemistry among these isolates, the probability of encountering more promising activity or useful lead structures through further studies of the chemistry of coprophilous fungi seems high. Indeed, other research teams have also had success in targeting coprophilous fungi as sources of bioactive metabolites (Ondeyka et al. 1998; Macias et al. 2001; Segeth et al. 2003; Singh et al. 2003; Ridderbusch et al. 2004; Weber et al. 2005; Lehr et al. 2006).

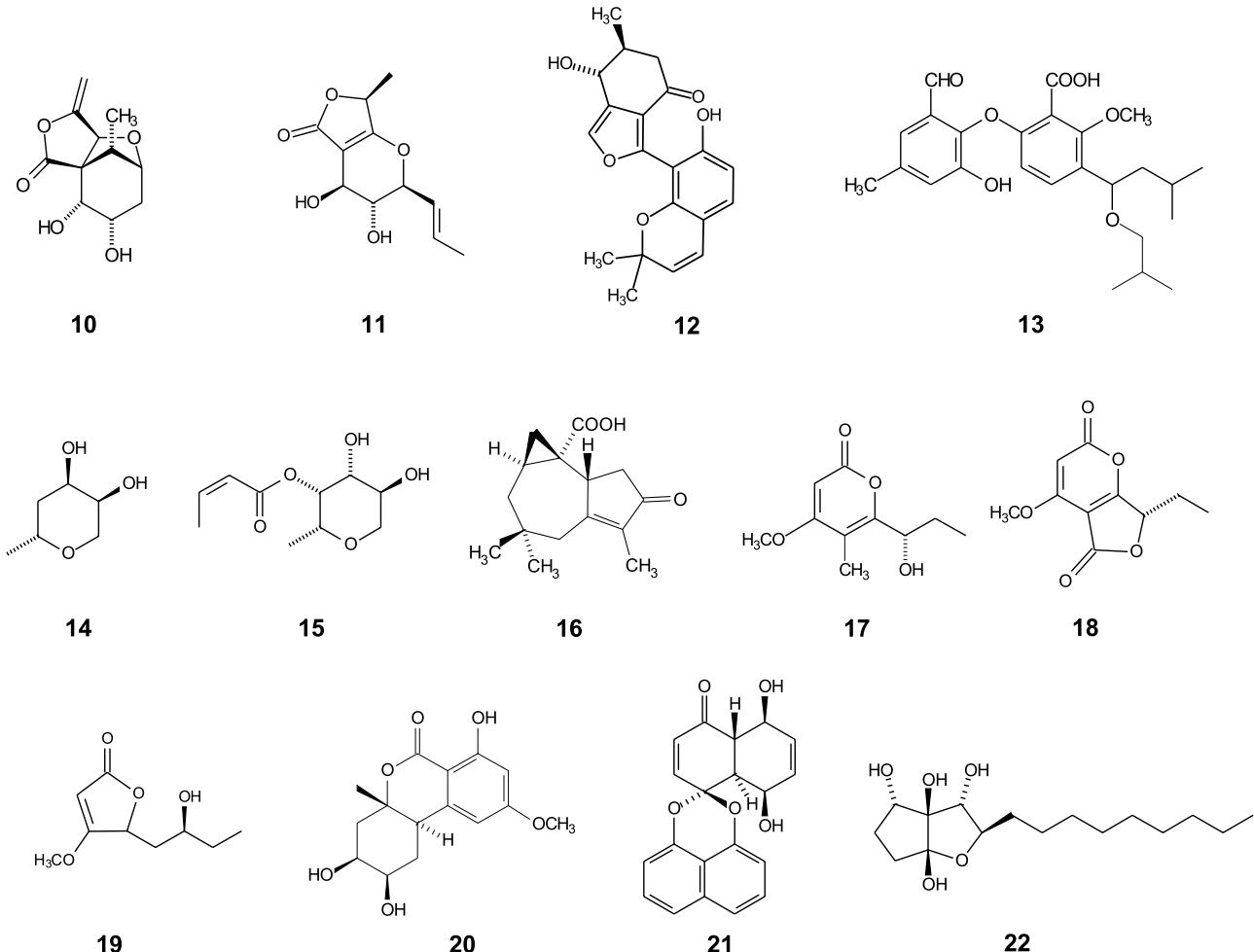
Although the results above demonstrate that antifungal activity is a logical and effective primary target assay, agents with antifungal activity often exhibit other potentially useful biological effects. For example, cyclosporin was originally isolated from a *Tolypocladium* sp. as an antifungal agent (Masurekar 2005). Cyclosporin is now a vitally important immunosuppressive drug, and a valuable pharmacological tool.

The studies summarized above involved only a small subset of organisms isolated from only one of many niches in which interference competition among fungal competitors has been reported. Naturally, the success of this project led us to consider exploration of antagonistic fungi from other ecosystems. Another underexplored ecological group that parallels the coprophilous niche as a reasonable choice in many respects consists of the freshwater aquatic fungi. Freshwater aquatic fungi are similarly underexplored from a chemical standpoint, have been reported to show antagonistic activity toward competitors in some instances (Shearer and Zare-Maivan 1988), and are taxonom-

ically and morphologically distinctive (Goh and Hyde 1996). Initial investigations reviewed previously led to the discovery of several new bioactive natural products (Gloer 1997). Further studies of antagonistic freshwater aquatic fungal isolates in our laboratory have continued to afford novel metabolites with unusual chemical structures and/or significant biological activities.

While a small number of other research groups have also published in this area (Schlingmann and Roll 2005; Dong et al. 2005), some recent examples from our own ongoing work are shown in Fig. 15.2. An isolate of *Massarina tunicata* was particularly prolific, producing multiple new metabolites representing different polyketide-derived structural classes (Oh et al. 1999a, 2001, 2003) exemplified by structures 10–12. *Dendrospora tenella* produced a series of new antibacterial diphenyl ether derivatives (e.g., 13; Oh et al. 1999b). *Ophioceras venezuelense* was found to produce a series of monocyclic tetrahydropyran-type compounds (ophiocerins A–D; e.g., 14, 15; Reátegui et al. 2005). Surprisingly, despite their simplicity and abundance in culture, these compounds had not been previously encountered as natural products. This species also afforded a new africanene sesquiterpenoid (16) that was of interest not only because it has a rare skeleton, but also because it is the first member of this terpenoid group to be encountered from a microbial source. The new species *Annulatasca triseptatus* produces a series of simple, yet previously unreported, polyketide-derived pyrones and furanones (annularins; 17–19), some of which show antibacterial activity (Li et al. 2003a). The fused pyrone-furanone system in annularin F (18) had not been previously reported. An unidentified member of the family Tubeufiaceae afforded several new altenuene analogs (e.g., 20; Jiao et al. 2006a), while investigations of an isolate of *Decaisnella thyridioides* yielded a series of five new palmarumycin-type compounds called decaspirones (e.g., decaspirone A, 21) with significant activity against *A. flavus* (Jiao et al. 2006b). Studies of the aero-aquatic fungus *Helicodendron giganteum* led to the discovery of three more new antifungal metabolites (heliconols; e.g., 22) possessing another unusual ring system (Mudur et al. 2006a).

Many of the fungal isolates explored during the course of this project represent rare and/or previously undescribed genera and species. Our results to date, only a portion of which have been published, include the first secondary metabolite structures ever described from members of the genera



**Fig. 15.2.** Some representative new metabolites from freshwater aquatic fungi (10–22)

*Anguillospora*, *Annulatascus*, *Decaisnella*, *Helicodendron*, *Kirschsteinothelia*, *Pseudoproboscispora*, *Dendrospora*, *Ophioceras*, *Massarina*, *Aniptodera*, and *Pyramidospora*.

Analogous studies of other niche groups that commonly display interspecies antagonism can be similarly fruitful. Studies of wood-decay fungi that show antagonistic effects have afforded a number of agents that could be useful in controlling economically important wood-rotting fungi (Strunz et al. 1972; Ayer and Miao 1993; Ayer and Kawahara 1995). In other cases, individual niche groups are targeted for chemical studies mainly because of the distinctiveness of the niche, rather than because of specific indications of antagonistic effects. This is exemplified by the rapidly increasing number of reports of chemistry from marine fungal isolates (Lin et al. 2001; Proksch et al. 2003; Rowley et al. 2003; Bugni and Ireland 2004; Oh et al. 2005; Bhadury

et al. 2006; Boot et al. 2006; Cueto et al. 2006; Koenig et al. 2006), and from other, narrower niche groups, such as mangrove (Lin et al. 2001, 2002; Chen et al. 2003) and lichenicolous (Hawksworth et al. 1993; Bills et al. 2000; Seephonkai et al. 2002; He et al. 2005; Lin et al. 2005) fungi.

It is interesting to consider whether antifungal metabolites produced by antagonistic fungal species occur under natural conditions, since this would be a requirement in order for them to play some role in the ecology of the producing species. Studies of this kind of issue are unfortunately rare, but there are exceptions. For example, it has been demonstrated that the antifungal agent sordarin (Odds 2001), produced by a coprophilous fungus, is indeed produced in the natural substrate (Weber et al. 2005). It has also been shown that some species produce active metabolites under a certain set of laboratory culture conditions only when chal-

lenged with microbial competitors (Oh et al. 2005). However, it is difficult to demonstrate true roles for such compounds in nature with confidence. Detailed individual case studies would be particularly valuable in shedding light on the ecology of interspecies interactions, and relating ecological observations directly to chemistry produced by individual organisms under specific circumstances. Nevertheless, efforts in this area must be balanced with the broader interest in discovery of potentially useful new chemistry. Because our primary focus is on the search for new chemistry of potential value in medicine or agriculture, it is unfortunately impractical for us to investigate in depth the roles of the metabolites we encounter in most instances. However, the following section describes a project wherein the potential roles of metabolites were investigated in somewhat greater detail.

### B. Resistance of Key Fungal Structures to Fungivory or Microbial Attack

The vast majority of known fungal metabolites are produced in liquid fermentation cultures under conditions very different from those encountered by the fungi in nature. Although little is known about possible functions these compounds may have within the producing organisms, there is frequently a close correlation between secondary metabolite production and morphological differentiation in liquid culture (Bennett 1983). Interestingly, many fungi produce morphological structures under natural conditions (or on solid substrates in the laboratory) that are not generally formed in liquid cultures. Such structures include various fruiting bodies, sclerotia, and stromata. If there is secondary chemistry associated with such structures, it is reasonable to expect that such chemistry may not manifest itself under conditions under which these bodies are not formed.

Our own studies in this area focused on important fungal survival structures (sclerotia and ascostromata) that are exposed to potential predators (fungivorous insects) under natural conditions. Our results indicated that fungal sclerotia and ascostromata often contain unique antiinsectan metabolites that may help to protect them from predation (see below). This work was characterized by a particularly high incidence of previously undescribed natural products, even in cases where the producing fungi had significant histories of prior chemical investigation.

A considerable body of direct and circumstantial evidence for the production by fungi of chemical defenses against predation had been previously compiled and reviewed (Wicklow 1988a). Fungal toxins are often implicated as possible defenses in a general sense, but relatively few detailed studies of the chemistry involved have been reported. There are many reports of grazing preferences among fungivores. For example, mycelia or fruiting structures of certain fungi are known to be avoided by fungivorous insects or arthropods (Curl et al. 1985; Shaw 1992; Wicklow 1992a). The presence of bioactive secondary metabolites is often invoked or implicated in these phenomena, and agents responsible for the observed effects have occasionally been identified (Wicklow 1988a; Bernillon et al. 1989; Koshino et al. 1989). Some fungivores appear to have evolved detoxification mechanisms allowing them to consume, and sometimes specialize in consuming, toxin-producing fungi (Wicklow 1988a; Dowd and VanMiddlesworth 1989; Shaw 1992). There is also evidence that some advantages may be conferred on certain fungi upon moderate grazing by fungivores (e.g., inoculum dispersal; Stevenson and Dindal 1987; Shaw 1992). Many other instances of selective grazing or refusal of fungi by individual mycophagists could be cited.

#### 1. *Claviceps* Ergot

Considering the observation that the concentration of plant defensive metabolites is often highest in reproductively important plant parts (Rhodes 1985), it seems logical to initiate discussion of fungal "chemical defenses" by considering physiological structures that are particularly important to fungal survival. One precedent provides a particularly useful introduction. The ergot alkaloids comprise a class of medicinally useful compounds that were originally isolated from the ergot (sclerotia) of *Claviceps purpurea* (Mantle 1978; Masurekar 1992), a parasitic fungus found on many species of cereal plants. This class of compounds exhibits a wide array of physiological activities, and many ergot alkaloids have found medicinal uses (Floss 1976; Stadler and Giger 1984). Chemical studies of *Claviceps* were not based on a general interest in sclerotial metabolites, but rather were stimulated by the long-term implication of ergots in poisonings of humans and livestock. It is especially significant that the ergot alkaloids were originally found only in the sclerotia of the fungus (Mantle 1978). *Claviceps* sclerotia are not formed in liquid fermentation cultures, and

many of the fungal ergot alkaloids would not have been discovered through screening of liquid cultures alone. The medicinal importance of the ergot alkaloids has led to the gradual development by the pharmaceutical industry of *Claviceps* strains that produce some of the compounds in liquid fermentations, but even in these cultures, alkaloid production is associated with "sclerotial-like" cells (Mantle 1978). It has been proposed that the evolutionary development of the ergot alkaloids may have been guided at least partly by selection pressures exerted by herbivores (Kendrick 1986; Wicklow 1988a).

## 2. Sclerotia of Other Fungi

There is, of course, no reason to believe that production of bioactive sclerotial metabolites is limited to *Claviceps*. Several literature reports afford circumstantial evidence that sclerotia produced by other fungi contain biologically active metabolites. These fungi include species of *Sclerotinia*, *Sclerotium*, *Verticillium*, *Macrophomina*, and *Aspergillus* (Tanda et al. 1968; Morrall et al. 1978; Wicklow and Cole 1982; Wicklow et al. 1988).

Sclerotia, in general, are specially adapted, multicellular structures produced by certain fungi as a survival mechanism (Willets 1971, 1978; Coley-Smith and Cooke 1971). These durable resting bodies can survive periods of dry, nutrient-poor conditions that other fungal parts cannot withstand. They then serve as vital sources of primary inoculum for the fungi when conditions again become favorable for growth. Generally, sclerotia survive under more severe conditions and for longer periods than any other kinds of fungal bodies, sometimes remaining viable in soil for periods of several years. Sclerotia are by far the largest fungal propagules, ranging in size from 30 µm to several cm, depending on species, and their germination frequently results in the generation of very large quantities of inoculum. Thus, sclerotia represent a substantial metabolic investment for the producing fungi.

It is interesting to consider how the presence of bioactive metabolites could impact on sclerotial survival (Wicklow 1988a). Sclerotia typically form in fungus-infected plant tissues, and are separately dispersed onto the soil surface or remain attached to decaying plant parts. Soil is heavily populated with insects and other invertebrates (Keven 1965), many of which are known to consume fungi (Wicklow et al. 1988). A dormant (or germinating) sclerotium would represent a substantial

nutrient reward for an insect predator, especially since sclerotia possess a much higher nutrient content than the unorganized mycelium (Willets 1971). Sclerotia damaged by insect larvae are much more susceptible to microbial decay than are undamaged sclerotia (Baker and Cook 1974). Thus, if sclerotia commonly contain metabolites that somehow limit feeding by insects, this property could clearly influence the longevity of these important fungal bodies. Based on parallel observations in plant chemical ecology (e.g., allocation of defensive metabolites to seeds; Rhodes 1985), there is reason to suspect that selection pressures exerted by insect predation may have led to the evolution of sclerotial chemical defenses (Wicklow and Cole 1982; Wicklow et al. 1988).

Our efforts in this area were initially stimulated by the fact that the sclerotia of *Aspergillus flavus* are avoided by the common detritivorous beetle *Carpophilus hemipterus*, an insect that feeds on the conidia and mycelia of the same fungus (Wicklow et al. 1988). In light of this observation, we investigated the secondary metabolites of *A. flavus* sclerotia, and encountered a number of antiinsectan compounds not found in the conidia or mycelia of the fungus (Gloer et al. 1988; Wicklow et al. 1988). These compounds were not produced in simple liquid shake cultures of *A. flavus*, and most of them had not been previously reported (Gloer et al. 1988). Furthermore, the most potent antiinsectan metabolite was nontoxic to vertebrates at 300 mg/kg (Cole et al. 1981), but was found to be present in sclerotia at levels that would effectively deter feeding in *C. hemipterus*. These findings led us to initiate general studies of the chemistry of *Aspergillus* sclerotia as sources of new antiinsectan natural products (Gloer 1995a, b). Assays for activity against *C. hemipterus* and the important crop pest *Helicoverpa zea* (corn earworm) were employed to guide isolation procedures. *H. zea* is unlikely to be ecologically relevant to the sclerotia of *Aspergillus* spp., but the discovery of agents with potent activity against *H. zea* could be important from a more practical perspective.

Most of the results from this project were summarized in detail in the earlier volume in this series (Gloer 1997). Briefly, our bioassay-guided chemical studies of sclerotia afforded over 70 new metabolites and approximately 30 known compounds, the majority of which display some degree of activity against insects (Gloer 1995b). Indole diterpenoids, a unique class of structurally complex fungal metabolites that display a broad

array of biological activities (Parker and Scott 2005), were particularly common among these samples, although other metabolite-types were also encountered. In most cases where detailed analyses were performed, the compounds of interest were found to be concentrated at least to some degree in the sclerotia. The success rate in finding new compounds was exceptionally high, despite extensive prior studies of the chemistry of *Aspergillus* spp. A few additional new *Aspergillus* sclerotial metabolites have been published since the previous review (Oh et al. 1998; Whyte et al. 2000). In addition, these studies were extended to a limited number of *Penicillium* spp. that are also known to produce sclerotia, and some new bioactive compounds were obtained in these investigations as well (Belofsky et al. 1998a; Joshi et al. 1999a).

The existence of antiinsectan sclerotial metabolites does not conclusively prove that they have a role in chemical defense – these studies provide only circumstantial evidence. However, the success of these investigations argues for chemical studies of other fungal structures that serve similar functions. These results also demonstrate that assumptions about the identity of compounds causing ecological effects should not be based on prior knowledge of the chemistry of a fungal species or genus. Given the vast prior knowledge about *Aspergillus* metabolites, such assumptions might have seemed warranted in the case of *Aspergillus* sclerotia, but would not have been valid based on the results described above.

### 3. Ascostromata

Other fungi produce morphological structures that are analogous to sclerotia in function. Certain ascomycetes, for example, produce ascostromata, which are proposed to play ecological roles similar to those of sclerotia (Wicklow and Cole 1984; Horn and Wicklow 1986). Studies of such structures have shown that they also can contain bioactive metabolites. Examples include ascomata of *Epichloe*, *Petromyces*, *Eurotium*, *Eupenicillium*, *Daldinia*, and *Talaromyces* spp. (Wicklow 1988a; Koshino et al. 1989; Nozawa et al. 1994; Belofsky et al. 1995; Wang et al. 1995; Ooike et al. 1997a, b; Suzuki et al. 1999; Stadler et al. 2001).

We therefore undertook what we viewed as a logical extension of the project described above by expanding our studies to include the sclerotoid ascostromata produced by species of *Eupenicil-*

*lum*. Initial bioassays showed an incidence of antiinsectan activity comparable to that observed among *Aspergillus* sclerotial extracts, although the potency of activity was somewhat lower on average. Studies of several *Eupenicillium* spp. afforded interesting results, including several more new compounds with antiinsectan activity. Interestingly, several of the compounds encountered were either identical to those obtained from sclerotia of *Aspergillus* spp. (e.g., aflavinine analogs and other indole alkaloids; Belofsky et al. 1995; Wang et al. 1995), or structurally similar, and in cases where analysis of ascostromata vs. other fungal material was carried out, the compounds were again heavily concentrated in the ascostromata. Based on these findings, it appears that at least some of the ascostroma-producing *Eupenicillium* spp. have evolved (or retained) chemical defense systems similar to those of *Aspergillus*. Studies of other *Eupenicillium* spp. led to isolation of other active metabolite types, some of which displayed antiinsectan effects (Belofsky et al. 1998b; Wang et al. 1998).

### 4. Other Fungal Structures

Other fungal structures such as spores, cleistothecia, or fruiting bodies may also be chemically defended. Selective chemical studies of very small structures are complicated by the difficulty in separating them in quantity from other fungal material, while studies of fruiting bodies are often hindered by an inability to form them in laboratory cultures, or to reliably predict when and where they will occur in nature. Some researchers have proposed defensive roles for metabolites found in fruiting bodies of basidiomycetes (Sterner et al. 1985; Shaw 1992). The amanitins and phalloidins, well-known toxic peptides found in fruiting bodies of the genus *Amanita*, are often cited as examples of possible chemical defenses. These compounds are toxic to a variety of insects, although some insect species, either coincidentally or through selection processes, have evolved the capacity to detoxify or tolerate these metabolites (Jaenike et al. 1983; Shaw 1992). Metabolites of edible mushrooms may also display potentially useful biological activities. The strobilurins are produced by a common edible mushroom, and have proven to be important as fungicides and leads thereto (Anke 1995; Peláez 2005). These compounds have also been proposed to play an ecological role, and are reportedly produced on their natural

substrate (Anke 1995), although most, if not all, of the strobilurins have been isolated from laboratory cultures. Other researchers have discussed the potential value of mycophagy to the fungi because of its importance as a mechanism of spore dispersal (Shaw 1992), citing examples of metabolites that attract fungivores. These reports are illustrative of the complexity of interpreting aspects of fungus-fungivore interactions as they might relate to secondary metabolite production. Sporadic reports suggest that smaller structures may also contain unique bioactive metabolites. Brevianamide A was found to be localized in the penicilllus of *Penicillium brevicompactum* and proposed to deter feeding in fungivorous arthropods (Wicklow 1986). Ascomata of *Chaetomium bostrychodes* were reportedly avoided by certain fly larvae, and this behavior was presumed to be due to the presence of secondary metabolites (Wicklow 1988a).

### C. Fungi that May Confer Host Resistance to Herbivory or Disease

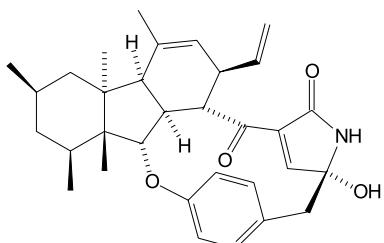
It has been proposed that the capability of fungi to produce mycotoxins evolved and has been retained partly because they render fungal substrates (e.g., fruits, seeds, etc.) unpalatable to herbivores (Janzen 1977; Kendrick 1986; Wicklow 1988a). While this could be viewed as a defense of substrate resources from competing consumers, it is too general a concept to be particularly helpful in targeting fungi for chemical investigation, and does not lend itself to field investigations. On the other hand, there are documented instances where secondary metabolites play roles in mutualisms between certain fungi and their hosts. One of the best known examples of such a system is the production by grass endophytes of fungal ergot alkaloids that influence feeding by herbivores (Clay 1988; Carroll 1992; Scott 2001, 2004). Evidence for parallel effects in other systems is also available. For example, fungal endophytes found in conifer needles produce metabolites that confer some level of resistance to herbivory by insects (Miller 1991; Findlay et al. 1995). Numerous industrial and academic groups have targeted endophytic fungi in general for inclusion in their screening programs (Caporale 1995; Lee et al. 1995), and several reviews of endophyte secondary metabolites have appeared recently (Tan and Zou 2001; Strobel 2002; Schulz et al. 2002; Gunatilaka 2006).

Although some researchers have argued that other endophytic fungi are likely to play roles in the ecology of the producing species, at least in some instances, the in-depth studies required to demonstrate this are seldom carried out, as the targeting of endophytes is in most cases viewed primarily as a means of enhancing the diversity of species being screened for activity, rather than any acknowledgment that such fungi might play defensive roles through metabolite production.

A related, intriguing area of research involves efforts by some groups to explore the chemistry of endophytes from specific plant hosts that are targeted because the plants produce compounds of industrial importance. The main objective of such work is to find fungal strains that produce compounds isolated from the plant, thereby enabling production of the compounds of interest by fermentation processes, and also perhaps enabling more ready access to the corresponding secondary metabolic genes. Reports of the important plant metabolites taxol, podophyllotoxin, and camptothecin as trace constituents of endophytes from the corresponding plant hosts have appeared (Li et al. 1998; Puri et al. 2005; Eyberger et al. 2006).

Recently, we have initiated preliminary studies of a subgroup of endophytes that do not appear to have been chemically explored by others to a significant extent. This work stems from an interest in the antagonistic properties of *Acremonium zeae*; an endophyte of corn that is widespread in agroecosystems in the USA and elsewhere. *A. zeae* is one of the two most common seed-borne endophytes of pre-harvest maize, typically producing symptomless kernel infections (Wicklow 1988b; Arino and Bullerman 1994), but the significance of its occurrence is not understood (Munkvold et al. 1997). Patterns of fungal occurrence within plant seeds can sometimes be indicative of interactions among the corresponding fungal species (Wicklow 1988a).

Based on several experimental observations, including exhibition of antagonistic activity toward other fungi in our laboratories (including *Aspergillus flavus*), our colleague D.T. Wicklow hypothesized that *A. zeae* produces antifungal metabolites that could interfere with mycotoxicogenic, destructive fungal pathogens of maize. Chemical investigations led to the discovery that isolates of *A. zeae* from across the USA produce a series of unusual antifungal and antibacterial antibiotics (e.g., pyrrocidine A, 23; Fig. 15.3; Wicklow et al. 2005). Although the lead compounds in the series were published first by an

**23**

**Fig. 15.3.** Structure of pyrrocidine A (23), a bioactive metabolite from the corn endophyte *Acremonium zeae*

industrial group from another fungal source (He et al. 2002), we continued our studies of the natural occurrence and potential significance of these *A. zeae* metabolites, and have found that they occur commonly as major metabolites of isolates of *A. zeae* from various geographical locations, and that the most active component shows potent activity against mycotoxigenic fungi, as well as fungal and bacterial pathogens of corn.

There are many other endophytes that are known to occur and grow asymptotically in healthy crop tissues, including latent plant pathogens (Sinclair and Cerkauskas 1996), and most of these do not appear to have been studied chemically. There have been several reports of specific grass/cereal endophytes that show antagonism against plant pathogenic fungi, but for which no fungitoxic compounds have been described (Siegel and Latch 1991; Stovall and Clay 1991; Christensen 1996; Danielsen and Jensen 1999). On basis of our results with *A. zeae*, we view this group of endophytes as another potentially important set of targets for chemical investigation, not only because of their apparent potential to produce antagonistic (antifungal) metabolites, or because of interest in roles they might play in agroecosystems, but also because knowledge of the chemistry of fungi that occur widely in crop plants could be of importance to human health, even if these fungi do not produce obvious plant disease symptoms.

#### D. Fungi that Cause Diseases or Damage to Host Species

The concept of using biological control strategies in crop protection has been studied intensively for many years (Gardner and McCoy 1992; Pow-

ell 1993). These strategies involve the use of certain fungi as mycoherbicides (Boyette 2000; Ghorbani et al. 2005), mycoinsecticides, or mycoparasites (Harman 2006) to control weeds, insects, or fungal pathogens, respectively. Such approaches involve the deployment of a microbial disease agent effective against the pest, rather than (or as an adjunct to) the application of a chemical pesticide of some sort. Ultimately, damage to the pest is often caused by toxin(s) produced by the pathogen, and the use of measured and properly formulated quantities of the natural products themselves could provide an alternative control strategy. Use of the microorganism itself is appealing because it serves to selectively direct the toxin(s) to the target. Some toxin-producing microorganisms, most notably the bacterium *Bacillus thuringiensis*, have proven to be particularly effective in such applications. However, there are many specialized hurdles that must be overcome to implement an effective biocontrol strategy (Powell 1993), and the use of biocontrol agents will not necessarily eliminate the problem of resistance (Gardner and McCoy 1992). From a chemistry standpoint, knowledge of any metabolites involved in biocontrol effects is important as a means of avoiding unwanted side-effects, and precedents indicate that studies of fungi with mycopesticidal properties are likely to lead to discovery of pesticidal compounds. Some examples of such results are provided below.

#### 1. Plant Pathogens

Plant pathogenic fungi are well known as producers of diverse compounds with phytotoxic effects on host plants (Turner and Aldridge 1983; Harborne 1993; D'Mello and MacDonald 1998). The effects of the toxins are often principal causes of symptoms associated with the corresponding plant disease. In many cases, fungal phytotoxins produce damage that fosters fungal invasion and colonization of the plant. Typically, the compounds are general phytotoxins (Ballio 1991), but in some cases, a coevolutionary process has led to at least some degree of host selectivity (Walton and Panaccione 1993; Wolpert et al. 2002). The host-selective toxins are particularly interesting from a chemical standpoint because they tend to have structural features that distinguish them from commonly encountered fungal metabolites that might show more general toxicity. Notable examples include victorin, HC-toxin, and certain *Alternaria* toxins (Walton and Panaccione 1993; Wolpert et al. 2002; Masunaka

et al. 2005). Plant pathogenic fungi as a group could be viewed as logical sources to explore in search of biologically active metabolites in a general sense, since they have already demonstrated the capacity to produce bioactive compounds with distinctive chemical structures. Some fungal metabolites with phytotoxic effects are known to exhibit medically relevant activities as well, including antitumor and antibiotic effects. It is interesting to note that mevinolin is a rather potent herbicide (Hoagland 1990).

From a more specific viewpoint, fungi pathogenic to weeds have been proposed as rational sources of herbicides. Indeed, phytotoxins with novel and unusual structures have been isolated from weed pathogens, and subsequently shown to display herbicidal activity toward weeds (Hoagland 1990, 2001; Amalfitano et al. 2002; Evidente et al. 2004). This would seem to be a particularly worthy avenue of investigation in view of the fact that other microbial natural products, such as bialaphos (produced by a *Streptomyces* sp.), have already been used successfully as commercial herbicides (Hoagland 2001).

## 2. Entomopathogenic and Nematophagous Fungi

A number of entomopathogenic and nematophagous (nematode-trapping) fungi have been considered as biocontrol agents (Sun and Liu 2006), and the physiological effects associated with some fungi from these categories have been linked to fungal toxins (Wicklow 1988a). Such toxins could serve as leads to the development of new insecticides or nematocides. In view of this possibility, together with the association of a significant component of predicted fungal diversity with estimates of insect diversity, it is surprising that relatively few studies of entomopathogenic fungi as sources of bioactive metabolites have been reported. Several distinctive classes of insecticidal metabolites have been encountered through early studies of such species, including the beauverolides, destruxins, and viridoxins (Turner and Aldridge 1983; Gupta et al. 1993). Further studies have appeared more recently, and are thoroughly summarized in a recent review (Isaka et al. 2005). Some of these compounds display both dietary and topical activity against insects. Metabolites from nematopathogenic or nematophagous fungi are even more uncommon; however, a related approach involves investigation of nematophagous fungi as sources of nematocidal toxins that cause paralysis or mortality of the

nematode prey (Kwok et al. 1992; Stadler et al. 1994; Anderson et al. 1995; Anke et al. 1995).

## 3. Mycoparasitic and Fungicolous Fungi

Mycoparasitic fungi act as parasites of others, and the invaded organism often suffers negative effects from this interaction that are likely to be caused in at least some cases by fungal toxins. Fungi encountered as colonists of others (often termed fungicolous or mycophilic isolates) may also produce antifungal metabolites, even though a true parasitic relationship may not have been demonstrated. Some mycoparasitic fungi that have been used or proposed as biocontrol agents because of their anti-fungal effects (e.g., *Trichoderma*, *Verticillium* spp.) have been shown to produce agents that inhibit the growth of other fungi (Cardoza et al. 2005). Examples include peptide antibiotics, phenolics, and terpenoids (Huang et al. 1995a, b; Morris et al. 1995). Antifungal metabolites and compounds with other biological activities have also been reported from other fungicolous and mycoparasitic species (Ayer et al. 1980; Tezuka et al. 1994, 1997; Wagner et al. 1995, 1998; Breinholt et al. 1997, 1998; Schneider et al. 1997; Fabian et al. 2001; Feng et al. 2003; Wilhelm et al. 2004).

Our own interest in this area was in part an outgrowth arising from the project summarized above that involved studies of sclerotial metabolites. Our initial investigations were stimulated by the recognition that *Aspergillus* sclerotia collected from soil were often colonized by other fungi. In many cases, when these sclerotia were subjected to standard germination conditions, many of those colonized by others were nonviable. This raised the question of whether such "attacking" species might be producing compounds that were inhibitory or perhaps fungicidal to *Aspergillus*. Given the medical importance of *Aspergillus* spp. as opportunistic human pathogens, it seemed logical and particularly appealing to investigate the concept of whether species that attack *Aspergillus* sclerotia might serve as sources of new antifungal natural products specifically having anti-*Aspergillus* effects.

On the basis of this rationale, sclerotia of *A. flavus* were buried in soil for extended periods, re-collected, and examined for the presence of fungal colonists. Many isolates were obtained and screened, and a sizable number of these produced fermentation extracts that showed activity against *A. flavus*. Chemical studies of the

active extracts afforded a variety of antifungal metabolites, including a number of compounds with interesting new structures (Fig. 15.4). One of the first sclerotium-colonizing species investigated in detail was an isolate of *Humicola fuscoatra*. Initial studies of this organism afforded five known compounds – three representatives of the monocillin/monorden class, as well as two cerebroside analogs (Wicklow et al. 1998). Interestingly, monocillins had been previously isolated from *Monocillium nordinii*, a parasite of a tree rust, and are thus among the few previous examples of antifungal metabolites reported from mycoparasitic fungi (Ayer et al. 1980). This result provided an immediate, intriguing parallel to our findings from the sclerotial project, in that we again observed metabolic similarities among different fungi with similar adaptive requirements. The occurrence of cerebrosides in this organism was also interesting because they are known to potentiate the activity of other antifungal agents in assays against *Candida albicans* (Sitrin et al. 1988). Continued studies of this *H. fuscoatra* isolate led to the identification of several other, unrelated compounds (Joshi et al. 2002), including the new triterpenoid glycoside fuscoatroside (24), which displayed potent activity in assays against *A. flavus*, and a new, relatively simple, but previously unreported amide (fuscoatramide; 25).

Studies of a sclerotium-colonizing isolate of *Mortierella vinacea* afforded three new benzenoids called mortivinacins A–C (e.g., 26, 27; Soman et al. 1999b). Mortivinacin A (26) contains a thiol ester functionality that is rarely encountered in fungal secondary metabolites. Another rich source of active metabolites obtained as a colonist of *A. flavus* sclerotia was an isolate of *Gliocladium catenulatum*. This species is known to be mycoparasitic, as are certain other *Gliocladium* spp. Ten compounds were isolated, including new verticillin analogs (28–30) and a new glisoprenin analog (31; Joshi et al. 1999b). The anti-*Aspergillus* activity was ultimately ascribed to a series of unusual polyketide diglycosides (e.g., 32; Joshi 1999), although the same structures were published at approximately the same time by another group from a soil isolate of *G. catenulatum* as cytotoxic agents and referred to as TMC-151-A, E, F, and G (Kohno et al. 1999). Each of these metabolites contains a large, highly methylated polyketide linked to two different, unusual monosaccharide units. These compounds were quite potent in assays against *A. flavus*. Three more intriguing new antifungal compounds were

obtained from a sclerotium-colonizing isolate of *Podospora curvicolla*, a member of what is considered a typically coprophilous genus (Che et al. 2004b). Curvicollides A–C (e.g., 33) accounted for most of the antifungal activity of the extract, showing activity in our assays against *A. flavus* and *Fusarium verticillioides* at one-tenth the potency of nystatin. These compounds have no close known relatives, and are unusual in that they appear to consist of two different polyketide units linked via the central γ-lactone ring.

These efforts were successful and reasonably productive in leading to new antifungal metabolites, and other researchers have demonstrated that mycoparasites that colonize the sclerotia of other fungi can also produce metabolites active against the host (McQuilken et al. 2003). However, we felt that the strategy of burying sclerotia in soil for long periods as “bait” for fungal colonists to survey for antifungal metabolites was not particularly appealing due to the time required, the labor-intensive nature of the corresponding field work, and limitations that seemed likely to arise from the use of a single host-type. Consideration of other options led us to widen the scope of this work to include fungi that colonize other long-lived physiological structures, such as basidiomycete fruiting bodies. Of course, some such structures are much longer-lived than others, but many wood-decay fungi, for example, form stromata that last for considerable periods. Such bodies are widespread and macroscopic, making them relatively easy to find and collect, and would comprise a diverse array of different host-types that seem likely to lead to a diverse collection of colonizing fungal taxa. Initial collections of fungal sporocarps were made in the upper Midwest USA. Isolation of fungal colonists from their surfaces, followed by fermentation, bioassay, and chemical analysis again led to the discovery of a variety of new compounds, many with antifungal effects, thereby validating the concept and eventually leading to further collections in the southeastern USA and in Hawaii that have afforded many additional new bioactive natural products. These studies remain a major focus of our current research program.

Aside from interesting new chemistry, as will be highlighted below, this effort has also led to some intriguing contributions to mycology. As one might expect, these include discovery of a number of new taxa representing a variety of genera. However, some unanticipated findings also arose. One observation made early in this

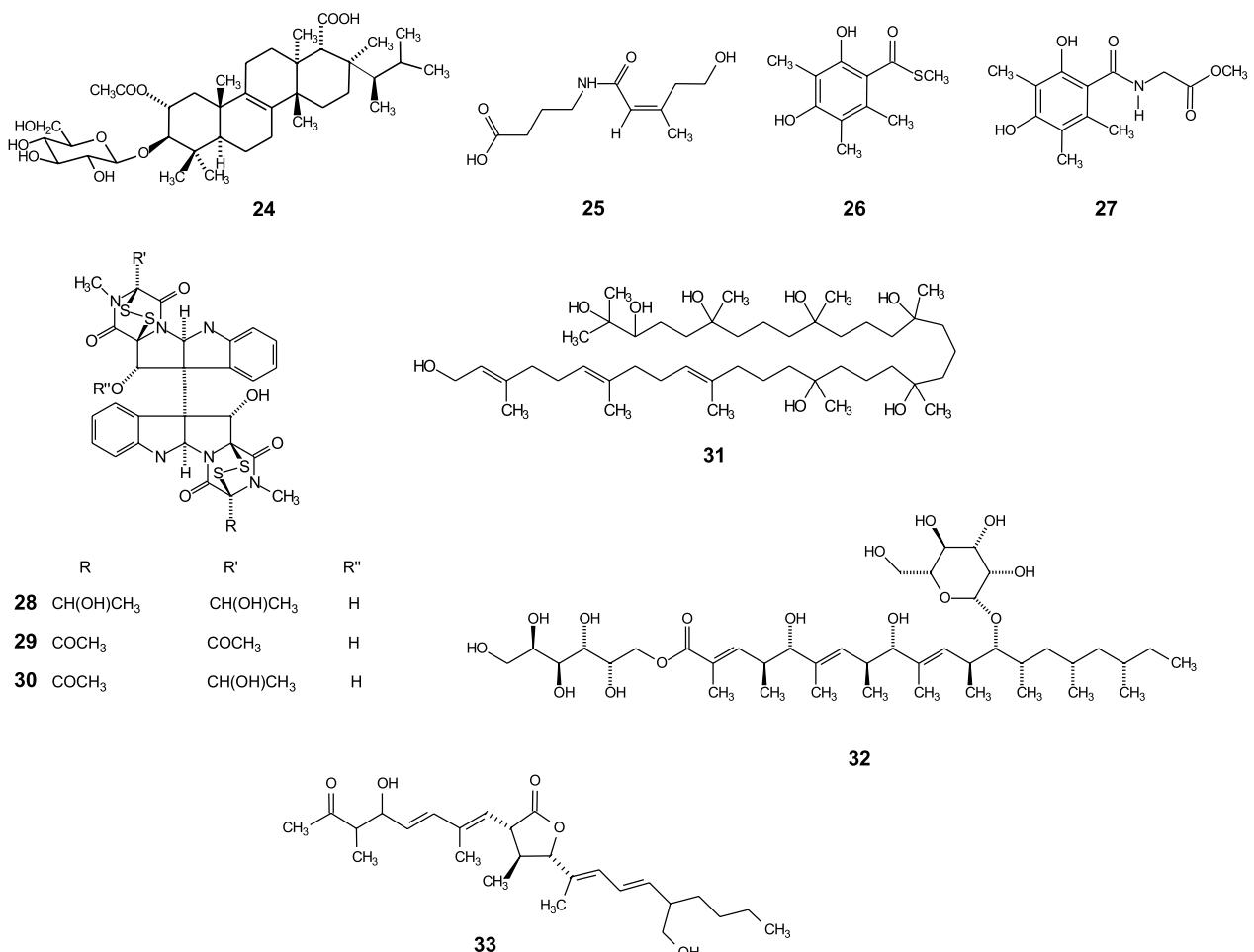


Fig. 15.4. Some representative new metabolites from fungi isolated as colonists of *Aspergillus flavus* sclerotia (24–33)

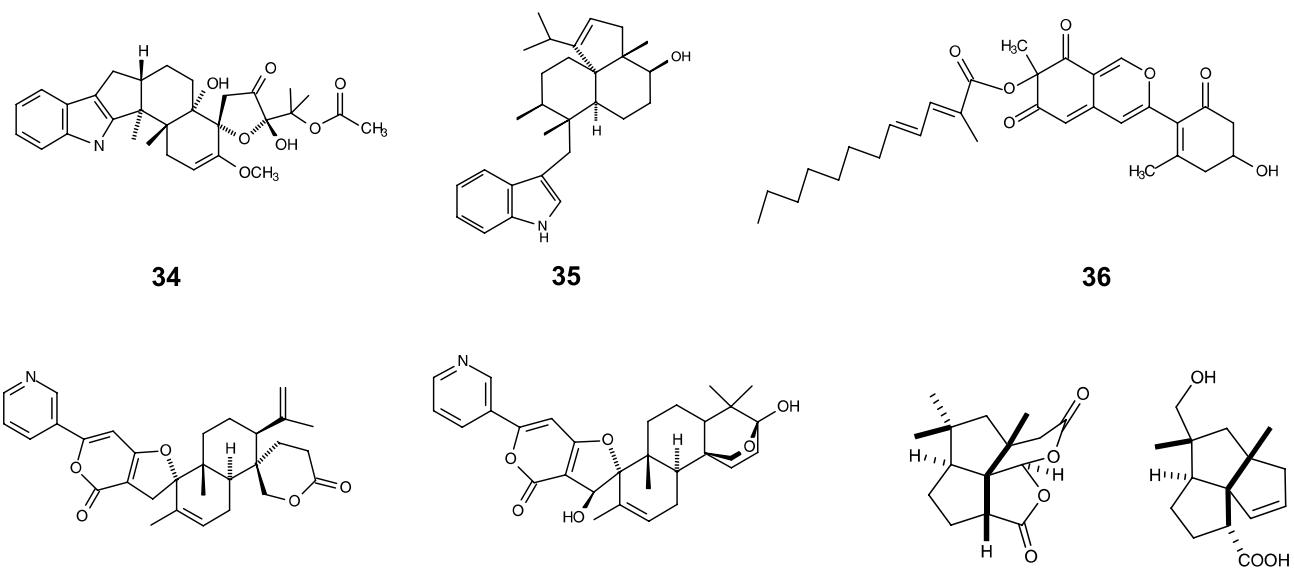
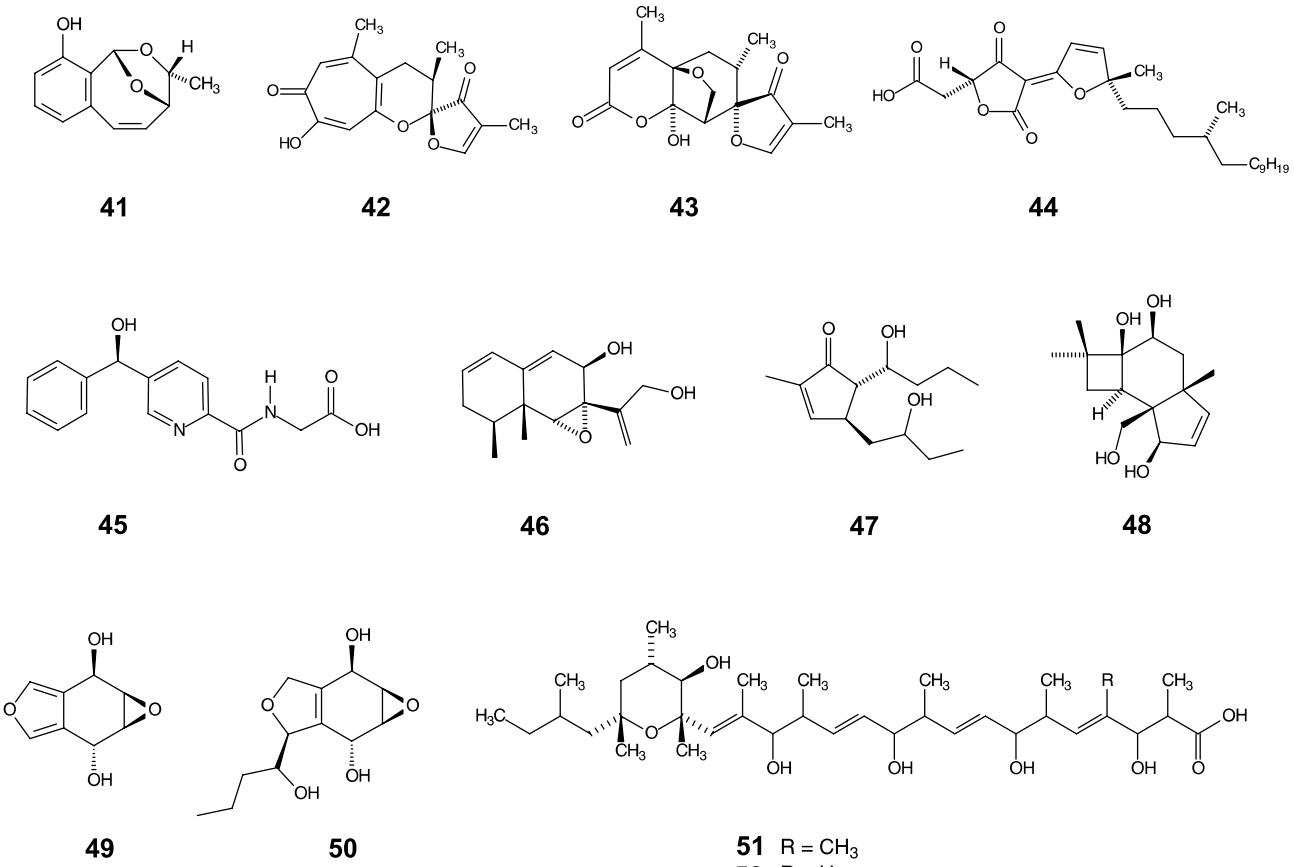


Fig. 15.5. Some representative new metabolites from fungicolous *Penicillium* isolates (34–40)

project was that *Penicillium* spp. were encountered with considerable frequency. Given the ubiquitous nature of *Penicillium* spp. in the environment, it was not particularly surprising that such species might be encountered among those occurring as colonists of the stromata of other fungi. However, it became clear upon continued investigation that some of the Penicillia being encountered appeared to be different from known species. Upon careful investigation of these isolates, accompanied by analysis of ITS and 26S rDNA sequences (ca. 1,200 bases; Peterson et al. 2004), it was discovered that many of them represented new species. This was an unexpected finding, and leads one to speculate about whether there is a subset of *Penicillium* species that have some predilection for colonization of other fungi. Encouragingly, chemical studies of some of these fungicolous *Penicillium* isolates have afforded interesting results (Fig. 15.5). While some of the new species produce previously known *Penicillium* metabolites, *Penicillium thiersii*, a new species isolated from a *Hypoxyylon* stroma collected in Wisconsin, produces a range of new compounds, including thiersinines (e.g., 34; Li et al. 2002) thiersindoles (e.g., 35; Li et al. 2003b), thiersilones (e.g., 36; Li 2003), and decaturins and new oxalicine analogs (e.g., 37, 38) that were similar to further new metabolites encountered during this project from another new species, *P. decaturense* (Zhang et al. 2003; Li et al. 2005). These metabolites include structures that incorporate several new or rarely encountered ring systems. A number of these metabolites show potent activity against the crop pest *Spodoptera frugiperda*, while compound 36 displays antifungal activity against *A. flavus*. Although not a new species, a Hawaiian isolate of *Penicillium griseofulvum* obtained as a colonist of a wood-decay fungus afforded a set of five new compounds called penifulvins (e.g., penifulvin A; 39) that incorporate a novel dioxafenestrane-type ring system (Shim et al. 2006a, b). Compound 39 showed significant antiinsectan activity against the fall armyworm *Spodoptera frugiperda*, causing a 74% reduction in growth rate relative to controls when tested at a 160 ppm dietary level. These unusual compounds appear to be derived from silphinene sesquiterpenoid precursors with an oxidative cleavage step occurring at some point in the biosynthesis, and this hypothesis was supported by the isolation of a new silphinene analog (40) from the same extract (Shim et al. 2006b).

Other fungicolous isolates obtained from various sporocarps collected through these studies have also been quite prolific as sources of new bioactive natural products. Only some recent examples that have already been published are cited here (Fig. 15.6). Polyketide-derived compounds possessing three additional new ring systems (cladoacetals, e.g., 41 and malettinins, e.g., 42, 43) were obtained from two unidentified fungicolous isolates (Höller et al. 2002; Angawi et al. 2003a, 2005). One of these colonists is a novel, but as yet unnamed hyphomycete resembling *Cladosporium*, and another is a *Mycelia sterilia*. Malettinin A (42) contains a relatively rare, naturally occurring tropolone unit, and shows potent activity against *A. flavus* (MIC 6 µg/ml). Lowdenic acid (44) is a similarly potent antifungal obtained from a new *Verticillium* species (Angawi et al. 2003b). Studies of an isolate of *Verticillium lecanii* encountered as a colonist of a basidiocarp of the mushroom *Amanita bisporigera* led to the isolation of several new pyridine derivatives (vertilecanins; e.g., 45; Soman et al. 2001). *V. lecanii* is known as an insect pathogen, and the extract showed activity against *Helicoverpa zea*, but the antiinsectan constituents were not isolated. Several antibacterial sesquiterpenoid metabolites (e.g., 46) and a cyclopentenone derivative (47) were obtained from a *Phoma* sp. isolated from stromata of *Hypoxyylon* sp. (Che et al. 2002a), while further antibacterial sesquiterpenoids (e.g., 48) were also obtained from a culture of *Pestalotiopsis disseminata* isolated from stromata of an unidentified pyrenomycete (Deyrup et al. 2006). A *Phaeoacremonium* sp. isolated from stromata of *Hypoxyylon truncatum* afforded two new isobenzofuranoids (49, 50), with 49 being somewhat distinctive among members of this class in that it retains the aromaticity of the furan moiety, rather than the benzene ring. However, the antifungal activity of the fermentation extract was ascribed to the presence of more common sorbicillin-type compounds (Reátegui et al. 2006). An isolate of *Sporormiella minimoides* was found to produce a pair of antifungal agents ( $IC_{50}$  vs. *A. flavus* ca. 6 µg/ml; MIC ca. 10 µg/ml) with intriguing structures (51, 52) of presumed polyketide origin (Mudur et al. 2006b).

Many additional new compounds have already been isolated and identified from further fungicolous isolates in our laboratory, but have not yet been reported. In summary, this niche is proving to be a particularly prolific one from the standpoint of



**Fig. 15.6.** Some representative new metabolites from other fungicolous fungal isolates (41–52)

new chemistry, and many of the agents encountered (both new and previously known) display antifungal activity, as was proposed at the outset of the project.

### III. Perspectives and Future Directions

The results reviewed here do not prove that fungi have evolved chemical defenses, or that the antagonistic agents produced by certain fungi render a competitive advantage. Carefully controlled studies are needed to determine whether these compounds are truly significant in the life histories of the producing species. Even so, it is clear that observations in fungal ecology can be employed to generate strategies that have proven effective in the discovery of novel bioactive fungal metabolites. From a mycological perspective, these findings help to validate the application of fundamental princi-

ples of chemical ecology to studies of fungi, provide additional tools with which to study the ecology of the fungi involved, foster the discovery of new taxa, and supply information that could be useful in exploring genetic relationships.

Investigations based on observations in fungal ecology are appealing from a chemistry standpoint because it is more attractive to seek bioactive metabolites with a hypothesis in mind than to screen organisms at random. As demonstrated here, such studies can lead to the discovery of fascinating new chemistry, and often provide unexpected analytical or structural challenges. Although unambiguous identification of natural products, even previously reported metabolites, can be a lengthy process, continuing technical improvements in methodology for isolation and structure determination (Harris 2005) now allow identification of milligram- or sub-milligram quantities of complex, unknown natural products in many cases.

The frequency of occurrence of novel compounds in our studies to date continues to be relatively high. This is due in part to the assay systems employed. The use of "ecologically relevant", whole-organism assays, in addition to more sophisticated medically and agriculturally relevant test systems, would understandably lead to some discoveries not likely to be made by those employing only the latter assays. However, the less random selection of organisms for study, and the relatively unexplored nature of many of these fungi and their interactions are also contributing factors. Strategies of this type cannot replace random screening programs in the search for new bioactive natural products, but can offer insights that could increase the diversity of compounds encountered in such programs. For example, these results argue strongly for employment of solid-substrate fermentation in studies of fungal metabolites, for consideration of habitat and ecological characteristics when selecting fungi for study, and for allowing longer fermentation times to access metabolites produced by slower-growing species.

There is no shortage of current and future challenges that fungal natural products chemistry can help to confront. Adequate treatments are still conspicuously lacking for many viral diseases, fungal infections, and human cancers. The long-term failure to find effective therapeutic agents in these areas does not mean that further, more effective agents cannot be found among natural sources. Discovery of suitable drugs may simply await the development of appropriate assays or the discovery of appropriate molecular targets. A newer, developing challenge is posed by the increasing occurrence of antibiotic resistance among bacterial infections, as well as the emergence of drug-resistant tuberculosis and malaria. The development of new agents effective against such diseases, particularly those having novel modes of action, is becoming an urgent priority. On the agricultural front, the demand for new pesticides is compounded by the fact that problematic insect pests, e.g., are developing resistance to many commonly used commercial pesticides. In addition, the use of some effective pesticides has been curtailed due to concerns about undesirable environmental impact. Similar concerns about other agrochemicals imply that new alternatives are needed, and that products of natural origin would be particularly appealing (Rimando and Duke 2006).

Another promising avenue of investigation can make use of advances in molecular biology and bio-

chemistry. Knowledge of metabolite biosynthesis has been extended to an awareness of genes linked to regulation and production of the enzymes associated with the biosynthesis of some important fungal metabolites (Calvo et al. 2004; Demain et al. 2005; Keller et al. 2005; Wang et al. 2005). Some fungal genes involved in antibiotic production are known to be clustered, as is the case with many actinomycetes (Demain 1992; Cary 2004). Potential applications of this technology include the recognition and cloning of genes involved in metabolite biosynthesis, improvement in metabolite production techniques, production of novel hybrid metabolites, and the use of fungal DNA libraries to aid in the discovery process.

The rapidly increasing capacity of biological screening systems has outpaced the capability of even the most prolific microbiology programs to provide adequate numbers of samples for assay. The pressure for short-term results, together with competition from other approaches to discovery of bioactive lead compounds (e.g., combinatorial chemistry, molecular modeling-based design) played a significant role in the termination and/or contraction of several industrial natural products programs in the USA over the past 25 years. Considering the diversity of fungi (and other sources) that remain unexplored, it is unfortunate that some institutions have had to abandon a line of research that has been instrumental to their success. Obviously, such occurrences increase the pressure on remaining natural products research programs to come up with promising leads, while decreasing the total effort underway in the field. At this stage, there is still no substitute for natural products chemistry as a source of truly novel lead structures, and it appears that recognition of this fact is beginning to stimulate a resurgence of interest in this field (Rouhi 2003). The unsurpassed chemical diversity of natural products is widely recognized, and there is considerable interest in construction of natural product libraries for general screening purposes (Bindseil et al. 2001). Interestingly, a current mainstream objective in combinatorial chemistry involves the development of so-called natural-product-like synthetic chemical libraries (Shang and Tan 2005; Messer et al. 2005). In any event, practical successes are more important than ever to continued support for fundamental studies of fungal taxonomy, ecology, and natural products chemistry. In this climate, the value and importance of cooperation between mycologists and chemists cannot be

overemphasized. The results described and cited in this chapter are illustrative of the potential rewards of such collaborative efforts.

**Acknowledgements.** Generous support for our research in this area over the past 10 years from the National Institutes of Health (AI 27436 and GM 60600) and the National Science Foundation (CHE-0315591, CHE-0079141, and CHE-9708316) is gratefully acknowledged. The author also wishes to express his appreciation to Drs. D.T. Wicklow, P.F. Dowd, D. Malloch, and C.A. Shearer, as well as the many graduate students and other research associates whose collaboration on projects described here has been both enjoyable and invaluable.

## References

- Amalfitano C, Pengue R, Andolfi A, Vurro M, Zonno MC, Evidente A (2002) HPLC analysis of fusaric acid, 9,10-dehydrofusaric acid and their methyl esters, toxic metabolites from weed pathogenic *Fusarium* species. *Phytochem Anal* 13:277–282
- Anderson MG, Jarman TB, Rickards RW (1995) Structures and absolute configurations of antibiotics of the oligosporon group from the nematode-trapping fungus *Arthrobotrys oligospora*. *J Antibiot* 48:391–8
- Anderson MB, Roemer T, Fabrey R (2003) Progress in antifungal drug discovery. *Annu Rep Med Chem* 38:163–172
- Angawi RF, Swenson DC, Gloer JB, Wicklow DT (2003a) Malettinin A: a new antifungal tropolone from an unidentified fungal colonist of *Hypoxyylon* stromata (NRRL 29110). *Tetrahedron Lett* 44:7593–7596
- Angawi RF, Swenson DC, Gloer JB, Wicklow DT (2003b) Lowdenic acid: a new antifungal polyketide-derived metabolite from a new fungicolous *Verticillium* sp. (NRRL 29280). *J Nat Prod* 66:1259–1262
- Angawi RF, Swenson DC, Gloer JB, Wicklow DT (2005) Malettinins B-D: new polyketide metabolites from an unidentified fungal colonist of *Hypoxyylon* stromata (NRRL 29110). *J Nat Prod* 68:212–216
- Angel K, Wicklow DT (1983) Coprophilous fungal communities in semiarid to mesic grasslands. *Can J Bot* 61:594–602
- Anke T (1995) The antifungal strobilurins and their possible ecological role. *Can J Bot* 73 suppl 1:S940–S945
- Anke H, Sterner O (2002) Insecticidal and nematicidal metabolites from fungi. *Mycota* 10:109–127
- Anke H, Stadler M, Mayer A, Sterner O (1995) Secondary metabolites with nematicidal and antimicrobial activity from nematophagous fungi and ascomycetes. *Can J Bot* 73 suppl 1:S932–S939
- Anonymous (1995) SCRIP World Pharmaceutical News Review of 1995. PJ Publications, London
- Arino AA, Bullerman LB (1994) Fungal colonization of corn growth in Nebraska in relation to year, genotype, and growing conditions. *J Food Prot* 57:1084–1087
- Ayer WA, Kawahara N (1995) Lecythophorin, a potent inhibitor of blue-stain fungi, from the hyphomycetous fungus *Lecythophora hoffmannii*. *Tetrahedron Lett* 36:7953–7956
- Ayer WA, Miao S (1993) Secondary metabolites of the aspen fungus *Stachybotrys cylindrospora*. *Can J Chem* 71:487–493
- Ayer WA, Lee SP, Tsuneda A, Hiratsuka Y (1980) The isolation, identification, and bioassay of the antifungal metabolites produced by *Monocillium nordinii*. *Can J Microbiol* 26:766–773
- Baker KF, Cook RJ (1974) Biological control of plant pathogens. W.H. Freeman, San Francisco, CA
- Baker SJ, Hui X, Maibach HI (2005) Progress on new therapeutics for fungal nail infections. *Annu Rep Med Chem* 40:322–335
- Balandrin MF, Kinghorn AD, Farnsworth NR (1993) Plant-derived natural products in drug discovery and development: an overview. In: Balandrin MF, Kinghorn AD (eds) Human medicinal agents from plants. American Chemical Society, Washington, DC, pp 2–12
- Balkovec JM (1998) Non-azole antifungal agents. *Annu Rep Med Chem* 33:173–182
- Ballio A (1991) Non-host-selective fungal phytotoxins: biochemical aspects of their mode of action. *Experientia* 47:783–790
- Barrett D (2002) From natural products to clinically useful antifungals. *Biochim Biophys Acta* 1587:224–233
- Barton JH, Emanuel EJ (2005) The patents-based pharmaceutical development process. Rationale, problems, and potential reforms. *J Am Med Assoc* 294:2075–2082
- Belofsky GN, Gloer JB, Wicklow DT, Dowd PF (1995) Antiinsectan alkaloids: shearinines A-C and a new paxilline derivative from the ascostromata of *Eupenicillium shearii*. *Tetrahedron* 51:3959–3968
- Belofsky GN, Gloer KB, Gloer JB, Wicklow DT, Dowd PF (1998a) New *p*-terphenyl and polyketide metabolites from the sclerotia of *Penicillium raistrickii*. *J Nat Prod* 61:1115–1119
- Belofsky GN, Gloer JB, Wicklow DT, Dowd PF (1998b) Shearamide A: a new cyclic peptide from the ascostromata of *Eupenicillium shearii*. *Tetrahedron Lett* 39:5497–5500
- Bennett JW (1983) Differentiation and secondary metabolism in mycelial fungi. In: Bennett JW, Ciegler A (eds) Secondary metabolism and differentiation in fungi. Marcel Dekker, New York, pp 1–35
- Bernillon J, Favre-Bonvin J, Pommier MT, Arpin N (1989) First isolation of (+)-epipentenomycin I from *Peziza* sp. carpophores. *J Antibiotics* 42:1430–1432
- Bhadury P, Mohammad BT, Wright PC (2006) The current status of natural products from marine fungi and their potential as anti-infective agents. *J Indust Microbiol Biotechnol* 33:325–337
- Bills GF (1995) Analysis of microfungal diversity from a user's perspective. *Can J Bot* 73 suppl 1:S33–S41
- Bills G, Dombrowski A, Morris SA, Hensens O, Liesch JM, Zink DL, Onishi J, Meinz MS, Rosenbach M, Thompson JR, Schwartz RE (2000) Hyalodendrosides A and B, antifungal triterpenoid glycosides from a lignicolous hyphomycete, *Hyalodendron* species. *J Nat Prod* 63:90–94
- Bindseil KU, Jakupovic J, Wolf D, Lavayre J, Leboul J, van der Pyl D (2001) Pure compound libraries, a new perspective for natural product based drug discovery. *Drug Discovery Today* 6:840–847

- Boddy L (2000) Interspecific combative interactions between wood-decaying basidiomycetes. *FEMS Microbiol Ecol* 31:185–194
- Boot CM, Tenney K, Valeriote FA, Crews P (2006) Highly N-methylated linear peptides produced by an atypical sponge-derived *Acremonium* sp. *J Nat Prod* 69:83–92
- Boyette CD (2000) The bioherbicide approach: using phytopathogens to control weeds. In: Cobb AH, Kirkwood RC (eds) *Herbicides and their mechanisms of action*. Sheffield Academic Press, Sheffield, pp 134–152
- Breinholt J, Jensen GW, Kjaer A, Olsen CE, Rosendahl CN (1997) Hypomycetin – an antifungal, tetracyclic metabolite from *Hypomyces aurantius*: production, structure and biosynthesis. *Acta Chem Scand* 51:855–860
- Breinholt J, Jensen HC, Kjaer A, Olsen CE, Rassing BR, Rosendahl CN, Sotofte I (1998) Cladobotryal: a fungal metabolite with a novel ring system. *Acta Chem Scand* 52:631–634
- Bugni TS, Ireland CM (2004) Marine-derived fungi: a chemically and biologically diverse group of microorganisms. *Nat Prod Rep* 21:143–163
- Calvo AM, Bok J, Brooks W, Keller NP (2004) *veA* is required for toxin and sclerotial production in *Aspergillus parasiticus*. *Appl Environ Microbiol* 70:4733–4739
- Caporale LH (1995) Chemical ecology: a view from the pharmaceutical industry. *Proc Natl Acad Sci USA* 92:75–82
- Cardoza RE, Hermosa MR, Vizcaino JA, Sanz L, Monte E, Gutierrez S (2005) Secondary metabolites produced by *Trichoderma* and their importance in the biocontrol process. *Microorganisms for Industrial Enzymes and Biocontrol, Research Signpost*, Trivandrum, India, pp 207–228
- Carroll GC (1992) Fungal mutualism. In: Carroll GC, Wicklow DT (eds) *The fungal community: its organization and role in the ecosystem*, 2nd edn. Marcel Dekker, New York, pp 327–354
- Cary JW (2004) Secondary metabolic gene clusters in filamentous fungi. In: Arora DK, Bridge PD, Bhatnagar D (eds) *Mycology Series 20. Handbook of Fungal Biotechnology*, 2nd edn. Marcel Dekker, New York, pp 81–94
- Chakravarti R, Sahai V (2004) Compactin – a review. *Appl Microbiol Biotechnol* 64:18–24
- Che Y, Swenson DC, Gloer JB, Koster B, Malloch D (2001) Pseudodestruxins A and B: new cyclic depsipeptides from the coprophilous fungus *Nigrosabulum globosum*. *J Nat Prod* 64:555–558
- Che Y, Gloer JB, Wicklow DT (2002a) Phomadecalins A-D and phomapentenone a: new metabolites from *Phoma* sp. NRRL 25697, a fungal colonist of *Hypoxyylon* stromata. *J Nat Prod* 65:399–402
- Che Y, Gloer JB, Koster B, Malloch D (2002b) Decipinins A and decipienolides A and B: new bioactive metabolites from the coprophilous fungus *Podospora decipiens*. *J Nat Prod* 65:916–919
- Che Y, Gloer JB, Scott JA, Malloch D (2004a) Communiols A-D: new mono- and bis-tetrahydrofuran derivatives from the coprophilous fungus *Podospora communis*. *Tetrahedron Lett* 45:6891–6894
- Che Y, Gloer JB, Wicklow DT (2004b) Curvicollides A-C: new polyketide-derived lactones from a sclerotium-colonizing isolate of *Podospora curvicolla* (NRRL 25778). *Org Lett* 6:1249–1252
- Che Y, Araujo AR, Gloer JB, Scott JA, Malloch D (2005) Communiols E-H: new polyketide metabolites from the coprophilous fungus *Podospora communis*. *J Nat Prod* 68:435–438
- Chen G, Lin Y, Wen L, Vrijmoed LLP, Jones EBG (2003) Two new metabolites of a marine endophytic fungus (No. 1893) from an estuarine mangrove on the South China Sea coast. *Tetrahedron* 59:4907–4909
- Christensen MJ (1996) Antifungal activity in grasses infected with *Acremonium* and *Epichloe* endophytes. *Aust J Plant Pathol* 25:186–191
- Clay K (1988) Fungal endophytes of grasses: a defensive mutualism between plants and fungi. *Ecology* 69:10–16
- Cole RJ, Schweikert MA (2003) *Handbook of Secondary Fungal Metabolites*. Academic Press, New York
- Cole RJ, Dorner JW, Springer JP, Cox RH (1981) Indole metabolites from a strain of *A. flavus*. *J Agr Food Chem* 29:293
- Coley-Smith JR, Cooke RC (1971) Survival and germination of fungal sclerotia. *Annu Rev Phytopathol* 9:65–92
- Corley DG, Durley RC (1994) Strategies for database dereplication of natural products. *J Nat Prod* 57:1484–1490
- Cragg GM, Newman DJ (2000) Antineoplastic agents from natural sources: achievements and future directions. *Expert Opin Inv Drugs* 9:2783–2797
- Cragg GM, Newman DJ (2005) Biodiversity: a continuing source of novel drug leads. *Pure Appl Chem* 77:7–24
- Cueto M, MacMillan JB, Jensen PR, Fenical W (2006) Tropolactones A-D, four meroterpenoids from a marine-derived fungus of the genus *Aspergillus*. *Phytochemistry* 67:1826–1831
- Curl EA, Gudauskas RT, Peterson CM (1985) Effects of soil insects on populations and germination of fungal propagules. In: Parker CA, Rovira AD, Moore KJ, Wong PTW, Kollmorgen JF (eds) *Ecology and management of soil-borne plant pathogens*. American Phytopathological Society, St. Paul, MN, pp 20–23
- Daly JW (1998) Thirty years of discovering arthropod alkaloids in amphibian skin. *J Nat Prod* 61:162–172
- Danielsen S, Jensen DF (1999) Fungal endophytes from stalks of tropical maize and grasses: isolation, identification, and screening for antagonism against *Fusarium verticillioides* in maize stalks. *Biocontrol Sci Technol* 9:545–553
- Demain AL (1992) Regulation of secondary metabolism. In: Finkelstein DB, Ball C (eds) *Biotechnology of filamentous fungi: technology and products*. Butterworth-Heinemann, Boston, MA, pp 89–112
- Demain AL, Velasco J, Adrio JL (2005) Industrial mycology: past, present, and future. In: An Z (ed) *Mycology Series 22. Handbook of Industrial Mycology*. Marcel Dekker, New York, pp 1–25
- Deyrup ST, Swenson DC, Gloer JB, Wicklow DT (2006) Caryophyllene sesquiterpenoids from a fungicolous isolate of *Pestalotiopsis disseminata*. *J Nat Prod* 69:608–611
- DiMasi JA, Hansen RW, Grabowski HG (2003) The price of innovation: new estimates of drug development costs. *J Health Econ* 22:151–186
- Dinan L (2005) Dereplication and partial identification of compounds. In: Sarker SD, Latif Z, Gray AI (eds) *Methods in Biotechnology 20. Natural products isolation*, 2nd edn. Humana Press, Totowa, NJ, pp 297–321

- D'Mello JPF, MacDonald AMC (1998) Fungal toxins as disease elicitors. *Environ Topics* 7:253–289
- Dong J-Y, Ru L, He H-P, Zhang K-Q (2005) Nematicidal sphingolipids from the freshwater fungus *Paraniesslia* sp. YMF 1.01400. *European J Lipid Sci Technol* 107:779–785
- Dowd PF, VanMiddlesworth FL (1989) In vitro metabolism of the trichothecene 4-monoacetoxyscirpenol by fungus- and non-fungus-feeding insects. *Experientia* 45:393–395
- Dreyfus MM, Chapela IH (1994) Potential of fungi in the discovery of novel low-molecular weight pharmaceuticals. In: Gullo VP (ed) *The discovery of natural products with therapeutic potential*. Butterworth-Heinemann, Boston, pp 49–80
- Du LC, Cheng YQ, Ingenhorst G, Tang GL, Huang Y, Shen B (2003) Hybrid peptide-polyketide natural products: biosynthesis and prospects towards engineering novel molecules. In: Setlow JK, Hollaender A (eds) *Genetic engineering: principles and methods*. Kluwer/Plenum Press, New York, pp 227–267
- Duke SO, Baerson SR, Dayan FE, Rimando AM, Scheffler BE, Tellez MR, Wedge DE, Schrader KK, Akey DH, Arthur FH, De Lucca AJ, Gibson DM, Harrison HF Jr, Peterson JK, Gealy DR, Tworkoski T, Wilson CL, Morris JB (2003) United States Department of Agriculture-Agricultural Research Service research on natural products for pest management. *Pest Manage Sci* 59:708–717
- Eckerman SJ, Graham, KJ (2000) Using chemical ecology to locate new antifungal natural products. In: Rahman A (ed) *Studies in Natural Products Chemistry* 22. Bioactive natural products, part C. Elsevier, Amsterdam, pp 55–92
- Evidente A, Andolfi A, Abouzeid MA, Vurro M, Zonno MC, Motta A (2004) Ascosonchine, the enol tautomer of 4-pyridylpyruvic acid with herbicidal activity produced by *Ascochyta sonchi*. *Phytochemistry* 65:475–480
- Eyberger AL, Dondapati R, Porter JR (2006) endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. *J Nat Prod* 69:1121–1124
- Fabian K, Anke T, Sternert O (2001) Mariannaeapyrone – a new inhibitor of thromboxane A2 induced platelet aggregation. *Zeitschr Naturforsch C Biosci* 56:106–110
- Feng Y, Blunt JW, Cole ALJ, Cannon JF, Robinson WT, Munro MHG (2003) Two novel cytotoxic cyclodepsipeptides from a mycoparasitic *Cladobotryum* sp. *J Org Chem* 68:2002–2005
- Findlay JA, Buthelezi S, Lavoie R, Pena-Rodriguez I, Miller JD (1995) Bioactive isocoumarins and related metabolites from conifer endophytes. *J Nat Prod* 58:1759–1766
- Floss HG (1976) Biosynthesis of ergot alkaloids and related compounds. *Tetrahedron* 32:873–912
- Fokkema NJ (1976) Antagonism between fungal saprophytes and pathogens on aerial plant surfaces. In: Dickinson CH, Preece TF (eds) *Microbiology of aerial plant surfaces*. Academic Press, New York, pp 487–506
- Frisvad JC (1989) The connection between the Penicillia and Aspergilli and mycotoxins with special emphasis on misidentified isolates. *Arch Environ Contam Toxicol* 18:452–467
- Gardner WA, McCoy CW (1992) Insecticides and herbicides. In: Finkelstein DB, Ball C (eds) *Biotechnology of filamentous fungi: technology and products*. Butterworth-Heinemann, Boston, pp 335–359
- Ghorbani R, Leifert C, Seel W (2005) Biological control of weeds with antagonistic plant pathogens. *Adv Agron* 86:191–225
- Gloer JB (1995a) The chemistry of fungal antagonism and defense. *Can J Bot* 73 suppl 1:S1265–S1274
- Gloer JB (1995b) Antiinsectan natural products from fungal sclerotia. *Accounts Chem Res* 28:343–350
- Gloer JB (1997) Applications of fungal ecology in the search for new bioactive natural products. In: Wicklow DT, Soderstrom BE (eds) *The Mycota* vol IV, 1st edn. Environmental and microbial relationships. Springer, Berlin Heidelberg New York, pp 249–268
- Gloer JB, TePaske MR, Sima J, Wicklow DT, Dowd PF (1988) Antiinsectan aflavinine derivatives from the sclerotia of *Aspergillus flavus*. *J Org Chem* 53:5457–5460
- Goh TK, Hyde KD (1996) Biodiversity of freshwater fungi. *J Indust Microbiol Biotechnol* 17:328–345
- Gunatilaka AAL (2006) Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. *J Nat Prod* 69:509–526
- Gupta S, Krasnoff SB, Renwick JAA, Roberts DW, Steiner JR, Clardy J (1993) Viridoxins A and B: Novel toxins from the fungus *Metarhizium flavoviride*. *J Org Chem* 58:1062–1067
- Hansen ME, Smedsgaard J, Larsen TO (2005) X-Hitting: an algorithm for novelty detection and dereplication by UV spectra of complex mixtures of natural products. *Anal Chem* 77:6805–6817
- Harborne JB (1987) Natural fungitoxins. In: Hostettmann K, Lea PJ (eds) *Biologically active natural products*. Oxford University Press, Oxford, pp 195–211
- Harborne JB (1993) Recent advances in chemical ecology. *Nat Prod Rep* 10:327–348
- Harmann GE (2006) Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology* 96:190–194
- Harper JE, Webster J (1964) An experimental analysis of the coprophilous fungal succession. *Trans Br Mycol Soc* 47:511–530
- Harper MK, Bugni TS, Copp BR, James RD, Lindsay BS, Richardson AD, Schnabel PC, Tasdemir D, VanWagoner RM, Verbitski SM, Ireland CM (2001) Introduction to the chemical ecology of marine natural products. In: McClintock JB, Baker BJ (eds) *Marine chemical ecology*. CRC Press, Boca Raton, FL, pp 3–69
- Harris GH (2005) The isolation and structure elucidation of fungal metabolites. In: An Z (ed) *Mycology Series* 22. *Handbook of Industrial Mycology*. Marcel Dekker, New York, pp 187–268
- Hawksworth DL (1991) The fungal dimension of biodiversity: magnitude, significance, and conservation. *Mycol Res* 95:641–655
- Hawksworth DL (2001) The magnitude of fungal diversity: the 1.5 million species estimate revisited. *Mycol Res* 105:1422–1432
- Hawksworth DL, Paterson RM, Vote N (1993) An investigation into the occurrence of metabolites in obligately lichenicolous fungi from thirty genera. *Bibliotheca Lichenologica (Phytochem Chemotaxon Lichenized Ascomycetes)* 53:101–108

- He H, Yang HY, Bigelis R, Solum EH, Greenstein M, Carter GT (2002) Pyrrocidines A and B, new antibiotics produced by a filamentous fungus. *Tetrahedron Lett* 43:1633–1636
- He H, Bigelis R, Yang HY, Chang L-P, Singh MP (2005) Lichenicolins A and B, new bisnaphthopyrones from an unidentified lichenicolous fungus, strain LL-RB0668. *J Antibiot* 58:731–736
- Hein SM, Gloer JB, Koster B, Malloch D (2001) Bombardolides: new antifungal and antibacterial metabolites from the coprophilous fungus *Bombardioidea anartia*. *J Nat Prod* 64:809–812
- Hoagland RE (1990) Microbes and microbial products as herbicides: an overview. In: Hoagland RE (ed) *Microbes and microbial products as herbicides*. American Chemical Society, Washington, DC, pp 2–52
- Hoagland RE (2001) Microbial allelochemicals and pathogens as bioherbicidal agents. *Weed Technol* 15:835–857
- Höller U, Gloer JB, Wicklow DT (2002) Biologically active polyketide metabolites from an undetermined fungicolous hyphomycete resembling *Cladosporium*. *J Nat Prod* 65:876–882
- Horn BW, Wicklow DT (1986) Ripening of *Eupenicillium ochrosalmoneum* ascostromata on soil. *Mycologia* 78:248–252
- Huang Q, Tezuka Y, Hatanaka Y, Kikuchi T, Nishi A, Tubaki K (1995a) Studies on metabolites of mycoparasitic fungi. III. New sesquiterpene alcohol from *Trichoderma koningii*. *Chem Pharmcol Bull* 43:1035–1038
- Huang Q, Tezuka Y, Hatanaka Y, Kikuchi T, Nishi A, Tubaki K (1995b) Studies on metabolites of mycoparasitic fungi. IV. Minor peptaibols of *Trichoderma koningii*. *Chem Pharmcol Bull* 43:1663–1667
- Ikediugwu FEO, Webster J (1970a) Antagonism between *Coprinus heptemerus* and other coprophilous fungi. *Trans Br Mycol Soc* 54:181–204
- Ikediugwu FEO, Webster J (1970b) Hyphal interference in a range of coprophilous fungi. *Trans Br Mycol Soc* 54:205–210
- Isaka M, Kittakoop P, Kirtikara K, Hywel-Jones NL, Thebtaranonth Y (2005) Bioactive substances from insect pathogenic fungi. *Accounts Chem Res* 38:813–823
- Jaenike J, Grimaldi D, Shuder AE, Greenleaf AL (1983) α-Amanitin tolerance in mycophagous *Drosophila*. *Science* 221:165–167
- Janzen DH (1977) Why fruits rot, seeds mold, and meat spoils. *Am Nat* 111:691–713
- Jarvis BB (2003) Analysis for mycotoxins: the chemist's perspective. *Arch Environ Health* 58:479–483
- Jarvis BB, Miller JD (2005) Mycotoxins as harmful indoor contaminants. *Appl Microbiol Biotechnol* 66:367–372
- Jiao P, Gloer JB, Campbell J, Shearer CA (2006a) Altenuene derivatives from an unidentified freshwater fungus in the family Tuberaceae. *J Nat Prod* 69:612–615
- Jiao P, Swenson DC, Gloer JB, Campbell J, Shearer CA (2006b) Decaspiroles A-E: new bioactive spirodioxynaphthalenes from the freshwater aquatic fungus *Decaisnella thyridioides*. *J Nat Prod* 69:1667–1671
- Joshi BK (1999) New bioactive natural products from coprophilous and mycoparasitic fungi. PhD Thesis, University of Iowa, Iowa City, IA
- Joshi BK, Gloer JB, Wicklow DT, Dowd PF (1999a) Sclerotigenin: a new antiinsectan benzodiazepine from the sclerotia of *Penicillium sclerotigenum*. *J Nat Prod* 62:650–652
- Joshi BK, Gloer JB, Wicklow DT (1999b) New verticillin and glisoprenin analogs from *Gliocladium catenulatum* NRRL 22970, a mycoparasite of *Aspergillus flavus* sclerotia. *J Nat Prod* 62:730–733
- Joshi BK, Gloer JB, Wicklow DT (2002) Antifungal natural products from a sclerotium-colonizing isolate of *Humicola fuscoatra*. *J Nat Prod* 65:1734–1737
- Keating GM, Figgitt DP (2003) Caspofungin – a review of its use in esophageal candidiasis, invasive candidiasis and invasive aspergillosis. *Drugs* 63:2235–2263
- Keller NP, Turner G, Bennett JW (2005) Fungal secondary metabolism: from biochemistry to genomics. *Nature Rev Microbiol* 3:937–947
- Kendrick B (1986) Biology of toxigenic anamorphs. *Pure Appl Chem* 58:211–218
- Kevan DK (1965) The soil fauna: its nature and biology. In: Baker KF, Snyder WC (eds) *Ecology of soil-borne plant pathogens*. University of California Press, Berkeley, CA, pp 33–50
- Khosla C, Keasling JD (2003) Timeline – metabolic engineering for drug discovery and development. *Nature Rev Drug Discov* 2:1019–1025
- Koenig GM, Kehraus S, Seibert SF, Abdel-Lateff A, Mueller D (2006) Natural products from marine organisms and their associated microbes. *ChemBioChem* 7:229–238
- Kohno J, Nishio M, Sakurai M, Kawano K, Hiramatsu H, Kameda N, Kishi N, Yamashita T, Okuda T, Komatsura S (1999) Isolation and structure determination of TMC-151s: novel polyketide antibiotics from *Gliocladium catenulatum* Gilman & Abbott TC 1280. *Tetrahedron* 55:7771–7786
- Koltin Y (1990) Targets for antifungal drug discovery. *Annu Rep Med Chem* 25:141–148
- Koshino H, Togiya S, Terada S, Yoshihara T, Sakamura S, Shimanuki T, Sato T, Tajimi A (1989) New fungitoxic sesquiterpenoids, chokols A-G, from stromata of *Epichloe typhina* and the absolute configuration of chokol E. *Agric Biol Chem* 53:789–796
- Kwok OCH, Plattner R, Wiesleder D, Wicklow DT (1992) A nematocidal toxin from *Pleurotus ostreatus* NRRL 3526. *J Chem Ecol* 18:127–130
- Larsen TO, Smedsgaard J, Nielsen KF, Hansen ME, Frisvad JC (2005) Phenotypic taxonomy and metabolite profiling in microbial drug discovery. *Nat Prod Rep* 22:672–695
- Lee JC, Lobkovsky E, Pliam NB, Strobel G, Clardy J (1995) Subglutinolins A and B: immunosuppressive compounds from the endophytic fungus *Fusarium subglutinans*. *J Org Chem* 60:7076–7078
- Lehr N-A, Meffert A, Antelo L, Sterner O, Anke H, Weber RWS (2006) Antiamoebins, myrocin B and the basis of antifungal antibiosis in the coprophilous fungus *Stilbella erythrocephala* (syn. *S. fimetaria*). *FEMS Microbiol Ecol* 55:105–112
- Li C (2003) Bioactive natural products from selected freshwater aquatic isolates and fungicolous *Penicillium* species. PhD Thesis, University of Iowa, Iowa City, IA
- Li JY, Sidhu RS, Bollon A, Strobel GA (1998) Stimulation of taxol production in liquid cultures of *Pestalotiopsis microspora*. *Mycol Res* 102:461–464

- Li C, Gloer JB, Wicklow DT, Dowd PF (2002) Thiersinines A and B: novel antiinsectan indole diterpenoids from a new fungicolous *Penicillium* species (NRRL 28147). *Org Lett* 4:3095–3098
- Li C, Gloer JB, Campbell J, Shearer CA (2003a) Annularins A-H: new polyketide metabolites from the freshwater aquatic fungus *Annulatascus triseptatus*. *J Nat Prod* 66:1302–1306
- Li C, Gloer JB, Wicklow DT (2003b) Thiersindoles A-C: new indole diterpenoids from *Penicillium thiersii*. *J Nat Prod* 66:1232–1235
- Li C, Gloer JB, Wicklow DT, Dowd PF (2005) Antiinsectan decaturin and oxalicine analogues from *Penicillium thiersii*. *J Nat Prod* 68:319–322
- Lin Y, Wu X, Feng S, Jiang G, Luo J, Zhou S, Vrijmoed LLP, Jones EBG, Krohn K, Steingroever K, Zsila F (2001) Five unique compounds: xyloketsals from mangrove fungus *Xylaria* sp. from the South China Sea coast. *J Org Chem* 66:6252–6256
- Lin Y, Wu X, Deng Z, Wang J, Zhou S, Vrijmoed LLP, Jones EBG (2002) The metabolites of the mangrove fungus *Verruculina enalia* No. 2606 from a salt lake in the Bahamas. *Phytochemistry* 59:469–471
- Lin X, Huang Y, Fang M, Wang J, Zheng Z, Su W (2005) Cytotoxic and antimicrobial metabolites from marine lignicolous fungus, *Diaporthe* sp. *FEMS Microbiol Lett* 251:53–58
- Liu X, Li S (2005) Fungal secondary metabolites in biological control of crop pests. In: An Z (ed) Mycology Series 22. Handbook of Industrial Mycology. Marcel Dekker, New York, pp 723–747
- Macias M, Gamboa A, Ulloa M, Toscano RA, Mata R (2001) Phytoxic naphthopyranone derivatives from the coprophilous fungus *Guanomyces polythrix*. *Phytochemistry* 58:751–758
- Malloch D (1981) Moulds. Their isolation, cultivation, and identification. University of Toronto Press, Toronto, pp 19–20
- Mantle PG (1978) Industrial exploitation of ergot fungi. In: Smith JE, Berry DR (eds) The Filamentous Fungi vol I. Industrial mycology. Wiley, New York, pp 281–300
- Masunaka A, Ohtani K, Peever TL, Timmer LW, Tsuge T, Yamamoto M, Yamamoto H, Akimitsu K (2005) An isolate of *Alternaria alternata* that is pathogenic to both tangerines and rough lemon and produces two host-selective toxins, ACT- and ACR- toxins. *Phytopathology* 95:241–247
- Masurekar PS (1992) Therapeutic metabolites. In: Finkelstein DB, Ball C (eds) Biotechnology of filamentous fungi: technology and products. Butterworth-Heinemann, Boston, pp 241–301
- Masurekar PS (2005) Strain improvement for the production of fungal secondary metabolites. In: An Z (ed) Mycology Series 22. Handbook of Industrial Mycology. Marcel Dekker, New York, pp 539–561
- McQuilken MP, Gemmell J, Hill RA, Whippes JM (2003) Production of macrosphelide A by the mycoparasite *Coniothyrium minitans*. *FEMS Microbiol Lett* 219:27–31
- Messer R, Fuhrer CA, Haener R (2005) Natural product-like libraries based on non-aromatic, polycyclic motifs. *Curr Opin Chem Biol* 9:259–265
- Miller JD (1991) Mycology, mycologists, and biotechnology. In: Hawksworth DL, Regensburg CA (eds) Frontiers in mycology. Honorary lectures 4th Int Mycological Congr. CABI, Wallingford, pp 225–240
- Miller JD, Trenholm HL (eds) (1994) Mycotoxins in grain: compounds other than Aflatoxin. Eagan Press, St. Paul, MN
- Monaghan RL, Tkacz JS (1990) Bioactive microbial products: focus upon mechanism of action. *Annu Rev Microbiol* 44:271–301
- Morrall RAA, Loew FM, Hayes MA (1978) Subacute toxicological evaluation of sclerotia of *Sclerotinia sclerotiorum* in rats. *Can J Comp Med* 42:473–477
- Morris RAC, Ewing DF, Whippes JM, Coley-Smith JR (1995) Antifungal hydroxymethyl-phenols from the mycoparasite *Verticillium biguttatum*. *Phytochemistry* 39:1043–1048
- Mudur SV, Swenson DC, Gloer JB, Campbell J, Shearer CA (2006a) Heliconols A-C: antimicrobial hemiketals from the freshwater aquatic fungus *Helicodendron giganteum*. *Org Lett* 8:3191–3194
- Mudur SV, Gloer JB, Wicklow DT (2006b) Spormarinins A and B: antifungal metabolites from a fungicolous isolate of *Sporormiella minimoides*. *J Antibiot* 59:500–506
- Munkvold GP, McGee DC, Carlton WM (1997) Importance of different pathways for maize kernel infection by *Fusarium moniliforme*. *Phytopathology* 87:209–217
- Newman DJ, Cragg GM, Snader KM (2000) The influence of natural products upon drug discovery. *Nat Prod Rep* 17:215–234
- Newman DJ, Cragg GM, Snader KM (2003) Natural products as sources of new drugs over the period 1981–2002. *J Nat Prod* 66:1022–1037
- Nozawa K, Nakajima S, Kawai K, Udagawa S, Miyaji M (1994) Bicoumarins from ascostromata of *Petromyces alliaeus*. *Phytochemistry* 35:1049–1051
- Odds FC (2001) Sordarin antifungal agents. *Expert Opin Therap Pat* 11:283–294
- Oh H, Gloer JB, Wicklow DT, Dowd PF (1998) Arenarins A-C: new cytotoxic metabolites from the sclerotia of *Aspergillus arenarius*. *J Nat Prod* 61:702–705
- Oh H, Gloer JB, Shearer CA (1999a) Massarinolins A-C: new bioactive sesquiterpenoids from the aquatic fungus *Massarina tunicata*. *J Nat Prod* 62:497–501
- Oh H, Kwon T, Gloer JB, Marvanová L, Shearer CA (1999b) Tenelic acids A-D: new bioactive diphenyl ether derivatives from the aquatic fungus *Dendrospora tenella*. *J Nat Prod* 62:580–583
- Oh H, Swenson DC, Gloer JB, Shearer CA (2001) Massarilactones A and B: novel secondary metabolites from the freshwater aquatic fungus *Massarina tunicata*. *Tetrahedron Lett* 42:975–977
- Oh H, Swenson DC, Gloer JB, Shearer CA (2003) New bioactive rosigenin analogs and aromatic polyketide metabolites from the freshwater aquatic fungus *Massarina tunicata*. *J Nat Prod* 66:73–79
- Oh DC, Jensen PR, Kauffman CA, Fenical W (2005) Libertellenones A-D: induction of cytotoxic diterpenoid biosynthesis by marine microbial competition. *Bioorg Med Chem* 13:5267–5273
- Ondeyka JG, Giacobbe RA, Bills GF, Cuadrillero C, Schmatz D, Goetz MA, Zink DL, Singh SB (1998) Coprophilin: an anticoccidial agent produced by a dung inhabiting fungus. *Bioorg Med Chem Lett* 8:3439–3442

- Ooike M, Nozawa K, Udagawa S, Kawai K (1997a) Bisindolylbenzenoids from ascostromata of *Petromyces muricatus*. *Can J Chem* 75:625–628
- Ooike M, Nozawa K, Udagawa S, Kawai K (1997b) Structures of a new type of indoloditerpene, petromindole, and a new asterriquinone derivative, PM-53, from the ascostromata of *Petromyces muricatus*. *Chem Pharmcol Bull* 45:1694–1696
- Parker EJ, Scott DB (2005) Indole-diterpene biosynthesis in ascomycetous fungi. In: An Z (ed) Mycology Series 22. Handbook of Industrial Mycology. Marcel Dekker, New York, pp 405–426
- Peláez F (2005) Biological activities of fungal metabolites. In: An Z (ed) Mycology Series 22. Handbook of Industrial Mycology. Marcel Dekker, New York, pp 49–92
- Peterson SW, Bayer EM, Wicklow DT (2004) *Penicillium thiersii*, *Penicillium angulare* and *Penicillium decaturense*, new species isolated from wood-decay fungi in North America and their phylogenetic placement from multilocus DNA sequence analysis. *Mycologia* 96:1280–1293
- Powell KA (1993) The commercial exploitation of microorganisms in agriculture. In: Jones DG (ed) Exploitation of microorganisms. Chapman and Hall, London, pp 441–459
- Proksch P, Ebel R, Edrada RA, Schupp P, Lin WH, Sudarson O, Wray V, Steube K (2003) Detection of pharmacologically active natural products using ecology. Selected examples from Indopacific marine invertebrates and sponge-derived fungi. *Pure Appl Chem* 75:343–352
- Puri SC, Verma V, Amna T, Qazi GN, Spiteller M (2005) An endophytic fungus from *Nothapodytes foetida* that produces campothecin. *J Nat Prod* 68:1717–1719
- Reátegui R, Gloer JB, Campbell J, Shearer CA (2005) Ophiocerins A-D and ophioceric Acid: new tetrahydropyran derivatives and an africane sesquiterpenoid from the freshwater aquatic fungus *Ophioceras venezuelense*. *J Nat Prod* 68:701–705
- Reátegui R, Gloer JB, Wicklow DT (2006) Phaeofurans and sorbicillin analogs from a fungicolous *Phaeoacremonium* species (NRRL 32148). *J Nat Prod* 69:113–117
- Rhodes DF (1985) Offensive-defensive interactions between herbivores and plants: their relevance in herbivore population dynamics and ecological theory. *Am Nat* 125:205–238
- Richardson K, MS Marriott (1987) Antifungal agents. *Ann Rep Med Chem* 22:159–167
- Ridderbusch DC, Weber RWS, Anke T, Sterner O (2004) Tulasnein and podospirone from the coprophilous xylariaceous fungus *Podosordaria tulasnei*. *Zeitschr Naturforsch C J Biosci* 59:379–383
- Rimando AM, Duke SO (2006) Natural products for pest management. In: ACS Symposium Series 927. Natural products for pest management. American Chemical Society, Columbus, OH, pp 2–21
- Rouhi AM (2003) Rediscovering natural products. *Chem Eng News* 81:77–91
- Rowley DC, Kelly S, Kauffman CA, Jensen PR, Fenical W (2003) Halovirins A-E, new antiviral agents from a marine-derived fungus of the genus *Scyphalidium*. *Bioorg Med Chem* 11:4263–4274
- Schlingmann G, Roll DM (2005) Absolute stereochemistry of unusual biopolymers from Ascomycete culture LL-W1278: examples that derivatives of (S)-6-hydroxymellein are also natural fungal metabolites. *Chirality* 17 suppl:S48–S51
- Schneider G, Anke H, Sterner O (1997) New secondary metabolites from a mycophilic *Hansfordia* species. *Nat Prod Lett* 10:133–138
- Schulz B, Boyle C, Draeger S, Roemmert A-K, Krohn K (2002) Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycol Res* 106:996–1004
- Scott B (2001) *Epichloe* endophytes: fungal symbionts of grasses. *Curr Opin Microbiol* 4:393–398
- Scott B (2004) Functional analysis of the perennial ryegrass – *Epichloe* endophyte interaction. *Dev Plant Breeding* 11:133–144
- Seephonkai P, Isaka M, Kittakoop P, Palittapongarnpim P, Kamchonwongpaisan S, Tantcharoen M, Thebtaranonth Y (2002) Evaluation of antimycobacterial, antiplasmodial and cytotoxic activities of preussomerins isolated from the lichenicolous fungus *Microsphaeropsis* sp. BCC 3050. *Planta Med* 68:45–48
- Segeth MP, Bonnefoy A, Bronstrup M, Knauf M, Schummer D, Toti L, Vertesy L, Wetzel-Raynal M-C, Wink J, Seibert G (2003) Coniosetin, a novel tetramic acid antibiotic from *Coniochaeta ellipoidea* DSM 13856. *J Antibiot* 56:114–122
- Shang S, Tan DS (2005) Advancing chemistry and biology through diversity-oriented synthesis of natural product-like libraries. *Curr Opin Chem Biol* 9:248–258
- Shaw PJA (1992) Fungi, fungivores, and food webs. In: Carroll GC, Wicklow DT (eds) The fungal community: its organization and role in the ecosystem, 2nd edn. Marcel Dekker, New York, pp 295–310
- Shearer CA (1995) Fungal competition. *Can J Bot* 73 suppl 1:S1259–S1264
- Shearer CA, Zare-Maivan H (1988) *In vitro* hyphal interactions among wood- and leaf-inhabiting ascomycetes and fungi imperfecti from freshwater habitats. *Mycologia* 80:31–37
- Shim SH, Swenson DC, Gloer JB, Dowd, PF, Wicklow DT (2006a) Penifulvin A: a sesquiterpenoid-derived metabolite containing a novel dioxo[5,5,5,6]fenestrane ring system from a fungicolous isolate of *Penicillium griseofulvum*. *Org Lett* 8:1225–1228
- Shim SH, Gloer JB, Wicklow DT (2006b) Penifulvins B-E and a silphinene analogue: sesquiterpenoids from a fungicolous isolate of *Penicillium griseofulvum*. *J Nat Prod* 69:1601–1605
- Siegel MR, Latch GCM (1991) Expression of antifungal activity in agar culture by isolates of grass endophytes. *Mycologia* 83:529–537
- Sinclair JB, Cerkauskas RF (1996) Latent infection vs. endophytic colonization by fungi. In: Redlin SC, Carris LM (eds) Endophytic fungi in grasses and woody plants. Systematics, ecology and evolution. APS Press, St. Paul, MN, pp 3–29
- Singh N, Webster J (1973) Antagonism between *Stilbella erythrocephala* and other coprophilous fungi. *Trans Br Mycol Soc* 61:487–495
- Singh SB, Jayasuriya H, Dewey R, Polishook JD, Domrowski AW, Zink DL, Guan Z, Collado J, Platas G, Pelaez F, Felock PJ, Hazuda DJ (2003) Isolation, structure, and HIV-1 integrase inhibitory activity of structurally diverse fungal metabolites. *J Indust Microbiol Biotechnol* 30:721–731

- Singh SB, Pelaez F, Hazuda DJ, Lingham RB (2005) Discovery of natural product inhibitors of HIV-1 integrase at Merck. *Drugs Future* 30:277–299
- Sitrin RD, Chan G, Dingerdissen J, DeBrosse C, Mehta R, Roberts G, Rottschaeffer S, Staiger D, Valenta J, Snader KM, Stedman RJ, Hoover JRE (1988) Isolation and structure determination of *Pachybasiun cerebrosides* which potentiate the antifungal activity of aculeacin. *J Antibiot* 41:469–480
- Soman AG, Gloer JB, Koster B, Malloch D (1999a) Sporovexins A-C and a new preussomerin analog: antibacterial and antifungal metabolites from the coprophilous fungus *Sporormiella vexans*. *J Nat Prod* 62:659–661
- Soman AG, Gloer JB, Wicklow DT (1999b) Antifungal and antibacterial metabolites from a sclerotium-colonizing isolate of *Mortierella vinacea*. *J Nat Prod* 62:386–388
- Soman AG, Angawi RF, Gloer JB, Wicklow DT (2001) Vertilecanins: new phenopicolinic acid analogs from *Verticillium lecanii*. *J Nat Prod* 64:189–192
- Stadler PA, Giger RKA (1984) Ergot alkaloids and their derivatives in medicinal chemistry and therapy. In: Krosgaard-Larsen P, Christensen SB, Kofod H (eds) Natural products and drug development. Proc 20th Alfred Benzon Symp. Munksgaard, Copenhagen, pp 463–485
- Stadler M, Sheldrick WS, Dasen-Brock J, Steglich W, Anke H (1994) Antibiotics from the nematode-trapping basidiomycete *Nematoctonus robustus*. *Nat Prod Lett* 4:209–16
- Stadler M, Baumgartner M, Grothe T, Muhlbauer A, Seip S, Wollweber H (2001) Concentricol, a taxonomically significant triterpenoid from *Daldinia concentrica*. *Phytochemistry* 56:787–793
- Stern O, Bergman R, Kihlberg J, Wickberg B (1985) The sesquiterpenes of *Lactarius vellereus* and their role in a proposed chemical defense system. *J Nat Prod* 48:279–288
- Stevenson BG, Dindal DL (1987) Functional ecology of coprophagous insects: a review. *Pedobiologia* 30:285–298
- Stovall ME, Clay K (1991) Fungitoxic effects of *Balansia cyperi*. *Mycologia* 83:288–295
- Strobel GA (2002) Rainforest endophytes and bioactive products. *Crit Rev Biotechnol* 22:315–333
- Strongman DB, Miller JD, Calhoun L, Findlay JA, Whitney NJ (1987) The biochemical basis for interference competition among some lignicolous marine fungi. *Bot Mar* 30:21–26
- Strunz GM, Kakushima M, Stillwell MA (1972) Scytalidin: A new fungitoxic metabolite produced by a *Scytalidium* species. *J Chem Soc Perkin Trans I* 2280–2283
- Sun MH, Liu XZ (2006) Carbon Requirements of some nematophagous, entomopathogenic and mycoparasitic hyphomycetes as fungal biocontrol agents. *Mycopathologia* 161:295–305
- Suzuki S, Hosoe T, Nozawa K, Yaguchi T, Udagawa S, Kawai K (1999) Mitorubrin derivatives on ascocarps of some *Talaromyces* species of ascomycetous fungi. *J Nat Prod* 62:1328–1329
- Tan RX, Zou WX (2001) Endophytes: a rich source of functional metabolites (1987 to 2000). *Nat Prod Rep* 18:448–459
- Tanda S, Tadakuma Y, Matsunami Y (1968) Fundamental studies on ergotial fungi. VIII. Poisonous experiments of ergot to mouse. *J Agric Sci (Tokyo)* 13:55–60
- Tezuka Y, Huang Q, Kikuchi T, Nishi A, Tubaki K (1994) Studies on the metabolites of mycoparasitic fungi: Metabolites of *Cladobotryum varium*. *Chem Pharmcol Bull* 42:2612–2617
- Tezuka Y, Tasaki M, Huang Q, Hatanaka Y, Kikuchi T (1997) Studies on metabolites of mycoparasitic fungi. Part 6. 15-Hydroxyacorenone. New acorene-type sesquiterpene from the culture broth of the mycoparasitic fungus *Trichoderma harzianum*. *Liebigs Ann/Recueil* 12:2579–2580
- Turner WB, Aldridge DC (1983) Fungal Metabolites II. Academic Press, New York
- Wagner C, Anke H, Besl H, Stern O (1995) Flavipucine and brunnescin, two antibiotics from cultures of the mycophilic fungus *Cladobotryum rubrobrunnescens*. *Zeitschr Naturforsch C Biosci* 50:358–64
- Wagner C, Anke H, Stern O (1998) Rubrobramide, a cytotoxic and phytotoxic metabolite from *Cladobotryum rubrobrunnescens*. *J Nat Prod* 61:501–502
- Walton JD, Panaccione DG (1993) Host-selective toxins and disease specificity: perspectives and progress. *Annu Rev Phytopathol* 31:275–303
- Wang HJ, Gloer JB, Wicklow DT, Dowd PF (1995) Aflavinines and other antiinsectan metabolites from ascostromata of *Eupenicillium crustaceum* and related species. *Appl Environ Microbiol* 61:4429–4435
- Wang HJ, Gloer JB, Wicklow DT, Dowd PF (1998) Mollenines A and B: new dioxomorpholines from the ascostromata of *Eupenicillium molle*. *J Nat Prod* 61:804–807
- Wang GY, Laidlaw RD, Marshall JH, Keasling JD (2005) Metabolic engineering of fungal secondary metabolite pathways. In: An Z (ed) Mycology Series 22. Handbook of Industrial Mycology. Marcel Dekker, New York, pp 635–666
- Weber HA, Gloer JB (1991) Preussomerins A-F: novel antifungal metabolites from the coprophilous fungus *Preussia isomera* Cain. *J Org Chem* 56:4355–4360
- Weber RWS, Meffert A, Anke H, Stern O (2005) Production of sordarin and related metabolites by the coprophilous fungus *Podospora pleiospora* in submerged culture and in its natural substrate. *Mycol Res* 109:619–626
- Webster J (1970) Coprophilous fungi. *Trans Br Mycol Soc* 54:161–180
- Whyte AC, Joshi BK, Gloer JB, Wicklow DT, Dowd PF (2000) New cyclic peptide and bis-indolyl benzenoid metabolites from the sclerotia of *Aspergillus sclerotiorum*. *J Nat Prod* 63:1006–1009
- Wicklow DT (1981) The coprophilous fungal community: a mycological system for examining ecological ideas. In: Wicklow DT, Carroll GC (eds) The fungal community: its organization and role in the ecosystem. Marcel Dekker, New York, pp 47–76, 351–385
- Wicklow DT (1986) Ecological adaptations and classification in *Aspergillus* and *Penicillium*. In: Sampson RA, Pitt JI (eds) Advances in *Penicillium* and *Aspergillus* systematics. Plenum Press, New York, pp 255–265
- Wicklow DT (1988a) Metabolites in the coevolution of fungal chemical defence systems. In: Pirozynski KA, Hawksworth D (eds) Coevolution of fungi with plants and animals. Academic Press, New York, pp 174–201
- Wicklow DT (1988b) Patterns of fungal association with maize kernels harvested in North Carolina. *Plant Disease* 72:113–115

- Wicklow DT (1992a) The coprophilous fungal community: an experimental system. In: Carroll GC, Wicklow DT (eds) *The fungal community: its organization and role in the ecosystem*, 2nd edn. Marcel Dekker, New York, pp 715–728
- Wicklow DT (1992b) Interference competition. In: Carroll GD, Wicklow DT (eds) *The fungal community: its organization and role in the ecosystem*, 2nd edn. Marcel Dekker, New York, pp 265–274
- Wicklow DT, Cole RJ (1982) Tremorgenic indole metabolites and aflatoxins in sclerotia of *Aspergillus flavus* Link: an evolutionary perspective. *Can J Bot* 60:525–528
- Wicklow DT, Cole RJ (1984) Citreoviridin in standing corn infested by *Eupenicillium ochrosalmoneum*. *Mycologia* 76:959–961
- Wicklow DT, Hirschfield BJ (1979a) Competitive hierarchy in post-fire ascomycetes. *Mycologia* 71:47–54
- Wicklow DT, Hirschfield BJ (1979b) Evidence of a competitive hierarchy among coprophilous fungal populations. *Can J Microbiol* 25:855–858
- Wicklow DT, Dowd PF, TePaske MR, Gloer JB (1988) Sclerotial metabolites of *Aspergillus flavus* toxic to a detritivorous maize insect (*Carpophilus hemipterus*, Nitidulidae). *Trans Br Mycol Soc* 91:433–438
- Wicklow DT, Joshi BK, Gamble WR, Gloer JB, Dowd PF (1998) Antifungal metabolites (monorden, monocillin IV, and cerebrosides) from *Humicola fuscoatra* Traaen NRRL 22980, a mycoparasite of *Aspergillus flavus* sclerotia. *Appl Environ Microbiol* 64:4482–4484
- Wicklow DT, Roth S, Deyrup ST, Gloer JB (2005) A protective endophyte of maize: *Acremonium zaeae* antibiotics inhibitory to *Aspergillus flavus* and *Fusarium verticillioides*. *Mycol Res* 109:610–618
- Wiederhold NP, Lewis RE (2003) The echinocandin antifungals: an overview of the pharmacology, spectrum and clinical efficacy. *Expert Opin Inv Drugs* 12(8):1313–1333
- Wilhelm C, Anke H, Flores Y, Sterner O (2004) New peptaiols from *Mycogone cervina*. *J Nat Prod* 67:466–468
- Willets HJ (1971) The survival of fungal sclerotia under adverse environmental conditions. *Biol Rev* 46:387–407
- Willets HJ (1978) Sclerotium formation. In: Smith JE, Berry DR (eds) *The Filamentous Fungi* vol III. Developmental mycology. Wiley, New York, pp 197–213
- Wolpert TJ, Dunkle LD, Ciuffetti LM (2002) Host-selective toxins and avirulence determinants: what's in a name? *Annu Rev Phytopathol* 40:251–285
- Wong-Beringer A, Kriengkauykiat J (2003) Systemic anti-fungal therapy: new options, new challenges. *Pharmacotherapy* 23:1441–1462
- Zhang Y, Li C, Swenson DC, Gloer JB, Wicklow DT, Dowd PF (2003) Novel antiinsectan oxalicine alkaloids from two undescribed fungicolous *Penicillium* spp. *Org Lett* 5:773–776

---

## **Decomposition, Biomass and Industrial Applications**

---

# 16 Nutrient Cycling by Saprotophobic Fungi in Terrestrial Habitats

J. DIGHTON<sup>1</sup>

## CONTENTS

I. Introduction .....	287
II. Derivation of Nutrients from Rock:	
Soil Formation .....	289
III. Leaf Litter Decomposition .....	290
A. Phylloplane Fungal Action .....	290
B. Litter Decomposition and Nutrient Mineralization .....	291
C. Nutrient Immobilization .....	293
D. Interactions Between Fungi and Other Microbes .....	295
IV. Impact of Pollutants on Nutrient Cycling .....	296
V. Conclusions.....	297
References .....	297

## I. Introduction

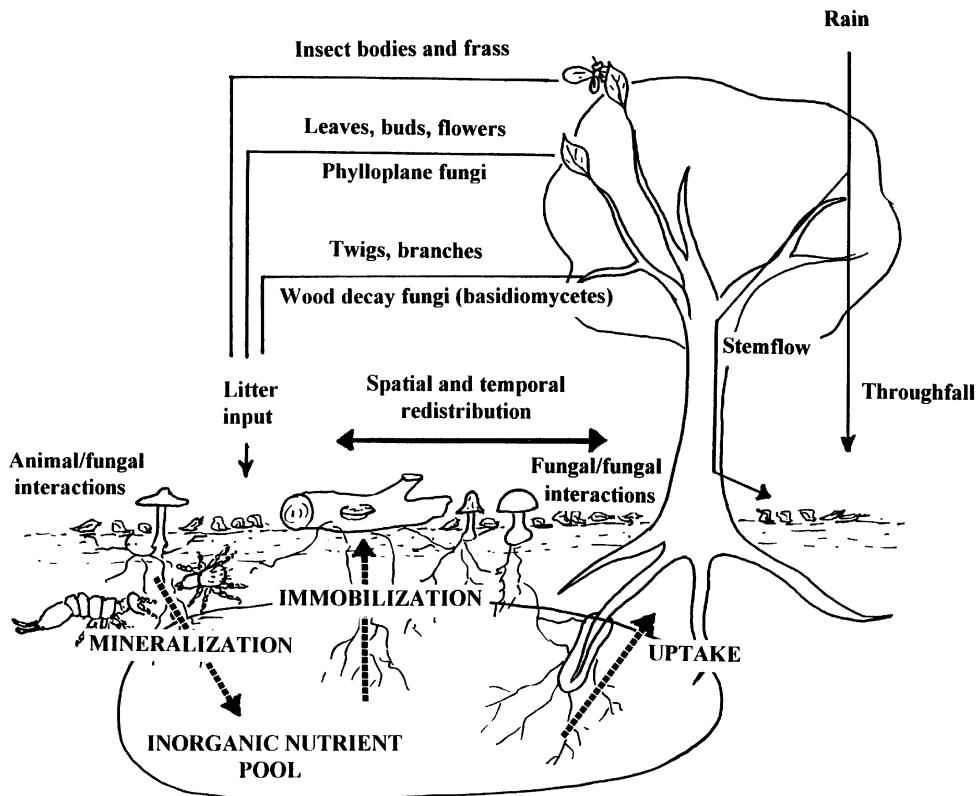
In his introduction to Carroll and Wicklow (1992), Rayner (1992) proposes that the potential role of fungi in regulating energy and nutrient fluxes through natural ecosystems is likely to be immense, via their involvement in soil development, decomposition and uptake of nutrients by plants. This is, in part, due to the structural adaptations of fungal mycelia that have indeterminate growth, presenting a large surface area to their environment for enzyme production and recovery of end products of enzymatic activity. They have the ability to translocate nutrients and energy from one location to another through their hyphal structures (Cairney 2005) and, due to their persistence, can endure stressful changes in the environment, which are less well tolerated by other organisms (Zak 1993; Zak et al. 1995). However, fungi do not work alone in the environment and more research is needed to understand the role of consortia of organisms in mediating processes in ecosystems. A wider view of the ecosystem services that fungi provide is given by Dighton (2003).

<sup>1</sup> Rutgers University Pinelands Field Station, P.O. Box 206, 501 Four Mile Road, New Lisbon, NJ 08064, USA

Terrestrial ecosystems present a wide diversity of habitats ranging from polar arctic conditions, through temperate coniferous and deciduous forests and grasslands, heathlands and tropical forests to deserts. Within one single chapter, it is not possible to describe the role of fungi in nutrient cycling in all of these systems; rather, I will provide some general principles that are common to all terrestrial systems, and that will be modified by the specific constraints of the system under consideration. A scheme for nutrient cycling in a forest ecosystem is given in Fig. 16.1. The saprotrophic condition of fungi is, possibly, the fundamental life strategy where dependence on excreted enzymes is enhanced by the presentation of a large surface area of hyphae to the environment, from which end products of decomposition are absorbed. However, other fungal groups, particularly necrotrophs and mycorrhizae, although specialized in their function, retain this general modus operandi and can be considered facultative saprotrophs when conditions dictate. As such, functional groups other than saprotrophs will be included in the following discussion where necessary.

## II. Derivation of Nutrients from Rock: Soil Formation

It is more common to think of the abiotic forces of freeze-thaw, water dissolution and wind erosion as the primary factors for producing the fine mineral particles from parent rock to create soil (Brady and Weil 1999). However, the action of plant roots is also important in both the production of organic acids to dissolve rock and hydrostatic pressure to fracture rock. The same can be said for fungal hyphae, which act either alone or in consortium with other organisms to effect similar processes, but at a smaller spatial scale. In particular, fungi in the symbiotic association with bacteria and al-



**Fig. 16.1.** Diagrammatic representation of nutrient cycling in woodland ecosystems and the role of fungi in that process (after Dighton and Boddy 1989)

gae, namely lichens, are an important contributor to early soil development. Indeed, Rayner (1992) says that lichens "clothe what might otherwise be bare parts of the planet".

Approximately 8% of terrestrial ecosystems are lichen dominated and, in many of these systems, the ground cover by lichens is often very high, up to 100% (Honegger 1991). Many of these systems are climatically extreme or oligotrophic (nutrient impoverished) environments, and it is here that lichens become important actors in the formation of soils. These fungal tissues, in association with symbiotic algae and non-photosynthetic bacteria (Banfield et al. 1999), play a major role in soil biogenesis.

Hydration of oxides of iron and aluminum is an important process in rock degradation; for example, hematite ( $\text{Fe}_2\text{O}_3$ ) is converted into ferrihydrate ( $\text{Fe}_{10}\text{O}_{15}9\text{H}_2\text{O}$ ) (Brady and Weil 1999). Hydrolysis is important in the release of essential nutrients for plant growth. For example, potassium is released from microcline, a feldspar, where dissolution allows the dissociation of anions and cations from complex materials, and gypsum dissolves to release

calcium and sulfate ions. Crustose lichens are often the first organisms to colonize outcrops of bare rock. They are able to scavenge water and nutrients from the atmosphere (Lange et al. 1994) that support their slow rate of growth, and by which they are able to tolerate complete desiccation. During their growth on the surface of rocks and in rock crevices, the organic acids they produce (oxalic, citric, lichenic and tartaric acids) solubilize the rock and assist in its physical breakdown. This action of lichens has been reported to cause significant damage to both buildings and sculptures made of rock (Chen et al. 2000), by hyphal penetration into the parent rock beneath the aerial structure of the lichen (Ascaso and Wierzchos 1995).

Fungi alone produce organic acids that are capable of breaking down rock. Ascaso and Wierzchos (1995) cite studies by Eckhardt (1985) that show that yeasts and filamentous fungi, such as *Aspergillus niger*, alone are involved in rock solubilization, releasing cations from amphibolite, biotite and orthoclase. *Penicillium* and yeasts were also found to be able to dissolve calcium-rich rocks, such as limestone, marble and calcium phosphate

(Chang and Li 1998). Solubilization of fluorapatite has also been shown in pure cultures of *Aspergillus niger* (Nahas et al. 1990). Small holes (3–10 µm diameter) in feldspars and hornblende (Jongmans et al. 1997) were attributed to the production of micromolar concentrations of organic acids (succinic, citric, oxalic, formate and malate) secreted by saprotrophic and ectomycorrhizal fungi associated with the overlying pine forest ecosystem. Thin sections of feldspars observed under the microscope have revealed fungal hyphae bearing cross walls in hyphal-generated tunnels in the rock (Hoffland et al. 2002, 2004). In addition, Connolly et al. (1998) showed that the white rot wood decay fungus, *Resinicum bicolor*, could solubilize strontianite sand to release the strontium contained within. Even in the absence of nutrient-rich substrates, fungi seem capable of obtaining energy and nutrients from minimalist media and scavenging from the atmosphere. Such fungi are known as oligotrophs, and are reviewed by Wainwright (2005).

In a similar manner to lichens, Hirsch et al. (1995) showed a loose relationship between fungi, bacteria and coccal cells (thought to be algae) that together form an endolithic community in sandstone and granite that produce organic acids for rock dissolution. Typical saprotrophic fungal species present included *Alternaria*, *Aspergillus*, *Aureobasidium*, *Candida*, *Cladosporium*, *Paecilomyces*, *Phoma*, *Penicillium* and *Sporobolomyces*.

The saprotrophic capabilities of mycorrhizal fungi have also been shown to play a role in the dissolution of parent rock material in more established soils. Azcon et al. (1976) showed that interactions between bacteria and arbuscular mycorrhizae of lavender facilitated acquisition of phosphorus by the host plant from rock phosphate, due to a synergism between the bacteria and mycorrhizal fungi and the differences in behavior between the two mycorrhizal fungal species. Berthelin and Leyval (1982) showed that in measures of maize growth (biomass), uptake of potassium, calcium and magnesium (derived from the breakdown of biotite) was similar between mycorrhizal fungi and rhizospheric bacterial communities, and there was no synergistic effect of the combination of mycorrhizae and bacteria. This suggests that arbuscular mycorrhizal fungi alone were capable of rock breakdown. Mojallala and Weed (1978) showed that mycorrhizal soybeans used weathered potassium from the biotites, phlogopite and muscovite, but

potassium release was insufficient to sustain the enhanced growth of the mycorrhizal plants. Plant acquisition of nutrients from insoluble or poorly soluble sources is also enhanced by consortia of mycorrhizae, saprotrophic fungi and bacteria. Singh and Kapoor (1998) showed that mung bean plants in association with a consortium of the arbuscular mycorrhizal fungus *Glomus fasciculatum*, fungal saprotroph *Cladosporium herbarum* and the bacterium *Bacillus circulans* grown under natural soil conditions could better obtain phosphorus from rock phosphate than each organism alone. Similar interactions were found by Vanlauwe et al. (2000) in the savanna zone of Nigeria.

April and Keller (1990) and Gobran et al. (1998) demonstrated the importance of rhizospheric microbial (fungal and bacterial) communities in the rhizosphere of forest trees for forest biogeochemistry. The abundance of weatherable minerals near the root surface was consistently less than in the bulk soil, due to increased hydrogen ion and carbon dioxide content and the presence of complexing organic acids produced by these rhizospheric communities. By observing the solubilization of limestone, marble and calcium phosphate by seven ectomycorrhizal, two saprotrophic fungal genera and *Pseudomonas fluorescens*, Chang and Li (1998) suggested that synergistic activities were important in mediating calcium availability in soils. Other interactions between ectomycorrhizae and nutrient element solubilization include potassium from biotite and microcline in Scots pine (Wallander and Wickman 1999), rock dissolution and K, Mg and Ca (Landeweert et al. 2001) and NH<sub>4</sub>-N from Miocene shales and, possibly, other rocks (Thompson et al. 2001).

To these embryonic soils, organic matter added by the death of fungi, lichens, bacteria, algae, etc. makes a more 'complete' soil in which other soil organisms can live. Here, the release of nutrients by decomposition as well as enhanced soil structure support the growth of cryptogamic and vascular plants, which further add their own floristic and chemical diversity to the decomposition cycle, leading to a more complex soil structure.

### III. Leaf Litter Decomposition

The nutrient component of soil comes partly from the dissolution of parent rock, and secondarily

from the decomposition of dead plant and animal remains. Dead plant parts (above- and below-ground) are returned to the soil where the activities of bacteria, saprotrophic fungi and soil fauna degrade the complex organic components. Extracellular enzyme activity of heterotrophic microorganisms, and the recovery of resulting solubilized nutrients is rarely 100% efficient, resulting in mineralized labile nutrient elements becoming available for plant uptake in soil solution (fertility). The activities of fungal extracellular enzymes has been reviewed by Sinsabaugh and Liptak (1997) and Sinsabaugh (2005). The activity of these saprotrophic fungi is neatly summarized by Forsythe and Miyata (1984) ... "under the silent, relentless chemical jaws of the fungi, the debris of the forest floor quickly disappears".

Part of the structure of different soils is related to the balance between rates of decomposition and mineralization and the rate of input of dead plant parts to soil. Where decomposition is very slow, organic matter accumulates and peaty soils develop. In contrast, where plant litter turnover rates are high, resulting soils have a more mineral profile with low organic matter content. Much of the regulation of plant litter decomposition is related to the relative availabilities of carbon and nutrient elements within the plant resource. Melillo et al. (1982) showed that the carbon to nitrogen ratio in plant resources and, particularly, the lignin:N ratio could predict the rate of decomposition of litter, which, in turn, influences the saprotrophic fungal community capable of decomposing them.

The process of decomposition starts in the phylloplane when leaves senesce, and progressively continues in the soil in the litter and organic horizons, where fungi interact with bacteria and soil fauna (Ponge 1991, 2005).

### A. Phylloplane Fungal Action

In wet tropical forests, some 7% of the total expected leaf litter fall never reaches the forest floor, but is trapped in the canopy of the trees or in the canopy of the understory shrub community (Hedger et al. 1993). Plant litter trapped in the canopies is held there by fungal hyphae and, particularly, rhizomorphs formed by species of the genera *Marasmius* and *Marasmiellus*, which effect the decomposition of the plant litter. The balance between the amount of leaves trapped is a function of leaf weight, tensile strength of the retaining fungal structures, and weight loss due to decomposition. As the leaves decompose, mineralization will release nutrients that will wash to the forest floor in throughfall rain. Thus, the formation of 'soil' in the tree canopy is a reality and, probably, has a significant impact on the fertility of the tropical forest ecosystem. Under crop canopies, Beare et al. (1989) showed enhanced microbial activity and litter decomposition of low-resource quality materials in the presence of throughfall rainfall.

The magnitude of effects of rainfall volume and leaching rates from canopy plant parts in wet tropical forests is much greater than that of other ecosystems. However, even in temperate forest ecosystems, the changes occurring in the stemflow water chemistry is enough to provide a suitable habitat for epiphytic lichen communities. The extensive biomass of the epiphytic lichen, *Ramalina menziesii*, ( $590 \text{ kg ha}^{-1}$ ) on blue oak (standing crop leaf biomass of  $958 \text{ kg ha}^{-1}$ ) had a higher deposition of total N, organic N, Ca, Mg, Na and Cl in throughfall rain than was the case for trees without lichens (Knops et al. 1996). Trees with lichens had a lower throughfall of  $\text{SO}_4$ , and the concentrations of  $\text{NO}_3$ ,  $\text{NH}_4$ , K and total P were not different because lichen litter reduced the decomposition of oak leaf litter,

**Table 16.1.** Resource quality and rates of decomposition of contrasting plant litters in relation to ecosystem succession (from left to right, these components become more

important contributors to the decomposition pool), and the increased dependency of consortia of soil organisms to effect their decomposition (after Heal and Dighton 1986)

	Lower plants	Herbaceous plants	Angiosperm leaves	Coniferous leaves	Wood
Cellulose (%)	16–35	20–37	6–22	20–31	36–63
Lignin (%)	7–36	3–30	9–42	20–58	17–35
C:N ratio	13–50	29–160	21–71	63–327	294–327
Decay (% year <sup>-1</sup> )	20	30–70	40–60	3–50	1–90
Faunal importance	Enchytraidae	Enchytraidae, Oligochaeta, Diptera	Oligochaeta, Collembola, Acari	Acari, Collembola, Oligochaeta	Insecta, other Arthropoda

**Table 16.2.** Regression analysis (regression coefficients) of decomposition (mass loss) and nitrogen release rate in relation to determinants of leaf litter resource quality (after Vanlauwe et al. 1997). Asterisk indicates a significant regression ( $a = 0.05$ )

	Litter mass loss	N mineralization
C:N ratio	0.74*	0.61
Lignin:N ratio	0.68	0.42
Polyphenol:N ratio	0.54	0.76*
(Lignin+polyphenol):N ratio	0.77*	0.68*

such that the release of N and P were reduced by 76 and 2%, respectively.

### B. Litter Decomposition and Nutrient Mineralization

The rate at which a resource is decomposed is dependent on its chemical composition (Heal and Dighton 1985; Heal et al. 1997), edaphic factors (available moisture and temperature), and the colonization of the resource by appropriate saprotrophic organisms (Table 16.1). Many of these factors are discussed by Cooke and Rayner (1984). The input of different types (chemical composition and, hence, resource quality) of plant litter varies with ecosystem type (Dickinson and Pugh 1974; Cadish and Giller 1997). The general consensus is that the carbon:nitrogen and lignin:nitrogen ratios can be used as determinants of the resistance of resources to decomposition, and ultimate mineralization of nutrients (Melillo et al. 1982). Where the C:N or lignin:N ratios are high, there are reduced rates of decomposition compared to resources containing lower ratios. However, other secondary chemicals produced by plants, particularly polyphenols and tannins, also inhibit rates of decomposition of plant material by soil microorganisms (Harborne 1997). Vanlauwe et al. (1997) showed that both rates of decomposition (mass loss) and nitrogen mineralization rates are strongly correlated to the (lignin+polyphenol):N ratio (Table 16.2).

Due to the variability in chemical composition of plant and animal remains, not all materials can be utilized by all fungal species. Fungal species differ in their access of simple or complex forms of carbohydrate and mineral nutrients. Decomposition is a product of enzyme activity, where the types of enzymes required are dependent on the substrates (chemical constituents) of the resource.

Sinsabaugh and Liptak (1997) give a description of the various ectoenzymes produced by fungi, and their biochemical effects on organic resources in plant litters. The ability of different species of fungi to produce specific enzymes dictates, in part, the succession of fungi colonizing resources. In addition to enzymatic competency, there are other factors, such as relative growth rates, the production of antibiotic secondary metabolites, and environmental constraints, which influence the ability of specific fungi to colonize resources in the face of competition against other fungi (Cook and Rayner 1984; Frankland 1992, 1998; Lockwood 1992; Wicklow 1992). Linkins et al. (1984) discussed some of the factors affecting the activity of extracellular cellulase, particularly the positive influence of temperature, and the cellulose to lignin ratio. Cellulose appears to become unavailable for microbial use when the cellulose:lignin ratio declines below 0.5.

The role of plant litter quality on the pattern of fungal colonization of resources has been discussed in Dickinson and Pugh (1974). Here, many examples of the change in species composition of fungal communities as different plant substrates undergo the cascade of decay are described. In general, there appears to be a succession of fungi utilizing different resources within the litter. The classic assumption is that the initial colonizers use soluble carbohydrate sources (sugars), and are later replaced by fungal species having greater enzymatic competence that are able to break down organic sources of carbon such as cellulose, and lignin. However, there are few clear distinctions in the succession and, in fact, many of the species overlap in time and space. The successional trends of fungi colonizing decomposing plant material have been described in more detail for the litter of the fern *Pteridium aquilinum* by Frankland (1992) in her discussion of fungal successions (Frankland 1998). She describes changes from lesion-forming *Rhizographus* and *Aureobasidium* on standing dead litter, through the colonization by basidiomycetes in relation to the rate of loss of cellulose and lignin, and the consequential decrease in C:N ratio from some 200:1 to 30:1. By microscopic observation of small samples of forest floor leaf litter, Ponge (1990, 1991) characterized the colonization of *Pinus sylvestris* needles into four stages (Table 16.3). Early stages of decomposition are characterized by minor changes to the leaf structure by fungi common in the phylloplane. Subsequent fungal colonization is associated with browning of the leaf, and decomposition of relatively available resources. This is fol-

**Table 16.3.** Fungal succession in relation to changes in resources during decomposition of Scots pine needles (after Ponge 1991)

Stage of decompositions	Fungal species	Litter characteristics
Phase I	<i>Lophodermium</i> and <i>Ceuthesopra</i>	Browning of cellulose walls, cytoplasm missing and replaced by hyphae
Phase II	<i>Verticillium</i> , <i>Marasmius</i> and <i>Cenococcum</i>	Melanized hyphae through stoma. Hyaline hyphae invade xylem tracheids and resin ducts. Lignocellulose walls disrupted
Phase III	<i>Cenococcum</i>	Invades fecal pellets of mites and enchytraeids as soil fauna, bacteria and algae invade
Phase IV	<i>Cenococcum</i> and <i>Hypodontia</i>	Penetration by mycorrhizal fungi. Needles become hollow

lowed by greater invasion of the leaf tissue by decomposing micro-fungi and basidiomycete fungi, culminating in the entry of soil arthropods, invasion by mycorrhizal fungi, and the establishment of a greater diversity of decomposer organisms. Within any group of fungi, however, there are considerable differences in efficiency of decomposition abilities. In a comparison of larch litter decomposition between 15 basidiomycete and 16 ascomycete fungi, Osono et al. (2003) showed litter weight loss of -2.0 to 7.8% by ascomycetes, and -0.8 to 14.2% by basidiomycetes. Thus, the generalization that basidiomycetes have greater enzyme diversity is not always correct, although the results suggested a stronger relationship to N mineralization due to lignin decomposition by basidiomycetes, in contrast with ascomycetes. A review of fungal succession during decomposition can be found in Ponge (2005).

These observations help to validate the model proposed by Swift et al. (1979) where changes from 'sugar' fungi to basidiomycetes in relation to changes in available resources and the influence of climatic stresses are presented. The model suggests that during initial decomposition, the carbohydrate component is used as an energy source until such a time that the C:nutrient ratio approaches that of the decomposer organism (around 15:1 for P, and 6:1 for N in fungi). Only then is there net conversion of organic nutrient to inorganic nutrient – net nutrient mineralization. In general, initial resource structure is chemically heterogeneous, thus supporting a variety of fungal species. As decomposition proceeds, only recalcitrant chemicals are left, requiring a specialized subset of fungal species that are capable of producing the enzymes necessary to degrade the complex resources. Hence, diversity is reduced. However, the colonization of decomposing plant material

in relation to resource quality has been presented only in reference to the chemical composition of the whole leaf. As fungal hyphae are of a small diameter ( $\sim 5 \mu\text{m}$ ), their patterns of growth, enzyme expression and the subsequent changes in leaf litter chemistry occur at a scale of resolution much smaller than that of a whole leaf. In recent studies (Mascarenhas et al. 2000; Dighton et al. 2001) using microscopic Fourier transform infrared (FT-IR) spectroscopy, real-time micro-scale ( $100 \times 100 \mu\text{m}$ ) changes in leaf surface carbohydrate chemistry resulting from fungal colonization are beginning to be revealed at the level of individual hyphae.

At the ecosystem level, plant tissue chemistry entering the decomposer pool changes during seral succession of vegetation from herbaceous to forest ecosystems (Heal and Dighton 1985). The initial seral stages are marked by an addition of high-quality resources to the decomposer community, consisting mainly of cellulose and a high C:N ratio and low lignin content. Following forest canopy closure, woody resources and more recalcitrant leaf litters dominate (Attiwill and Adams 1993). These litters have high lignin content and low C:N ratios and, therefore, decompose at a slower rate. In addition to changes in the dominance of fungal species or group with ecosystem succession, the degree of interaction between fungi and animals increases. There are increasingly intimate associations between fungi and fauna in the exploitation of more recalcitrant plant residues (Table 16.1).

In forested systems, much dead wood remains in the canopy prior to recruitment to the forest floor. This standing dead material may have a different fungal community than wood on the forest floor. The work of Boddy and Rayner (1983) on oak wood in canopies showed that 12 basidiomycete fungal species dominated in the community. Of these, *Phellinus ferreus*, *Sterium gausapatum*

and *Vullemnia comedens* were pioneer species of partially living branches, *Phlebia adiata* and *Coriolus versicolor* were secondary colonizers, and *Hypoderma setigerum* and *Sterium hirsutum* related to insect activity. In wood, the interactions between fungi can be most clearly observed. The zones of interaction between adjacent, competing fungal colonies have been mapped in three dimensions using wood as a resource (Rayner 1978; Rayner and Boddy 1988). Clear demarcation zones are set up when genetically incompatible strains or species meet in a relatively homogenous resource. In an environment where resources are patchily distributed, such as mixed litter on the forest floor, the colonization of individual resource units is more difficult to map. The colonization pattern of individual straw resource units by a range of fungal species was correlated to relative growth rates of the fungi on agar (Robinson et al. 1993a). These rates of growth allowed four species to be ranked in combative order. Mixtures of fungal significantly reduced growth rate of less combative fungal species, where enhanced respiration of mixed species communities (Robinson et al. 1993b) resulted from competition, rather than substrate utilization. Thus, the cascade of decomposition is related to colonization of a substrate by fungi based on their enzymatic competence in relation to the chemical resources available, and also on the outcome of interaction with other potential colonizers of that resource.

Sinsabaugh et al. (1993) demonstrated that most extracellular enzymes involved in wood decomposition are derived from fungal activity. Using standardized wood as a resource, they showed that the production of lignocellulase enzyme did not differ between different locations in a temperate forest ecosystem. However, the rate of immobilization (mainly fungal) of total nitrogen and total phosphorus into decomposing wood ranged from 2.2–4.4 µg g<sup>-1</sup> wood for P, and 43–139 µg g<sup>-1</sup> for N at the time when 80% mass loss was achieved. The spatial variability of this parameter was much greater than that for lignocellulase, but much less than for acid phosphatase and N-acetylglucosaminase activity. The process of decomposition is governed by the production of enzymes, which are, in turn, regulated by the availability of nitrogen or phosphorus. Thus, where nutrient elements are less available, the fungi expend greater amounts of energy to produce enzymes to sequester the nutrients from organic sources. These results suggest a large

degree of edaphic (soil condition) control over enzyme expression, which is closely related to the availability of inorganic N and P supplies in soil water. Thus, Sinsabaugh et al. (1993) and Sinsabaugh (2005) developed a model that contains both fungal (microbial) and soil nutrient controls over the expression of enzymes. Particularly, Sinsabaugh (2005) describes a successional loop through which the community composition of saprotrophic fungi and their enzyme expressions are tied to resource quality in organic matter decomposition. Use of models like this can help us to better understand the complexities of decomposition and nutrient cycling processes by allowing hypothesis development, leading to the design of experiments that can logically alter single or multiple parameters to investigate the key processes and organisms that are responsible for driving ecosystem processes.

Lodge and Asbury (1988) demonstrated that the ability of fungal hyphae and cords to bind leaf litter together on the forest floor is important in preventing down-slope loss of leaf litter in tropical forest ecosystems. The potential loss of organic matter, containing nutrients for plant growth, is prevented by the action of a number of, mainly, basidiomycete fungi that bind the leaf litter together. Species of *Collybia*, *Marasmiellus*, *Marasmius* and *Mycena* are the main fungi involved in forest floor litter trapping. The effect of litter binding by fungi increases with increasing ground slope. Lodge and Asbury (1988) concluded, from field manipulation experiments, that loss of litter was reduced by 35% from shallow slopes (<75% of angle) and 45% at greater slopes (75–90%). The reduction in leaf litter loss and subsequent incorporation of organic matter into the mineral soil is thought to prevent soil erosion and down-slope nutrient leaching during high-rainfall periods.

### C. Nutrient Immobilization

Mineral nutrients are sequestered by decomposer soil organisms by being incorporated into the organism's biomass. The residence time of these elements is usually equivalent to the turnover time (lifespan) of that organism. The amount of accumulation within the fungal component varies between ecosystems, depending on the chemical composition of the plant parts available for decomposition and the main fungal groups involved in the process. Thus, shorter-lived, ephemeral moulds, utilizing

ing simple carbohydrates, have lower investment in biomass than longer-lived basidiomycetes, growing on woody resources; by implication, the potential accumulation in basidiomycetes is greater (Frankland et al. 1982).

Where the C:nutrient ratio of a resource is very high, as in wood, the Swift et al. (1979) model proposes initial fungal immobilization and import of free nutrient during initial stages of decomposition until the fungal resource C:nutrient content is equivalent to that of the fungus. Fungal immobilization of nutrients can be considerable and is of economic concern in 'nitrogen lock-up' in post-harvest forest residues. To alleviate this competition, there has been the establishment of burning protocols to rid the site of both woody debris and, incidentally, leaf litter and the nutrients they contain (Dighton 1995). A greater understanding of the interactions between nutrient availability, temporary nutrient immobilization, and alternative applications for post-harvest residues could lead to a more rational use of residues to provide sustainable forestry without the loss of nutrients from the ecosystem from burning, and without the need for exogenous nutrients in the form of fertilizers (Jones et al. 1999). For example, Stark (1972) showed that hyphae had 193 to 272% greater N content, and 104 to 223% greater P content than the pine needle litter on which they were found, suggesting immobilization of these elements into fungal biomass. Data from Lodge (1993) show similar, but lower rates of fungal accumulation of nutrients in tropical forest ecosystems (N 1.6%, P 22%, K 3.7% and Ca 2%) from leaf litter. Fogel and Hunt (1983) estimated that saprotrophic fungal biomass accounted for 2% of the standing stock of a temperate Douglas-fir forest ecosystem, and Clinton et al. (1999) showed significant nutrient accumulation in fungal fruitbodies of a *Nothofagus* forest from both forest floor and dead woody material (Table 16.4).

**Table 16.4.** Accumulation of nutrients into fungal fruitbodies in a *Nothofagus* forest from either forest floor or dead woody material, expressed as a percentage of resource nutrient calculated as  $\text{mg kg}^{-1}$  (after Clinton et al. 1999)

Nutrient	% from forest floor	% from dead wood
N	402	2066
P	400	2500
K	628	19,000
Mg	800	400
Ca	7	89

Marumoto et al. (1982) suggested a longer turnover time for fungi than bacteria in their experimental decomposition study of killed bacteria, fungi, and combined bacteria and fungal cells. Using  $^{14}\text{C}$  and  $^{15}\text{N}$  labeling techniques for each of the cells, they showed that the rate of carbon loss, as  $\text{CO}_2$ , was similar between microbe sources, but that the rate of mineralization of nitrogen as both  $\text{NH}_4$  and  $\text{NO}_3\text{-N}$  was slower in the decomposition of fungal cells. In tropical ecosystems, fungal biomass can attain values of  $5\text{--}15 \text{ mg g}^{-1}$  litter (Lodge 1993), from which nutrients can be slowly released upon death and subsequent decomposition. Here, fungi may be important retainers of nutrients that would otherwise readily leach from the soil due to high precipitation volumes. Due to the high nutrient content, decomposition of fungal fruitbodies may be faster than that of surrounding forest floor material, providing localized spots of high rates of mineral nutrient release. However, the duration of immobilization of those nutrients into fungal hyphae before translocation to the fruitbody could be an important aspect of the control fungi have on the rates and timing of release of pulses of nutrients within the forest ecosystem. In temperate zones, the spring and fall abundance of fruitbody production may coincide with the high root growth and high nutrient demand by trees during these periods, a correlation that has yet to made by observation and measurement.

Fungi as non-discrete organisms are able to translocate elements within the fungal thallus (Cairney 1992), allowing for the spatial redistribution of elements. For example, if an element were always translocated away from dying regions, translocation would increase the length of time of immobilization into fungal components. Olsson and Jennings (1991) demonstrated that translocation of  $^{14}\text{C}$  and  $^{32}\text{P}$  through hyphal systems of *Rhizopus*, *Trichoderma* and *Stemphylium* occurred by diffusion. Real-time rates of translocation of carbon within the fungal thallus have been shown to react to provide directional flow to the building phases of the hyphae (Olsson 1995). This acropetal translocation is through cytoplasmic flow and diffusion in the cytoplasm and apoplasm. In contrast to this diffusion model of C and P translocation, Gray et al. (1995) demonstrated that translocation of  $^{137}\text{Cs}$  through hyphae of *Schizophyllum commune* was slower than diffusion, suggesting incorporation of the element into structural components of the cytoplasm or hyphal wall. This presents a plausible mechanism

**Table 16.5.** Velocities of translocation of elements within saprotrophic basidiomycete mycelia (after Cairney 2005)

Fungus	Element/material	Velocity (cm h <sup>-1</sup> )
<i>Armillaria mellea</i>	Glucose ( <sup>14</sup> C)	3.2
<i>A. mellea</i>	Glucose ( <sup>3</sup> H)	1.2
<i>A. mellea</i>	P	2.5
<i>Armillaria</i> spp.	Cs	14.2
<i>Armillaria</i> spp.	Cs	17.9
<i>A. gallica</i>	Cs	0.6
<i>A. ostoyae</i>	Cs	0.8
<i>Serpula lacrimans</i>	Glucose ( <sup>14</sup> C)	24.8
<i>Phanerochaete velutina</i>	Glucose ( <sup>14</sup> C)	336
<i>P. velutina</i>	Aminoisobutyric acid ( <sup>14</sup> C)	0.05
<i>Schizophyllum commune</i>	Cs	0.0002
<i>S. commune</i>	Aminoisobutyric acid ( <sup>14</sup> C/ <sup>32</sup> P)	0.0018
<i>Pleurotus ostreatus</i>	Aminoisobutyric acid ( <sup>14</sup> C/ <sup>32</sup> P)	0.0018

for long-term accumulation of radiocesium in basidiomycete fungi (Dighton and Horrill 1988; Yoshida and Muramatsu 1994).

Solute translocation through wood-colonizing basidiomycetes is important in facilitating colonization of low-resource quality substrates. Wells and Boddy (1990) showed that 75% (*Phanerochaete velutina*) and 13% (*Phallus impudicus*) of the phosphorus added to a decomposed wood resource is translocated to newly colonized wood resources through mycelial cord systems from previously colonized and partially decomposed wood. The maximum rate of P translocation is 7225 nmol P cm<sup>-2</sup> day<sup>-1</sup> through cords. These cords are formed only in unsterile soil, suggesting the trigger for cord formation is derived from other organisms. In field experimental manipulations, Wells and Boddy (1995a) showed that translocation could be conducted over distances of up to 75 cm between decomposing resources on the forest floor. Translocation of phosphorus in mycelial cords is temperature dependent, with greater rates of movement at higher temperatures (Wells and Boddy 1995b). A change from wet to dry soil conditions induces a thickening of the cord system of *Phanerochaete velutina* and a reduction in the translocation of phosphorus to a new wood resource; wetting appears to have no effect on cord structure or P movement (Wells et al. 2001). Velocity of translocation of material within basidiomycete mycelia can be high (Cairney 2005), although few studies have looked at nutrient elements other than phosphorus or cesium (Table 16.5). Changes in the source-sink relationships within the mycelium, and the degree of importance of translocation in non-basidiomycete

fungi in relation to nutrient cycling are as yet relatively unexplored.

#### D. Interactions Between Fungi and Other Microbes

It is important to remember at the outset that saprotrophic fungi involved with decomposition and nutrient cycling in soil do not perform that function in isolation. Plant and animal remains may be comminuted by soil fauna and subjected to enzyme attack by bacteria and actinomycetes. Interactions between these organisms are important in determining the rate of decomposition and diversity of soil biota. The decomposition process is also dynamic – for example, the same suite of organisms is not present on the plant or animal remains (resource) for the duration of the process of decomposition. It will be seen that different fungi have different enzymatic capabilities, so their appearance on a resource will be dictated by (i) their ability to utilize the resource, (ii) their rate of arrival at the resource either by growth or by transport as spores, etc., and (iii) their ability to compete against other fungal species with similar physiological competence.

The interactions between fungi and bacteria in the decomposition of leaf litter may also not always be synergistic. In an incubation study of beech leaves, Møller et al. (1999) showed that the cellulolytic fungus *Humicola* sp. caused double the carbon utilization from leaves than in combination with a mixed inoculum of soil bacteria. However, mixed species fungal inoculum significantly increased  $\beta$ -N-acetylglucosaminidase and endo-exo-

cellulase activity compared to the presence of bacteria alone, but the presence of bacteria decreased these enzyme activities in the presence of *Humicola* relative to *Humicola* alone. The quality of resultant dissolved organic carbon from decomposition also differed between fungal and fungal/bacterial decomposition of the leaf material.

As complexity in the system increases, the influence of trophic interactions alters the effectiveness of fungi to effect decomposition, and the subsequent mobility of nutrients. Nieminen and Setälä (2001) constructed microcosms of increasing complexity by sequentially adding fungi, bacteria and bacteria-feeding nematodes. Fungi alone had lower decomposition efficiency (higher respiration per unit biomass) when alone in the system, and effected lower C loss due to a smaller total biomass in the system, compared to more complex associations. Thus, generalizations of the role of fungi in nutrient cycling need to be modified when placed in a 'real world' context of fungal interactions with other soil biota. Further additive trophic interaction studies of this kind need to be done in order to better understand individual functional groups and their interactions.

#### IV. Impact of Pollutants on Nutrient Cycling

Changes in the environment caused by greenhouse gases and pollutants have been shown to directly and indirectly influence fungal communities and their activity in the decomposition process. Newsham et al. (1992a, b) and Boddy et al. (1996) showed that, although there was change in the fungal community on tree leaf litter exposed to sulfur dioxide, the leaf occupancy by fungi was not altered. These changes in fungal species had little effect on rate of decomposition of the leaf litter, probably due to the rapid leaching of sulfur from the system. In contrast, Dursun et al. (1996a, b) showed reduced hyphal extension and activity (measured as respiration) of fungi in leaf litter exposed to sulfur dioxide.

Enhanced carbon dioxide in the atmosphere alters plant chemistry. For example, Norby et al. (2001) show that lignin concentrations of leaf litter increase, whilst N concentrations decline. These changes in leaf litter chemistry influence the rate of litter decomposition, nutrient mineralization and affect the fungal communities that colonize

them (Treseder 2005). The effect of UV-B light (as enhanced by ozone depletion in the atmosphere) significantly reduced spore germination and hyphal growth of some phylloplane- and leaf litter-inhabiting fungi (*Aspergillus fumigatus*, *Penicillium hordei*, *P. janczewskii*, *P. purpurogenum*) but not others (*Verticillium* sp., *Mucor heimalis*, *Cladosporium cladosporioides*, *Leptosphaeria coniothyrum*, *Trichoderma viride*, *Ulocladium consortiale* and *Marasmius androsaceus*) (Moody et al. 1999). Changes in the species composition could affect the decomposition of leaf litter and subsequent nutrient mineralization.

Information on the role of fungi in nutrient cycling may also be gained by the interactions of fungi and heavy metals and radionuclides. Although these chemicals do not form the major nutrient elements that are essential for growth, their behavior may indicate the pathway of other elements within fungal-driven processes. Much of the interaction between fungi and heavy metals has recently been summarized by Fomina et al. (2005). The presence of heavy metals has been shown to alter growth patterns of foraging hyphae, which may influence their effectiveness in decomposition because hyphal aggregation shifts between assimilative and non-assimilative states as hyphal growth crosses 'hostile' chemical regions (Ritz 1995; Rayner et al. 1995). By the excretion of fungal acids and enzymes, fungi can change both the valency states and mobility of heavy metals, altering their toxicity to both fungi and other organisms in the decomposer food web. Ionizing radiation from radionuclides has been shown to affect the community composition of soil microfungi (Zhdanova et al. 1995, 2005) where tolerant species are capable of decomposing 'hot particles' containing radionuclides (Zhdanova et al. 1991), possibly by sensing the ionizing radiation and directing their growth toward it (Zhdanova et al. 2004). The fate of the radionuclides released by such action are unknown, but it is possible, based on information from radiocesium accumulation in saprotrophic fungi (Dighton et al. 1991) and mycorrhizal fungi (Oolbekkink and Kuyper 1989; Haselwandter and Berreck 1994; Guillette et al. 1994), that long-term accumulation in fungal tissues may result (Dighton and Horrill 1988). It is interesting to note that the role of melanin has been implicated in both the accumulation and sensing of the presence of heavy metals and radionuclides (Zhdanova et al. 2004; Fomina et al. 2005).

## V. Conclusions

Among the ecosystem services that fungi provide (Dighton 2003), plant litter decomposition and mineral nutrient cycling are among the most prominent processes regulated by saprotrophic fungi. Despite many years of research into this role of fungi, many of the subtle interactions with other organisms in the ecosystem have yet to be elucidated. Much of the role of these fungi is modified by environmental pollution and other environmental changes (global warming, etc.). Traditional techniques for measuring nutrient cycling as mediated by fungi will only tell us so much. Emerging technologies are poised to allow us to investigate new avenues of nutrient cycling. Among these is the use of natural abundance and labeling with radionuclides to follow the fate of nutrients and carbon in ecosystems, providing a new way of investigating fluxes in situ. Significant strides forward have been made in fungal ecology (see review by Hobbie 2005), in which isotopic discrimination between saprotrophic and mycorrhizal fungi can show where these two functional groups obtain both carbon and mineral nutrients. For example, saprotrophic fungi are several parts per mille more enriched in  $^{14}\text{C}$  compared to ectomycorrhizal fungi, and wood-decomposing fungi have a higher  $\delta^{15}\text{N}$  signature than leaf-litter decomposers (Hobbie 2005). Together with greater understanding of the communities of fungi involved in nutrient cycling and the physiology of the individual members of the community, these techniques are likely to lead to significant improvements in our understanding of the role of fungi in providing ecosystem services.

Traditional concepts of nutrient cycling rely on an understanding of leaf litter and wood decomposition. If we consider the greater dynamics of ecosystems, we can see that other, more transient components of the ecosystem may play an equally important role in driving nutrient cycling and impacting fungal communities. Writing the final part of this chapter during a gypsy moth outbreak in our local forest leads me to contemplate the following as potentially fruitful areas of future study:

1. What are the consequences of episodic litter inputs to the decomposer community (insect frass, plant parts other than leaves that provide short-term pulses of input, e.g., bracts, flowers)?
2. How do short-term pulses of interruption to ecosystem stability (fire, rainfall, drought) influence the rates of decomposition, fungal communities, and nutrient cycling?
3. How do food web dynamics change during such times as mentioned above in relation to fungal mediation of nutrient cycling?
4. How important are fungal mycelia and fruit-bodies in the temporary and long-term storage and release patterns of nutrients within diverse natural and agro-ecosystems?

These are but a few of the questions that still need to be addressed to complete our understanding of how we may best manage both natural and managed ecosystems to maintain diversity, effect conservation, and optimize our use of ecosystems.

## References

- April R, Keller D (1990) Mineralogy of the rhizosphere in forest soils of the eastern United States. *Biogeochemistry* 9:1–18
- Ascaso C, Wierzchos J (1995) Study of the biodeterioration zone between the lichen thallus and the substrate. *Cryptogamic Bot* 5:270–281
- Attiwill PM, Adams MA (1993) Nutrient cycling in forests. *New Phytol* 124:561–582
- Azcon R, Barea JM, Hayman DS (1976) Utilization of rock phosphate in alkaline soils by plants inoculated with mycorrhizal fungi and phosphate solubilizing bacteria. *Soil Biol Biochem* 8:135–138
- Banfield JF, Barker WW, Welch SA, Taunton A (1999) Biological impact of mineral dissolution: application of the lichen model to understanding mineral weathering in the rhizosphere. *Proc Natl Acad Sci USA* 96:3404–3411
- Beare MH, Blair JM, Parmelee RW (1989) Resource quality and trophic responses to simulated throughfall: effects on decomposition and nutrient flux in a no-tillage agroecosystem. *Soil Biol Biochem* 21:1027–1036
- Berthelin J, Leyval C (1982) Ability of symbiotic and non-symbiotic rhizospheric microflora of maize (*Zea mays*) to weather micas and to promote plant growth and plant nutrition. *Plant Soil* 68:369–377
- Boddy L, Rayner ADM (1983) Ecological roles of basidiomycetes forming decay communities in attached oak branches. *New Phytol* 93:177–188
- Boddy L, Frankland JC, Dursun S, Newsham K, Ineson P (1996) Effects of dry-deposited  $\text{SO}_2$  and sulphite on saprotrophic fungi and decomposition of tree leaf litter. In: Frankland JC, Magan N, Gadd GM (eds) *Fungi and environmental change*. Cambridge University Press, Cambridge, pp 70–89
- Brady NC, Weil RR (1999) *The nature and properties of soils*. Prentice Hall, Upper Saddle River, NJ
- Cadish G, Giller KE (1997) *Driven by nature: plant litter quality and decomposition*. CABI, Wallingford

- Cairney JWG (1992) Translocation of solutes in ectomycorrhizal and saprotrophic rhizomorphs. *Mycol Res* 96:135–141
- Cairney JWG (2005) Basidiomycete mycelia in forest soils: dimensions, dynamics and roles in nutrient distribution. *Mycol Res* 109:7–20
- Carroll G, Wicklow DT (1992) The fungal community: its organization and role in the ecosystem, 2nd edn. Marcel Dekker, New York
- Chang TT, Li CY (1998) Weathering of limestone, marble, and calcium phosphate by ectomycorrhizal fungi and associated microorganisms. *Taiwan J Forest Sci* 13:85–90
- Chen J, Blume H-P, Beyer L (2000) Weathering of rocks induced by lichen colonization – a review. *Catena* 39:121–149
- Clinton PW, Buchanan PK, Allen RB (1999) Nutrient composition of epigaeous fungal sporocarps growing on different substrates in a New Zealand mountain beech forest. *N Z J Bot* 37:149–153
- Connolly JH, Shortle WC, Jellison J (1998) Translocation and incorporation of strontium carbonate derived strontium into calcium oxalate crystals by the wood decay fungus *Resinicium bicolor*. *Can J Bot* 77:179–187
- Cooke RC, Rayner ADM (1984) Ecology of saprotrophic fungi. Longman, London
- Dickinson CH, Pugh GJF (1974) Biology of plant litter decomposition. Academic Press, London
- Dighton J (1995) Nutrient cycling in different terrestrial ecosystems in relation to fungi. *Can J Bot* 73 suppl 1:S1349–S1360
- Dighton J (2003) Fungi in ecosystem processes. Marcel Dekker, New York
- Dighton J, Boddy L (1989) Role of fungi in nitrogen, phosphorus and sulphur cycling in temperate forest ecosystems. In: Boddy L, Marchant R, Read DJ (eds) Nitrogen, phosphorus and sulphur utilization by fungi. Cambridge University Press, Cambridge, pp 267–298
- Dighton J, Horrill AD (1988) Radiocaesium accumulation in the mycorrhizal fungi *Lactarius rufus* and *Inocybe longicystis*, in upland Britain. *Trans Br Mycol Soc* 91:335–337
- Dighton J, Clint GM, Poskitt JM (1991) Uptake and accumulation of <sup>137</sup>Cs by upland grassland soil fungi: a potential pool of Cs immobilization. *Mycol Res* 95:1052–1056
- Dighton J, Mascarenhas M, Arbuckle-Keil GA (2001) Changing resources: assessment of leaf surface carbohydrate resource change at a microbial scale of resolution. *Soil Biol Biochem* 33:1429–1432
- Dursun S, Frankland JC, Boddy L, Ineson P (1996a) Sulphite and pH effects on CO<sub>2</sub> evolution by fungi growing on decomposing coniferous needles. *New Phytol* 134:155–166
- Dursun S, Ineson P, Frankland JC, Boddy L (1996b) Sulphur dioxide effects on fungi growing on leaf litter and agar media. *New Phytol* 134:167–176
- Eckhardt FEW (1985) Solubilization, transport, and deposition of mineral cations by microorganisms – efficient rock weathering agents. In: Drever JI (ed) The chemistry of weathering. D. Reidel Publishing, New York, pp 161–173
- Fogel R, Hunt G (1983) Contribution of mycorrhizae and soil fungi to nutrient cycling in a Douglas-fir ecosystem. *Can J Forest Res* 13:219–232
- Fomina M, Burford EP, Gadd GM (2005) Toxic metals and fungal communities. In: Dighton J, White JF, Oudemans P (eds) The fungal community: its organization and role in the ecosystem, 3rd edn. Taylor and Francis, Boca Raton, FL, pp 733–758
- Forsyth A, Miyata K (1984) Tropical nature. Charles Scribner's Sons, New York
- Frankland JC (1992) Mechanisms in fungal succession. In: Carroll G, Wicklow DT (eds) The fungal community: its organization and role in the ecosystem, 2nd edn. Marcel Dekker, New York, pp 383–401
- Frankland JC (1998) Fungal succession – unravelling the unpredictable. *Mycol Res* 102:1–15
- Frankland JC, Hedger JN, Swift MJ (1982) Decomposer basidiomycetes: their biology and ecology. Cambridge University Press, Cambridge
- Gobran GR, Clegg S, Courchesne F (1998) Rhizospheric processes influencing the biogeochemistry of forest ecosystems. *Biogeochemistry* 42:107–120
- Gray SN, Dighton J, Olsson S, Jennings DH (1995) Real-time measurement of uptake and translocation of <sup>137</sup>Cs within mycelium of *Schizophyllum commune* Fr. by autoradiography followed by quantitative image analysis. *New Phytol* 129:449–465
- Guillette O, Melin J, Wallberg L (1994) Biological pathways of radionuclides originating from the Chernobyl fallout in a boreal forest ecosystem. *Sci Total Environ* 157:207–215
- Harborne JB (1997) Role of phenolic secondary metabolites in plants and their degradation in nature. In: Cadisch G, Giller KE (eds) Driven by nature: plant litter quality and decomposition. CABI, Wallingford, pp 67–74
- Haselwandter K, Berreck M (1994) Accumulation of radionuclides in fungi. In: Winkelmann G, Winge DR (eds) Metal ions in fungi. Marcel Dekker, New York, pp 259–277
- Heal OW, Dighton J (1985) Resource quality and trophic structure in the soil system. In: Fitter AH, Atkinson D, Read DJ, Usher MB (eds) Ecological interactions in soil. Blackwell, Oxford, pp 339–354
- Heal OW, Anderson JM, Swift MJ (1997) Plant litter quality and decomposition: an historical overview. In: Cadish G, Giller KE (eds) Driven by nature: plant litter quality and decomposition. CABI, Wallingford, pp 3–30
- Hedger J, Lewis P, Gitay H (1993) Litter trapping by fungi in moist tropical forest. In: Isaac S, Frankland JC, Watling R, Whalley AJS (eds) Aspects of tropical mycology. Cambridge University Press, Cambridge, pp 15–35
- Hirsch P, Eckhardt FEW, Palmer RJ (1995) Fungi active in weathering of rock and stone monuments. *Can J Bot* 73 suppl 1: S13484–S1390
- Hobbie EA (2005) Using isotopic tracers to follow carbon and nitrogen. In: Dighton J, White JF, Oudemans P (eds) The fungal community: its organization and role in the ecosystem, 3rd edn. Taylor and Francis, Boca Raton, FL, pp 361–381
- Hoffland E, Giesler R, Jongmans T, van Breemen N (2002) Increasing feldspar tunneling by fungi across a north Sweden podzol chronosequence. *Ecosystems* 5:11–22
- Hoffland E, Kuyper TW, Wallander H, Plassard C, Gorbushina AA, Haselwandter K, Holmstrom S, Landeweert R, Lundstrom US, Rosling A, Sen R, Smits MM,

- Van Hees PAW, van Breemen N (2004) The role of fungi in weathering. *Frontiers Ecol Environ* 2:258–264
- Honegger R (1991) Functional aspects of the lichen symbiosis. *Annu Rev Plant Physiol Plant Mol Biol* 42:553–578
- Jones HE, Madeira M, Herraez L, Dighton J, Fabiao A, Gonzalez-Rio F, Fernandez Marcos M, Gomez C, Tome M, Feith H, Magalhaes MC, Howson G (1999) The effect of organic-matter management on the productivity of *Eucalyptus globulus* stands in Spain and Portugal: tree growth and harvest residue decomposition in relation to site and treatment. *Forest Ecol Manage* 122:73–86
- Jongmans AG, van Breemen, N, Lundstrom U, van Hees PAW, Findlay RD, Srinivasan M, Unestam T, Giesler R, Melkerud PA, Olsson M (1997) Rock-eating fungi. *Nature* 389:682–683
- Knops JMH, Nash TH, Schlesinger WH (1996) The influence of epiphytic lichens on the nutrient cycling of an oak woodland. *Ecol Monogr* 66:159–19
- Landeweert R, Hoffland E, Finlay RD, Kuyper TW, van Breemen N (2001) Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. *Trends Ecol Evol* 16:248–254
- Lange OL, Meyer A, Zellner H, Heber U (1994) Photosynthesis and water relations of lichen soil crusts: field measurements in the coastal fog zone of the Namib Desert. *Funct Ecol* 8:253–264
- Linkins AE, Melillo JM, Sinsabaugh RL (1984) Factors affecting cellulase activity in terrestrial and aquatic ecosystems. In: Klug MJ, Reddy CA (eds) Current perspective in microbial ecology. American Society for Microbiology, Washington, DC, pp 572–579
- Lockwood JL (1992) Exploitation competition. In: Carroll GC, Wicklow DT (eds) The fungal community: its organization and role in the ecosystem, 2nd edn. Marcel Dekker, New York, pp 243–263
- Lodge DJ (1993) Nutrient cycling by fungi in wet tropical forests. In: Isaac S, Frankland JC, Watling R, Whalley AJS (eds) Aspects of tropical mycology. Cambridge, University Press Cambridge, pp 37–57
- Lodge DJ, Asbury CE (1988) Basidiomycetes reduce export of organic matter from forest slopes. *Mycologia* 80:888–890
- Marumoto T, Anderson JPE, Domsch KH (1982) Decomposition of <sup>14</sup>C- and <sup>15</sup>N-labeled microbial cells in soil. *Soil Biol Biochem* 14:461–467
- Mascarenhas M, Dighton J, Arbuckle G (2000) Characterization of plant carbohydrates and changes in leaf carbohydrate chemistry due to chemical and enzymatic degradation measured by microscopic ATR FT-IR spectroscopy. *Appl Spectroscopy* 54:681–686
- Melillo JM, Aber JD, Muratore JF (1982) Nitrogen and lignin control of hardwood leaf litter decomposition dynamics. *Ecology* 63:621–626
- Mojallala H, Weed SB (1978) Weathering of micas by mycorrhizal soybean plants. *Soil Biol Biochem* 42:367–372
- Møller J, Miller M, Kjøller A (1999) Fungal-bacterial interaction on beech leaves: influence on decomposition and dissolved organic carbon quality. *Soil Biol Biochem* 31:367–374
- Moody SA, Newsham KK, Ayres PG, Paul ND (1999) Variation in the responses of litter and phylloplane fungi to UV-B radiation (290–315 nm). *Mycol Res* 103:1469–1477
- Nahas E, Banzatto DA, Assis LC (1990) Fluorapatite solubilization by *Aspergillus niger* in vinasse medium. *Soil Biol Biochem* 22:1097–1101
- Newsham KK, Frankland JC, Boddy L, Ineson P (1992a) Effects of dry-deposited sulphur dioxide on fungal decomposition of angiosperm tree leaf litter I. Changes in communities of fungal saprotrophs. *New Phytol* 122:97–116
- Newsham KK, Ineson P, Boddy L, Frankland JC (1992b) Effects of dry deposited sulphur dioxide on fungal decomposition of angiosperm leaf litter II. Chemical content of leaf litters. *New Phytol* 122:111–125
- Nieminen JK, Setälä H (2001) Bacteria and microbial-feeder modify the performance of a decomposer fungus. *Soil Biol Biochem* 33:1703–1712
- Norby RJ, Cotrufo MF, Ineson P, O'Neill EG, Canadell JG (2001) Elevated CO<sub>2</sub>, litter chemistry, and decomposition: a synthesis. *Oecologia* 93:153–165
- Olsson S (1995) Mycelial density profiles of fungi on heterogeneous media and their interpretation in terms of nutrient reallocation patterns. *Mycol Res* 99:143–153
- Olsson S, Jennings DH (1991) Evidence for diffusion being the mechanism of translocation in the hyphae of three moulds. *Exp Mycol* 15:302–309
- Oolbekkink GT, Kuyper TW (1989) Radioactive caesium from Chernobyl in fungi. *The Mycologist* 3:3–6
- Osono T, Fukasawa Y, Takeda H (2003) Roles of diverse fungi in larch needle-litter decomposition. *Mycologia* 95:820–826
- Ponge JF (1990) Ecological study of a forest humus by observing a small volume. I. Penetration of pine litter by mycorrhizal fungi. *Eur J Forest Pathol* 20:290–303
- Ponge JF (1991) Succession of fungi and fauna during decomposition of needles in a small area of Scots pine litter. *Plant Soil* 138:99–113
- Ponge JF (2005) Fungal communities: relation to resource succession. In: Dighton J, White JF, Oudemans P (eds) The fungal community: its organization and role in the ecosystem, 3rd edn. Taylor and Francis, Boca Raton, FL, pp 169–180
- Rayner ADM (1978) Interactions between fungi colonizing hardwood stumps and their possible role in determining patterns of colonization and succession. *Ann Appl Biol* 89:505–517
- Rayner ADM (1992) Introduction. In: Carroll GC, Wicklow DT (eds) The fungal community: its organization and role in the ecosystem, 2nd edn. Marcel Dekker, New York, pp xvii–xxiv
- Rayner ADM, Boddy L (1988) Fungal decomposition of wood. Wiley, Chichester
- Rayner ADM, Griffiths GS, Ainsworth AM (1995) Mycelial interconnectedness. In: Gow NAR, Gadd GM (eds) The growing fungus. Chapman and Hall, London, pp 21–40
- Ritz K (1995) Growth responses of some fungi to spatially heterogeneous nutrients. *FEMS Microbiol Ecol* 16:269–280
- Robinson CH, Dighton J, Frankland JC (1993a) Resource capture by interaction by fungal colonizers of straw. *Mycol Res* 97:547–558
- Robinson CH, Dighton J, Frankland JC, Coward PA (1993b) Nutrient and carbon dioxide release by interacting species of straw-decomposing fungi. *Plant Soil* 151:139–142

- Singh S, Kapoor KK (1998) Effects of inoculation of phosphate-solubilizing microorganisms and arbuscular mycorrhizal fungus on mungbean grown under natural soil conditions. *Mycorrhiza* 7:149–153
- Sinsabaugh RL (2005) Fungal enzymes at the community scale. In: Dighton J, White JF, Oudemans P (eds) *The fungal community: its organization and role in the ecosystem*. Taylor and Francis, Boca Raton, FL, pp 349–360
- Sinsabaugh RL, Liptak MA (1997) Enzymatic conversion of plant biomass. In: Wicklow DT, Soderström B (eds) *The Mycota IV. Environmental and microbial relationships*. Springer, Berlin Heidelberg New York, pp 347–357
- Sinsabaugh RL, Antibus RK, Linkins AE, McClaugherty CA (1993) Wood decomposition: nitrogen and phosphorus dynamics in relation to extracellular enzyme activity. *Ecology* 74:1586–1593
- Stark N (1972) Nutrient cycling pathways and litter fungi. *Bioscience* 22:355–360
- Swift MJ, Heal OW, Anderson JM (1979) *Decomposition in terrestrial ecosystems*. Blackwell, Oxford
- Thompson RM, Townsend CR, Craw D, Frew R, Riley R (2001) (Further) links from rocks to plants. *Trends Ecol Evol* 16:543
- Treseder KK (2005) Nutrient acquisition strategies of fungi and their relation to elevated atmospheric CO<sub>2</sub>. In: Dighton J, White JF, Oudemans P (eds) *The fungal community: its organization and role in the ecosystem*, 3rd edn. Taylor and Francis, Boca Raton, FL, pp 713–731
- Vanlauwe B, Diels J, Sangina N, Merckx R (1997) Residue quality and decomposition: an unsteady relationship? In: Cadish G, Giller KE (eds) *Driven by nature: plant litter quality and decomposition*. CABI, Wallingford, pp 157–166
- Vanlauwe B, Nwoke OC, Diels J, Sangina N, Carsky RJ, Dekers J, Merckz R (2000) Utilization of rock phosphate by crops on a representative toposequence in the Northern Guinea savanna zone of Nigeria: response by *Mucuna pruriens*, *Lallab purpureus* and maize. *Soil Biol Biochem* 32:2063–2077
- Wainwright M (2005) Oligotrophic growth of fungi. In: Dighton J, White JF, Oudemans P (eds) *The fungal community: its organization and role in the ecosystem*, 3rd edn. Taylor and Francis, Boca Raton, FL, pp 643–658
- Wallander H, Wickman T (1999) Biotite and microcline as potassium sources in ectomycorrhizal and non-mycorrhizal *Pinus sylvestris* seedlings. *Mycorrhiza* 9:25–32
- Wells JM, Boddy L (1990) Wood decay, and phosphorus and fungal biomass allocation, in mycelial cord systems. *New Phytol* 116:285–295
- Wells JM, Boddy L (1995a) Phosphorus translocation by saprotrophic basidiomycete mycelial cord systems on the floor of a mixed deciduous woodland. *Mycol Res* 99:977–999
- Wells JM, Boddy L (1995b) Effect of temperature on wood decay and translocation of soil-derived phosphorus in mycelial cord systems. *New Phytol* 129:289–297
- Wells JM, Thomas J, Boddy L (2001) Soil water potential shifts: developmental responses and dependence on phosphorus translocation by the saprotrophic, cord-forming basidiomycete *Phanerochaete velutina*. *Mycol Res* 105:859–867
- Wicklow DT (1992) Interference competition. In: Carroll GC, Wicklow DT (eds) *The fungal community: its organization and role in the ecosystem*, 2nd edn. Marcel Dekker, New York, pp 265–274
- Yoshida S, Muramatsu Y (1994) Accumulation of radiocesium in basidiomycetes collected from Japanese forests. *Sci Total Environ* 157:197–205
- Zak JC (1993) The enigma of desert ecosystems: the importance of interactions among the soil biota to fungal biodiversity. In: Isaac S, Frankland JC, Watling R, Whalley AJS (eds) *Aspects of tropical mycology*. Cambridge University Press, Cambridge, pp 59–71
- Zak JC, Sinsabaugh RL, MacKay W (1995) Windows of opportunity in desert ecosystems: their implications to fungal community development. *Can J Bot* 73:1407–1414
- Zhdanova NN, Lashko TN, Redchitz TI, Vasiliveskaya AI, Bosisyuk LG, Sinyavskaya OI, Gavril'yuk VI, Muzalev PN (1991) Interaction of soil micromycetes with 'hot' particles in the model system. *Microbiol Zhurnal* 53:9–17
- Zhdanova NN, Vasilevskaya AI, Artyshkova LV, Sadovnikov Yu S, Gavril'yuk VI, Dighton J (1995) Changes in the micromycete communities in soil in response to pollution by long-lived radionuclides emitted by the Chernobyl accident. *Mycol Res* 98:789–795
- Zhdanova NN, Tugay T, Dighton J, Zheteltonozhsky V, McDermott P (2004) Ionizing radiation attracts fungi. *Mycol Res* 108:1089–1096
- Zhdanova NN, Zakharchenko VA, Haselwandter K (2005) Radionuclides and fungal communities. In: Dighton J, White JF, Oudemans P (eds) *The fungal community: its organization and role in the ecosystem*, 3rd edn. Taylor and Francis, Boca Raton, FL, pp 759–768

---

# 17 Fungal Decomposers of Plant Litter in Aquatic Ecosystems

M.O. GESSNER<sup>1</sup>, V. GULIS<sup>2</sup>, K.A. KUEHN<sup>3</sup>, E. CHAUVET<sup>4</sup>, K. SUBERKROPP<sup>2</sup>

## CONTENTS

I. Introduction .....	301
II. Fungal Decomposers in Salt and Freshwater Marshes .....	303
A. Marshes as Fungal Habitat .....	303
B. Fungal Diversity on Emergent Wetland Plants .....	303
C. Fungal Biomass and Production .....	304
D. Enzymatic Capabilities .....	307
E. Respiratory Activities on Standing-Dead Wetland Plants .....	308
III. Fungal Decomposers in Streams .....	309
A. Streams as Fungal Habitat .....	309
B. Fungal Diversity on Decomposing Litter .....	309
C. Fungal Biomass and Production .....	311
D. Responses of Fungal Decomposers to Dissolved Nutrients .....	313
E. Enzymatic Capabilities .....	314
F. Significance of Fungal Diversity for Leaf Decomposition .....	315
IV. Importance of Fungal Decomposers in Aquatic Ecosystems .....	315
A. Fungal Biomass and Production at the Ecosystem Scale .....	315
B. Fungal vs. Bacterial Biomass and Production .....	316
C. Decomposition Budgets .....	317
V. Conclusions .....	318
References .....	319

## I. Introduction

Fungi are ubiquitous in the biosphere – from glacial forefields in high mountain to the deep sea (Jumpponen 2003; Gessner and Robinson 2003; Damare

et al. 2006) and from hot springs to the polar ice caps (Jones et al. 2000; Gunde-Cimerman et al. 2003; Magan, this volume). One of their basic functions in natural ecosystems is the decomposition of plant matter, such as leaves, wood and fruits (Harley 1971). The view that fungi are instrumental in plant litter decomposition has been a long-standing paradigm in terrestrial ecology (Dighton, this volume) but, for aquatic ecosystems, this notion has not gained wide acceptance. Nevertheless, there is now strong evidence that fungi are critically important, if not the predominant, decomposers of plant litter in marine and freshwater ecosystems (Newell 1993; Newell and Porter 2000; Gessner and Van Ryckegem 2003; Bärlocher 2005; Gulis et al. 2006b).

Fungal decomposers in aquatic ecosystems are particularly prominent at the interfaces between land and water, where dense growth of higher plants typically results in abundant litter supply when plants or plant parts senesce and die. These environments comprise coastal marine areas, inland wetlands such as mires (peatlands), freshwater swamps and marshes, including littoral zones of lakes and rivers, and forest ponds and streams, which receive plant litter from adjacent riparian vegetation. Plant matter decomposing in these environments may be constantly submerged, periodically or occasionally flooded, or permanently exposed to air as in the case of standing-dead shoots of emergent plants in salt and freshwater marshes. Since different types of plant matter may vary greatly in chemical composition, physical structure, particle size, and timing when they become available to microbial colonization, a range of opportunities exist for fungal development within these habitats. In contrast to terrestrial environments, the continual availability of water and, in many cases, the abundant supply of dissolved nutrients often create conditions that are particularly favourable for fungal growth and activity.

<sup>1</sup> Department of Aquatic Ecology, Eawag: Swiss Federal Institute of Aquatic Science and Technology, and Institute of Integrative Biology (IBZ), ETH Zurich, Überlandstrasse 133, 8600 Dübendorf, Switzerland

<sup>2</sup> Department of Biological Sciences, University of Alabama, Tuscaloosa, AL 35487-0206, USA

<sup>3</sup> Department of Biological Sciences, The University of Southern Mississippi, Hattiesburg, MS 39406-0001, USA

<sup>4</sup> Laboratoire d'écologie fonctionnelle - EcoLab, UMR 5245 CNRS, University of Toulouse 3, National Polytechnic Institute of Toulouse, 29 rue Jeanne Marvig, 31055 Toulouse Cedex, France

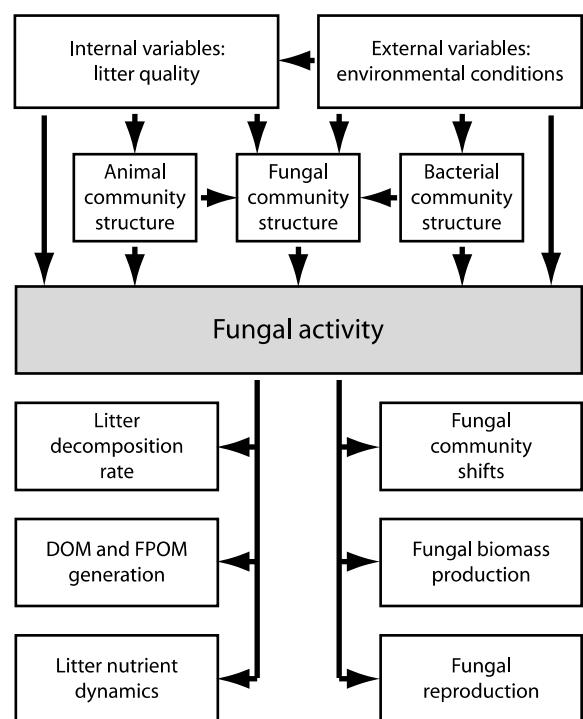
To demonstrate that fungi act as important decomposers in a given environment, several conditions should be met:

1. Fungi must be present in the natural system, where they may be detected by direct observation or indirect methods involving chemical indicator molecules (Newell 1992; Gessner and Newell 2002; Tsui and Hyde 2003; Foster et al. 2004; Graça et al. 2005). Culturing techniques also may be useful, although they carry a greater danger of introducing bias.
2. Identified species must be able to grow and reproduce under conditions prevailing in the natural habitat, i.e. the available substrate and under the environmental conditions defined by abiotic factors such as humidity, salinity, oxygen concentration, and temperature.
3. They should elaborate the enzymes necessary to degrade plant tissues and to produce them in amounts sufficient to cause significant litter degradation.
4. These activities should result in mass loss of organic matter or, when species are growing only in mixed assemblages, in an acceleration of mass loss.
5. Finally, the fungi should be successful in competing with other organisms present in the system and thus either rapidly colonize a resource and grow at a competitive rate or be able to oust established species.

Ultimate proof of fungal participation in decomposition consists in demonstrating fungus-specific degradative activity. This may be indicated when activities of a fungus grown on litter in microcosms (Hicks and Newell 1984; Suberkropp 1991; Treton et al. 2004) are similar to the activities observed *in situ*. Careful application of antibiotics and fungal inhibitors (Padgett 1993), coupled with the kind of measurements given above, may also be useful, although this approach is loaded with potential pitfalls (Oremland and Capone 1988). More powerful and currently promising methods appear to be the quantification of mRNA and/or enzymes, both of which are likely to provide important insights in the future as transcriptomics and proteomics are eventually applied to microbial assemblages associated with decomposing plant litter.

Decomposition of plant remains involves a range of biotic and abiotic transformations that result in the formation of carbon dioxide and other mineral substances, dissolved organic

matter (DOM), and fine particulate organic matter (FPOM), but also in the biomass production of microbial decomposers, such as fungi (Gessner et al. 1999). The rates of all these processes are governed by the response of decomposers to environmental conditions (external factors) and the intrinsic quality of litter (internal factors), and they are modulated by biotic interactions within and between different groups of decomposers and with other components of aquatic food webs. Outcomes can be divided into those affecting the decomposition process (Fig. 17.1, bottom left) and those affecting fungal performance (Fig. 17.1, bottom right). Outcomes pertaining to fungal performance relate to life-history patterns, often apparent as shifts in fungal community structure, mycelial growth, and allocation of resources to reproduction. Outcomes relevant at the ecosystem level include litter mass loss, nutrient dynamics including immobilization and mineralization, and generation of litter transformation products such as FPOM and DOM. Thus, plant litter decomposition may be viewed from an ecosystem process perspective or from a decomposer point of view (Fig. 17.1).



**Fig. 17.1.** Schematic representation of a fungus-dominated litter decomposition system as viewed from a process (*lower left cases*) and a fungal (*lower right cases*) perspective

This chapter will address both perspectives. Emphasis will be placed on two aquatic ecosystems in which fungal decomposers and plant litter decomposition have been studied to the greatest extent: standing-dead shoots of emergent vascular plants in salt and freshwater marshes (Sect. II.), and leaf litter that falls into forest streams from adjacent riparian vegetation (Sect. III). In addition, the importance of fungi as producers of biomass will be addressed at the ecosystem level; a comparison will be made with the biomass and production of bacteria, the other potentially important microorganisms associated with decomposing plant material; moreover, the role of fungi in decomposition budgets will be evaluated (Sect. IV.).

## II. Fungal Decomposers in Salt and Freshwater Marshes

### A. Marshes as Fungal Habitat

Salt and freshwater marshes are among the most productive ecosystems on the planet (Mitsch and Gosselink 2000). Emergent vascular plants, such as the smooth cord-grass *Spartina alterniflora* Loisel, the reed *Phragmites australis* (Cav.) Trin. ex Steud. and cattails (*Typha* spp.), are conspicuous features in these wetlands and often account for the greatest fraction of the total annual plant biomass produced (Dai and Wiegert 1996; Wetzel and Howe 1999). Since herbivore consumption is typically low (<1% of production; Wetzel and Howe 1999; Kreeger and Newell 2000), most of the plant biomass eventually enters into the detrital pool.

An important characteristic of most emergent wetland plants is that detachment of leaves and collapse of shoots does not immediately follow plant senescence and death (Newell 1993; Bärlocher 1997). Rather, shoots stay upright and leaves remain attached to the parent plant for extended periods, typically resulting in large amounts of standing-dead plant matter that accumulate in marshes (e.g. Asaeda et al. 2002). Fungi are a key component of the decomposer assemblage of this plant material (Newell 1993; Newell and Porter 2000; Gulis et al. 2006b), and may serve as an important food source for metazoan consumers (e.g. Silliman and Newell 2003). Thus, nutrient cycling and energy flow in these ecosystems are regulated to a significant extent by the metabolic activities of fungi associated with decaying plant

material. The following section focuses on fungi in salt and freshwater marshes with special emphasis on fungal dynamics associated with standing-dead shoots of emergent wetland plants.

### B. Fungal Diversity on Emergent Wetland Plants

Fungal communities associated with emergent plants in freshwater marshes are often taxonomically diverse. Early work by Saccardo (1898) reported 168 fungal taxa from shoots of a single species, *P. australis*, and a recent compilation of literature data now suggests that over 600 species have been recorded from this plant (Gessner and Van Ryckegem 2003). The most common fungal taxa identified from *P. australis* were members of the Ascomycota (94%, including 30% hyphomycetous and 22% coelomycetous anamorphs), with Basidiomycota (6%) occurring much less frequently. In a more limited survey, over 30 species were identified from standing-dead *Juncus effusus* (Kuehn and Suberkropp 1998a). Hyphomycetes and coelomycetes were the predominant taxa observed. However, examination of dead leaves attached to standing shoots in the field revealed large numbers of basidiomata of two white-rot fungi, *Panellus copelandi* and *Marasmiellus* sp., indicating that laboratory culturing techniques produced conditions that excluded sporulation of these and possibly other fungal species.

Several studies have reported successional changes of fungal taxa as shoot decomposition of emergent freshwater plants proceeds (Pugh and Mulder 1971; Apinis and Taligoola 1974; Van Ryckegem and Verbeken 2005). For example, based on observations of fruiting structures, Pugh and Mulder (1971) reported a succession of sporulating taxa during standing-dead and submerged decomposition of *Typha latifolia* leaves. Prevalent fungi observed during early decomposition stages (i.e. on standing-dead shoots) were similar to taxa observed on terrestrial plants, with primary colonization by phylloplane fungi such as *Aureobasidium pullulans*, *Cladosporium herbarum*, *Phoma typharum* and *Epicoccum nigrum*. This initial phase was followed by a secondary phase in which *Leptosphaeria* spp. were dominant. Predaceous nematode-trapping fungi, such as *Arthrobotrys* and *Dactylaria* spp., were most prevalent during the final submerged stages of decomposition when plant matter had collapsed to the sediment surface.

A particularly marked shift in sporulating fungal taxa appears to coincide with the transition of decay of standing-dead shoots to decomposition of fallen or collapsed plant parts on the sediment (Van Ryckegem et al. 2007).

In addition to temporal shifts in community structure, fungi may exhibit distinct spatial distribution patterns on standing-dead shoots (Pugh and Mulder 1971; Apinis and Taligoola 1974; Poon and Hyde 1998; Van Ryckegem and Verbeken 2005). Different taxa may occupy specific plant parts, such as leaf blades, leaf sheaths, or the nodes and internodes of culms. In addition, fungal taxa associated with *P. australis* in tidal marshes showed distinct vertical distribution patterns in the plant canopy, which appeared to be a primary factor determining fungal community composition (Van Ryckegem and Verbeken 2005; Van Ryckegem et al. 2007). These small-scale distribution patterns on shoots may be a result of small-scale spatial variation in environmental conditions and/or differences in the resource quality of plant litter, such as varying amounts of recalcitrant compounds within different plant tissues.

In salt marshes, observations of fungal reproductive structures associated with standing-dead *S. alterniflora* revealed that ascomycete species of *Phaeosphaeria*, *Mycosphaerella* and *Buergenerula* are typically the most frequently encountered (Gessner 1977; Newell 1993, 2001a; Newell et al. 2000). Studies using molecular methods to describe fungal communities on decomposing wetland plants, such as ITS rDNA sequencing and terminal restriction fragment length polymorphism (T-RFLP), largely concur with observations based on traditional microscopic techniques (Buchan et al. 2002, 2003; Lyons et al. 2005; Torzilli et al. 2006). In particular, on leaf blades of standing-dead *Spartina* shoots in a South-Eastern U.S. salt marsh, analysis of ascospore expulsion rates, ITS clone libraries and T-RFLPs provided a similar picture of fungal community composition, with *P. spartinicola*, *Mycosphaerella* sp. and *P. halima* being the dominant taxa encountered (Buchan et al. 2002). Overall, these data suggest that fungal communities associated with *Spartina* are not particularly complex, with a single species accounting for most of the fungal biomass (Newell et al. 1989) and reproductive output (Newell and Wasowski 1995; Newell 2001a) in some salt marshes. This degree of species dominance appears to be in contrast with the often much more diverse fungal communities associated with

standing-dead plants in freshwater marshes (see above), although strong dominance has also been found in freshwater marshes (Neubert et al. 2006).

Depending on characteristics of the habitat (i.e. degree and regularity of inundation by tides), fungi on standing-dead shoots in salt marshes may show vertical distribution patterns. Typical terrestrial fungi have often been observed on upper portions of standing-dead *S. alterniflora* shoots not exposed to tidal inundation (Gessner 1977), whereas marine taxa were most commonly observed on lower portions of plant shoots that were regularly submerged by tides (Gessner 1977). Similar observations have been reported for fungal communities on both standing-dead and collapsed plant parts of *P. australis* in a brackish tidal marsh (Poon and Hyde 1998; Van Ryckegem and Verbeken 2005; Van Ryckegem et al. 2007). Distinct fungal communities sporulated in different microhabitats (e.g. middle or basal canopy of standing-dead shoots), with greater numbers of terrestrial species associated with upper shoot portions. Flooding height and frequency influenced the vertical species distribution, presumably in response to not only water availability but also salinity.

### C. Fungal Biomass and Production

In addition to the wealth of qualitative evidence showing pervasive fungal colonization of emergent wetland plants, the productivity and functional role of fungi has been assessed in several systems (e.g. Newell and Porter 2000; Gulis et al. 2006b). Historically, the lack of suitable methods to quantify fungal biomass and rates of biomass production has been a major impediment to obtaining such data. A particular problem has been the intimate association of fungi with decomposing plant tissue (Newell 1992) – hyphae penetrate the plant tissue, rather than adhere to its surface (Fig. 17.2; Newell et al. 1996b). Consequently, earlier estimates of fungal biomass based on measurements of hyphal length (Table 17.1), in particular after clearing of leaves, are likely to be severe underestimates (Newell 1992; Gessner and Newell 2002). However, these methodological problems have been largely overcome by the use of indicator molecules such as ATP, chitin, certain fatty acids in phospholipids and, particularly, the membrane lipid, ergosterol, which is likely to provide the most accurate estimates (Newell 1992; Gessner and Newell 2002). ATP is not specific for fungi but can be used as a re-

**Table 17.1.** Some estimates of fungal biomass associated with decomposing plant litter in streams<sup>a</sup>

Fungal biomass (mg g <sup>-1</sup> detrital mass)	No. of streams	Litter type <sup>b</sup>	Method	Reference
0.12	1	LB(1)	Biovolume <sup>c</sup>	Iversen (1973)
8–49	1	LB(3)	Biovolume <sup>d</sup>	Findlay and Arsuffi (1989)
23	1	LB(1)	ATP	Findlay and Arsuffi (1989)
15–111	8 <sup>d</sup>	LB(1)	ATP	Suberkropp and Chauvet (1995)
47–83	2	LB(2)	ATP	Suberkropp et al. (1993)
127–158	2	LB(2)	Ergosterol	Suberkropp et al. (1993)
61–155	1 <sup>e</sup>	LB(7)	Ergosterol	Suberkropp et al. (1993), Gessner and Chauvet (1994)
78–226	4	LB(1)	Ergosterol	Methvin and Suberkropp (2003), Carter and Suberkropp (2004)
54–73	4	RCL	Ergosterol	Methvin and Suberkropp (2003), Carter and Suberkropp (2004)
1–175	10	WV	Ergosterol	Simon and Benfield (2001), Stelzer et al. (2003), Gulis et al. (2004)
2–25	4	WS	Ergosterol	Díez et al. (2002), Spähnhoff and Gessner (2004)
24–86	2	RCWS	Ergosterol	Gulis et al. (unpublished data)

<sup>a</sup> Hyphal lengths were converted mycelial biomass by assuming an average hyphal diameter of 3 µm and a density of 500 fg µm<sup>-3</sup> (cf. Findlay and Arsuffi 1989; Newell 1992). ATP was converted fungal biomass assuming that fungal ATP accounted for 90% of the total ATP (cf. Findlay and Arsuffi 1989), at an average ATP concentration of 1.75 mg g<sup>-1</sup> mycelial dry mass (Suberkropp 1991; Suberkropp et al. 1993). Ergosterol was converted to fungal biomass assuming an average concentration of 5.5 mg g<sup>-1</sup> dry mass (Gessner and Chauvet 1993), unless more specific data were available

<sup>b</sup> LB, leaves in litter bags, with the number of leaf types in parentheses (maximum fungal biomass from decomposition experiments is given); RCL, randomly collected naturally occurring leaf litter (mean annual fungal biomass); WV, wood veneers and WS, wood sticks (range of fungal biomass from decomposition experiments); RCWS, randomly collected wood sticks (mean fungal biomass)

<sup>c</sup> Hyphal length determined after clearing of whole leaf material

<sup>d</sup> Hyphal length determined after grinding and collecting leaf fragments on membrane filters

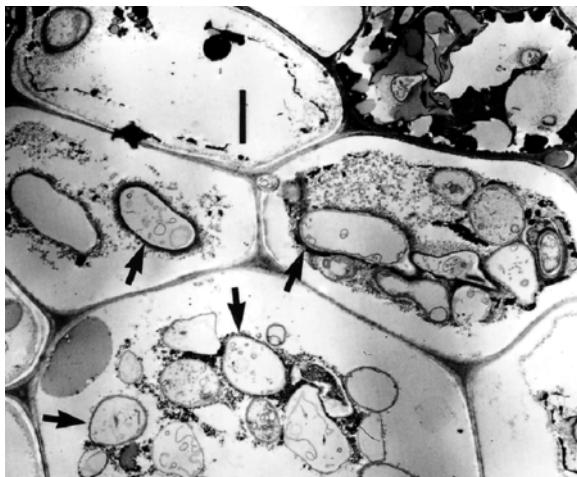
<sup>e</sup> Different sites or years or both in the same stream

liable index of fungal biomass because, in terms of biomass, fungi commonly outweigh bacteria associated with plant litter (see Sect. IV.B.), and major sources of ATP other than bacteria are usually absent (Golladay and Sinsabaugh 1991; Suberkropp et al. 1993). Phospholipid fatty acid (PLFA) profiling has been used to quantify fungi in terrestrial habitats (e.g. Klamer and Bååth 2004) but has not yet been applied to fungi in aquatic habitats. Beyond the use of these biomass indicators, measurement of [<sup>14</sup>C]acetate incorporation into ergosterol facilitates the determination of *in situ* fungal growth rates and production to assess the dynamics of fungal biomass accrual and loss (Newell and Fallon 1991; Gessner and Newell 2002).

Application of these and other quantitative methods has provided compelling evidence that fungi are a key component of microbial assemblages within standing-dead shoots of emergent wetland plants, suggesting an overall important contribution to carbon and nutrient cycling in

marsh ecosystems (Gulis et al. 2006b). In pioneering studies involving a range of methods, fungal biomass in leaves of *S. alterniflora* ranged from 1.8% (microscopic determination of mycelial biovolume) to as much as 20% (immunosorbent assay, ELISA) of the total organic matter (Newell et al. 1989). Estimates based on the determination of ergosterol concentrations were intermediate (5%) but would have been about twice as high if a more realistic conversion factor (see Newell 1994) had been used. A large proportion of this biomass can be in the form of ascocarps, which sometimes account for as much as 31% of the total fungal biomass in decomposing *Spartina* leaves (Newell and Wasowski 1995).

Assessment of multiyear patterns of fungal biomass associated with salt-marsh plants corroborates these earlier observations. Fungi associated with both standing-dead *S. alterniflora* (Newell 2001b) and *Juncus roemerianus* (Newell 2001c) accumulated substantial levels of biomass, with



**Fig. 17.2.** Transmission electron micrograph of a cross-section of a yellow poplar leaf (*Liriodendron tulipifera* L.) that had been decomposing in a hardwater stream for 3 weeks. The leaf was fixed in 2% glutaraldehyde in 10 mM Na cacodylate, pH 7, immediately after it was removed from the stream. It was then post-fixed in 2% osmium tetroxide, dehydrated, embedded and sectioned. Note the masses of fungal hyphae within leaf cells. Four hyphae are indicated by arrows. Scale bar indicates 2  $\mu\text{m}$

estimated peak values exceeding 5% of the total organic mass of dead plant matter. On both plant species, fungal biomass was generally higher during the winter and spring and low during the summer, and *S. alterniflora* supported  $\sim$ 1.5-fold higher fungal biomass than *J. roemerianus*. In *S. alterniflora* leaves, ergosterol concentrations were negatively correlated with rainfall and tidal height (Newell 2001b), which supports findings from manipulative field experiments suggesting that prolonged water saturation has a negative effect on fungi associated with standing-dead wetland plants (Newell et al. 1996a).

Similar to the consistent temporal pattern over several years, a large-scale comparative study along the eastern coast of North America revealed no significant latitudinal differences in fungal biomass in leaves of standing-dead *Spartina* or *Juncus* in salt marshes between 29 and 43°N latitude (Newell et al. 2000). This suggests that substantial fungal production derived from the aboveground primary production of these salt-marsh plants is the rule. However, Samiaji and Bärlocher (1996) found much lower fungal biomass associated with *S. alterniflora* leaves in the Bay of Fundy (Canada) at 45°N, indicating that harsher environmental conditions at more northern latitudes may begin to limit fungal biomass accrual in standing-dead shoots.

Substantial fungal biomass accumulations have also been reported from standing-dead shoots decaying in freshwater marshes (Newell et al. 1995; Bärlocher and Biddiscombe 1996; Kuehn and Suberkropp 1998a; Gessner 2001; Findlay et al. 2002a; Newell 2003; Welsch and Yavitt 2003). For example, in *Erianthus giganteus*, a reed-like grass, fungal biomass increased gradually during shoot senescence and early decomposition to reach 7 and 4% of total detrital mass in leaves and culms respectively (Kuehn et al. 1999). However, fungal biomass varied spatially along the culm axis of this plant, resulting in a maximum concentration in the lower portion of only about 1.5%.

The observed accrual of substantial fungal biomass in shoots of wetland plants is consistent with high rates of biomass production that fungi can exhibit in this type of habitat. Newell (2001b) reported production rates of  $70\text{--}329 \mu\text{g g}^{-1}$  organic mass  $\text{h}^{-1}$  on decomposing leaf blades of *S. alterniflora* over a 3-year period, corresponding to growth rates of  $\sim 0.1\text{--}0.3\% \text{ h}^{-1}$ . Higher rates were generally observed in winter and spring and lower rates during the summer, consistent with patterns of fungal biomass accrual. Similar dynamics were observed for fungi associated with *J. roemerianus* (Newell 2001c), where rates ranged from  $66\text{--}366 \mu\text{g g}^{-1} \text{ h}^{-1}$  and were lower in summer than in spring and autumn. High rates of fungal production are not restricted to salt marshes in subtropical climate and, as with fungal biomass accrual, no significant latitudinal difference in production rates was observed in either *Spartina* or *Juncus* (Newell et al. 2000), suggesting that appreciable fungal production is a common feature of standing-dead plant shoots in salt marshes. On *S. alterniflora*, fungal production rates were negatively correlated with the C:N but not the C:P ratio of leaves (Newell 2001b), indicating that nitrogen may be the critical nutrient controlling fungal growth in this system (e.g. Newell et al. 1996a). In *J. roemerianus*, however, no significant relationship was observed between rates of fungal production and C:N or C:P ratios of the plant material.

Fungal production rates in freshwater wetlands can also be comparable to those measured in salt marshes (Newell et al. 1995; Kuehn et al. 2000; Findlay et al. 2002a; Su et al. 2007). For example, fungal production rates on leaves of standing-dead *T. angustifolia* ranged from 12 to  $359 \mu\text{g g}^{-1} \text{ h}^{-1}$  (Fig. 17.3), corresponding to growth rates of  $\sim 0.02\text{--}0.2\% \text{ h}^{-1}$  (K.A. Kuehn et al., unpublished

data), very similar to the ranges reported by Newell (2001b, c). Fungi associated with *Typha* stems showed similar seasonal patterns in biomass production but rates were only 11–108 µg g<sup>-1</sup> h<sup>-1</sup> (corresponding growth rates ~0.05–0.4% h<sup>-1</sup>), consistent with a much lower fungal biomass in stems (Fig. 17.3). Similar fungal production rates were observed for *Typha* and *Phragmites* stems (between 2 and 70 µg g<sup>-1</sup> h<sup>-1</sup>) in a tidal freshwater marsh (Findlay et al. 2002a). These data indicate that fungal production rates on standing-dead shoots of emergent wetland plants may vary significantly, depending on both environmental conditions and the intrinsic quality of plant litter.

Detachment of leaf blades from their parent plant, or collapse of standing-dead shoots to the sediments or overlying surface waters often lead to distinct changes in the biomass and productivity of the fungi associated with this plant material. In a study with *J. effusus*, fungal biomass and production rates declined rapidly following submergence of plant material colonized during the standing-dead phase (Kuehn et al. 2000). Similar declines of fungal biomass have been observed for fungi on the salt-marsh grass *S. alterniflora* (Newell et al. 1989), and the emergent freshwater plant *P. australis* in both lakes (Tanaka 1991; Komíková et al. 2000) and a tidal freshwater marsh (Van Ryclegem et al. 2007). These concordant patterns suggest that fungi

colonizing standing-dead shoots of wetland plants are poorly adapted to the abrupt changes in environmental conditions associated with the transition of plant material from an aerial, standing-dead to an aquatic or semi-aquatic decay phase. However, despite the strong decline in fungal biomass following collapse or detachment of the plant material, fungi continued to account for a major portion of total microbial biomass (Newell et al. 1989; Sinsabaugh and Findlay 1995; Komíková et al. 2000; Kuehn et al. 2000; Su et al. 2007).

#### D. Enzymatic Capabilities

Fungi associated with emergent wetland plants can produce a variety of extracellular enzymes that are involved in the degradation of plant cell walls (Gessner 1980; Torzilli 1982; Pointing and Hyde 2000). Much of the current knowledge comes from laboratory-based studies of fungi isolated from decomposing shoots of the salt-marsh grass *S. alterniflora*. These isolates have typically been found to produce enzymes that degrade cellulose and hemicelluloses, including those containing xylose and arabinose (Gessner 1980; Torzilli 1982). Mixed results have been obtained concerning the ability of isolates to degrade pectin. Gessner (1980) found that only five of 20 isolates tested produced polygalacturonases. Torzilli (1982) detected pectinolytic activity when assays were carried out at pH 8 (pectin lyase) for all three species tested, but only one species produced pectinolytic activity when assayed at pH 5 (polygalacturonase). When four fungal species were provided with isolated cell walls from *S. alterniflora*, all of them grew, and filtrates from these cultures caused release of reducing sugars, indicating that these fungi were able to degrade polysaccharides embedded in native cell walls (Torzilli 1982). Three species grown in culture also caused losses of both the cellulose and hemicellulose fractions but not the lignin fraction of *Spartina* tissue (Torzilli and Andrykovitch 1986).

Although less efficient than typical terrestrial white-rot fungi (Basidiomycota), there is evidence that fungal decomposers associated with emergent wetland plants are often capable of degrading lignin. When provided with *Spartina* lignocellulose in which the lignin had been specifically radiolabelled, the ascomycete *Phaeosphaeria spartinicola* caused a loss of 6% (3.3% mineralized and 2.7% solubilized) in the lignin fraction within 45 days (Bergbauer and Newell 1992). In the same

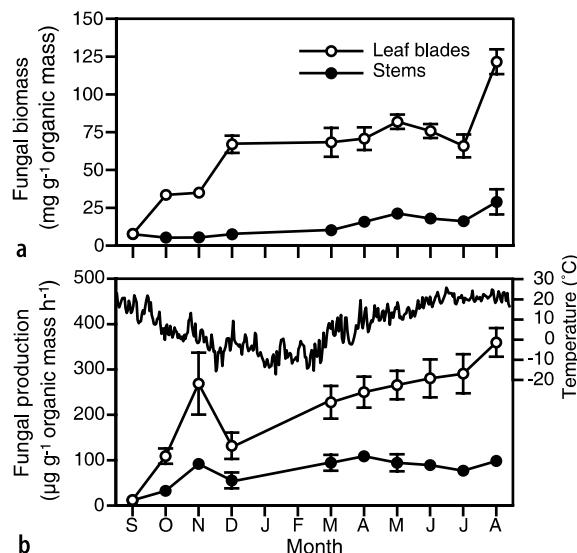


Fig. 17.3. Fungal biomass (a) and temperature trend and fungal production (b) associated with standing-dead shoots of *T. angustifolia* in a temperate littoral marsh (K.A. Kuehn et al., unpublished data). Symbols indicate means±1 SE ( $n=6$ )

time interval, *P. spartincola* caused 26% loss in total lignocellulose (22% mineralized and 4% solubilized). Further, transmission electron micrographs of decaying *Spartina* leaves collected in the field revealed symptoms of soft rot from each of four ascomycete species examined, demonstrating the fungal ability to degrade cell wall material in its native state (Newell et al. 1996b). In line with this enzymatic and microscopic evidence, a high diversity of DNA sequences encoding laccase, a key enzyme in lignin degradation, has been demonstrated within the natural fungal community on *S. alterniflora* (Lyons et al. 2003). Laccase sequences amplified directly from decaying leaf blades were dominated by sequences characteristic of *P. spartincola*, *Mycosphaerella* sp. and *P. halima*, which were previously identified as the principal fungal colonizers of standing *S. alterniflora* leaves (Newell 1993; Buchan et al. 2002, 2003). Thus, dominant fungi colonizing standing-dead *S. alterniflora* have the enzymes needed to degrade lignocellulosic tissues and can elaborate these enzymes when growing in their natural habitat. Fungi on other emergent wetland plants, both in salt and freshwater marshes, may have similar enzymatic capabilities, given the frequent occurrence of Ascomycota, including their anamorphs, on plant matter in these habitats.

#### E. Respiratory Activities on Standing-Dead Wetland Plants

If fungi are the dominant component of microbial assemblages associated with standing-dead wetland plants, as comparative estimates of fungal and bacterial biomass and productivity suggest (see below), then general microbial activities, such as CO<sub>2</sub> release resulting from microbial respiration, should largely be attributable to fungi (e.g. Kuehn et al. 2004). The rationale behind the arguments and conclusions in this section are based on this premise.

As in most terrestrial ecosystems, moisture availability is the single most important factor limiting microbial activity on and, thus, mineralization (CO<sub>2</sub> evolution) of dead emergent wetland plants (Gallagher et al. 1984; Newell et al. 1985; Kuehn and Suberkropp 1998b; Kuehn et al. 2004). Fungi are well adapted to this situation due to their ability to respond quickly (within 5 min or less) to wetting, permitting them to take advantage of even short favourable periods in an environment

characterized by strongly fluctuating environmental conditions (Newell et al. 1985; Kuehn and Suberkropp 1998b; Kuehn et al. 1998, 2004). During desiccation stress, these fungi survive by accumulating intracellular compatible solutes (polyols and trehalose; Kuehn et al. 1998). As a result of these adaptations, microbial assemblages associated with standing-dead wetland plants, particularly fungi, are capable of mineralizing a significant portion of the plant carbon before the collapse of leaves or shoots to the sediment (Kuehn et al. 2004).

Respiratory activities of microbial assemblages associated with standing-dead *S. alterniflora* and *J. roemerianus* shoots fluctuate rapidly after exposure to wetting or drying conditions (Newell et al. 1985). During periods of desiccation (i.e. water content <30%, < -6.0 MPa, see Newell et al. 1991), CO<sub>2</sub> is released at very low rates (<10 µg CO<sub>2</sub>-C g<sup>-1</sup> dry mass h<sup>-1</sup>). However, upon exposure to water (water content >50%, < -2.5 MPa), rates of CO<sub>2</sub> evolution increase greatly (to >100 µg CO<sub>2</sub>-C g<sup>-1</sup> h<sup>-1</sup>) and are maintained at high rates until exposure to drying conditions (Newell et al. 1985). Frequent wetting of standing *S. alterniflora* shoots had a negative effect on fungal growth and ascospore production of the dominant fungal species (*P. spartincola*) colonizing leaves. This counter-intuitive result suggests that fungi on *S. alterniflora* shoots are specifically adapted to fluctuating water availability, and are dependent upon the cyclic episodes of desiccation and wetting for optimal growth and reproduction (Newell et al. 1996a).

Similar respiratory patterns have been reported for microbial assemblages associated with standing-dead plant shoots in freshwater marshes (Kuehn and Suberkropp 1998b; Kuehn et al. 1998, 1999, 2004; Welsch and Yavitt 2003). For example, rates of CO<sub>2</sub> evolution from standing-dead *P. australis* exhibited a pronounced diel periodicity, with the highest rates occurring at night when cooling increased relative humidity (to the point of dew formation) and thus water potentials of the plant material (Fig. 17.4). By contrast, respiratory activities virtually ceased during the day as a result of desiccation. This indicates that diel fluctuations in water availability play a key role in controlling microbial metabolic activities during the standing-decay phase of emergent vascular plants (Kuehn et al. 2004). Results of this study in a temperate wetland were remarkably similar to earlier observations from *J. effusus* in a subtropical freshwater marsh (Kuehn and Suberkropp 1998b), suggesting

that pronounced diel shifts in microbial carbon mineralization of standing-dead wetland plants may be a geographically widespread phenomenon.

As with fungal biomass and production (Fig. 17.3), large differences in microbial respiration patterns have been observed among plant litter types (species and organ) in terms of microbial colonization and metabolic response to water availability. Respiration rates associated with different *P. australis* shoot fractions varied considerably (Kuehn et al. 2004). Maximum respiration rates from standing-dead leaf blades were 24–42% higher than those from leaf sheaths under the same environmental conditions, and maximum respiration rates from standing-dead culms were always an order of magnitude lower. These differences in respiration rates were consistent with differences in water absorption patterns, known structural characteristics (e.g. lignocellulose concentration), and degree of fungal colonization among shoot fractions (Kuehn et al. 2004). Maximum rates of microbial respiration were positively correlated ( $r = 0.72, p < 0.001$ ) with litter-associated fungal biomass. Similar correlations were found for rates of microbial CO<sub>2</sub> evolution from decaying standing *J. effusus* shoots ( $r = 0.65$ ; Kuehn and Suberkropp 1998b), and a variety of other plant species from salt and freshwater marshes ( $r = 0.77$ ,

Newell 2003). These consistent patterns suggest that fungi are likely to be responsible for most of the respiratory carbon release from standing-dead marsh plant shoots.

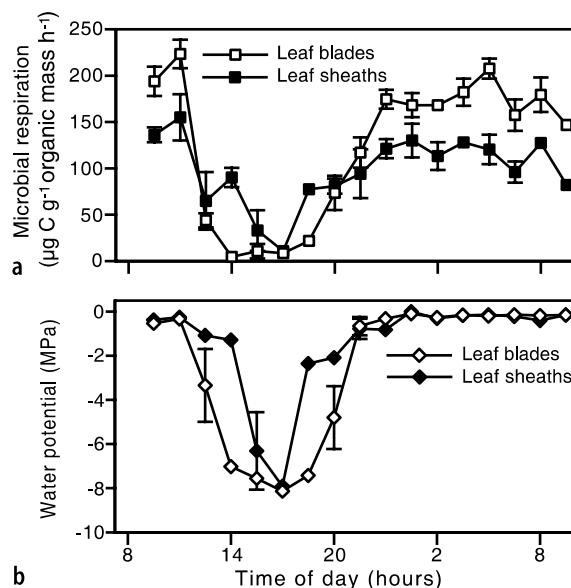
### III. Fungal Decomposers in Streams

#### A. Streams as Fungal Habitat

The most striking feature of stream and river ecosystems is the unidirectional flow of water. Streams and rivers have therefore long been considered mere transport systems – for water, solutes and particulate matter. Current concepts emphasize, however, that running waters are actively metabolizing ecosystems with strong longitudinal and vertical coupling and intimate terrestrial linkages (e.g. Fisher et al. 2004). In forest streams, much of the resources available to decomposers and consumers enter the wetted channel in the form of leaf litter and wood derived from riparian vegetation (Webster and Meyer 1997). Total litter inputs are typically on the order of 500 g dry mass m<sup>-2</sup> year<sup>-1</sup> but may exceed 2000 g m<sup>-2</sup> year<sup>-1</sup> (Webster and Meyer 1997; Abelho 2001). Decomposition of this organic matter is a key process in streams that is driven by both microorganisms and invertebrates (Webster and Benfield 1986; Maltby 1992; Suberkropp 1998b; Gessner and Van Ryckegem 2003). Fungi rapidly establish as key components of decomposer assemblages on submerged litter (Bärlocher and Kendrick 1974; Suberkropp and Klug 1976), thus mediating to a large extent not only litter decomposition (Gessner and Chauvet 1994) but also the transfer of energy and nutrients to other trophic levels in stream food webs (Bärlocher 1985; Suberkropp 1992a; Graça 2001). This section focuses on fungal decomposers of leaf litter in streams. Emphasis is placed on a particularly important and well-studied group commonly referred to as aquatic hyphomycetes but also known as Ingoldian fungi or amphibious hyphomycetes (Webster and Descals 1981; Bärlocher 1992).

#### B. Fungal Diversity on Decomposing Litter

The fungi associated with decomposing plant litter in streams have received more attention than any other fungi in aquatic ecosystems. Representatives of all major fungal phyla (Chytridiomycota,



**Fig. 17.4.** Diel changes in rates of CO<sub>2</sub> evolution from leaf blades and sheaths on standing-dead *P. australis* (a) and leaf blade and sheath water potential (b) in a temperate littoral marsh (data from Kuehn et al. 2004). Symbols indicate means  $\pm$  1 SE ( $n = 3$ ).

Zygomycota, Ascomycota and Basidiomycota), in addition to the fungus-like Oomycota (Kingdom Stramenopila), can be detected on submerged leaves or wood by means of both traditional and molecular techniques (e.g. Tsui and Hyde 2003; Nikolcheva and Bärlocher 2004; Shearer et al. 2007). Casual observations suggest that Chytridiomycota, Zygomycota and Oomycota probably play a minor role as decomposers of plant litter, while Ascomycota and their anamorphs, particularly hyphomycetes, assume the greatest importance (Bärlocher 1992). Using phyla-specific primers for the ITS region of rDNA, Nikolcheva and Bärlocher (2004) found that fungal communities of submerged leaves and wood were consistently dominated by Ascomycota in terms of species numbers and abundance, followed by Basidiomycota (on wood) and Chytridiomycota (in winter). With such a molecular approach, the Ascomycota also included most of the mitosporic fungi or anamorphs commonly detected by conventional methods, i.e. aquatic and terrestrial hyphomycetes, the majority of which appear to have ascomycetous affinities (Webster 1992). Although a diverse assemblage of Ascomycota (over 500 species) is associated with wood in freshwaters, sexual (teleomorphic) stages of Ascomycota are uncommon on decomposing leaves in streams (Shearer 1993; Cai et al. 2003). Terrestrial hyphomycetes are part of the phylloplane microbiota in plant canopies and thus colonize leaves before they enter streams (Bärlocher 1992). Their role in decomposition once leaves have fallen into streams is not certain, but their activity appears to be limited at the low winter temperatures that prevail in temperate regions after leaf fall (Bärlocher 1992; Maltby 1992). Dematiaceous and other hyphomycetes that do not produce intricately shaped conidia are less common on leaves in streams but are frequently isolated from submerged wood (Goh and Tsui 2003).

The most active fungal decomposers of leaf litter in streams are the aquatic hyphomycetes (Webster and Descals 1981; Bärlocher 1992; Suberkropp 1998b). The group includes at least 320 species (Descals 2005). Aquatic hyphomycetes are well adapted to the stream environment (Bärlocher 1992; Suberkropp 1992b; Gessner and Van Ryckegem 2003), and produce tetraradiate, sigmoid or variously branched conidia that have been interpreted as traits that facilitate attachment to the substrate in flowing water (Webster and Descals 1981; Webster 1987; Descals 2005; Dang et al. 2007). These fungi are able to quickly colonize ephemeral

resources, such as leaf litter in streams, to grow and rapidly produce spores, and thus to complete their life cycle within a few weeks. At the low water temperatures prevailing after leaf fall in temperate climates, they are also able to outcompete other fungi, mainly of terrestrial origin (Bärlocher 1992).

Colonization of leaf litter by aquatic hyphomycetes is initiated by the impacting and trapping of conidia on leaf surfaces after the leaves enter a stream. Subsequent germination is rapid, within 2–6 h in most species (Read et al. 1992). Once established, the fungal hyphae extend inside the leaf matrix (Fig. 17.2), so that significant quantities of mycelial mass are built up within a few weeks after leaf colonization (Fig. 17.5a, see below). A striking feature of aquatic hyphomycete life cycle is that mycelial growth is closely followed by the production of conidiophores, which may start to release conidia in as little as 6–10 days after leaves are submerged. This has been demonstrated both in microcosm experiments (Suberkropp 1991; Gulis and Suberkropp 2003b; Treton et al. 2004) and under field conditions where sporulation rates of natural communities often peaked earlier than fungal biomass (Fig. 17.5b; Suberkropp et al. 1993; Baldy et al. 1995; Gulis and Suberkropp 2003c; Ferreira et al. 2006a). Sporulation rates rapidly

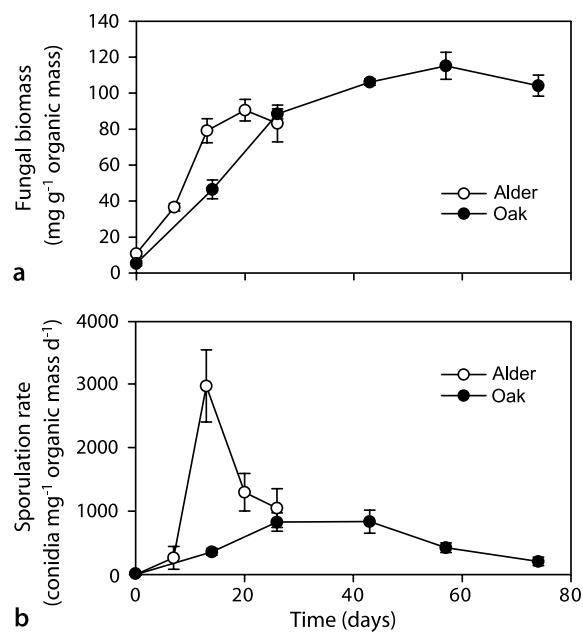


Fig. 17.5. Fungal biomass (a) and sporulation rates of aquatic hyphomycetes (b) associated with alder and oak leaves decomposing in a Portuguese woodland stream (data from Ferreira et al. 2006a). Symbols indicate means  $\pm 1$  SE ( $n = 4$  to 6).

increase to maxima and then decline (Fig. 17.5b). Maximum rates can reach seven conidia  $\mu\text{g}^{-1}$  litter dry mass day $^{-1}$ , although rates of at least an order of magnitude lower are frequently observed (Bärlocher 1982; Suberkropp et al. 1993; Gessner and Chauvet 1994; Suberkropp and Chauvet 1995; Bärlocher et al. 1995). The maximum rates of sporulation are controlled by both internal factors, such as litter quality (Gessner and Chauvet 1994; Ferreira et al. 2006b), and external factors such as temperature, alkalinity, pH and nutrient availability in stream water (e.g. Jenkins and Suberkropp 1995; Suberkropp and Chauvet 1995; Sridhar and Bärlocher 1997; Chauvet and Suberkropp 1998; Gulis and Suberkropp 2003c; see Sect. III.D. for nutrient effects).

Identification of many aquatic hyphomycetes is facilitated by characteristic conidial shapes. Diversity and community structure of these fungi is therefore often inferred from the relative abundances of released conidia that are captured either after incubation of leaf material in the laboratory, by filtering stream water or by examining foam collected from streams, in which conidia tend to be trapped (Suberkropp 1992b; Gessner et al. 2003; Bärlocher 2005). This approach is based on the assumption that sporulation rates correlate with the biomass of fungal species inside a leaf. Although this is not necessarily the case, much of the current knowledge on aquatic hyphomycete communities in streams has been derived from these types of studies. Individual leaves are colonized by typically 4–10 and up to 23 species (Shearer and Webster 1985; Suberkropp 1992b). On a stream scale, richness varies dramatically from just a few to >70 species, depending on collection effort and stream characteristics (Bärlocher 2005). A range of factors affect the composition of aquatic hyphomycete communities. These include latitude and altitude, season, water chemistry (pH, alkalinity, concentrations of inorganic nutrients, degree of pollution), composition of riparian vegetation, possibly interspecific competition, competition with and predation by invertebrates, and type of substrate. These factors are discussed in greater detail elsewhere (Bärlocher 1992, 2005; Suberkropp 1992b; Gessner and Van Ryckegem 2003).

Alternative approaches to studying aquatic hyphomycete communities make use of immunological or molecular techniques. Monoclonal antibodies raised against individual species and detected by enzyme-linked immunosorbent assay (ELISA) or immunofluorescence enable *in situ*

identification and, to some extent, quantification of mycelial biomass of individual species (Bermingham et al. 1996, 1997). Even though the technique is highly specific and gives different insight into species abundances than the traditional approach based on counting conidia, it has not been developed to a point where it is practical for ecological investigations. Antibodies need to be available for each species in a community but to date they have been developed for only four species. DNA-based approaches to analyse aquatic hyphomycete communities include development of fluorescently labelled oligonucleotide probes for *in situ* detection of fungal mycelia (FISH) (Baschien et al. 2001; McArthur et al. 2001) and PCR-based techniques. Denaturing gradient gel electrophoresis (DGGE) and T-RFLP analyses of amplified fungal DNA from submerged leaf litter indicate that dominant phylotypes belong to aquatic hyphomycetes. These analyses also suggest higher species richness during the initial stages of decomposition than have been detected with the traditional microscopic identification of conidia, and some decline in the number of phylotypes as decomposition progresses (Nikolcheva et al. 2003, 2005). These results corroborate previous conclusions that aquatic hyphomycetes replace phylloplane/terrestrial fungi during early stages of decomposition (Bärlocher and Kendrick 1974; Suberkropp and Klug 1976), and they also suggest that some germinated aquatic conidia are unable to establish long-lasting viable colonies (Nikolcheva et al. 2005), pointing to a possible role of interspecific competition in structuring fungal communities on decomposing leaves in streams.

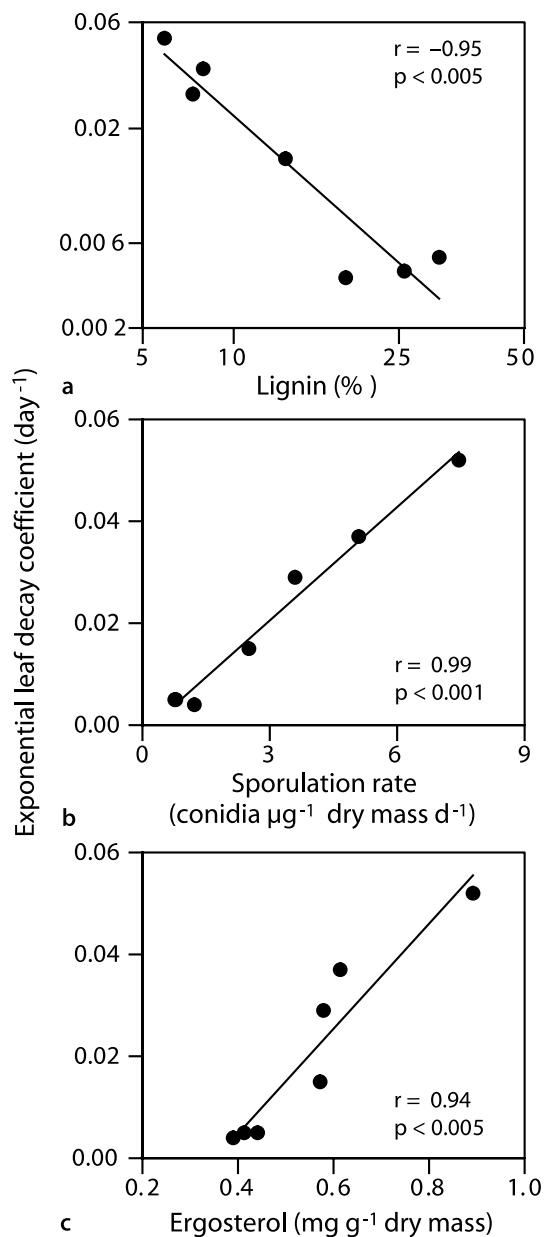
### C. Fungal Biomass and Production

Following submergence of leaves in streams, fungal biomass usually increases gradually during a few weeks to months and then levels off or decreases slightly (Fig. 17.5a). The rate of fungal biomass accrual and maximum values attained largely depend on plant litter quality and stream water chemistry, and can vary dramatically among systems (Table 17.1). From a stoichiometric perspective, the lower C:N or C:P ratio of fungal biomass in comparison to leaf litter, and especially wood, should result in better fungal growth on substrates high in N and P (Stelzer et al. 2003; Gulis et al. 2006b). Indeed, slower fungal biomass accrual on low-N oak than high-N alder leaves (Fig. 17.5a; Gessner

and Chauvet 1994; Nikolcheva et al. 2003; Gulis et al. 2006a), on wood than on leaves (Nikolcheva et al. 2003; Stelzer et al. 2003), and generally lower levels attained on wood (Table 17.1) support this idea. However, both oak leaves and wood have high lignin concentration, which could be more important in determining fungal activity and decomposition rates than C:N or C:P ratios. In line with this argument, initial lignin rather than N or P concentration of leaf litter was strongly correlated with litter decomposition rate in a comparative study across seven leaf species, as were maximum fungal biomass and sporulation rate of aquatic hyphomycetes (Fig. 17.6), suggesting that leaf litter decomposition was controlled through a kinetic carbon limitation of fungal growth (Gessner and Chauvet 1994).

Estimates of fungal growth rate and production (Suberkropp and Weyers 1996; Gessner and Chauvet 1997) give a better understanding of carbon flow from plant litter through fungal compartment than estimates of fungal biomass alone, since losses of fungal biomass as conidia or mycelial fragments, through respiration or as a result of detritivore feeding and hyphal death can be extensive. Growth rate (or ratio of daily production to biomass, P:B) and production of fungi colonizing leaves during decomposition in litter bags peak very early following leaf submergence in streams when fungal biomass is still relatively low, and gradually decrease as decomposition progresses (Suberkropp 1995; Weyers and Suberkropp 1996; Baldy and Gessner 1997; Suberkropp 2001; Baldy et al. 2002). Growth rates attain maxima of 0.01 to 0.42 day<sup>-1</sup> in decomposition experiments with litter placed in mesh bags, and vary from 0.01 to 0.17 day<sup>-1</sup> from randomly collected leaf litter where the stages of decomposition are unknown (Suberkropp 1997; Methvin and Suberkropp 2003; Carter and Suberkropp 2004). In decomposition experiments with litter bags, fungal production has been found to peak at 0.6–16 mg g<sup>-1</sup> organic litter mass day<sup>-1</sup> (Suberkropp 1995, 2001; Weyers and Suberkropp 1996; Baldy and Gessner 1997; Pascoal and Cássio 2004; Pascoal et al. 2005), very similar to the range (0.8–10 mg g<sup>-1</sup> day<sup>-1</sup>) observed on randomly collected leaves in streams (Suberkropp 1997; Methvin and Suberkropp 2003; Carter and Suberkropp 2004). While average fungal biomass in randomly collected leaves is relatively constant throughout the year, fungal growth rates and production on a leaf mass basis (mg g<sup>-1</sup> organic litter mass day<sup>-1</sup>) are seasonal and peak in summer,

probably in response to elevated temperature. Fungal biomass and daily production calculated on an areal basis (g m<sup>-2</sup> of stream bed) peak in autumn and winter following leaf fall in temperate regions and sharp increases in the amounts of litter in stream channels (Suberkropp 1997; Methvin and



**Fig. 17.6.** Relationships between decay coefficients of litter from seven deciduous leaf species and concentrations of refractory leaf constituents (a), maximum sporulation rates of aquatic hyphomycetes on leaves (b), and maximum fungal biomass in leaves expressed as ergosterol concentration (c) (data from Gessner and Chauvet 1994). Symbols indicate means  $\pm 1$  SE ( $n = 3$ ).

Suberkropp 2003; Carter and Suberkropp 2004). Similar data on fungi associated with submerged wood are scarce, but a recent estimate from randomly collected wood sticks suggests that both fungal growth rate and production ( $\text{mg g}^{-1}$  detrital mass  $\text{d}^{-1}$ ) are about an order of magnitude lower than those on leaves in the same streams, while biomass is only about twofold lower (Gulis et al., unpublished data).

Increases in fungal biomass associated with leaf litter often correlate with increases in nitrogen concentration, suggesting that fungi immobilize N from the stream water (Gulis et al. 2006b). Phosphorus concentration of leaf litter also often increases during decomposition, concomitant with fungal biomass accrual (e.g. Robinson and Gessner 2000; Gulis et al. 2006a; Stallcup et al. 2006). These increases in fungal biomass and elemental concentrations enhance the palatability and nutritional quality of litter as a food source for stream invertebrates (Bärlocher 1985; Suberkropp 1992a).

#### D. Responses of Fungal Decomposers to Dissolved Nutrients

A variety of factors influence fungal activity and decomposition of plant litter in aquatic ecosystems. The most important ones are plant litter quality (e.g. concentrations of nutrients and refractory and inhibitory plant constituents), biotic parameters (e.g. fungal community structure, presence of detritivores), and environmental variables (e.g. temperature, pH, oxygen availability, and dissolved nutrient concentrations) (Fig. 17.1). The critical importance of dissolved nutrients in regulating fungal activity and fungal-mediated decomposition in streams was recognized about a decade ago (Suberkropp and Chauvet 1995), following earlier discovery that elevated nutrient concentrations can stimulate litter decomposition (e.g. Elwood et al. 1981), and has received much attention in recent years (e.g. Robinson and Gessner 2000; Rosemond et al. 2002; Gulis and Suberkropp 2003c; Ferreira et al. 2006b). Stream fungi can obtain inorganic nutrients (e.g. N and P) from both the plant litter they grow in and stream water (Suberkropp and Jones 1991; Suberkropp 1995). Since plant litter is typically low in N and P (i.e. C:N and C:P are much higher than those of mycelium), fungi are often nutrient-limited in oligotrophic streams, and their activity is significantly higher in streams with high dissolved nutrient concentra-

tions (Suberkropp and Chauvet 1995) or following experimental nutrient addition (Grattan and Suberkropp 2001; Gulis and Suberkropp 2003c; Ferreira et al. 2006b). Strong positive correlations between dissolved nitrogen and/or phosphorus concentrations and fungal biomass, sporulation of aquatic hyphomycetes, respiration and/or exponential decay rates of leaves have been observed in various streams (e.g. Suberkropp and Chauvet 1995; Niyogi et al. 2003; Gulis et al. 2006a). In addition to this correlational evidence, microcosm studies have clearly shown stimulation of fungal activity and litter decomposition by dissolved N and/or P (Sridhar and Bärlocher 1997; Suberkropp 1998a; Gulis and Suberkropp 2003a, b). The most convincing results on the importance of dissolved nutrients, however, came from whole-stream nutrient enrichment experiments that demonstrated stimulation of microbial activity, and acceleration of leaf and wood decomposition in a variety of streams in different geographic settings (Gulis and Suberkropp 2003c; Stelzer et al. 2003; Gulis et al. 2004; Benstead et al. 2005; Ferreira et al. 2006b; Stallcup et al. 2006).

A few studies have not found evident effects of nutrient addition on either fungal activity or litter decomposition. This could happen when background levels of dissolved nutrients in streams are high and therefore not limiting to fungi (Royer and Minshall 2001; Simon and Benfield 2001), or when a non-limiting nutrient such as N is experimentally added to streams (Newbold et al. 1983) when another nutrient such as P is limiting (Elwood et al. 1981). Furthermore, fungal activity in nutrient-poor streams may be co-limited by N and P and, thus, the addition of either nutrient alone has no effect (Tank and Webster 1998; Grattan and Suberkropp 2001).

The shape of the dose-response curve between dissolved nutrient concentrations in water and fungal activity or litter decomposition rate depends on the range of concentrations examined. Studies in streams with low to moderate N and P concentrations strongly support a linear relationship (Suberkropp and Chauvet 1995; Niyogi et al. 2003). However, as the range of dissolved nutrient concentrations is increased to include high-nutrient streams, the relationship with fungal biomass, sporulation rate of aquatic hyphomycetes, microbial respiration, and leaf litter decomposition rather follows a Michaelis-Menten-type saturation model (Fig. 17.7; Rosemond et al. 2002; Ferreira et al. 2006b; Gulis et al. 2006a, b; Baldy et al. 2007).

Therefore, the linear responses observed in earlier experiments are likely to represent only the rising limb of the saturation model.

Nutrient stoichiometry of plant litter may modify the response of fungi to dissolved nutrients. A stimulating effect of exogenous nitrogen, for example, would be less pronounced when fungal demands can be met by nitrogen sources within decomposing plant material. Consistent with this idea, the effect of stream water nutrients appears to be greater on wood, which has very high C:N and C:P ratios, than on leaves (Stelzer et al. 2003; Gulis et al. 2004; Ferreira et al. 2006b). However, nutrient availability, whether external or internal, would be less critical when labile carbon is in limited supply, as may often be the case in leaf species with high concentrations of refractory carbon compounds such as lignin (Gessner and Chauvet 1994). Thus, the regulation of fungal activity and plant litter decomposition by dissolved nutrients may vary according to the relative impact and interactions of

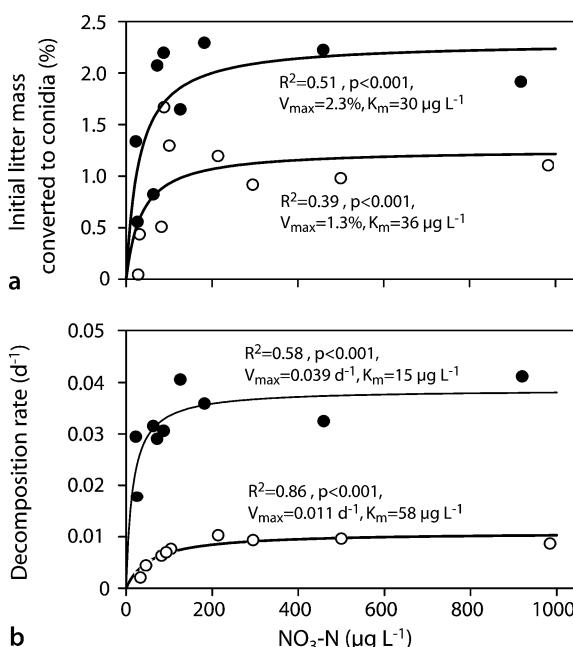
a range of controlling factors, both external ones related to the environment and factors intrinsic to the decomposing plant material.

### E. Enzymatic Capabilities

Aquatic hyphomycetes produce a variety of extracellular enzymes that degrade the structural polysaccharides of leaves (Chamier 1985; Suberkropp 1992b). Enzymes that hydrolyze cellulose (endoglucanases, exoglucanases and exoglucosidase) and hemicelluloses (xylanases, xylosidase and arabinosidase) are produced by a number of species in culture growing on pure substrates or leaf material. Aquatic hyphomycetes also typically produce several enzymes that degrade pectin (Suberkropp and Klug 1980; Chamier and Dixon 1982). Pectin degradation leads to the softening and maceration of plant tissue, resulting in the release of mesophyll cells (Chamier 1985; Suberkropp 1992b). Both polygalacturonase and pectin lyase depolymerize pectin, and both are produced by aquatic hyphomycetes, but the latter appears to play a greater role in leaf maceration (Suberkropp and Klug 1980; Jenkins and Suberkropp 1995). Since aquatic hyphomycetes also produce enzymes to degrade proteins and lipids (Zemek et al. 1985; Zare-Maivan and Shearer 1988; Abdullah and Taj-Aldeen 1989), it appears that most plant polymers can be metabolized by the majority of these fungi.

Lignin may be an important exception. Evidence from laboratory studies indicates that aquatic hyphomycetes can degrade lignin-like substrates (Fisher et al. 1983; Zemek et al. 1985; Zare-Maivan and Shearer 1988; Abdullah and Taj-Aldeen 1989), and some freshwater fungi have been reported to solubilize lignin in wood (Bucher et al. 2004). However, ligninolytic capabilities of aquatic hyphomycetes appear to be limited, and the general difficulty to assess lignin degradation still hinders our understanding of this process under natural circumstances (Chamier 1985).

The general picture that emerges is, aside from quantitative difference in activity (Suberkropp et al. 1983), an apparent lack of specialization among species in terms of enzymatic capabilities. This indicates that aquatic hyphomycetes are a rather homogenous and generalist group with respect to nutritional niche breadth (Suberkropp 1992b), even though some substrate preferences and quite distinct communities associated with



**Fig. 17.7.** Relationship between nitrate concentrations in stream water and percentage of initial litter mass converted into aquatic hyphomycete conidia (a) or litter decomposition rate (b). Data are fitted to a Michaelis-Menten saturation-type model:  $V=V_{max}[S]/(K_m+[S])$ , where  $V_{max}$  is the maximum parameter value,  $K_m$  is the nutrient concentration at which half of the maximum parameter value is achieved, and  $[S]$  is the nutrient concentration. Open symbols denote high-nutrient leaf litter (alder) and closed symbols indicate low-nutrient balsa wood veneers (data from Ferreira et al. 2006b)

leaves vs. wood have been reported (Gulis 2001; Bärlocher 2005; Ferreira et al. 2006b).

Little information is available on fungal enzyme activities associated with plant litter in streams, because fungi are not the only microbes colonizing this material. Nevertheless, since fungi typically dominate microbial biomass and production on leaf litter (see Sect. V.B.), they are likely to make a substantial contribution to the enzymatic activities associated with decomposing leaves in streams. For example, Golladay and Sinsabaugh (1991) found that exocellulase activity on maple leaves was closely correlated with fungal biomass, suggesting this hydrolytic activity was due to fungi. Similarly, the activity of four lignocellulose-degrading enzymes on wood showed generally positive relationships with fungal biomass (Tank et al. 1998). However, in another study, leaf-associated activities of three hydrolytic enzymes (xylanase, endocellulase and galacturonase) were lower in a hardwater than a softwater stream, whereas leaf softening and decay were faster, and fungal biomass accrual and sporulation of aquatic hyphomycetes were higher in the hardwater stream (Jenkins and Suberkropp 1995). It was therefore concluded that the hydrolytic enzymes examined were poor indicators of decomposition. Pectin lyase activity, by contrast, was higher in the hardwater stream, concomitant with faster leaf breakdown and greater fungal activity (Jenkins and Suberkropp 1995). These and similar results by Griffith et al. (1995) suggest that pectin degradation mediated by fungi is a key mechanism promoting leaf decomposition in streams (Suberkropp and Klug 1980; Chamier and Dixon 1982).

#### F. Significance of Fungal Diversity for Leaf Decomposition

In view of possible consequences of species extinction to ecosystem processes, the effects of fungal diversity on litter decomposition in streams have been examined in several studies. Results from field surveys suggest that species-poor fungal communities in streams affected by forestry practices or water pollution do not result in altered leaf decomposition rates (Raviraja et al. 1998; Bärlocher and Graça 2002). Similarly, varying species richness of aquatic hyphomycetes in microcosms had no effect on average leaf decomposition rates in mixed communities with up to eight species (Dang et al.

2005; Duarte et al. 2006). This points to a high degree of functional redundancy among aquatic hyphomycetes.

However, mixed cultures of two early colonizers enhanced decomposition by 73% compared to values expected from decomposition rates of single-species cultures (Treton et al. 2004). This outcome, in contrast to results from multispecies experiments (Dang et al. 2005; Duarte et al. 2006), is strong evidence of niche complementarity resulting in faster litter decomposition. In a similar vein, Bärlocher and Corkum (2003) reported a tendency towards faster decomposition with increasing fungal richness (1–5 species), although mixed communities never caused greater mass loss than the most effective species alone. Raviraja et al. (2006) also found that both species richness and identities affected leaf mass loss in microcosms, although again the most effective fungal species degraded leaves faster than did species mixtures.

There is also evidence that richness of aquatic hyphomycete communities can indirectly enhance decomposition through a positive effect on resource quality for invertebrate detritivores (Lecerf et al. 2005). Further, ecosystem processes other than litter decomposition (e.g. fungal biomass production) may be enhanced by diverse communities (Duarte et al. 2006). Lastly, even when average rates of decomposition are independent of species richness, variability of rates has been found to strongly decline with increasing fungal richness, as predicted from theoretical models (Dang et al. 2005). All else being equal, this should lead to higher predictability of litter decomposition rates when aquatic hyphomycete communities in streams are diverse.

### IV. Importance of Fungal Decomposers in Aquatic Ecosystems

#### A. Fungal Biomass and Production at the Ecosystem Scale

When periodic estimates of fungal biomass or production per gram of litter are accompanied by data on the amount of plant litter present per m<sup>2</sup> of habitat, then fungal importance can be estimated at the ecosystem scale. Such estimates of fungal production in streams range from 16 to 193 g m<sup>-2</sup> year<sup>-1</sup>, and are generally comparable with estimates of bacterial and macro invertebrate

production (Suberkropp 1997; Methvin and Suberkropp 2003; Carter and Suberkropp 2004; Gulis et al. 2006b). Fungal production on an areal basis correlates well with the mean annual amount of leaf litter in streams. Amounts of benthic litter, in turn, are a function of litter input, downstream transport, and decomposition by microbes and invertebrates. Small woodland streams receive high litter input per  $\text{m}^2$  of stream bed because they are intimately linked to their riparian zones, and also often retain litter effectively during high flows, because they are shallow and tend to have rough stream bottoms and other retention structures. Accordingly, annual fungal production per  $\text{m}^2$  in these streams is particularly high (Gulis et al. 2006b).

Consistent with generally lower fungal activity on submerged wood than on leaves, fungal production on wood (randomly collected naturally occurring sticks, 5–40 mm in diameter) in two headwater streams was estimated at  $9\text{--}11 \text{ g m}^{-2} \text{ year}^{-1}$  (Gulis et al., unpublished data). Depending on stick size and stream water nutrient concentration, this translates into 2–13% of wood carbon assimilated by fungi per year, which is considerably lower than the estimated amounts of leaf carbon assimilated by fungi. However, taking into account the longer residence times of wood compared to leaves, the importance of wood-colonizing fungi in many streams is likely to be significant as well.

Fungal production associated with standing-dead plants in marshes is also sizeable and further points to the quantitative significance of fungi at the ecosystem scale. For example, in a subtropical coastal salt marsh, fungal biomass on standing-dead shoots of *S. alterniflora* ranged from 9 (summer–autumn) to  $37 \text{ g C m}^{-2}$  of marsh area (winter–spring) (assuming 43% C in fungal dry mass). Estimated annual fungal production totalled  $230 \text{ g C m}^{-2} \text{ year}^{-1}$ , equivalent to roughly 40% of the annual plant production (Newell 2001b). This estimate is based on the assumption that fungal communities of standing-dead *S. alterniflora* shoots are metabolically active (i.e. released from water stress) for 12 h per day (see Sect. II.E. above). Even if this were an overestimate, it indicates that conversion of plant biomass to fungal biomass can be substantial.

Substantial fungal production has also been observed in freshwater wetlands. Annual fungal biomass and production associated with leafblades and stems of standing-dead *T. angustifolia* shoots in a north-temperate lake littoral marsh was 70 and

$45 \text{ g C m}^{-2} \text{ year}^{-1}$  respectively (K.A. Kuehn et al., unpublished data). This production estimate takes into account the diel periodicity in water availability (i.e. dew formation) that regulates microbial activities (see Sect. II.E. above). Substantial additional fungal production can occur on submerged litter in freshwater marshes. An annual production of nearly  $100 \text{ g C m}^{-2}$  has been estimated in the submerged litter layer of another littoral marsh dominated by *P. australis* in a temperate lake (Buesing and Gessner 2006). Thus, all systems studied so far (i.e. submerged leaf litter in streams, and both submerged litter and standing-dead shoots in salt and freshwater marshes) have revealed very high potential for fungal production, suggesting a great importance of fungi in food webs and organic matter turnover at the ecosystem scale.

## B. Fungal vs. Bacterial Biomass and Production

Studies in diverse streams (Sanzone et al. 2001; Findlay et al. 2002b) and salt and freshwater marshes (Sinsabaugh and Findlay 1995; Newell and Porter 2000) suggest that fungal biomass exceeds bacterial biomass on coarse submerged organic particles such as leaves, wood and other plant litter, whereas bacteria assume greater importance on finer organic particles and possibly on decaying floating-leaved macrophytes (Mille-Lindblom et al. 2006). In streams, fungi typically account for 88–99.9% of the microbial biomass (i.e. the combined fungal and bacterial biomass) developing on decomposing leaves (e.g. Findlay and Arsuffi 1989; Baldy et al. 1995; Weyers and Suberkropp 1996; Baldy and Gessner 1997; Hieber and Gessner 2002; Gulis and Suberkropp 2003a). Given these ratios of fungal and bacterial biomass, and the experimentally demonstrated preference of stream detritivores for fungal-colonized leaf patches (Arsuffi and Suberkropp 1985; Suberkropp 1992a), fungi appear to play a much greater role than bacteria in altering the palatability and food quality of decaying leaf litter in streams, and provide a much larger fraction to the nutrition of invertebrate detritivores (Suberkropp 1992a). Fungi appear to dominate microbial communities also on submerged wood in streams (67–97% in terms of biomass; Findlay et al. 2002b; Stelzer et al. 2003) but information is still very limited at present.

Fungal dominance of microbial biomass (typically >90%) associated with standing-dead plant

shoots and submerged litter in freshwater marshes (Sinsabaugh and Findlay 1995; Newell et al. 1995; Komíková et al. 2000; Kuehn et al. 2000; Findlay et al. 2002a; Su et al. 2007) and salt marshes (Newell 1992, 1993; Newell and Porter 2000) is well established. For example, microbial biomass associated with naturally standing-dead shoots of the freshwater sedge, *Carex walteriana*, was dominated by fungi, with bacterial biomass often less than 0.5% that of fungi (Newell et al. 1995). Bacterial biomass increased significantly once standing-dead plant material fragmented and fell to the sediment surface. However, despite the change in decay conditions, fungal biomass still accounted for 97% of the total microbial biomass (Newell et al. 1995).

Bacteria may have higher growth rates and shorter turnover times than fungi, suggesting that comparisons between both groups are more meaningful on the basis of production than biomass. However, outcomes of both types of comparisons have generally been similar. In particular, fungal production greatly exceeded bacterial production ( $1\text{--}627\times$ ) associated with leaves in streams in all studies when both microbial groups were followed simultaneously (Suberkropp and Weyers 1996; Weyers and Suberkropp 1996; Baldy et al. 2002; Pascoal and Cássio 2004; Pascoal et al. 2005). This consistent finding further emphasizes the key importance of fungi colonizing leaf litter in stream ecosystems. One exception from the general pattern is an experiment with fresh green leaves collected in summer where fungal and bacterial production estimates were comparable (Baldy and Gessner 1997).

Similar findings have been reported for fungi colonizing standing-dead shoots and submerged litter in salt and freshwater marshes, where fungal production accounted for >93% of the total microbial production (Newell et al. 1995; Newell and Porter 2000; Kuehn et al. 2000; Findlay et al. 2002a; Su et al. 2007). By contrast, bacterial production outweighed fungal production (>8:1) on submerged *P. australis* litter in a littoral marsh of a lake (Buesing and Gessner 2006). The inverse relationship between fungi and bacteria in this marsh was due to a particularly high bacterial production (average of  $660\text{ g C m}^{-2}\text{ year}^{-1}$ ), rather than a low fungal production ( $93\text{ g C m}^{-2}\text{ year}^{-1}$ ), and it is possible that this very high bacterial production was an overestimate caused by the high concentration of leucine used to determine protein synthesis rates as a measure of bacterial production (Gillies et al. 2006).

### C. Decomposition Budgets

Estimates of the different fates of decomposing plant material in addition to conversion into fungal and bacterial biomass have been made in several aquatic ecosystems. However, most budgets considering these fates are partial and have been calculated for a particular period, usually advanced decomposition stages. Consequently, they do not reflect the dynamic changes that characterize the entire decomposition sequence. Since much of the fungal biomass produced during litter decomposition is transient and eventually lost as  $\text{CO}_2$  or in other forms (Gessner et al. 1999; see below), fungi often appear more important when budgets are calculated at the time of maximum fungal biomass, rather than at final decomposition stages when the remaining mycelial biomass is relatively low (e.g. in streams, 0.5–3.9% of the initial organic litter mass; Gessner et al. 1997). Aquatic hyphomycetes on leaves in streams channel a substantial proportion of their production (1.0–7.3% of initial organic litter mass) into the formation of conidia (Findlay and Arsuffi 1989; Suberkropp 1991; Baldy et al. 1995; Hieber and Gessner 2002; Ferreira et al. 2006b), and two species grown on leaves in microcosms even allocated 46 and 81% of their production to conidia, equivalent to 7 and 12% of leaf mass loss respectively (Suberkropp 1991).

Estimates of fungal reproductive output are also available for fungi growing on standing-dead *Spartina* shoots in a salt marsh. Like aquatic hyphomycetes in streams, these salt-marsh fungi allocate substantial amounts of fungal biomass to reproductive structures (ascocarps of *Phaeosphaeria spartinicola* and *Mycosphaerella* sp.). During periods of leaf wetness, an average of 59 ascospores per hour were found to be released per  $\text{cm}^2$  of the upper two thirds of decaying leaf blades attached to standing-dead shoots. This value was conservatively estimated to represent 7.5 g fungal biomass per  $\text{m}^2$  of salt marsh per year, and nearly 5% of the total mycelial production in these leaves (Newell 2001a). Since fungal spores typically contain high concentrations of nutrients (Dowding 1976), spore release is likely to be a more significant pathway of N and P loss from decomposing leaves than of carbon loss.

$\text{CO}_2$  fluxes from standing-dead plant shoots as a result of microbial (presumably, mostly fungal) respiration can represent an important pathway of carbon flow in wetlands (Kuehn and Suberkropp 1998b; Kuehn et al. 2004). Taking

into account diel fluctuations in respiration rates (see Sect. II.E.) and estimates of litter standing crops, daily fluxes from standing-dead *J. effusus* were estimated at 1.4–3.6 g C m<sup>-2</sup> (Kuehn and Suberkropp 1998a), which generally exceeded CO<sub>2</sub> fluxes from sediments in the same wetland (0.12–2.4 g C m<sup>-2</sup> day<sup>-1</sup>; see Roden and Wetzel 1996). CO<sub>2</sub> fluxes from standing-dead *P. australis* shoots were lower (0.05–0.57 g C m<sup>-2</sup> day<sup>-1</sup>) but still within the range of those from wetland sediments in north-temperate climates (Kuehn et al. 2004). As a result, fungi and, to a smaller extent, other microorganisms could mineralize a significant portion of *J. effusus* leaf (~28%), and *Phragmites* leaf (~8%) and sheath (~29%) annual production under standing-dead conditions (Gulis et al. 2006b). CO<sub>2</sub> fluxes from decomposing leaf litter in streams are also sizeable, and were estimated in decomposition experiments to range from 17 to 56% of total leaf carbon losses (Elwood et al. 1981; Findlay and Arsuffi 1989; Baldy and Gessner 1997; Gulis and Suberkropp 2003c).

Estimates of the fraction of litter assimilated by fungi can be calculated as the sum of fungal production and respiration, or from fungal production when fungal growth efficiency is known. For stream fungi, growth efficiency ranges from 24 to 60% (Suberkropp 1991; Gulis and Suberkropp 2003b). This translates into a fungal assimilation of 5 to 97% of the annual leaf input (Gulis et al. 2006b). Estimates for freshwater marshes suggest that at least ~10% of the annual aboveground *Typha* production (K.A. Kuehn et al., unpublished data) and 15% of aboveground *Phragmites* production (Buesing and Gessner 2006) go into the production of fungal biomass. Both estimates consider only part of the fungal production per m<sup>2</sup> of marsh, because either the standing-dead or submerged decomposition phase was ignored. Total fungal production therefore is likely to be much higher in both cases.

One of the fates of leaves degraded by fungi in streams is the conversion to dissolved and fine particulate organic matter (DOM and FPOM; Suberkropp and Klug 1980; Gessner et al. 1999; Baldy et al. 2007). The ratio of released DOM to FPOM is variable but typically greater than one (Gessner et al. 1997), and the amounts of the two released fractions combined (FPOM+DOM; 36% of leaf mass loss in Findlay and Arsuffi 1989, and 8% in Baldy and Gessner 1997) may be on the order of the fraction released as CO<sub>2</sub> (40 and 17% respectively). Greater release of DOM compared to FPOM (barely detectable) has been reported from

*Phragmites* leaves, with DOM representing some 39% of the initial leaf mass (Komíková et al. 2000). FPOM and DOM can also be generated by feeding, and defecation or excretion by leaf-shredding macroinvertebrates (Wallace and Webster 1996). However, even where detritivore-mediated leaf conversion to other forms of organic matter and CO<sub>2</sub> is high (e.g. >50% of total leaf mass loss vs. 14–18% for fungi; Hieber and Gessner 2002), fungi may significantly contribute to litter conversion in an indirect way by stimulating litter consumption by detritivores (Suberkropp 1992b).

## V. Conclusions

A diversity of aquatic habitats occurs at land–water interfaces where the productivity of plants is often high and large amounts of plant matter enter the detrital pool. Environmental conditions (e.g. temperature, salinity, nutrient availability) vary widely within and across these systems where different types of plant matter from both aquatic and terrestrial sources are decomposing. The diversity of fungi present and potentially active in these systems is high. However, given the paucity of data for many systems, the overall importance of fungi as decomposers across aquatic ecosystems remains difficult to assess. Identification of the fungi present, by either traditional or molecular methods, is a prerequisite but not sufficient to ascertain an important functional role of these organisms in ecosystems. However, quantitative data are becoming increasingly available to evaluate the significance of fungi as agents of decomposition and nutrient cycling, producers of biomass, and mediators of organic matter transfer in aquatic food webs.

In a few types of aquatic ecosystems, particularly the marshes and streams discussed in this chapter, the role of fungi as decomposers of organic matter and producers of biomass has been demonstrated to be substantial. Fungi are clearly the key decomposers of standing-dead emergent plants in freshwater wetlands and salt marshes, and of terrestrial leaf litter in streams. The dominant species in these ecosystems possess the enzymatic potential necessary to degrade the structural compounds of litter, although fungal lignin degradation in streams is not well documented. Fungal biomass associated with decomposing plant material can easily exceed 10% of total litter mass in these systems, and typically outweighs bacterial biomass.

Comparisons of fungi and bacteria on a production basis generally yield similar results. Fungal biomass production at an ecosystem scale varies among systems and sites but can approach and even surpass  $100 \text{ g C m}^{-2} \text{ year}^{-1}$ . Evidence from various sites suggests, furthermore, that fungal activity can be responsible for a large proportion of leaf mass loss during decomposition, leading to the mineralization of plant organic matter to  $\text{CO}_2$  as well as conversion into DOM and FPOM.

Fungal activity and, consequently, leaf decomposition rates are regulated both by internal (e.g. litter nutrient concentration and carbon quality) and external (e.g. temperature, dissolved nutrient concentrations) factors. As fungi grow in leaf litter, their production is partitioned between the mycelium and reproductive structures. A significant fraction of biomass is ultimately channelled into spores. Fungi growing in decomposing leaves can immobilize nutrients such as nitrogen and phosphorus, thereby increasing the palatability and nutritional value of plant litter to invertebrate consumers. Thus, fungal decomposers assume multiple key roles in the aquatic ecosystems presented in this chapter. In other aquatic ecosystems, fungi may be important as well. However, these have not received sufficient attention to make assessments with any confidence, especially in view of some data (e.g. from mangrove swamps, seagrass beds or floating-leaved macrophytes; Gessner et al. 1997; Mille-Lindblom et al. 2006) that suggest notable differences may exist among aquatic systems in the roles of fungal litter decomposers.

## References

- Abdullah SK, Taj-Aldeen SJ (1989) Extracellular enzymatic activity of aquatic and aero-aquatic conidial fungi. *Hydrobiologia* 174:217–223
- Abelho M (2001) From litterfall to breakdown in streams: a review. *The ScientificWorld* 1:656–680
- Apinis AE, Taligoola HK (1974) Biodegradation of *Phragmites communis* Trin. by fungi. In: Kilbertus G, Reisinger O, Concela Da Fonseca JA (eds) *Biodégradation et humification*. Pierron, Sarreguemines, pp 24–32
- Arsuffi TL, Suberkropp K (1985) Selective feeding by stream caddisfly (Trichoptera) detritivores on leaves with fungal-colonized patches. *Oikos* 45:50–58
- Asaeda T, Nam LH, Hietz P, Tanaka N, Karunaratne S (2002) Seasonal fluctuations in live and dead biomass of *Phragmites australis* as described by a growth and decomposition model: implications of duration of aerobic conditions for litter mineralization and sedimentation. *Aquat Bot* 73:223–239
- Baldy V, Gessner MO (1997) Towards a budget of leaf litter decomposition in a first-order woodland stream. *C R Acad Sci Paris Sér III* 320:747–758
- Baldy V, Gessner MO, Chauvet E (1995) Bacteria, fungi and the decomposition of leaf litter in a large river. *Oikos* 74:93–102
- Baldy V, Chauvet E, Charcosset JY, Gessner MO (2002) Microbial dynamics associated with leaves decomposing in the mainstem and a floodplain pond of a large river. *Aquat Microbial Ecol* 28:25–36
- Baldy V, Gobert V, Guerold F, Chauvet E, Lambrigot D, Charcosset JY (2007) Leaf litter breakdown budgets in streams of various trophic status: effects of dissolved inorganic nutrients on macroorganisms and invertebrates. *Freshwater Biol* 52:1322–1335
- Bärlocher F (1982) Conidium production from leaves and needles in four streams. *Can J Bot* 60:1487–1494
- Bärlocher F (1985) The role of fungi in the nutrition of stream invertebrates. *Bot J Linn Soc* 91:83–94
- Bärlocher F (ed) (1992) *The ecology of aquatic hyphomycetes*. Ecological Studies vol 94. Springer, Berlin Heidelberg New York
- Bärlocher F (1997) Pitfalls of traditional techniques when studying decomposition of vascular plant remains in aquatic habitats. *Limnetica* 13:1–11
- Bärlocher F (2005) Freshwater fungal communities. In: Dighton J, White JF, Oudemans P (eds) *The fungal community. Its organization and role in the ecosystem*, 3rd edn. CRC Press, Boca Raton, FL, pp 39–59
- Bärlocher F, Biddiscombe NR (1996) Geratology and decomposition of *Typha latifolia* and *Lythrum salicaria* in a freshwater marsh. *Arch Hydrobiol* 136:309–325
- Bärlocher F, Corkum M (2003) Nutrient enrichment overwhelms diversity effects in leaf decomposition by stream fungi. *Oikos* 101:247–252
- Bärlocher F, Graça MAS (2002) Exotic riparian vegetation lowers fungal diversity but not leaf decomposition in Portuguese streams. *Freshwater Biol* 47:1123–1135
- Bärlocher F, Kendrick B (1974) Dynamics of the fungal populations on leaves in a stream. *J Ecol* 62:761–791
- Bärlocher F, Canhoto C, Graça MAS (1995) Fungal colonization of alder and eucalypt leaves in two streams in Central Portugal. *Arch Hydrobiol* 133:457–470
- Baschien C, Manz W, Neu TR, Szewzyk U (2001) Fluorescence in situ hybridization of freshwater fungi. *Int Rev Hydrobiol* 86:371–381
- Benstead JP, Deegan LA, Peterson BJ, Huryn AD, Bowden WB, Suberkropp K, Buzby KM, Green AC, Vacca JA (2005) Responses of a beaded Arctic stream to short-term N and P fertilisation. *Freshwater Biol* 50:277–290
- Bergbauer M, Newell SY (1992) Contribution to lignocellulose degradation and DOC formation from a salt marsh macrophyte by the ascomycete *Phaeosphaeria spartinicola*. *FEMS Microbiol Ecol* 86:341–348
- Birmingham S, Maltby L, Dewey FM (1996) Monoclonal antibodies as tools to quantify mycelium of aquatic hyphomycetes. *New Phytol* 132:593–601
- Birmingham S, Maltby L, Dewey FM (1997) Use of immunoassays for the study of natural assemblages of aquatic hyphomycetes. *Microbial Ecol* 33:223–229
- Buchan A, Newell SY, Moreta JIL, Moran MA (2002) Analysis of internal transcribed spacer (ITS) regions of rRNA genes in fungal communities in a Southeastern US salt marsh. *Microbial Ecol* 43:329–340

- Buchan A, Newell SY, Butler M, Biers EJ, Hollibaugh JT, Moran MA (2003) Dynamics of bacterial and fungal communities on decaying salt marsh grass. *Appl Environ Microbiol* 69:6676–6687
- Bucher VVC, Pointing SB, Hyde KD, Reddy CA (2004) Production of wood decay enzymes, loss of mass, and lignin solubilization in wood by diverse tropical freshwater fungi. *Microbial Ecol* 48:331–337
- Buesing N, Gessner MO (2006) Benthic bacterial and fungal productivity and carbon turnover in a freshwater marsh. *Appl Environ Microbiol* 72:596–605
- Cai L, Zhang KQ, Hyde KD (2003) Freshwater ascomycetes. In: Tsui CKM, Hyde KD (eds) *Freshwater mycology*. Fungal Diversity Press, Hong Kong, pp 275–324
- Carter MD, Suberkropp K (2004) Respiration and annual fungal production associated with decomposing leaf litter in two streams. *Freshwater Biol* 49:1112–1122
- Chamier A-C (1985) Cell-wall-degrading enzymes of aquatic hyphomycetes: a review. *Bot J Linn Soc* 91:67–81
- Chamier A-C, Dixon PA (1982) Pectinases in leaf degradation by aquatic hyphomycetes: the enzymes and leaf maceration. *J Gen Microbiol* 128:2469–2483
- Chauvet E, Suberkropp K (1998) Temperature and sporulation of aquatic hyphomycetes. *Appl Environ Microbiol* 64:1522–1525
- Dai T, Wiegert RG (1996) Ramet population dynamics and net aerial primary productivity of *Spartina alterniflora*. *Ecology* 77:276–288
- Damare S, Raghukumar C, Raghukumar S (2006) Fungi in deep-sea sediments of the Central Indian Basin. *Deep-Sea Res Part I-Oceanogr Res Pap* 53:14–27
- Dang CK, Chauvet E, Gessner MO (2005) Magnitude and variability of process rates in fungal diversity-litter decomposition relationships. *Ecol Lett* 8:1129–1137
- Dang CK, Gessner MO, Chauvet E (2007) Influence of conidial traits and leaf structure on attachment success of aquatic hyphomycetes on leaf litter. *Mycologia* 99:24–32
- Descals E (2005) Diagnostic characters of propagules of Ingoldian fungi. *Mycol Res* 109:545–555
- Díez J, Elosegi A, Chauvet E, Pozo J (2002) Breakdown of wood in the Agüera stream. *Freshwater Biol* 47:2205–2215
- Dowding P (1976) Allocation of resources, nutrient uptake and release by decomposer organisms. In: Anderson JM, Macfadyen A (eds) *The role of terrestrial and aquatic organisms in decomposition processes*. Blackwell, Oxford, pp 169–183
- Duarte S, Pascoal C, Cássio F, Bärlocher F (2006) Aquatic hyphomycete diversity and identity affect leaf litter decomposition in microcosms. *Oecologia* 147:658–666
- Elwood JW, Newbold JD, Trimble AF, Stark RW (1981) The limiting role of phosphorus in a woodland stream ecosystem: effects of P enrichment on leaf decomposition and primary producers. *Ecology* 62:146–158
- Ferreira V, Elosegi A, Gulis V, Pozo J, Graça MAS (2006a) *Eucalyptus* plantations affect fungal communities associated with leaf litter decomposition in Iberian streams. *Arch Hydrobiol* 166:467–490
- Ferreira V, Gulis V, Graça MAS (2006b) Whole-stream nitrate addition affects litter decomposition and associated fungi but not invertebrates. *Oecologia* 149:718–729
- Findlay SEG, Arsuffi TL (1989) Microbial growth and detritus transformations during decomposition of leaf litter in a stream. *Freshwater Biol* 21:261–269
- Findlay SEG, Dye S, Kuehn KA (2002a) Microbial growth and nitrogen retention in litter of *Phragmites australis* compared to *Typha angustifolia*. *Wetlands* 22:616–625
- Findlay S, Tank J, Dye S, Valett HM, Mulholland PJ, McDowell WH, Johnson SL, Hamilton SK, Edmonds J, Bowd WB (2002b) A cross-system comparison of bacterial and fungal biomass in detritus pools of headwater streams. *Microbial Ecol* 43:55–66
- Fisher PJ, Davey RA, Webster J (1983) Degradation of lignin by aquatic and aero-aquatic hyphomycetes. *Trans Br Mycol Soc* 80:166–168
- Fisher SG, Sponseller RA, Heffernan JB (2004) Horizons in stream biogeochemistry: flowpaths to progress. *Ecology* 85:2369–2379
- Foster MS, Bills GF, Mueller GM (eds) (2004) *Biodiversity of fungi: inventory and monitoring methods*. Elsevier, Amsterdam
- Gallagher JL, Kirby HV, Skirvin KW (1984) Community respiration of decomposing plants in Oregon estuarine marshes. *Estuarine Coastal Shelf Sci* 18:421–431
- Gessner RV (1977) Seasonal occurrence and distribution of fungi associated with *Spartina alterniflora* from Rhode Island estuary. *Mycologia* 69:477–491
- Gessner RV (1980) Degradative enzyme production by salt-marsh fungi. *Bot Mar* 23:133–139
- Gessner MO (2001) Mass loss, fungal colonisation and nutrient dynamics of *Phragmites australis* leaves during senescence and early decay in a standing position. *Aquat Bot* 69:325–339
- Gessner MO, Chauvet E (1993) Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. *Appl Environ Microbiol* 59:502–507
- Gessner MO, Chauvet E (1994) Importance of stream microfungi in controlling breakdown rates of leaf litter. *Ecology* 75:1807–1817
- Gessner MO, Chauvet E (1997) Growth and production of aquatic hyphomycetes in decomposing leaf litter. *Limnol Oceanogr* 42:496–595
- Gessner MO, Newell SY (2002) Biomass, growth rate, and production of filamentous fungi in plant litter. In: Hurst CJ, Crawford RL, Knudsen G, McInerney M, Stettenbach LD (eds) *Manual of Environmental Microbiology*. American Society for Microbiology, Washington, DC, pp 390–408
- Gessner MO, Robinson CT (2003) Aquatic hyphomycetes in alpine streams. In: Ward JV, Uehlinger U (eds) *Ecology of a glacial flood plain*. Kluwer, Dordrecht, pp 123–137
- Gessner MO, Van Ryckegem G (2003) Water fungi as decomposers in freshwater ecosystems. In: Bitton G (ed) *Encyclopedia of Environmental Microbiology*. Wiley, New York (online edn DOI: 10.1002/0471263397.env314)
- Gessner MO, Chauvet E, Suberkropp K (1997) Decomposition of plant litter by fungi in marine and freshwater ecosystems. In: Wicklow DT, Söderström B (eds) *The Mycota: a Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research* vol V, 1st edn. Environmental and microbial relationships. Springer, Berlin Heidelberg New York, pp 303–322

- Gessner MO, Chauvet E, Dobson M (1999) A perspective on leaf litter breakdown in streams. *Oikos* 85:377–384
- Gessner MO, Bärlocher F, Chauvet E (2003) Qualitative and quantitative analyses of aquatic hyphomycetes in streams. In: Tsui CKM, Hyde KD (eds) *Freshwater mycology*. Fungal Diversity Press, Hong Kong, pp 127–157
- Gillies JE, Kuehn KA, Francoeur SN, Neely RK (2006) Application of the  $^3\text{H}$ -leucine incorporation technique for quantifying rates of bacterial secondary production associated with decaying wetland plant litter. *Appl Environ Microbiol* 72:5948–5956
- Goh TK, Tsui CKM (2003) Key to common dematiaceous hyphomycetes from freshwater. In: Tsui CKM, Hyde KD (eds) *Freshwater mycology*. Fungal Diversity Press, Hong Kong, pp 325–343
- Golladay SW, Sinsabaugh RL (1991) Biofilm development on leaf and wood surfaces in a boreal river. *Freshwater Biol* 25:437–450
- Graça MAS (2001) The role of invertebrates on leaf litter decomposition in streams: a review. *Int Rev Hydrobiol* 86:383–393
- Graça MAS, Bärlocher F, Gessner MO (eds) (2005) Methods to study litter decomposition: a practical guide. Springer, Dordrecht
- Grattan RM, Suberkropp K (2001) Effects of nutrient enrichment on yellow poplar leaf decomposition and fungal activity in streams. *J N Am Benthol Soc* 20:33–43
- Griffith MB, Perry SA, Perry WB (1995) Leaf litter processing and exoenzyme production on leaves in streams of different pH. *Oecologia* 102:460–466
- Gulis V (2001) Are there any substrate preferences in aquatic hyphomycetes? *Mycol Res* 105:1088–1093
- Gulis V, Suberkropp K (2003a) Effect of inorganic nutrients on relative contributions of fungi and bacteria to carbon flow from submerged decomposing leaf litter. *Microbial Ecol* 45:11–19
- Gulis V, Suberkropp K (2003b) Interactions between stream fungi and bacteria associated with decomposing leaf litter at different levels of nutrient availability. *Aquat Microbial Ecol* 30:149–157
- Gulis V, Suberkropp K (2003c) Leaf litter decomposition and microbial activity in nutrient-enriched and unaltered reaches of a headwater stream. *Freshwater Biol* 48:123–134
- Gulis V, Rosemond AD, Suberkropp K, Weyers HS, Benstead JP (2004) Effects of nutrient enrichment on the decomposition of wood and associated microbial activity in streams. *Freshwater Biol* 49:1437–1447
- Gulis V, Ferreira V, Graça MAS (2006a) Stimulation of leaf litter decomposition and associated fungi and invertebrates by moderate eutrophication: implications for stream assessment. *Freshwater Biol* 51: 1655–1669
- Gulis V, Kuehn KA, Suberkropp K (2006b) The role of fungi in carbon and nitrogen cycles in freshwater ecosystems. In: Gadd GM (ed) *Fungi in biogeochemical cycles*. Cambridge University Press, Oxford, pp 404–435
- Gunde-Cimerman N, Sonjak S, Zalar P (2003) Extremophilic fungi in arctic ice: a relationship between adaptation to low temperature and water activity. *Physics Chem Earth* 28:1273–1278
- Harley JL (1971) Fungi in ecosystems. *J Appl Ecol* 8:627–642
- Hicks RE, Newell SY (1984) The growth of bacteria and the fungus *Phaeosphaeria typharum* (Desm.) Holm (Eurocytota: Ascomycotina) in salt-marsh microcosms in the presence and absence of mercury. *J Exp Mar Biol Ecol* 78:143–155
- Hieber M, Gessner MO (2002) Contribution of stream detritivores, fungi, and bacteria to leaf breakdown based on biomass estimates. *Ecology* 83:1026–1038
- Iversen TM (1973) Decomposition of autumn-shed beech leaves in a spring brook and its significance for the fauna. *Arch Hydrobiol* 72:305–312
- Jenkins CC, Suberkropp K (1995) The influence of water chemistry on the enzymatic degradation of leaves in streams. *Freshwater Biol* 33:245–253
- Jones B, Renaud RW, Rosen MR (2000) Stromatolites forming in acidic hot-spring waters, North Island, New Zealand. *Palaios* 15:450–475
- Jumpponen A (2003) Soil fungal community assembly in a primary successional glacier forefront ecosystem as inferred from rDNA sequence analyses. *New Phytol* 158:569–578
- Klammer M, Bååth E (2004) Estimation of conversion factors for fungal biomass determination in compost using ergosterol and PLFA 18:2ω6,9. *Soil Biol Biochem* 36:57–65
- Komínková D, Kuehn KA, Büsing N, Steiner D, Gessner MO (2000) Microbial biomass, growth, and respiration associated with submerged litter of *Phragmites australis* decomposing in a littoral reed stand of a large lake. *Aquat Microbial Ecol* 22:271–282
- Kreeger DA, Newell RIE (2000) Trophic complexity between producers and invertebrate consumers in salt marshes. In: Weinstein P, Kreeger DA (eds) *Concepts and controversies in tidal marsh ecology*. Kluwer, Dordrecht, pp 187–220
- Kuehn KA, Suberkropp K (1998a) Decomposition of standing litter of the freshwater macrophyte *Juncus effusus*. *L. Freshwater Biol* 40:717–727
- Kuehn KA, Suberkropp K (1998b) Diel fluctuations in microbial activity associated with standing-dead litter of the freshwater emergent macrophyte *Juncus effusus*. *Aquat Microbial Ecol* 14:171–182
- Kuehn KA, Churchill PF, Suberkropp K (1998) Osmoregulatory strategies of fungal populations inhabiting standing dead litter of the emergent macrophyte *Juncus effusus*. *Appl Environ Microbiol* 64:607–612
- Kuehn KA, Gessner MO, Wetzel RG, Suberkropp K (1999) Standing litter decomposition of the emergent macrophyte *Erianthus giganteus*. *Microbial Ecol* 38:50–57
- Kuehn KA, Lemke MJ, Suberkropp K, Wetzel (2000) Microbial biomass and production associated with decaying leaf litter of the emergent macrophyte *Juncus effusus*. *Limnol Oceanogr* 45:862–870
- Kuehn KA, Steiner D, Gessner MO (2004) Diel mineralization patterns of standing-dead plant litter: implications for CO<sub>2</sub> flux from wetlands. *Ecology* 85:2504–2518
- Lecerf A, Dobson M, Dang CK, Chauvet E (2005) Riparian plant species loss alters trophic dynamics in detritus-based stream ecosystems. *Oecologia* 146:432–442
- Lyons JI, Newell SY, Buchan A, Moran MA (2003) Diversity of ascomycete laccase gene sequences in a southeastern US salt marsh. *Microbial Ecol* 45:270–281
- Lyons JI, Newell SY, Brown RP, Moran MA (2005) Screening for bacterial-fungal associations in a south-eastern US salt marsh using pre-established fungal monocultures. *FEMS Microbiol Ecol* 54:179–187

- Maltby L (1992) Heterotrophic microbes. In: Calow P, Petts GE (eds) *The River Handbook: Hydrological and Ecological Principles* vol 1. Blackwell, Oxford, pp 165–194
- McArthur FA, Bärlocher MO, MacLean NAB, Hiltz MD, Bärlocher F (2001) Asking probing questions: can fluorescent *in situ* hybridization identify and localise aquatic hyphomycetes on leaf litter? *Int Rev Hydrobiol* 86:429–438
- Methvin BR, Suberkropp K (2003) Annual production of leaf-decaying fungi in 2 streams. *J N Am Benthol Soc* 22:554–564
- Mille-Lindblom C, Fischer H, Tranvik LJ (2006) Litter-associated bacteria and fungi – a comparison of biomass and communities across lakes and plant species. *Freshwater Biol* 51:730–741
- Mitsch WJ, Gosselink JG (2000) *Wetlands*, 3rd edn. Wiley, New York
- Neubert K, Mendgen K, Brinkmann H, Wirsöl SGR (2006) Only a few fungal species dominate highly diverse mycofloras associated with the common reed. *Appl Environ Microbiol* 72:1118–1128
- Newbold JD, Elwood JW, Schulze MS, Stark RW, Barnecker JC (1983) Continuous ammonium enrichment of a woodland stream: uptake kinetics, leaf decomposition, and nitrification. *Freshwater Biol* 13:193–204
- Newell SY (1992) Estimating fungal biomass and productivity in decomposing litter. In: Carroll GC, Wicklow DT (eds) *The fungal community: its organization and role in the ecosystem*, 2nd edn. Marcel Dekker, New York, pp 521–561
- Newell SY (1993) Decomposition of shoots of a saltmarsh grass: methodology and dynamics of microbial assemblages. *Adv Microbial Ecol* 13:301–326
- Newell SY (1994) Total and free ergosterol in mycelia of saltmarsh ascomycetes with access to whole leaves or aqueous extracts of leaves. *Appl Environ Microbiol* 60:3479–3482
- Newell SY (2001a) Spore-expulsion rates and extents of blade occupation by ascomycetes of smooth-cordgrass standing-decay system. *Bot Mar* 44:277–285
- Newell SY (2001b) Multiyear patterns of fungal biomass dynamics and productivity within naturally decaying smooth cordgrass shoots. *Limnol Oceanogr* 46:573–583
- Newell SY (2001c) Fungal biomass and productivity in standing-decaying leaves of black needle rush (*Juncus roemerianus*). *Mar Freshwater Res* 52:249–255
- Newell SY (2003) Fungal content and activities in standing-decaying leaf blades of plants of the Georgia coastal ecosystems research area. *Aquat Microbial Ecol* 32:95–103
- Newell SY, Fallon RD (1991) Toward a method for measuring fungal instantaneous growth rates in field samples. *Ecology* 72:1547–1559
- Newell SY, Porter D (2000) Microbial secondary production from salt marsh-grass shoots, and its known potential fates. In: Weinstein MP, Kreger DA (eds) *Concepts and controversies in tidal marsh ecology*. Kluwer, Dordrecht, pp 159–186
- Newell SY, Wasowski J (1995) Sexual productivity and spring intramarsch distribution of a key saltmarsh microbial secondary producer. *Estuaries* 18:241–249
- Newell SY, Fallon RD, Cal Rodriguez RM, Groene LC (1985) Influence of rain, tidal wetting and relative humidity on release of carbon dioxide by standing-dead salt-marsh plants. *Oecologia* 68:73–79
- Newell SY, Fallon RD, Miller JD (1989) Decomposition and microbial dynamics for standing, naturally positioned leaves of the salt-marsh grass *Spartina alterniflora*. *Mar Biol* 101:471–481
- Newell SY, Arsuffi TL, Kemp PF, Scott LA (1991) Water potential of standing-dead shoots of an intertidal grass. *Oecologia* 85:321–326
- Newell SY, Moran MA, Wicks R, Hodson RE (1995) Productivities of microbial decomposers during early stages of decomposition of leaves of a freshwater sedge. *Freshwater Biol* 34:135–148
- Newell SY, Arsuffi TL, Palm LA (1996a) Misting and nitrogen fertilization of shoots of a saltmarsh grass: effects upon fungal decay of leaf blades. *Oecologia* 108:495–502
- Newell SY, Porter D, Lingle WL (1996b) Lignocellulolysis by ascomycetes (fungi) of a saltmarsh grass (smooth cordgrass). *Microsc Res Techn* 33:32–46
- Newell SY, Blum LK, Crawford RE, Dai T, Dionne M (2000) Autumnal biomass and potential productivity of salt marsh fungi from 29° to 43° north latitude along the United States Atlantic coast. *Appl Environ Microbiol* 66:180–185
- Nikolcheva LG, Bärlocher F (2004) Taxon-specific fungal primers reveal unexpectedly high diversity during leaf decomposition in a stream. *Mycol Progr* 3:41–49
- Nikolcheva LG, Cockshutt AM, Bärlocher F (2003) Determining diversity of freshwater fungi on decaying leaves: comparison of traditional and molecular approaches. *Appl Environ Microbiol* 69:2548–2554
- Nikolcheva LG, Bourque T, Bärlocher F (2005) Fungal diversity during initial stages of leaf decomposition in a stream. *Mycol Res* 109:246–253
- Niyogi DK, Simon KS, Townsend CR (2003) Breakdown of tussock grass in streams along a gradient of agricultural development in New Zealand. *Freshwater Biol* 48:1698–1708
- Oremland RS, Capone DG (1988) Use of “specific” inhibitors in biogeochemistry and microbial ecology. In: Marshall KC (ed) *Advances in Microbial Ecology* vol 10. Plenum Press, New York, pp 285–383
- Padgett DE (1993) Distinguishing bacterial from non-bacterial decomposition of *Spartina alterniflora* by respirometry. In: Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds) *Handbook of methods in aquatic microbial ecology*. Lewis, Boca Raton, FL, pp 465–469
- Pascoal C, Cássio F (2004) Contribution of fungi and bacteria to leaf litter decomposition in a polluted river. *Appl Environ Microbiol* 70:5266–5273
- Pascoal C, Cássio F, Marcotequi A, Sanz B, Gomes P (2005) Role of fungi, bacteria, and invertebrates in leaf litter breakdown in a polluted river. *J N Am Benthol Soc* 24:784–797
- Pointing SB, Hyde KD (2000) Lignocellulose-degrading marine fungi. *Biofouling* 15:221–229
- Poon MOK, Hyde KD (1998) Evidence for vertical distribution of saprophytic fungi on senescent *Phragmites australis* culms at Mai Po marshes. *Bot Mar* 41:285–292
- Pugh GJF, Mulder JL (1971) Mycoflora associated with *Typha latifolia*. *Trans Br Mycol Soc* 57:273–282

- Raviraja NS, Sridhar KR, Bärlocher F (1998) Breakdown of *Ficus* and *Eucalyptus* leaves in an organically polluted river in India: fungal diversity and ecological functions. *Freshwater Biol* 39:537–545
- Raviraja NS, Nikolcheva LG, Bärlocher F (2006) Fungal growth and leaf decomposition are affected by amount and type of inoculum and by external nutrients. *Sydowia* 58:91–104
- Read SJ, Moss ST, Jones EBG (1992) Attachment and germination of conidia. In: Bärlocher F (ed) *The ecology of aquatic hyphomycetes. Ecological Studies* vol 94. Springer, Berlin Heidelberg New York, pp 135–151
- Robinson CT, Gessner MO (2000) Nutrient addition accelerates leaf breakdown in an alpine springbrook. *Oecologia* 122:258–263
- Roden EE, Wetzel RG (1996) Organic carbon oxidation and suppression of methane production by microbial Fe(III) oxide reduction in vegetated and unvegetated freshwater wetland sediments. *Limnol Oceanogr* 41:1733–1748
- Rosemond AD, Pringle CM, Ramírez A, Paul MJ, Meyer JL (2002) Landscape variation in phosphorus concentration and effects on detritus-based tropical streams. *Limnol Oceanogr* 47:278–289
- Royer TV, Minshall GW (2001) Effects of nutrient enrichment and leaf quality on the breakdown of leaves in a headwater stream. *Freshwater Biol* 46:603–610
- Saccardo PA (1898) *Sylloge Fungorum Omnitum Hucusque cognitorum* 13, *Index Universalis. Fratres Borntraeger, Lipsiae*
- Samiaji J, Bärlocher F (1996) Geratology and decomposition of *Spartina alterniflora* Loisel in a New Brunswick saltmarsh. *J Exp Mar Biol Ecol* 201:233–252
- Sanzone DM, Tank JL, Meyer JL, Mulholland PJ, Findlay SEG (2001) Microbial incorporation of nitrogen in stream detritus. *Hydrobiologia* 464:27–35
- Shearer CA (1993) The freshwater Ascomycetes. *Nova Hedwigia* 56:1–33
- Shearer CA, Webster J (1985) Aquatic hyphomycete communities in the River Teign. III. Comparison of sampling techniques. *Trans Br Mycol Soc* 84:509–518
- Shearer CA, Descals E, Kohlmeyer J, Marvanová L, Padgett D, Porter D, Raja HA, Schmit JP, Thorton HA, Voglmeyer H (2007) Fungal biodiversity in aquatic habitats. *Biodivers Conserv* 16:49–67
- Silliman BR, Newell SY (2003) Fungal farming in a snail. *Proc Natl Acad Sci USA* 100:15643–15648
- Simon KS, Benfield EF (2001) Leaf and wood breakdown in cave streams. *J N Am Benthol Soc* 20:550–563
- Sinsabaugh RL, Findlay S (1995) Microbial production, enzyme activity, and carbon turnover in surface sediments of the Hudson River estuary. *Microbial Ecol* 30:127–141
- Spähnhoff B, Gessner MO (2004) Slow initial decomposition and fungal colonization of pine branches in a nutrient-rich lowland stream. *Can J Fish Aquat Sci* 61:2007–2013
- Sridhar KR, Bärlocher F (1997) Water chemistry and sporulation by aquatic hyphomycetes. *Mycol Res* 101:591–596
- Stallcup LA, Ardón M, Pringle CM (2006) Does nitrogen become limiting under high-P conditions in detritus-based tropical streams? *Freshwater Biol* 51:1515–1526
- Stelzer RS, Heffernan J, Likens GE (2003) The influence of dissolved nutrients and particulate organic matter quality on microbial respiration and biomass in a forest stream. *Freshwater Biol* 48:1925–1937
- Su R, Lohner RN, Kuehn KA, Sinsabaugh RL, Neely RK (2007) Microbial dynamics associated with decomposing *Typha angustifolia* litter in two contrasting Lake Erie coastal wetlands. *Aquat Microbial Ecol* 46:295–307
- Suberkropp K (1991) Relationships between growth and sporulation of aquatic hyphomycetes on decomposing leaf litter. *Mycol Res* 95:843–850
- Suberkropp K (1992a) Interactions with invertebrates. In: Bärlocher F (ed) *The ecology of aquatic hyphomycetes. Ecological Studies* vol 94. Springer, Berlin Heidelberg New York, pp 118–133
- Suberkropp K (1992b) Aquatic hyphomycete communities. In: Carroll GC, Wicklow DT (eds) *The fungal community: its organization and role in the ecosystem*, 2nd edn. Marcel Dekker, New York, pp 729–747
- Suberkropp K (1995) The influence of nutrients on fungal growth, productivity, and sporulation during leaf breakdown in streams. *Can J Bot* 73 suppl 1:S1361–S1369
- Suberkropp K (1997) Annual production of leaf-decaying fungi in a woodland stream. *Freshwater Biol* 38:169–178
- Suberkropp K (1998a) Effect of dissolved nutrients on two aquatic hyphomycetes growing on leaf litter. *Mycol Res* 102:998–1002
- Suberkropp K (1998b) Microorganisms and organic matter processing. In: RJ Naiman, Bilby RE (eds) *River ecology and management: lessons from the Pacific Coastal Ecoregion*. Springer, Berlin Heidelberg New York, pp 120–143
- Suberkropp K (2001) Fungal growth, production, and sporulation during leaf decomposition in two streams. *Appl Environ Microbiol* 67:5063–5068
- Suberkropp K, Chauvet E (1995) Regulation of leaf breakdown by fungi in streams: influences of water chemistry. *Ecology* 76:1433–1445
- Suberkropp K, Jones EO (1991) Organic phosphorus nutrition of some aquatic hyphomycetes. *Mycologia* 83:665–668
- Suberkropp K, Klug MJ (1976) Fungi and bacteria associated with leaves during processing in a woodland stream. *Ecology* 57:707–719
- Suberkropp K, Klug MJ (1980) The maceration of deciduous leaf litter by aquatic hyphomycetes. *Can J Bot* 58:1025–1031
- Suberkropp K, Weyers H (1996) Application of fungal and bacterial production methodologies to decomposing leaves in streams. *Appl Environ Microbiol* 62:1610–1615
- Suberkropp K, Arsuffi TL, Anderson JP (1983) Comparison of degradative ability, enzymatic activity, and palatability of aquatic hyphomycetes grown on leaf litter. *Appl Environ Microbiol* 46:237–244
- Suberkropp K, Gessner MO, Chauvet E (1993) Comparison of ATP and ergosterol as indicators of fungal biomass associated with decomposing leaves in streams. *Appl Environ Microbiol* 59:3367–3372
- Tanaka Y (1991) Microbial decomposition of reed (*Phragmites communis*) leaves in a saline lake. *Hydrobiologia* 220:119–129

- Tank JL, Webster JR (1998) Interaction of substrate and nutrient availability on wood biofilm processes in streams. *Ecology* 79:2168–2179
- Tank JL, Webster JR, Benfield EF, Sinsabaugh RL (1998) Effect of leaf litter exclusion on microbial enzyme activity associated with wood biofilms in streams. *J N Am Benthol Soc* 17:95–103
- Torzilli AP (1982) Polysaccharidase production and cell wall degradation by several salt marsh fungi. *Mycologia* 74:297–302
- Torzilli AP, Andrykovich G (1986) Degradation of *Spartina* lignocellulose by individual and mixed cultures of salt marsh fungi. *Can J Bot* 64:2211–2215
- Torzilli AP, Sikaroodi M, Chalkley D, Gillevet PM (2006) A comparison of fungal communities from four salt marsh plants using automated ribosomal intergenic spacer analysis (ARISA). *Mycologia* 95:690–698
- Treton C, Chauvet E, Charcosset JY (2004) Competitive interaction between two aquatic hyphomycete species and increase in leaf litter breakdown. *Microbial Ecol* 48:439–446
- Tsui CKM, Hyde KD (eds) (2003) Freshwater mycology. Fungal Diversity Press, Hong Kong
- Van Ryckegem G, Verbeken A (2005) Fungal diversity and community structure on *Phragmites australis* (Poaceae) along a salinity gradient in the Scheldt estuary (Belgium). *Nova Hedwigia* 80:173–197
- Van Ryckegem G, Gessner MO, Verbeken A (2007) Fungi on leaf blades of *Phragmites australis* in a brackish tidal marsh: diversity, succession and leaf decomposition. *Microbial Ecol* 53:600–611
- Wallace JB, Webster JR (1996) The role of macroinvertebrates in stream ecosystem function. *Annu Rev Entomol* 41:115–139
- Webster J (1987) Convergent evolution and the functional significance of spore shape in aquatic and semi-aquatic fungi. In: Rayner ADM, Brasier CM, Moore D (eds) *Evolutionary biology of the fungi*. Cambridge University Press, Cambridge, pp 191–200
- Webster J (1992) Anamorph-teleomorph relationships. In: Bärlocher F (ed) *The ecology of aquatic hyphomycetes*. Ecological Studies vol 94. Springer, Berlin Heidelberg New York, pp 99–117
- Webster JR, Benfield EF (1986) Vascular plant breakdown in freshwater ecosystems. *Annu Rev Ecol Syst* 17:567–594
- Webster J, Descals E (1981) Morphology, distribution, and ecology of conidial fungi in freshwater habitats. In: Cole GT, Kendrick B (eds) *Biology of Conidial Fungi* vol 1. Academic Press, New York, pp 295–355
- Webster JR, Meyer JL (eds) (1997) Stream organic matter budgets. *J N Am Benthol Soc* 16:3–161
- Welsch M, Yavitt JB (2003) Early stages of decay of *Lythrum salicaria* L. and *Typha latifolia* L. in a standing-dead position. *Aquat Bot* 75:45–57
- Wetzel RG, Howe MJ (1999) High production in a herbaceous perennial plant achieved by continuous growth and synchronized population dynamics. *Aquat Bot* 64:111–129
- Weyers HS, Suberkropp K (1996) Fungal and bacterial production during the breakdown of yellow poplar leaves in two streams. *J N Am Benthol Soc* 15:408–420
- Zare-Maivan H, Shearer CA (1988) Extracellular enzyme production and cell wall degradation by freshwater lignicolous fungi. *Mycologia* 80:365–375
- Zemek J, Marvanová L, Kuniak L, Kadlecíková B (1985) Hydrolytic enzymes in aquatic Hyphomycetes. *Folia Microbiol* 30:363–372

---

# 18 Degradation of Plant Cell Wall Polymers by Fungi

C. GAMAUF<sup>1</sup>, B. METZ<sup>1</sup>, B. SEIBOTH<sup>1</sup>

## CONTENTS

I. Introduction .....	325
II. Structure and Composition of Plant Cell Walls .....	326
III. The Degradation of the Plant Cell Wall .....	327
A. Biodegradation of Cellulose .....	327
B. Biodegradation of Hemicelluloses .....	329
1. Xylans .....	330
2. Xyloglucan .....	330
3. (Galacto-)glucomannan .....	331
C. Biodegradation of Pectins .....	331
D. Accessory Enzymes .....	334
IV. Biodegradation of Lignin .....	335
A. Brown-Rot Fungi .....	335
B. White-Rot Fungi .....	336
V. Conclusions .....	338
References .....	338

## I. Introduction

The main producers of primary biomass on earth are plants, through the photosynthetic fixation of carbon dioxide into organic matter. Fungi are heterotrophic organisms which depend on pre-formed organic compounds as energy and nutrient source for the biosynthesis of their own cellular components. They are today the most important and widespread group of organisms responsible for the recycling of plant material back into the ecosystem and are therefore essential components of the global carbon cycle. A large group of fungi is in particular specialized to degrade the complex cell wall produced by plants. One of the reasons for this seems to be the “side by side” evolution of plants and fungi. Symbiotic associations of fungi and photosynthetic plant hosts have most probably facilitated the colonization of land. The first fossil land plants and fungi appeared 480 to 460 million years ago. These fossil fungi are found closely associated with rhizomes, the primitive

root systems of the first vascular land plants. However, the time when colonization occurred remains speculative; molecular clock estimates suggest colonization of land about 600 million years ago whereas protein sequence analyses indicate that land plants appeared as early as 700 million years ago (Simon et al. 1993; Remy et al. 1994; Heckman et al. 2001). Today, symbiotic associations are still found in the arbuscular mycorrhizal fungi, which are partners of 70% or more of today's higher plants (Smith and Read 1997). During their further co-evolution, the fungal partner took advantage of the increasing availability of plant materials and adapted to the decomposition of these plants by either a parasitic or saprotrophic way of life.

Saprotrophic or saprobic fungi grow on dead material and are particularly important for the breakdown of various polymers in the plant cell wall. However, fungi are also adapted to grow as parasites or pathogens on living plants, and can be subdivided into biotrophic and necrotrophic fungi. Biotrophic fungi feed from living cells without killing the host plant whereas necrotrophic parasites kill the host organism as part of their attack. A number of economically important plants are attacked by fungi, leading to crop losses either by plant decomposition or by the production of toxic substances.

The importance of fungi in the global cycling of carbon, the significance of enzymes produced by fungi to degrade the plant cell wall, and the biotechnological applications of fungi and their enzymes have promoted research interest towards understanding their role in the degradation of plant material. Today, the use of fungal lignocellulose-degrading enzymes for total hydrolysis of plant biomass to sugars is under intensive study. The sugar monomers formed thereby could serve as raw material in the bioproduction of chemicals and fuels by microbes. Production of bio-ethanol derived from agricultural waste

<sup>1</sup> FB Gentechnik und Angewandte Biochemie, Institut für Verfahrenstechnik, Umwelttechnik und Technische Biowissenschaften, TU Wien, Getreidemarkt 9/166/5/1, 1060 Vienna, Austria

materials could partly replace fossil fuels. Further applications of the enzymes and organisms can be found in various industries including the food, feed, textile, and pulp and paper industries. The general use of sustainable and environmentally friendly technologies which have introduced lignocellulolytic enzymes at different stages of pulp and paper manufacturing as pretreatment for pulping (biopulping), bleaching (biobleaching) or wastewater treatment has led to considerable energy savings and an overall reduction of pollutants in wastewater from these industries.

The fungi best studied with respect to biopolymer-degrading enzyme formation are species of *Aspergillus*, *Trichoderma* and *Penicillium* or – as an example of lignin degradation – the white-rot fungus *Phanerochaete chrysogenum*. In nature, most of these fungi produce high amounts of extracellular enzymes due to an efficient secretory machinery. Some are easy and inexpensive to grow in large bioreactors, and suitable for genetic recombination technologies. Industrial strains of *Aspergillus* and *Trichoderma* can yield more than one hundred grams of these extracellular enzymes per litre (Durand et al. 1988; Berka et al. 1991; Cherry and Fidantsef 2003) and are therefore also used in the production of recombinant proteins and enzymes. Indeed, it is not surprising that these fungi are among the first for which a genome sequence became available. The recent advances in the sequencing and annotation of different fungal genomes of *Aspergillus nidulans*, *Aspergillus niger*, *Hypocreah jecorina* or *Phanerochaete chrysogenum* (<http://genome.jgi-psf.org>; <http://www.broad.mit.edu/annotation/fgi/>) give a first impression of the hidden enzymatic potential of these organisms.

This review summarizes our current knowledge on the diversity of the plant cell wall-degrading enzymes of these saprobic fungi. As an example for the complex degradation of wood, lignocellulolytic degradation in brown- and white-rot decay is highlighted in more detail. A complete listing of all enzymes involved in the breakdown of plant cell wall materials and which have been characterized in the last few years is beyond the scope of this review but web-based databases are available. One example is the CAZy database, which describes the families of structurally related catalytic and carbohydrate-binding modules of enzymes which degrade, modify or create glycosidic bonds ([www.cazy.org/CAZY/](http://www.cazy.org/CAZY/); also see Coutinho and Henrissat 1999).

## II. Structure and Composition of Plant Cell Walls

Plant cells are usually enclosed by a cell wall which provides rigidity to the cell for structural and mechanical support, maintains and determines cell shape, counterbalances osmotic pressure, determines the direction of growth and, ultimately, the architecture and form of the plant. In addition, the plant cell wall protects against pathogens and other environmental factors. The predominant polymers in plant cell walls are polysaccharides such as cellulose, hemicelluloses and pectins. Together with the aromatic polymer lignin and proteins, these polysaccharides can form a complex and rigid structure termed lignocellulose.

The middle lamella, the primary wall, and the secondary wall are the three major regions to be distinguished. The middle lamella is the outermost layer and is composed primarily of pectin. The primary wall is expanded inside this middle lamella and consists of several interconnected matrices composed of polysaccharides and (glyco)proteins. In these matrices, cellulose microfibrils are aligned at all angles and cross-linked via hemicellulosic tethers to form the cellulose–hemicellulose network (e.g. xyloglucan and galactoglucomannan). This network is embedded in the gelatinous pectin matrix composed of homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II (O'Neill and York 2003). Two different types of primary cell walls are distinguished (Carpita and Gibeaut 1993): type I is found in all dicotyledons, non-graminaceous monocotyledons and gymnosperms, and typically contains xyloglucan and/or glucomannan and 20–35% pectin. Type II is found in the monocotyledons of the *Poaceae* family and is rich in arabinoxylan but contains less than 10% pectin.

Some plants synthesize a secondary wall which is constructed between the plant cell and the primary wall. The secondary wall mainly provides support and is comprised primarily of cellulose and lignin. The cellulose microfibrils are generally aligned in the same direction but, with each additional layer, the orientation of the microfibrils changes slightly. The secondary wall is altered during development by successive encrustation and deposition of cellulose fibrils and other components. Non-structural components of the secondary wall represent generally less than 5% of the dry weight of wood, and include compounds extractable with organic solvents which can be either polar (e.g. phe-

nols and tannins) or apolar (e.g. fats and sterols), water-soluble compounds (e.g. sugars and starch) as well as proteins and ashes. Whereas the primary cell wall structure is of similar type for all species studied to date, species and cell-type-specific differences are typical for the secondary cell wall.

The polysaccharides found in the cell wall are either linear polymers composed of a single type of glycosyl residue (e.g. cellulose is composed of 1,4-linked  $\beta$ -glucosyl residues), polymers with a regular branching pattern (e.g. xyloglucan and rhamnogalacturonan II) or, as in the case of rhamnogalacturonan I, substituted with a diverse range of arabinosyl and galactosyl-containing oligosaccharide side chains. Understanding the structures of these polymers and determining their primary structures remains a major challenge, especially because their biosynthesis is not template driven (O'Neill and York 2003).

### III. The Degradation of the Plant Cell Wall

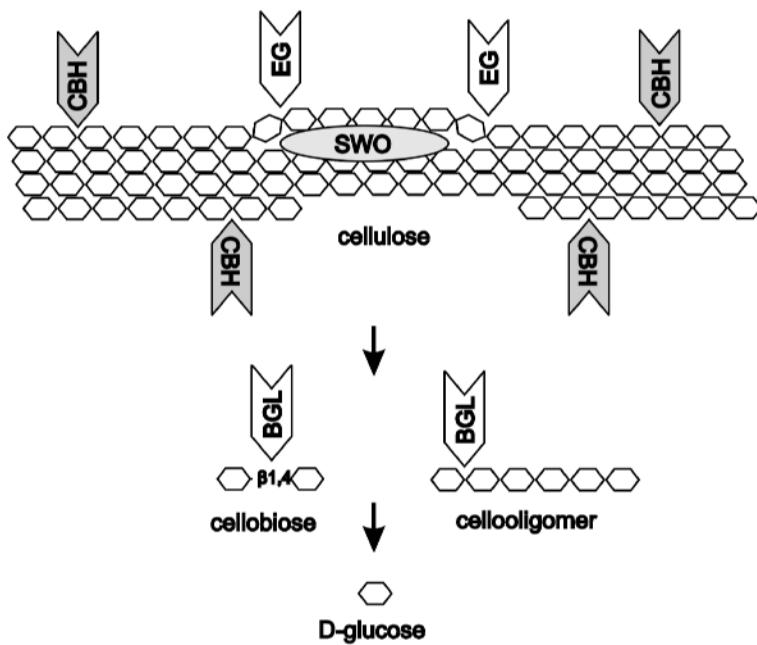
Due to the overall structural and chemical complexity of many plant cell walls, a complete breakdown of the different components is, in nature, brought about only by a wide range of organisms acting in a consortium, following a characteristic decomposition sequence which starts with organisms colonizing living plants and ends with the production of highly persistent soil humus. The fungi found in these generalized decomposition sequences live in complex and diverse communities and are often specialized to degrade only certain types of polymers, reflecting their genetic and enzymatic capabilities. The efficient breakdown of the plant cell wall by fungi is linked to their hyphal growth, which provides penetrating power, and to highly specialized extracellular plant cell wall-degrading enzyme systems. The enzymatic decomposition of plant cell walls is normally synergistic: individual, highly specialized enzymes operate as components of multi-enzyme systems to efficiently degrade specific polymers. For the synthesis and export of these enzymes, a sophisticated gene regulation system and highly productive secretory machinery has developed. All these characteristics enable fungi to successfully compete with other microorganisms in their environment, and they are today the main agents of decomposition in terrestrial and aquatic ecosystems.

The diversity of substrates has contributed to the difficulties encountered in enzymatic studies. The degradation has to occur extracellularly, since the substrates are usually large polymers which are also often insoluble or even crystalline. Two principal types of extracellular enzymatic systems for the degradation of the polymeric fraction have developed: the hydrolytic system, which degrades the polysaccharides mainly by hydrolases, and a unique oxidative ligninolytic system which depolymerizes lignin. Even for the degradation of the chemically simple polysaccharide cellulose, however, several enzymes are necessary. In general, three classes of enzymes can be distinguished: (i) exo-acting enzymes, which release mainly mono- and dimers of the ends of the polymeric chain, (ii) endo-acting enzymes, which cleave in the middle of the sugar chain and (iii) enzymes (often exo-acting) which are specialized in cleaving the resulting oligosaccharides into their monomers. Substituted polysaccharides require additional sets of enzymes for a complete hydrolysis. The resulting di- and monosaccharides are then readily taken up into the cell by different permeases and further degraded by a wide range of specialized catabolic pathways. In contrast to polysaccharides, lignin is a complex heteropolymer with no stereochemical regularity and must, therefore, involve a non-specific and non-stereoselective mechanism.

#### A. Biodegradation of Cellulose

Cellulose constitutes the most abundant biopolymer on earth, and accounts for about 50% of the primary biomass production by plants due to photosynthetic fixation of carbon dioxide. It is estimated that approximately  $4 \times 10^9$  tons of cellulose are formed annually (Eriksson et al. 1990).

Cellulose is a linear and highly ordered polymer consisting of about 8,000–12,000  $\beta$ -1,4-linked D-glucopyranose units. The polymeric chains are packed together by hydrogen bonds to form highly insoluble microfibrils. Although cellulose has a tendency to crystallize shortly after biosynthesis, less-ordered amorphous regions can also occur in plant tissues. The complete hydrolysis of cellulose to D-glucose requires a minimal set of three synergistically acting enzymes: cellobiohydrolases (1,4- $\beta$ -D-glucan cellobiohydrolases; EC 3.2.1.91), which act processively from the ends of the cellulose chain to generate mainly the glucose



**Fig. 18.1.** Enzymatic degradation of cellulose. Different cellobiohydrolases (CBH) act on either the reducing or non-reducing end of the chain. Endoglucanases (EG) hydrolyze internal glycosidic bonds, thereby providing additional sites for the CBHs. Finally, smaller oligomers and the dimer cellobiose are cleaved by  $\beta$ -glucosidases (BGL) into D-glucose. Swollenin supports the enzymatic degradation of cellulose by disrupting the microfibril structure

disaccharide cellobiose. Based on their primary sequence, the cellobiohydrolases are classified in different glycosyl hydrolase (GH) families. Cellobiohydrolases of GH family 6 are characterized by an inverting mechanism and cleave from the non-reducing cellulose ends. By contrast, GH 7 family members cleave cellobiose from the reducing end by a retaining mechanism. Endoglucanases (1,4- $\beta$ -D-glucan 4-glucanohydrolase; EC 3.2.1.4) attack the cellulose chains internally in the amorphous regions, thereby generating additional sites for the attack of the cellobiohydrolases. Finally,  $\beta$ -glucosidases (EC 3.2.1.21) degrade oligosaccharides and cellobiose to D-glucose (Fig. 18.1; Beguin 1990; Teeri 1997).

A large number of fungi are able to grow on amorphous cellulose or water-soluble derivates but relatively few are able to produce a complete enzyme system necessary to hydrolyze crystalline cellulose (Mandels and Weber 1969). Numerous genes encoding cellulases have been isolated and the respective enzymes studied in detail. One of the best studied cellulolytic fungi is *Hypocrea jecorina* (*Trichoderma reesei*), which was discovered during World War II on the Solomon Islands as a severe degrader of cellulosic material of the US Army (Kubicek and Harman 1998). Numerous cellulase-encoding genes have been isolated from a wide variety of fungi. Detailed analyses of the enzymes and gene regulation are available for, e.g. *Aspergillus niger*, *Hypocrea jecorina* and

*Phanerochaete chrysogenum* (de Vries 2003; Aro et al. 2005).

A structural comparison of the different cellulases shows that these proteins comprise – besides the domain responsible for the actual enzymatic reaction – often several other conserved functional domains. One of these domains is involved in substrate binding and is usually about 40 aa in size. This domain can be found N- or C-terminal and was originally described as the cellulose-binding domain (CBD). Removal of this domain leads to enzymes which are still able to cleave glycosyl linkages from smaller oligosaccharides but the binding to cellulose and, therefore, the action on crystalline cellulose is impaired. Later, these CBDs were also found in other carbohydrate-degrading enzymes, e.g. in the *H. jecorina* mannase, acetyl xylan esterase, and in the *Humicola* xylanase; to date, more than 300 putative sequences have been identified. Therefore, such domains with carbohydrate-binding activity were renamed as carbohydrate binding modules (CBMs). CBMs are structurally similar, and their carbohydrate-binding capacity can be attributed to several amino acids constituting the hydrophobic surface. Extensive data, classification and applications of these CBMs can be found in the Carbohydrate-Binding Module Family Server ([http://afmb.cnrs-mrs.fr/CAZY/fam/acc\\_CBM.html](http://afmb.cnrs-mrs.fr/CAZY/fam/acc_CBM.html)) and in a recent review by Shoevery et al. (2006). The CBM domain is often spatially separated from the

catalytic core domain by a linker region. This linker region is rich in prolines, serins and threonines, and the latter two amino acids are highly O-glycosylated.

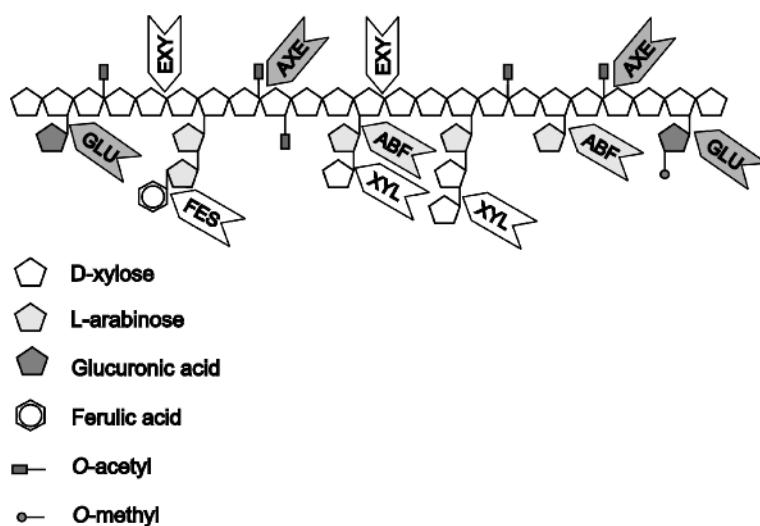
The catalytic domain structure of the cellobiohydrolase II of *Hypocrea jecorina* was the first cellulase crystal structure resolved at the atomic level (Rouvinen et al. 1990) and has explained why the enzyme is able to attack the cellulose chains only from the end and not from the middle. The crystal structure shows a tunnel-shaped active site which is so tight that it can incorporate only one cellulose chain. Despite a similar overall structure, endoglucanases have in general a more open active site and allow, therefore, an attack of the cellulose chains from the middle. The active site topology of such polymer-degrading enzymes shows that these enzymes have extended active sites which provide binding sites for a number of sugar units. These subsites position the substrate tightly and correctly with respect to the catalytic amino acids.

In addition to these classical cellulase enzymes, novel types of proteins have recently been described which are involved in plant cell wall degradation: Swollenin (*Swo1*) from *H. jecorina* shows amino acid similarity to plant expansins. Expansins induce the extension of isolated cell walls (McQueen-Mason et al. 1992) and induce non-hydrolytic activity on cell wall polymers, e.g. pectins and xyloglucans, which are tightly bound to the cellulose microfibrils (McQueen-Mason and Cosgrove 1995). Swollenins are able to disrupt the cellulose microfibrils without any hydrolytic

activity and would, in this way, make the cellulose fibres more accessible for the cellulases to act upon (Saloheimo et al. 2002). Comparisons of the annotated genomes from cellulolytic and hemi-cellulolytic organisms with EST data generated under appropriate conditions have led to the identification of new proteins involved in cellulose degradation (Foreman et al. 2003; Schmoll et al. 2003; Schmoll and Kubicek 2005). One example is the isolation of a novel type of endoglucanase which can be attached to the membrane via a glycosylphosphatidylinositol anchor. Regulation of cellulases and hemicellulases has been reviewed by de Vries et al. (2002) and Aro et al. (2005).

## B. Biodegradation of Hemicelluloses

Hemicelluloses are heterogeneous polymers and the second most abundant natural polysaccharide, accounting for around 20–30% of plant cell wall biomass (Eriksson et al. 1990). These plant cell wall polysaccharides have a backbone of 1,4-linked  $\beta$ -D-pyranosyl residues, with the exception of arabinogalactan, and are solubilized by aqueous alkali (O'Neill and Selvendran 1985; O'Neill and York 2003). The backbone can consist of xylosyl-, glucosyl-, galactosyl-, arabinosyl- or mannosyl residues and, depending on the dominant sugar, these are trivially named xylans or arabinogalactans, for example, if both sugars occur in near-equal amounts. The chemical diversity of the hemicelluloses produced by the plants requires a larger set of enzymes which either act on



**Fig. 18.2.** Enzymatic degradation of hemicelluloses. The main chain of xylan is degraded by endo-1,4- $\beta$ -xylanases (EXY). Accessory enzymes necessary for side group removal are  $\beta$ -xylosidase (XYL),  $\alpha$ -glucuronidase (xylan- $\alpha$ -1,2-glucuronosidase, GLU), feruloyl esterase (FES), acetyl xylan esterase (AXE) and  $\alpha$ -L-arabinofuranosidase (arabinoxylan arabinofuranohydrolase, ABF)

the main chain or attack the side chains. The main chain is internally cleaved by endo-acting enzymes whereas exo-acting enzymes liberate the respective monomers. In a synergistic degradation pattern, additional enzymes attack the side chains, leading to the release of various mono- and disaccharides; in this way, the main chain becomes more accessible for the endo-acting enzymes. Most extensively studied is the enzymatic degradation of xylan, which involves endoxylanases,  $\beta$ -xylosidases and accessory enzymes (Fig. 18.2).

## 1. Xylans

Xylans are a highly heterogeneous group characterized by a  $\beta$ -1,4-linked  $\beta$ -D-xylosepyranose backbone and including arabino-, glucurono- and glucuronoarabinoxylans. Xylans are abundant in the walls of cereals (*Poaceae*) and in hardwood, e.g. the secondary walls of woody plants, and are minor components of the walls of dicotyledons and non-graminaceous monocotyledons (Darvill et al. 1980; Ebringerova and Hienze 2000). Xylans found in cereals are highly substituted with single residues or short side chains of  $\alpha$ -1,2- or  $\alpha$ -1,3-linked L-arabinofuranose residues and are therefore commonly referred to as arabinoxylans (Izydorczyk and Biliaderis 1995). Glucuronoxylylans are typical hardwood xylans and contain large amounts of  $\alpha$ -1,2- and  $\alpha$ -1,3-linked 4-O-methyl- $\alpha$ -D-glucuronic acid and acetyl groups at O-2 or O-3. In softwood, glucuronoarabinoxylans are found which are substituted with a higher content of  $\alpha$ -1,2-linked 4-O-methyl- $\alpha$ -D-glucuronic acid than in hardwood and, in addition, contain  $\alpha$ -L-arabinosefuranose but no acetyl groups. The L-arabinose residues may be esterified at O-5 with feruloyl or *p*-coumaroyl residues, and a number of other minor residues have been detected, too.

The hydrolysis of the xylan backbone involves endo-1,4- $\beta$ -xylanases (endo-1,4- $\beta$ -D-xylan xylohydrolases; EC 3.2.1.8) and  $\beta$ -xylosidases (1,4- $\beta$ -D-xylan xylohydrolase; EC 3.2.1.37). Endoxylanases cleave the main sugar chain depending on the type of xylan, the degree of branching, and the presence of different substituents (Polizeli et al. 2005). The main hydrolysis products are substituted or non-substituted oligomers which are further converted by  $\beta$ -xylosidases into tri-, di- and monomers. Endoxylanases can be classified according to their end product into debranching and non-debranching enzymes, based on their

ability to release L-arabinose from arabinoxylan (Wong et al. 1988). Some enzymes cut randomly between unsubstituted D-xylose residues whereas the cleavage site of some endoxylanases is dependent on the neighbouring substituents of the side chains.  $\beta$ -Xylosidases can be classified according to their relative affinities for xylobiose or larger xylooligosaccharides, and release  $\beta$ -D-xylopyranose by a retaining mechanism from the non-reducing end.  $\beta$ -Xylosidases are in general highly specific for small unsubstituted D-xylose oligosaccharides, and the activity decreases with increasing polymerization of the substrates. Accumulation of the short oligosaccharides would inhibit the action of the endoxylanases but the hydrolysis of these products by  $\beta$ -xylosidases removes this possible cause of inhibition, thereby increasing the efficiency of xylan hydrolysis (Andrade et al. 2004). Similar to cellulases, most of the genes encoding endoxylanases and  $\beta$ -xylosidases have been characterized in different *Aspergillus*, *Trichoderma* and *Penicillium* spp. as well as in *Agaricus bisporus* and *Magnaporthe grisea*.

## 2. Xyloglucan

Xyloglucan is quantitatively the predominant hemicellulosic polysaccharide of dicotyledons and non-graminaceous monocotyledons, constituting up to 20% of the plant cell wall. Xyloglucans are strongly associated with cellulose microfibrills and support the structural integrity of the cell wall. The backbone is composed of 1,4-linked  $\beta$ -D-glucopyranose residues which are substituted by D-xylopyranose via an  $\alpha$ -1,6-linkage.

Xyloglucans are classified as XXXG or XXGG type, depending on the number of backbone residues with branches; e.g. XXXG have three consecutive backbone residues which are substituted with D-xylopyranose, and a fourth unbranched backbone residue. XXXG-type glucans are present in numerous plant species whereas the XXGG type occurs in solanaceous plants. Sugars bound to the D-xylopyranose include  $\alpha$ -1,2-L-fucopyranose,  $\beta$ -1,2-D-galactopyranose,  $\alpha$ -1,2-L-galactopyranose or  $\alpha$ -1,2-L-arabinose residues. Some of these residues can also contain O-linked acetyl groups (O'Neill and York 2003). Some of the endoglucanases active against cellulose are also active on xyloglucans; in addition, xyloglucan hydrolases (EC 3.2.1.151) which are specific for xyloglucan have been reported (Pauly et al. 1999; Hasper et al. 2002; Grishutin et al. 2004).

### 3. (Galacto-)glucomannan

Mannan consists of a  $\beta$ -1,4-linked  $\beta$ -D-mannopyranose backbone whereas, in glucomannans, the backbone comprises both  $\beta$ -1,4-linked  $\beta$ -D-glucopyranose and  $\beta$ -D-mannopyranose residues which are randomly distributed. It can be substituted by  $\alpha$ -1,6-linked  $\alpha$ -D-galactopyranose residues which can be substituted further by  $\alpha$ -1,2-linked  $\alpha$ -D-galactopyranose. These polysaccharides are usually referred to as galactomannans and galactoglucomannans (Brett and Waldren 1996). Galactomannans and galactoglucomannans are the major hemicellulose structures of softwoods, and glucomannan of hardwood (Aspinall 1980; Stephen 1982). The D-glucose or D-mannose residues are partially substituted with acetyl residues linked to O-2 or O-3. The backbone is degraded by endo-1,4- $\beta$ -mannanases (Mannan endo-1,4- $\beta$ -mannosidase, EC 3.2.1.78) and  $\beta$ -mannosidases (EC 3.2.1.25). The ability of the endo-1,4- $\beta$ -mannanases to degrade these polymers depends on the number and position of the side chain substituents. The enzymes releasing the glucose and galactose residues act in synergism with endo-1,4- $\beta$ -mannanases and  $\beta$ -mannosidases.  $\beta$ -Mannosidases split off the  $\beta$ -D-mannose residue from the non-reducing end of the manno-oligosaccharides and are characterized by a retaining mechanism.

## C. Biodegradation of Pectins

Pectins are a complex and heterogeneous group of polysaccharides characterized by a significant content of  $\alpha$ -1,4-linked D-galacturonic acids. They are found mainly in the middle lamella and in the primary cell wall, their proportion ranging from 5–10% in grasses to 30% in dicotyledons. The carbohydrate composition and, hence, the structure vary depending on the species and cell type. Pectin is made up of several distinct domains which, depending on the side chains, are called either “smooth” or “hairy” regions (Pérez et al. 2000; Ridley et al. 2001).

The “smooth” region, or homogalacturonan (HG), consists of linear chains of  $\alpha$ -1,4-linked D-galacturonic acid residues which can carry methyl esters at the terminal carboxyl group and acetyl esters at the O-2 or O-3 position. Homogalacturonan with a high degree of methyl esterification is referred to as pectin whereas pectic acid (pectate) has a low degree of ester-

ification. The esterification of the uronic acid group results in the elimination of the negative charge, which is of great significance for the gelling process of pectin, since the complexes between the carboxyl groups and Ca<sup>2+</sup> ions in addition to borate and uronyl esters are involved in this (Vincken et al. 2003). Additionally, the number of methyl- and acetyl esters has a strong influence on the susceptibility to cleavage by the different pectinolytic enzymes. Rhamnogalacturonan I+II and xylogalacturonan (XGA) are known as “hairy” regions due to the abundant and often branched side chains. Rhamnogalacturonan I distinguishes itself from the other domains in that its backbone consists of D-galacturonic acid and L-rhamnose in a [1->2]- $\alpha$ -L-Rha-(1->4)- $\alpha$ -D-GalA-(1->)n linkage. Whereas D-galacturonic acid can be substituted with either methyl- or acetyl esters similar to those in homogalacturonan, 20–80% of the L-rhamnose residues are substituted at the O-4 position. The substituents contain L-arabinose and D-galactose, and vary in size from monomers up to branched, heterogeneous oligomers. They can be terminated with  $\alpha$ -L-fucose and (4-O-methyl)- $\beta$ -D-glucuronic acid. The arabinan chain has an  $\alpha$ -1,5-linked L-arabinose backbone which can be substituted with  $\alpha$ -1,3-linked L-arabinose residues. Two types of arabinogalactan side chains have been identified. Type I consists of a chain of  $\beta$ -1,4-linked D-galactopyranose whereas type II contains a backbone of  $\beta$ -1,3-linked D-galactopyranose residues which can be substituted with  $\beta$ -1,6-linked D-galactopyranose residues. Both are occasionally substituted with L-arabinose at O-3. In addition, ferulic acid and p-coumaric acids have been identified in the pectic hairy regions attached to O-2 of L-arabinose and O-6 of D-galactose.

Rhamnogalacturonan II consists of a short backbone of  $\alpha$ -1,4-linked D-galacturonic acid which is substituted either at the O-2 or O-3 position (Vidal et al. 2000). The side chains have been found to be either dimers or branched oligomers, and to contain rare sugars such as D-apiose and L-fucose in addition to L-arabinose, D-galactose and L-rhamnose.

The backbone of another substructure found in hairy regions, the xylogalacturonans, is similar to that of the homogalacturonans but a major part of the D-galacturonic residues carry  $\beta$ -D-xylose substituents at the O-3 position. It has so far been found only in reproductive tissues, including soybean seed, apple fruit and pine pollen (Schols et al.

1995). Whereas the composition and structure of the individual subunits are mostly established, the manner in which they make up the pectin polymer is still under investigation. For a long time, pectin was thought to consist of linear chains of homogalacturonan interspersed with hairy regions. Recently, however, Vincken et al. (2003) proposed a new model in which rhamnogalacturonan I alone forms the backbone, substituted with homogalacturonan and the abovementioned arabinan and galactan side chains.

To efficiently degrade pectin, fungi have developed a broad spectrum of pectinolytic enzymes. The term "pectinases" usually refers to enzymes which act on the pectin backbone. The degradation of pectin has, besides in saprobic fungi, been most extensively studied in plant pathogenic fungi (*Botrytis cinerea*, *Fusarium oxysporum*, *Sclerotium sclerotiorum*). Enzymatic depolymerization of pectin weakens the cell wall and exposes the other cell wall polymers to degradation by other plant cell wall-degrading enzymes. As a protection against this enzymatic attack, numerous plants have developed specific plant defence proteins – the so-called polygalacturonase-inhibiting proteins (PGIPs), which reduce specifically the hydrolytic activity of fungal polygalacturonases (Cervone et al. 1990). PGIPs are leucine-rich repeat glycoproteins associated with the cell wall of both monocotyledons and dicotyledons (D'Ovidio et al. 2004).

Pectinases can be subdivided, in terms of their reaction mechanism, into hydrolases and lyases and further according to their substrate specificity into, e.g. polygalacturonases and rhamnogalacturonases. Pectin lyases (EC 4.2.2.10), pectate lyases (EC 4.2.2.2) and rhamnogalacturonan lyases (EC 4.2.2.-) cleave polysaccharide chains via a  $\beta$ -elimination mechanism resulting in the formation of a  $\Delta$ -4,5-unsaturated bond at the newly formed non-reducing end.

The three-dimensional structure of several pectinases has been determined. The results show that – in contrast to the cellulases and hemicellulases, which are characterized by a high diversity of protein structures – pectinases share a common conserved structure. Although their overall sequence similarity is low, pectinases possess a central core consisting of parallel  $\beta$ -strands forming a large, right-handed helix defined as parallel  $\beta$ -helix (Jenkins and Pickersgill 2001). Whereas the catalytic mechanism differs between the hydrolases and lyases, the substrate-binding sites are all found in a similar location within a cleft

formed on the exterior of the parallel  $\beta$ -helix. This structure facilitates the binding and cleaving of the buried pectin polymers in the undamaged cell wall. The parallel  $\beta$ -helix fold confers the stability needed by these enzymes for efficient aggressive action in a variety of hostile extracellular environments. An exception to this rule is the rhamnogalacturonan lyase from *A. aculeatus*, which displays a unique arrangement of these three distinct modular domains (McDonough et al. 2004).

In saprobes, the pectinolytic system has been studied in great detail in *Aspergillus* spp. including *A. niger* (de Vries and Visser 2001). Polygalacturonases (PGAs) are the most extensively studied class of pectinases and have been isolated from various saprotrophic and plant pathogenic fungi. Endopolygalacturonases catalyze the hydrolytic cleavage of  $\alpha$ -1,4 D-galacturonic bonds within the chain (EC 3.2.1.15), and exopolygalacturonases (Galacturan 1,4- $\alpha$ -galacturonidase. EC 3.2.1.67) cleave from the non-reducing end. Both endo- and exopolygalacturonases belong to glycoside hydrolase family 28, and have similar reaction mechanisms and substrate specificities, but their level of sequence identity is surprisingly low (Herrissat and Bairoch 1993; Biely et al. 1996; Markovic and Janecek 2001). Among the endopolygalacturonases, some enzymes cleave only once per chain (single attack or non-processive) whereas others attack the pectin polymer multiple times per strand (processive behaviour). Single-attack polygalacturonases generally produce longer fragments, which are only gradually degraded into dimers, trimers or short oligomers, providing possible sites for exopolygalacturonases. Digestion with processive enzymes, on the other hand, results in an accumulation of these short oligomers from the very beginning of the reaction. The circumstances under which the one or the other pathway of degradation is preferred are not completely clear to date, and may be related to the biological significance of the resulting fragments. A factor which significantly influences the activity of polygalacturonases is the number (and distribution) of methyl- and acetyl ester groups. In general, most endo- and exoPGAs prefer substrates with a low degree of esterification, although some exceptions exist (Parenicova et al. 2000). In most cases, the activity of a methyl/acetyl esterase is required to prepare the pectin molecule for PGA digestion. Genes encoding PGAs are organized into families the members of which exhibit a high degree of poly-

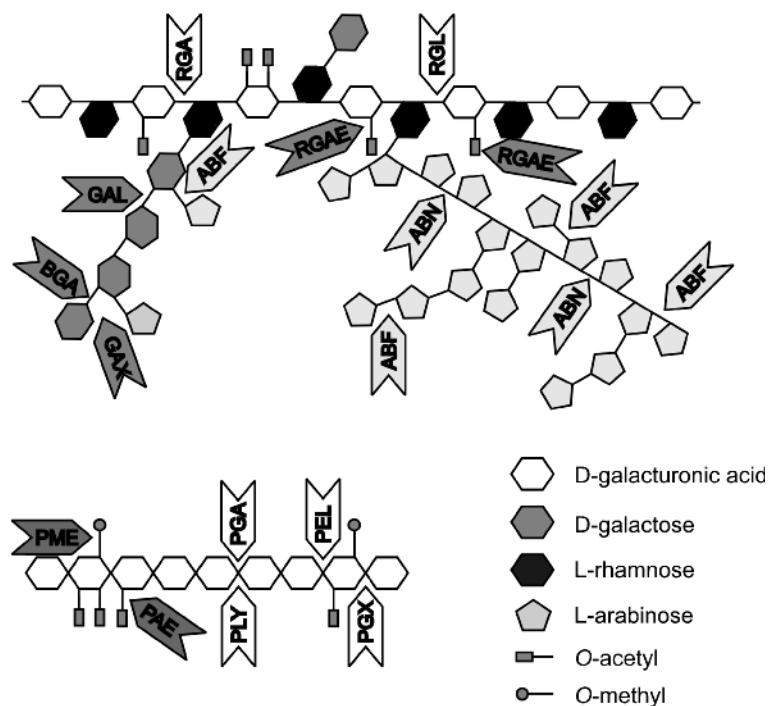
morphism (Annis and Goodwin 1997; Markovic and Janecek 2001). Most fungi produce multiple isozymes with a wide range of enzymatic properties, substrate specificities and pH optima, which may reflect the complexity of the pectin molecule in plant cell walls and the need for enzymes capable of cleaving the homogalacturonan backbone in a variety of structural contexts. Another structural feature which may determine the functional diversification of these enzymes is the presence or absence and type of N-terminal extension, which has been suggested to influence their substrate specificity and to play a role in their interaction with particular regions of the pectin polymer (Parenicova et al. 2000; Gotesson et al. 2002).

The degree of esterification is also important for the functional classification of lyases. Pectate lyases prefer substrates with a low degree of methyl esterification, which therefore have a more acidic character, and are strictly dependent on  $\text{Ca}^{2+}$  for catalysis. Pectin lyases, on the other hand, favour highly methyl-esterified substrates and do not require  $\text{Ca}^{2+}$  ions (Jurnak et al. 1996).

The pectinolytic enzyme system of *A. niger* serves as an example of how a microorganism can degrade a pectin molecule. *A. niger* produces seven polygalacturonases, two of them (PgaA and PgaB) constitutively. These two are most active on pectins

containing 22% methyl esters (Parenicova et al. 2000), thus making them suitable for an initial attack on the native substrate. Enzymes subsequently induced at this early stage during growth on pectin are pectin methyl esterase PmeA, an exopolysaccharide lyase PgxA, and pectin lyases (PelA and PelD; de Vries et al. 2002). The action of the methyl esterase renders the substrate accessible to polygalacturonases, which are expressed at a later stage after removal of the methyl esters, while the pectin lyases contribute to the breakdown of the still esterified polymer. The exopolysaccharide lyase cleaves D-galacturonic acid monomers from the homogalacturonan poly- and oligomers, which may serve as inducers for the other pectinases (Fig. 18.3).

Similar to homogalacturonans, the degradation of the rhamnogalacturonan I backbone is catalyzed by hydrolases and lyases (Fig. 18.3). Endorhamnogalacturonases have been isolated from *A. aculeatus* and *A. niger* (de Vries and Visser 2001), and were shown to hydrolyze the  $\alpha$ -1,4 glycosidic bonds in saponified hairy regions. The resulting fragments were tetra- and hexamers of the backbone, which partly were still substituted with D-galactose. This suggests that – similar to some exoPGAs ability to cleave XGA (van den Broek et al. 1996) – endorhamnogalacturonases are tolerant towards monomeric



**Fig. 18.3.** Enzymatic degradation of rhamnogalacturonan I and homogalacturonan. The main chain of rhamnogalacturonan I (shown above) is degraded by rhamnogalacturonan hydrolase (RGA) and rhamnogalacturonan lyase (RGL). The side chains are degraded by rhamnogalacturonan acetyl esterase (RGAE), endoarabinase (ABN), endo- $\beta$ -1,6-galactanases (GAL) and exogalactanases (GAX). Terminal monosaccharides are removed by  $\alpha$ -L-arabinofuranosidases (arabinofuranan arabinofuranohydrolases, ABF) and  $\beta$ -galactosidases (BGA). The main chain of homogalacturonan (below) is degraded by endopolygalacturonases (PGA), exopolysaccharide lyases (PGX), pectin lyases (PLY), pectate lyases (PEL). Pectin methyl esterase (PME) and pectin acetyl esterase (PAE) act on the side groups

substituents. Depending on the monosaccharide cleaved from the non-reducing end of the rhamnogalacturonan, two kinds of exorhamnogalacturonases have been described: rhamnogalacturonan  $\alpha$ -D-galactosyluron-hydrolases and rhamnogalacturonan  $\alpha$ -L-rhamnohydrolases. All rhamnogalacturonan hydrolases were classified as members of GH family 28. Rhamnogalacturonan lyases cleave the rhamnogalacturonan backbone via  $\beta$ -elimination. Unlike the hydrolases, they act on the Rha-(1->4)- $\alpha$ -D-GalA bond, resulting in the formation of  $\Delta$ -4,5-unsaturated D-galacturonic acid residues at the non-reducing end.

#### D. Accessory Enzymes

Most of the plant cell wall-degrading enzymes described above act on the backbone of the respective polysaccharide, but usually their activity is impaired by monomeric substituents or larger side chains which are present in hemicelluloses and pectins. To ensure an efficient and complete breakdown of such polysaccharides, these substituents have to be removed and degraded by different families of accessory enzymes. These accessory enzymes work in synergism with the enzymes attacking the main chain and often depend on each other for an efficient breakdown of the substrate. The substrate specificity of these enzymes varies; some of the enzymes can hydrolyze the intact polymer whereas others show maximum activity only in the presence of shorter breakdown products (cf. Puls and Schuseil 1993; Tenkanen and Siika-aho 2000). A detailed list of the enzymes involved in the degradation of hemicelluloses and pectinases and their mode of action has been reviewed for *Aspergillus* spp. by de Vries and Visser (2001), and only some of the major enzymes are listed here (Figs. 18.2 and 18.3).

Side groups in xylans are generally small (mono-, di- and trimers) but can consist of several different sugars and acids (e.g. acetic acid, L-arabinose, ferulic acid, D-galactose, D-glucuronic acid) and, consequently, multiple enzymes are required to make the backbone fully accessible for the xylanases.  $\alpha$ -L-Arabinofuranosidases (EC 3.2.1.55) remove terminal L-arabinose residues but differences in the specificity towards  $\alpha$ -1,2-,  $\alpha$ -1,3- or  $\alpha$ -1,5-arabinosidic bounds and towards the substrata themselves have been observed. Whereas several representatives are also able to release L-arabinose from pectins and xylans, others – also

called arabinoxylan arabinofuranohydrolases – are strictly specific for L-arabinose bound to xylan. In addition, some arabinofuranosidases are inhibited by the presence of D-glucuronic acid residues adjacent to the targeted L-arabinose. This D-glucuronic acid and its 4-O-methyl ethers are removed by  $\alpha$ -glucuronidases (EC 3.2.1.139) and by xylan- $\alpha$ -1,2-glucuronosidases (EC 3.2.1.131). In addition to carbohydrate substituents, two types of esters are found in xylans – those with ferulic and those with acetic acid – which are both hydrolyzed by specific esterases. Several types of feruloyl esterases (EC 3.1.1.73) have been described, their activity varying with the presence of additional methoxy or hydroxyl substituents on the ferulic acid's aromatic ring, and with the type of linkage (O-2, O-5 or O-6) to the carbohydrate chain. Acetyl xylan esterases (EC 3.1.1.72) participate in the breakdown of the xylan backbone by removing acetyl ester groups from O-2 and O-3 of the D-xylose chain, thereby facilitating the action of the endoxylanases. As is the case with arabinofuranosidases, synergistic effects with main chain cleaving enzymes have been reported (Kormelink et al. 1993; Tenkanen 1998; de Vries et al. 2000). So far, only two types of substituents have been described in galacto(gluco)mannans: D-galactose mono- and dimers, and acetyl esters. The former are removed from the backbone by  $\alpha$ -galactosidases (EC 3.2.1.22) whereas the latter are hydrolyzed by acetylglucosidase esterases (EC 3.1.1.-). Both reactions result in an increased activity of the endomannases and  $\beta$ -mannosidases (Tenkanen 1998; de Vries et al. 2000). For the removal of  $\alpha$ -1,6-linked D-xylose side groups of the xyloglucan, specific  $\alpha$ -D-xylosidases are required.

Pectin contains two different types of substituents. On the one hand, acetyl esters at the O-2 or O-3 and methyl esters at the carboxy group are bound to the D-galacturonic acid residues in the smooth regions. On the other hand, polymeric side chains consisting mainly of L-arabinose and D-galactose are found in the hairy regions. Pectin methyl esterases (EC 3.1.1.11) and pectin acetyl esterases (EC 3.1.1.-) have been isolated from different fungal species and they show a prominent synergism with polygalacturonases (de Vries et al. 2000). Similar results were achieved with rhamnogalacturonan acetyl esterases (EC 3.1.1.-) and rhamnogalacturonase or rhamnogalacturonan lyase (de Vries et al. 2000). The D-galactose or L-arabinose side chains are often

substituted with several other carbohydrates or acids. These chains are cleaved by endoarabinases (EC 3.2.1.99) and exoarabinases (EC 3.2.1.-), and by endo- $\beta$ -1,4-galactanases (EC 3.2.1.89), endo- $\beta$ -1,6-galactanases (EC 3.2.1.-) and exogalactanases (EC 3.2.1.23). Terminal monosaccharides are removed by  $\alpha$ -L-arabinofuranosidases (EC 3.2.1.55) and  $\beta$ -galactosidases (EC 3.2.1.23) but additional enzymes (e.g.  $\alpha$ -L-fucosidases,  $\alpha$ -glucuronidases) are required to completely degrade the side chains.

#### IV. Biodegradation of Lignin

Next to cellulose, lignin is the most abundant polymer in nature and accounts for 15 to 36% of the lignocellulosic material. It forms an extensively cross-linked network within the cell wall, and confers structural support and decreases water permeability. It protects the other, more easily degradable cell wall components and is therefore the main obstacle for an efficient saccharification of cellulose and hemicellulose. The aromatic polymer is synthesized from the three substituted phenyl-propanoid alcohols coniferyl (guaiacyl propanol), sinapyl (syringyl propanol) and *p*-coumaryl (*p*-hydroxyphenylpropanol). The softwood of gymnosperms contains mainly coniferyl alcohols, some *p*-coumaryl but no sinapyl alcohol whereas, in the hardwood of the angiosperms, coniferyl and sinapyl alcohols are found in equal amounts (46%), with a minor proportion of *p*-coumaryl (8%). The lignin polymer is synthesized by the generation of free phenoxy radicals, which is initiated by plant peroxidases-mediated dehydrogenation of the three precursor alcohols. The result of this polymerization is a highly insoluble, complex-branched and amorphous heteropolymer joined together by different types of linkages such as carbon–carbon and ether bonds. The chemical complexity and structural variability of the lignin polymer makes it resistant to breakdown by conventional enzymatic hydrolysis and therefore the initial attack is oxidative, non-specific, non-hydrolytic and extracellular (Kirk and Farrell 1987; Higuchi 1990; Hatakka 1994).

Fungi are the most efficient group of organisms able to decompose or, at least, alter the wood structure. Based on macroscopic characteristics, different types of decay have been distinguished, including white, brown and soft rot. White-rot basidiomycetes are the major group of wood rots to-

day and the only group which can completely degrade lignin into CO<sub>2</sub> and H<sub>2</sub>O. They can overcome difficulties in wood decay, including the low nitrogen content of wood (a C:N ratio of about 500:1) and the presence of toxic and antibiotic compounds. Besides this group, brown-rot fungi are also able to degrade wood extensively. Some ascomycetes also colonize wood in contact with soil but alter the lignin component only slightly. Their action leads to a decrease in the mechanical properties of wood, giving rise to so-called soft rot, a process which often involves bacteria. Soft-rot fungi can degrade wood under extreme environmental conditions (extreme wetness or frequent dryness) which prohibit the activity of other wood-degrading fungi. Soft-rot fungi are relatively unspecialized (hemi-)cellulolytic ascomycetes in the genera *Chaetomium*, *Ceratostysis* and *Philaophora*, and some basidiomycetes can also cause a soft rot-type of decay pattern. In soft rot, decay by fungi is closely associated with penetration by the fungal hyphae, because the enzymes cannot diffuse through the plant cell wall. Two distinct types of soft rot are currently recognized. Type 1 is characterized by longitudinal cavities formed within the secondary wall of wood cells, and type 2 by an erosion of the entire secondary wall (Martinez et al. 2005). Although many white rots and brown rots secrete oxidative and hydrolytic enzymes, it is generally recognized that their enzymes are unable to diffuse through healthy wood and that smaller, non-proteinaceous molecules are involved in the initiation of decay.

##### A. Brown-Rot Fungi

Brown-rot fungi degrade mainly cellulose and hemicellulose of coniferous softwoods and partially modify the lignin mainly by demethylation (Eriksson et al. 1990). They attack cellulose in wood, which promotes rapid loss of mechanical strength – ultimately, extensively brown-rotten wood consists almost entirely of modified lignin. The term “brown rot” refers to the characteristics of this decayed wood: a reddish-brown material consisting of oxidized lignin, which cracks into characteristic brick-like pieces. Representatives of brown-rot basidiomycetes comprise *Schizophyllum commune*, *Fomes fomentarius*, *Serpula lacrimans* and *Gloeophyllum trabeum*. They are also the major cause of decay of woods in commercial use, and have an important role in coniferous ecosystems through their contribution to humus

formation. These fungi grow mainly in the cell lumen of the woody cells, and the degradation is not localized to the fungal hyphae but found at greater distances from these. The extracellular enzymes formed are too large to penetrate healthy cell walls and therefore – as noted above – degradation of cellulose by brown-rot fungi must involve diffusible low-molecular agents. The degradation process is still not completely understood but it has been suggested that the brown-rot fungi use both an oxidative and hydrolytic attack. Although some brown rots possess cellobiohydrolases, they generally lack the ability to hydrolyze crystalline cellulose enzymatically. However, crystalline cellulose can be disrupted when classical endoglucanases act together with an oxidative degradation system such as extracellular reactive oxygen species. Reactive oxygen species (ROS), such as hydroxyl radicals ( $\bullet\text{OH}$ ) and the less reactive peroxyl ( $\text{ROO}\bullet$ ) and hydroperoxyl ( $\bullet\text{OOH}$ ) radicals, have been discussed as the agents which initiate degradation (Hammel et al. 2002). There is a well-established pathway for the generation of these radicals via the Fenton reaction ( $\text{H}_2\text{O}_2 + \text{Fe}^{2+} + \text{H}^+ \rightarrow \text{H}_2\text{O} + \text{Fe}^{3+} + \bullet\text{OH}$ ). In order not to destroy the fungal hyphae and to act in the lignified parts of the secondary cell wall, the  $\bullet\text{OH}$  production has to occur at a distance from the hyphae, and the fungal reductants should be stable enough to diffuse before they react to reduce  $\text{Fe}^{3+}$  and  $\text{O}_2$  to  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$ . The production of  $\bullet\text{OH}$  radicals can take place in several ways and different systems are being discussed, including secreted hydroquinones, cellobiose dehydrogenases, low molecular-weight glycopeptides and phenolate chelators.

The principle of the quinone redox cycling for  $\bullet\text{OH}$  production is that the fungus reduces the quinone extracellularly to its hydroquinone which then reacts with  $\text{Fe}^{3+}$  to give  $\text{Fe}^{2+}$  and a semiquinone radical. The semiquinone reduces  $\text{O}_2$  to  $\bullet\text{OOH}$ , which is a source for  $\text{H}_2\text{O}_2$ , and is in this way recycled to quinone. *Gloeophyllum trabeum* produces extracellular quinones including 2,5-dimethoxy-1,4-benzoquinone and 4,5-dimethoxy-1,2-benzoquinone which can reduce  $\text{Fe}^{3+}$  and  $\text{O}_2$  rapidly under physiological conditions, thereby generating both  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$ . Moreover, the fungus was shown to reduce the resulting dimethoxyquinones back to hydroquinones, possibly by the action of an intracellular quinone reductase. Another non-enzymatic system includes phenolate or catechol-

chelators (Goodell 2003). These were isolated from culture filtrates of the brown-rot fungus *Gloeophyllum trabeum* and termed Gt chelator. They have a high affinity for the binding of iron and have the ability to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ . The two compounds 4,5-dimethoxy-1,2-benzenediol and 2,5-dimethoxy-1,4-benzenediol were identified in the Gt chelator fraction, and also their oxidized benzoquinone forms (see above).  $\bullet\text{OH}$  radicals can also be produced by the extracellular flavohaemoprotein cellobiose dehydrogenase (CDH). CDH production has been reported for all types of wood-rotting fungi (Zamocky et al. 2006), and CDH can act as cellobiose oxidase by reducing  $\text{O}_2$  to  $\text{H}_2\text{O}_2$ . However,  $\text{Fe}^{3+}$  is a better electron acceptor than  $\text{O}_2$  and, thus, CDHs are actually  $\text{Fe}^{3+}$  reductases. Glycopeptides, implicated in wood degradation, have been isolated from *G. trabeum* and *Tyromyces palustris*. They reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  and bind  $\text{Fe}^{2+}$  (Goodell 2003; Enoki et al. 2003). In the presence of  $\text{H}_2\text{O}_2$ , the glycopeptide generates one-electron oxidation and possesses the ability to oxidize NADH in the presence of oxygen, and thereby produces  $\text{H}_2\text{O}_2$ .

Most brown rots secrete oxalic acid, which is a strong chelator of  $\text{Fe}^{3+}$  and  $\text{Fe}^{2+}$  but also reduces the pH. The pH of wood itself is generally in the range 3–6, and is lowered to pH values between 2.5 and 1.7. The reduction of pH is important for the function of the extracellular enzymes and has been identified as a key factor in several hypotheses related to molecular weight degradation systems, as discussed in the reviews listed above.

## B. White-Rot Fungi

White rots are the most frequently found wood-rotting organisms and are mainly basidiomycetes but also some ascomycetes are able to cause white rot. They are characterized by their ability to completely degrade lignin, hemicelluloses and cellulose, thereby often giving rise to a cellulose-enriched white wood material. Two different white-rot patterns have been described:

- (i) Simultaneous (non-selective) delignification attacks mainly hardwood and degrades cellulose, lignin and hemicellulose simultaneously. The cell wall is attacked progressively from the cell lumen towards the middle lamella. Degradation is associated with the fungal hyphae and substantial amounts of undecayed wood remains. Basidiomycetes (e.g. *Tram-*

*etes versicolor*, *Irpex lacteus*, *Phanerochaete chrysosporium*, *Heterobasidion annosum* and *Phlebia radiata*) and some ascomycetes (e.g. *Xylaria hypoxylon*) perform this type of degradation.

- (ii) Selective delignification, or sequential decay, is found in hardwood and softwood. The initial attack is selective for lignin and hemicellulose, and the cellulose is attacked later. Lignin is degraded in the middle lamella, which is dissolved by a diffusion mechanism, and in the secondary wall. This type of degradation is performed exclusively by various basidiomycetes (e.g. *Ganoderma australe*, *Phlebia tremellosa*, *C. subvermispora*, *Pleurotus* spp. and *Phellinus pini*).

Many white-rot fungi cause both types of rot, and the amount of simultaneous or selective decayed wood varies even among different strains of the same species and depends also on the substrate (Eriksson et al. 1990; Martinez et al. 2005). To date, *P. chrysosporium* is the most intensively studied white-rot fungus (Cullen and Kersten 2004; Martinez et al. 2004).

White-rot fungi degrade lignin via an oxidative process involving peroxidases and laccases (phenol oxidases) which act non-specifically by generating lignin free radicals, which then undergo spontaneous cleavage reactions. Peroxidases, such as the lignin peroxidases (LiPs; EC 1.11.1.14), manganese peroxidases (MnPs; EC 1.11.1.13) and the versatile peroxidase (VP), have been described as true ligninases due to their high redox potential which enables them to oxidize non-phenolic aromatic substrates constituting up to 90% of the lignin structure. Peroxidases require the presence of  $\text{H}_2\text{O}_2$  as acceptor. LiPs are often produced as isoenzymes with a heme group in their active centre, and oxidize phenolic and non-phenolic compounds (Hammel et al. 1986; Kersten 1990; Hatakka 1994; Eggert et al. 1997). The catalytic, oxidative cycle of LiP is similar to those of other peroxidases. LiP becomes highly oxidized when  $\text{H}_2\text{O}_2$  is reduced to  $\text{H}_2\text{O}$ , and a two-electron reaction allows the activated enzyme to oxidize two substrate units before it is reduced to the peroxidase resting state once again. Veratryl alcohol is oxidized to the short-lived VA cation radical which may oxidize the lignin directly or pass on the charge to other, more stable carriers which can act as diffusible mediators. Since enzymes such as LiPs are too large to enter

the plant cell, direct degradation is carried out only in exposed regions of the cell lumen (simultaneous delignification). Microscopic studies of selective lignin biodegradation revealed that white-rot fungi remove the polymer from inside the cell wall, which can be performed only by indirect oxidation mediated by LiP low molecular-weight diffusible compounds capable of penetrating the cell wall. MnPs are closely related to LiPs. They have the same catalytic cycle involving a two-electron oxidation of the heme by  $\text{H}_2\text{O}_2$ , followed by two subsequent one-electron reductions. MnPs oxidize  $\text{Mn}^{+2}$  to  $\text{Mn}^{+3}$ , which is stabilized by organic acids such as oxalate, fumarate and malate.  $\text{Mn}^{+3}$  acts as diffusible oxidizer on phenolic substrates and oxidizes non-phenolic substrata via lipid peroxidation reactions (Martinez et al. 2005).

The more recently discovered versatile peroxidase (VP) combines the enzymatic properties of LiP and MnP and oxidizes both  $\text{Mn}^{+2}$  and veratryl alcohol. It oxidizes hydroquinone in the absence of exogenous  $\text{H}_2\text{O}_2$  when  $\text{Mn}^{+2}$  is present in the reaction as well as dimethoxybenzenes. The crystal structures have been resolved for both LiP and MnP (Piontek et al. 1993; Poulos et al. 1993; Sundaramoorthy et al. 1994). The prosthetic group (iron protoporphyrin IX) of LiPs is accessible only through a narrow pore (Piontek et al. 2001). The catalytic cycle is common to other peroxidases. However, the position of the iron-binding histidine residue in ligninolytic peroxidases is displaced farther away from the heme iron, and, which leads to an increased redox potential, and the existence of specific binding sites for substrate oxidation are unique (Martinez 2002).

The substrate-binding sites have been identified for LiP, MnP and VP, and explain the dual catalytic properties of VP.  $\text{Mn}^{+2}$  oxidation occurs at a binding site near the cofactor which enables direct electron transfer. By contrast, veratryl alcohol is oxidized at the surface of the protein by a long-range electron transfer mechanism. The rationale of the existence of this electron transfer mechanism is related to the fact that many of the aromatic substrates cannot penetrate inside the LiP/VP and, therefore, these substrates are oxidized at the enzyme surface, and electrons are transferred to the heme by a protein pathway (Sundaramoorthy et al. 1997; Doyle et al. 1998; Gold et al. 2000).

Laccases are multicopper phenoloxidases and generally larger than peroxidases. They perform four one-electron oxidations by reducing  $\text{O}_2$  to

$\text{H}_2\text{O}$ , and are only able to directly oxidize phenols and aromatic amines. The phenolic nucleus is oxidized by removal of one electron, generating phenoxy-free-radical products, which can lead to polymer cleavage. Due to their low redox potential, non-phenolic substrates have to be oxidized by other mediators. Metabolites such as 3-hydroxyanthranilate can mediate oxidation in *Pycnoporus cinnabarinus* (Eggert et al. 1997), and also lignin degradation products can act as such redox charge transfer molecules (ten Have and Teunissen 2001).

Other extracellular enzymes involved in wood lignin degradation are  $\text{H}_2\text{O}_2$ -generating enzymes which are essential for the peroxidases. Fungal oxidases which can reduce  $\text{O}_2$  to  $\text{H}_2\text{O}_2$  include glyoxal oxidase, glucose 1-oxidase, methanol-oxidase and aryl-alcohol oxidase (Zhao and Janse 1996). Flavin is often used as cofactor, with the exception of, e.g. copper-containing glyoxal oxidase from *P. chrysosporium*. Cellobiose dehydrogenase oxidizes soluble cello- and mannodextrine and uses a wide spectrum of electroacceptors ( $\text{Fe}^{3+}$ ,  $\text{Cu}^{2+}$ , quinone and phenoxy radicals). Proposed roles of CDH in the ligninolytic system which have been discussed comprise (i) the reduction of aromatic radicals formed by ligninolytic enzymes, thereby preventing repolymerization and supporting lignin degradation, (ii) the production of  $\bullet\text{OH}$  radicals via a Fenton-type reaction to modify cellulose hemicellulose and lignin and (iii) a cooperation with the manganese peroxidases to make the abundant non-phenolic components of lignin accessible for MnP and laccases. The role of ROS in the initial attack of lignin has also been discussed (Hammel et al. 2002) and was reviewed already in the brown-rot section.

## V. Conclusions

Fungal recycling of abundant plant cell wall polymers is an essential process for life on earth. Organisms, enzymes and non-enzymatic components involved have been identified and characterized within the last years. Still, a major challenge for future research is to understand the complex multi-enzyme process of lignocellulose decomposition, and to establish the contribution of each individual component in the overall lignin degradation system.

## References

- Andrade SV, Polizeli MLTM, Terenzi HF, Jorge JA (2004) Effect of carbon source in the biochemical properties of the  $\beta$ -xylosidase produced by *Aspergillus versicolor*. *Process Biochem* 39:1931–1938
- Annis SL, Goodwin PH (1997) Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. *EJPP* 103:1–14
- Aro N, Pakula T, Penttilä M (2005) Transcriptional regulation of plant cell wall degradation by filamentous fungi. *FEMS Microbiol Rev* 29:719–739
- Aspinall GO (1980) Chemistry of cell wall polysaccharides. In: Preiss J (ed) *The biochemistry of plants*. Academic Press, New York, pp 473–500
- Begin P (1990) Molecular biology of cellulose degradation. *Annu Rev Microbiol* 44:219–248
- Berka RM, Kodama KH, Rey MW, Wilson LJ, Ward M (1991) The development of *Aspergillus niger* var. *awamori* as a host for the expression and secretion of heterologous gene products. *Biochem Soc Trans* 19:681–685
- Biely P, Benen J, Heinrichova K, Kester HC, Visser J (1996) Inversion of configuration during hydrolysis of  $\alpha$ -1,4-galacturonidic linkage by three *Aspergillus* polygalacturonases. *FEBS Lett* 382:249–255
- Brett CT, Waldren K (1996) Physiology and biochemistry of plant cell walls. Chapman & Hall, London
- Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J* 3:1–30
- Cervone F, De Lorenzo G, Pressey R, Darvill AG, Albersheim P (1990) Can phaseolus PGIP inhibit pectic enzymes from microbes and plants? *Phytochemistry* 29:447–449
- Cherry JR, Fidantsef AL (2003) Directed evolution of industrial enzymes: an update. *Curr Opin Biotechnol* 14:438–443
- Coutinho PM, Henrissat B (1999) Carbohydrate-active enzymes: an integrated database approach. In: Gilbert HJ, Davies G, Henrissat B, Svensson B (eds) *Recent advances in carbohydrate bioengineering*. Royal Society of Chemistry, Cambridge, pp 3–12
- Cullen D, Kersten PJ (2004) Enzymology and molecular biology of lignin degradation. In: Brambl R, Marzluf GA (eds) *The Mycota vol III. Biochemistry and molecular biology*. Springer, Berlin Heidelberg New York, pp 249–273
- Darvill JE, McNeil M, Darvill AG, Albersheim P (1980) Structure of plant cell walls. XI. Glucuronoarabinogalactan, a second hemicellulose in the primary cell walls of suspension-cultured sycamore cells. *Plant Physiol* 66:1135–1139
- de Vries RP (2003) Regulation of *Aspergillus* genes encoding plant cell wall polysaccharide-degrading enzymes; relevance for industrial production. *Appl Microbiol Biotechnol* 61:10–20
- de Vries RP, Visser J (2001) *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol Mol Biol Rev* 65:497–522
- de Vries RP, Kester HC, Poulsen CH, Benen JA, Visser J (2000) Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharides. *Carbohydr Res* 327:401–410

- de Vries RP, Jansen J, Aguilar G, Parenicova L, Joosten V, Wulfert F, Benen JA, Visser J (2002) Expression profiling of pectinolytic genes from *Aspergillus niger*. FEBS Lett 530:41–47
- D'Ovidio R, Mattei B, Roberti S, Bellincampi D (2004) Polygalacturonases, polygalacturonase-inhibiting proteins and pectic oligomers in plant-pathogen interactions. Biochim Biophys Acta 1696:237–244
- Doyle WA, Blodig W, Veitch NC, Piontek K, Smith AT (1998) Two substrate interaction sites in lignin peroxidase revealed by site-directed mutagenesis. Biochemistry 37:15097–15105
- Durand H, Clanet H, Tiraby G (1988) Genetic improvement of *Trichoderma reesei* for large scale cellulase production. Enzyme Microbiol Technol 10:341–346
- Ebringerova A, Hienze T (2000) Xylan and xylan derivatives – biopolymers with valuable properties. 1. Naturally occurring xylyans, isolation procedures and properties. Macromol Rapid Commun 21:542–556
- Eggert C, Temp U, Eriksson KE (1997) Laccase is essential for lignin degradation by the white-rot fungus *Pycnoporus cinnabarinus*. FEBS Lett 407:89–92
- Enoki A, Tanaka H, Itakura S (2003) Physical and chemical characteristics of glycopeptide from wood decay fungi. In: Goodell B, Nicholas DD, Schultz TP (eds) Wood deterioration and preservation: advances in our changing world. American Chemical Society Publication, Washington, DC, pp 140–153
- Eriksson K-EL, Blanchette RA, Ander P (1990) Microbial and enzymatic degradation of wood components. Springer, Berlin Heidelberg New York
- Foreman PK, Brown D, Dankmeyer L, Dean R, Diener S, Dunn-Coleman NS, Goedegebuur F, Houfek TD, England GJ, Kelley AS, Meerman HJ, Mitchell T, Mitchinson C, Olivares HA, Teunissen PJ, Yao J, Ward M (2003) Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma reesei*. J Biol Chem 278:31988–31997
- Gold MH, Youngs HL, Gelpke MD (2000) Manganese peroxidase. Metal Ions Biol Syst 37:559–586
- Goodell B (2003) Brown-rot fungal degradation of wood: our evolving view. In: Goodell B, Nicholas DD, Schultz TP (eds) Wood deterioration and preservation: advances in our changing world. American Chemical Society Publication, Washington, DC, pp 97–118
- Gotesson A, Marshall JS, Jones DA, Hardham AR (2002) Characterization and evolutionary analysis of a large polygalacturonase gene family in the oomycete plant pathogen *Phytophthora cinnamomi*. Mol Plant Microbe Interact 15:907–921
- Grishutin SG, Gusakov AV, Markov AV, Ustinov BB, Semenova MV, Sinitsyn AP (2004) Specific xyloglucanases as a new class of polysaccharide-degrading enzymes. Biochim Biophys Acta 1674:268–281
- Hammel KE, Kalyanaraman B, Kirk TK (1986) Oxidation of polycyclic aromatic hydrocarbons and dibenz[*p*]-dioxins by *Phanerochaete chrysosporium* ligninase. J Biol Chem 261:16948–16952
- Hammel KE, Kapich AN, Jensen KA, Ryan ZC (2002) Reactive oxygen as agents of wood decay by fungi. Enzyme Microbial Technol 30:445–453
- Hasper AA, Dekkers E, van Mil M, van de Vondervoort PJ, de Graaff LH (2002) EgIC, a new endoglucanase from *A. pergillus niger* with major activity towards xyloglucan. Appl Environ Microbiol 68:1556–1560
- Hatakk A (1994) Lignin-modifying enzymes from selected white-rot fungi-production and role in lignin degradation. FEMS Microbiol Rev 13:125–135
- Heckman DS, Geiser DM, Eidell BR, Stauffer RL, Kardos NL, Hedges SB (2001) Molecular evidence for the early colonization of land by fungi and plants. Science 293:1129–1133
- Henrissat B, Bairoch A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 293:781–788
- Higuchi T (1990) Lignin biochemistry: biosynthesis and biodegradation. Wood Sci Technol 24:23–63
- Izydorczyk MS, Biliaderis CD (1995) Cereal arabinoxylans: advances in structure and physicochemical properties. Carbohydr Polym 28:33–48
- Jenkins J, Pickersgill R (2001) The architecture of parallel  $\beta$ -helices and related folds. Progr Biophys Mol Biol 77:111–175
- Jurnak F, Kita N, Garrett M, Heffron SE, Scavetta R, Boyd C, Keen N (1996) Functional implications of the three-dimensional structures of pectate lyases. In: Visser J, Voragen AGJ (eds) Pectin and pectinases. Elsevier, Amsterdam, pp 295–308
- Kersten PJ (1990) Glyoxal oxidase of *Phanerochaete chrysosporium*: its characterization and activation by lignin peroxidase. Proc Natl Acad Sci USA 87:2936–2940
- Kirk TK, Farrell RL (1987) Enzymatic “combustion”: the microbial degradation of lignin. Annu Rev Microbiol 41:465–505
- Kormelink RJ, Lefebvre B, Strozyk F, Voragen AG (1993) The purification and characterisation of an acetyl xylan esterase from *Aspergillus niger*. J. Biotechnol 27:267–282
- Kubicek CP, Harman GE (1998) *Trichoderma & Gliocladium*. Taylor & Francis, London
- Mandels M, Weber J (1969) The production of cellulases. Adv Chem Ser 95:391–413
- Markovic O, Janecek S (2001) Pectin degrading glycoside hydrolases of family 28: sequence-structural features, specificities and evolution. Protein Eng 14:615–631
- Martinez AT (2002) Molecular biology and structure-function of lignin-degrading heme peroxidases. Enz Microb Technol 30:425–444
- Martinez D, Larondo LF, Putnam N, Gelpke MD, Huang K, Chapman J, Helfenbein KG, Ramaiya P, Detter JC, Larimer F, Coutinho PM, Henrissat B, Berka R, Cullen D, Rokhsar D (2004) Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nature Biotechnol 22:695–700
- Martinez AT, Speranza M, Ruiz-Duenas FJ, Ferreira P, Camarero S, Guillen F, Martinez MJ, Gutierrez A, del Rio JC (2005) Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int Microbiol 8:195–204
- McDonough MA, Kadirvelraj R, Harris P, Poulsen JC, Larsen S (2004) Rhamnogalacturonan lyase reveals a unique three-domain modular structure for polysaccharide lyase family 4. FEBS Lett 565:188–194
- McQueen-Mason SJ, Cosgrove DJ (1995) Expansin mode of action on cell walls. Analysis of wall hydrolysis, stress relaxation, and binding. Plant Physiol 107:87–100

- McQueen-Mason S, Durachko DM, Cosgrove DJ (1992) Two endogenous proteins that induce cell wall extension in plants. *Plant Cell* 4:1425–1433
- O'Neill MA, Selvendran RR (1985) Hemicellulosic complexes from the cell walls of runner bean (*Phaseolus coccineus*). *Biochem J* 227:475–481
- O'Neill MA, York WS (2003) The composition and structure of plant primary walls. In: Rose JKC (ed) *The plant cell wall*. Blackwell, Oxford, pp 1–54
- Parenicova L, Kester HC, Benen JA, Visser J (2000) Characterization of a novel endopolygalacturonase from *Aspergillus niger* with unique kinetic properties. *FEBS Lett* 467:333–336
- Pauly M, Albersheim P, Darvill A, York WS (1999) Molecular domains of the cellulose/xyloglucan network in the cell walls of higher plants. *Plant J* 20:629–639
- Pérez S, Mazeau K, du Penhoat CH (2000) Pectins: structure, biosynthesis, and oligogalacturonide-related signalling. *Plant Physiol Biochem* 38:37–55
- Piontek K, Glumoff T, Winterhalter K (1993) Low pH crystal structure of glycosylated lignin peroxidase from *Phanerochaete chrysosporium* at 2.5 Å resolution. *FEBS Lett* 315:119–124
- Piontek K, Smith AT, Blodig W (2001) Lignin peroxidase structure and function. *Biochem Soc Trans* 29:111–116
- Polizeli ML, Rizzatti AC, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. *Appl Microbiol Biotechnol* 67:577–591
- Poulos TL, Edwards SL, Wariishi H, Gold MH (1993) Crystallographic refinement of lignin peroxidase at 2 Å. *J Biol Chem* 268:4429–4440
- Puls J, Schuseil J (1993) Chemistry of hemicelluloses: relationship between hemicellulose structure and enzymes required for hydrolysis. In: Coughlan MP, Hazlewood GP (eds) *Hemicellulose and hemicellulase*. Portland Press, London, pp 1–28
- Remy W, Taylor TN, Hass H, Kerl H (1994) Four hundred-million-year-old vesicular arbuscular mycorrhizae. *Proc Natl Acad Sci USA* 91:11841–11843
- Ridley BL, O'Neill MA, Mohnen D (2001) Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* 57:929–967
- Rouvinen J, Bergfors T, Teeri T, Knowles JK, Jones TA (1990) Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. *Science* 249:380–386
- Saloheimo M, Paloheimo M, Hakola S, Pere J, Swanson B, Nyssonen E, Bhatia A, Ward M, Penttilä M (2002) Swollenin, a *Trichoderma reesei* protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. *Eur J Biochem* 269:4202–4211
- Schmoll M, Kubicek CP (2005) *ooc1*, a unique gene expressed only during growth of *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) on cellulose. *Curr Genet* 48:126–133
- Schmoll M, Zeilinger S, Mach RL, Kubicek CP (2003) Identification of genes involved in cellulose signalling to cellulase gene expression in *Hypocrea jecorina* using a rapid subtraction hybridization protocol RaSH. *Fungal Genet Biol* 41:877–887
- Schols HA, Vierhuis E, Bakx EJ, Voragen AG (1995) Different populations of pectic hairy regions occur in apple cell walls. *Carbohydr Res* 275:343–360
- Shoseyov O, Shani Z, Levy I (2006) Carbohydrate binding modules: biochemical properties and novel applications. *Microbiol Mol Biol Rev* 70:283–295
- Simon L, Bousquet J, Lévesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. *Nature* 363:67–69
- Smith SE, Read DJ (1997) *Mycorrhizal symbiosis*. Academic Press, San Diego, CA
- Stephen (1982) Other plant polysaccharides. In: Aspinall GO (ed) *The polysaccharides*. Academic Press, New York, pp 97–123
- Sundaramoorthy M, Kishi K, Gold MH, Poulos TL (1994) The crystal structure of manganese peroxidase from *Phanerochaete chrysosporium* at 2.06-Å resolution. *J Biol Chem* 269:32759–32767
- Sundaramoorthy M, Kishi K, Gold MH, Poulos TL (1997) Crystal structures of substrate binding site mutants of manganese peroxidase. *J Biol Chem* 272:17574–17580
- Teeri T (1997) Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. *Trends Biotechnol* 15:160–167
- ten Have R, Teunissen PJ (2001) Oxidative mechanisms involved in lignin degradation by white-rot fungi. *Chem Rev* 101:3397–3413
- Tenkanen M (1998) Action of *Trichoderma reesei* and *Aspergillus oryzae* esterases in the deacetylation of hemicelluloses. *Biotechnol Appl Biochem* 27:19–24
- Tenkanen M, Siika-aho M (2000) An α-glucuronidase of *Schizophyllum commune* acting on polymeric xylan. *J Biotechnol* 75:149–161
- van den Broek LAM, Schols HA, Searle-van Leeuwen MJF, van Laere KMJ, Voragen AGJ (1996) An exogalacturonase from *Aspergillus aculeatus* able to degrade xylogalacturonan. *Biotechnol Lett* 18:707–712
- Vidal S, Doco T, Williams P, Pellerin P, York WS, O'Neill MA, Glushka J, Darvill AG, Albersheim P (2000) Structural characterization of the pectic polysaccharide rhamnogalacturonan II: evidence for the backbone location of the aceric acid-containing oligoglycosyl side chain. *Carbohydr Res* 326:277–294
- Vincken JP, Schols HA, Oomen RJ, McCann MC, Ulvsbo P, Voragen AG, Visser RG (2003) If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiol* 132:1781–1789
- Wong KK, Tan LU, Saddler JN (1988) Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. *Microbiol Rev* 52:305–317
- Zamocky M, Ludwig R, Peterbauer C, Hallberg BM, Divine C, Nicholls P, Haltrich D (2006) Cellobiose dehydrogenase – a flavocytochrome from wood-degrading, phytopathogenic and saprotropic fungi. *Curr Protein Pept Sci* 7:255–280
- Zhao J, Janse B (1996) Comparison of H<sub>2</sub>O<sub>2</sub> producing enzymes in selective white rot fungi. *FEMS Microbiol Lett* 139:215–221

---

## Biosystematic Index

- Acari, 202  
*Acremonium*, 216  
*Adorphorus couloni*, 175  
*Agaricus*, 78  
    *arvensis*, 78  
    *bisporus*, 77, 330  
    *campestris*, 78  
    *edulis*, 78  
    *haemorrhoidarius*, 78  
    *xanthodermus*, 78  
*Agrocybe rivulosa*, 115  
*Alternaria*, 289  
    *alternata*, 74  
    *solani*, 193  
*Amanita*, 209  
    *caesarea*, 115  
    *muscaria*, 78, 113, 116, 237, 238, 240  
    *phalloides*, 116  
    *rubescens*, 78  
    *strobiliformis*, 78  
*Ampelomyces quisqualis*, 130  
Angiosperms, 232, 244  
Annelida, 202, 203  
*Anoplophora glabripennis*, 178  
*Anthurus archeri*, 116  
*Antrodia vaillantii*, 72  
*Aphelenchoides*, 209  
*Aphis gossypii*, 175  
Archaea, 7  
*Armillaria*, 61, 77, 209  
    *ectypa*, 119  
    *mellea*, 106  
*Arthrobotrys*, 303  
Arthropoda, 202  
*Aschersonia aleyrodis*, 160, 171  
*Ascobolus*, 109, 112  
Ascomycota, 9, 11, 139, 230–233, 303, 310  
*Aspergillus*, 7, 74, 220, 289, 326, 332, 334  
    *aculeatus*, 332  
    *flavus*, 163, 177  
    *fumigatus*, 296  
    *niger*, 288, 289, 328, 332, 333  
    *wentii*, 58  
Astigmata, 203  
*Aureobasidium*, 289, 291  
    *pullulans*, 70, 75, 303  
  
*Bacillus*, 191, 193  
    *amyloliquefaciens*, 192–193  
    *cereus*, 193  
    *circulans*, 289  
  
*subtilis*, 192–193, 223  
*thuringiensis*, 164, 165  
*Balansia*, 216  
    *cyperi*, 218  
Basidiomycota, 9, 11, 230, 232, 236  
    heterobasidiomycetes, 232  
    homobasidiomycetes, 231, 232  
*Bdellidae*, 203  
*Beauveria*, 174, 175  
    *bassiana*, 160, 174, 175, 177–180  
    *brongniartii*, 163, 70, 175–176, 178  
    *sulfurescens*, 179  
    *bassiana*, 170, 171, 176  
*Bjerkandera*, 71  
*Boletus*, 78, 209  
    *obscurecoccineus*, 113  
    *edulis*, 120  
*Bombyx mori*, 177  
*Botryodiplodia theobromae*, 193  
*Botryosphaeria*, 221  
*Botrytis*, 207  
    *cinerea*, 196, 332  
*Bovista*, 109, 112  
    *verrucosa*, 112  
*Brassica napus*, 189  
*Buergerula*, 304  
  
*Cadophora* (syn. *Phialophora*) *finlandia*, 232  
*Calluna*, 75  
*Candida*, 7, 289  
*Cantharellus formosus*, 121  
*Carex walteriana*, 317  
*Castanea dentata*, 116  
*Cenococcum geophilum*, 231  
*Cephalanthera*, 246  
    *damasonium*, 246  
*Ceratobasidium cornigerum*, 246  
*Ceratocystis*, 335  
    *ulmi*, 116  
*Cercospora*, 116  
*Ceriporiopsis subvermispora*, 337  
*Chaetomium*, 335  
Chironomidae, 203  
Chytridiomycota, 8, 10, 13  
*Cladosporium*, 75, 206, 220, 289  
    *cladosporiooides*, 74, 296  
    *herbarum*, 289, 303  
*Claviceps*, 216  
    *purpurea*, 218  
Clavicipitaceae, 216  
*Coelomomyces*, 159

- Collembola*, 202, 203, 206  
*Colletotrichum*, 221  
  *musae*, 222  
*Collybia*, 293  
  *johNSTONII*, 61  
*Conidiobolus*, 159  
*Coprinus comatus*, 78  
*Cordyceps*, 159, 160, 171, 216  
*Coriolopsis*, 71  
*Coriolus versicolor*, 293  
*Corniothyrium minitans*, 131  
*Cortinarius*, 59  
  *metallicus*, 113  
*Cryptonectria parasitica*, 116  
*Cunninghamella*, 71  
*Curtobacterium flaccumfaciens*, 223  
*Curvularia*, 222  
*Cydia pomonella*, 170  
*Cymatoderma elegans*, 113  
*Cyperus rotundus*, 218  
*Cypripedium*, 245
- Dactylaria*, 303  
*Delia floralis*, 178  
  *radicum*, 178  
*Dendrobaena octaedra*, 59  
*Dermocybe*, 59  
*Dermoloma*, 119  
*Dichanthelium lanuginosum*, 222  
*Disciseda*, 119  
*Discula quercina*, 220  
  *umbrinella*, 220  
*Dorylaimida*, 202
- Encephalitozoon*, 7  
*Enchytraeidae*, 202  
*Endogone*, 231  
*Entomophthorales*, 171  
*Entamoeba*, 7  
*Entoloma*, 106, 119  
*Entomophaga*, 159  
  *aulicæ*, 163, 176  
  *grylli*, 163  
  *maimaiga*, 164, 200, 201  
*Entomophthora muscae*, 163  
*Epichlöe*, 216  
  *typhina*, 216  
*Epicoccum*, 206  
  *nigrum*, 303  
*Epipactis*, 246  
*Erianthus giganteus*, 306  
*Erica*, 75  
*Ericales*  
  *Arbutoideae*, 232  
  *Ericaceae*, 232  
  *Monotropoideae*, 232, 245  
*Eremaeidae*, 203  
*Erwinia amylovora*, 191  
*Erynia*, 159  
  *radicans*, 163  
*Eucalyptus urophylla*, 195  
*Eurygaster integriceps*, 178
- Flammulina*, 209
- Folsomia*, 206–208  
*Fomes fomentarius*, 115, 335  
*Fusarium*, 7, 61, 71, 74, 208  
  *graminearum*, 219  
  *moniliforme*, 219  
  *oxysporum*, 193, 332
- Gamasina*, 203  
*Gaeumannomyces graminis*, 54  
*Ganoderma australe*, 337  
*Geastrum*, 119  
*Gibberella fujikuroi*, 219  
*Gigaspora* spp., 56  
*Gliocladium*, 7, 132  
*Globodera*, 147, 153  
*Gloeophyllum trabeum*, 335, 336  
*Glomerales*, 232  
*Glomeromycota*, 9, 230–232  
*Glomus*, 56, 236  
  *constrictum*, 56  
  *etunicatum*, 56  
  *fasciculatum*, 289  
  *intraradices*, 231, 236, 239, 240, 243  
  *mosseae*, 236, 239, 240  
  *versiforme*, 236  
*Goodyera repens*, 246  
*Guignardia*, 221  
*Gymnosperms*  
  *Pinaceae*, 232  
*Gynoxis oleifolia*, 220
- Hebeloma cylindrosporum*, 237, 240  
*Hemileia vastatrix*, 116  
*Heterobasidion annosum*, 337  
*Heterodera*, 147  
*Histiostomatidae*, 203  
*Humicola*, 295, 296, 328  
*Hygrocybe*, 119  
*Hymenoscyphus ericae*, 232  
*Hyphoderma setigerum*, 293  
*Hypholoma*, 208  
*Hypoerea*, 132  
  *jecorina*, 328, 329  
*Hypoxyylon fragiforme*, 220
- Irpex*, 71  
  *lacteus*, 337
- Juncus*, 306  
  *effusus*, 303, 307, 308, 318  
  *roemerianus*, 305, 306
- Laccaria*, 238  
  *amethystina*, 77  
  *bicolor*, 236  
  *accata*, 78  
*Lagenidium giganteum*, 159, 163, 171  
*Larix decidua*, 115  
*Lecanicillium*, 160  
  *longisporum*, 160, 170, 171, 174–176, 179  
  *muscarium*, 170, 174, 179  
*Lentinus*, 109  
*Leptinotarsa decemlineata*, 170  
*Leptosphaeria*, 189, 303

- coniothyrum*, 296  
*Limodorum*, 246  
*Littorina*, 206  
*Locustana pardalina*, 175  
*Lolium*, 218  
*Lophiodermium piceae*, 220  
*Lumbricus*, 203  
  *terrestris*, 59  
*Lumbricidae*, 202  
*Lycoperdon*, 78  
  *americanum*, 113  
  *echinatum*, 113  
  *perlatum*, 78  
*Lycoriella*, 207  
*Lymantria dispar*, 164
- Macrophomina phaseolina*, 193  
*Manduca sexta*, 180  
*Marasmiellus*, 290, 293, 303  
*Marasmius*, 204, 290, 293  
  *androsaceus*, 296  
  *oreadus*, 78  
*Medicago truncatula*, 236  
*Meloidogyne*, 147  
*Melolontha melolontha*, 175  
*Metarhizium*, 172, 174, 177, 178  
  *anisopliae*, 159, 160, 170, 171, 175, 176, 178–180  
  var. *acridum*, 175  
  var. *majus*, 160  
  *flavoviride*, 173  
*Mesostigmata*, 203  
*Monotropa*, 245  
*Mortierella*, 208  
*Mucor*, 71, 74  
  *haemalis*, 159  
  *heimalis*, 296  
*Mutinus ravenelii*, 116  
  *elegans*, 116  
*Mycena*, 204, 293  
  *pura*, 78  
*Mycetophilidae*, 203  
*Mycosphaerella*, 304, 308, 317
- Nectria invertum*, 74  
*Nematoda*, 202, 203  
*Neocallimastix*, 7, 8  
*Neotyphodium*, 216  
  *lolii*, 218  
*Nilaparvata lugens*, 178  
*Nuclearia*, 8  
*Nomuraea rileyi*, 160, 163, 170, 171
- Oniscus*, 203  
*Onychiurus*, 205, 207, 208  
*Oomycota*, 139  
*Oppidae*, 203  
*Orchidaceae*, 245  
*Ostrinia nubilalis*, 175  
*Otiorhynchus sulcatus*, 160  
*Oxyporus nobilissimus*, 119
- Paecilomyces*, 7, 205, 289  
  *arinosus*, 173  
  *fumosoroseus*, 170, 172, 173, 176, 180
- lilacinus*, 74  
*Pandora*, 159  
  *neoaphidis*, 160, 171  
*Panellus copelandi*, 303  
*Pantoea agglomerans*, 192, 193  
*Paxillus*, 205, 206, 234  
*Penicillium*, 71, 74, 206, 220, 288, 289, 326  
  *hordei*, 296  
  *janczewskii*, 296  
  *janthinellum*, 72  
  *purpurogenum*, 296  
*Pestalotia*, 221  
*Phaeosphaeria*, 304  
  *halima*, 304, 308  
  *nodorum*, 107  
  *spartincola*, 304, 307, 308, 317  
*Phalansterium*, 7  
*Phallus hadriani*, 112  
*Phanerochaete*, 71  
  *chrysogenum*, 326, 328  
  *chrysosporium*, 70, 72, 337, 338  
*Phellinus pini*, 337  
  *weiri*, 61  
*Philaophora*, 335  
*Phlebia adiata*, 293  
  *radiata*, 337  
  *remellosa*, 337  
*Phoma*, 130, 206, 289  
  *fimetri*, 74  
  *typharum*, 303  
*Phoridae*, 203  
*Phthiracaridae*, 202  
*Phragmites*, 307, 318  
  *australis*, 303, 304, 307–309, 316–318  
*Phyllosticta*, 221  
  *multicorniculata*, 220  
*Phytophthora*, 61  
*Phytophthora infestans*, 116, 222, 224  
*Picea*, 206  
  *abies*, 115  
*Pichia*, 7  
*Pinus*, 206  
  *sylvestris*, 237  
*Piromyces*, 7  
*Pisolithus*, 234, 238  
  *tinctorius*, 238  
*Pleurotus*, 71, 337  
  *nebrodensis*, 118  
  *ostreatus*, 72  
*Plicaturopsis crispa*, 115  
*Plutella xylostella*, 176  
*Podaxis pistillaris*, 113  
*Populus fremontii*, 220  
  *tremula x tremuloides*, 238  
*Prostigmata*, 203  
*Pseudomonas*, 191  
  *aeruginosa*, 196  
  *chlororaphis*, 193  
  *fluorescens*, 191, 195  
*Psilocybe cyanescens*, 115  
*Puccinia chondrillina*, 116  
  *triticina*, 224  
*Pycnoporus cinnabarinus*, 115, 338  
*Pyrenophora tritici-repentis*, 224

- Pythium nunn*, 130
- Ralstonia solanacearum*, 195
- Ramalina menziesii*, 290
- Resinicum bicolor*, 289
- Rhabdocline parkeri*, 220
- Rhizoctonia solani*, 54, 55, 193
- Rhizographus*, 291
- Rhizopogon*, 234
- Rhizopus*, 74, 294
- Rhodospirillum*, 13
- Rhytismataceae*, 221
- Rozella*, 8  
    *allomycis*, 8
- Russula*, 78  
    *castanopsisidis*, 115  
    *densifolia*, 115  
    *laurocerasi*, 115  
    *lepidia*, 115  
    *lilacea*, 115
- Saccharomyces*, 7
- Saccobolus*, 109
- Sarcodon imbricatus*, 117
- Schistocerca gregaria*, 160, 172, 175
- Schizophyllum commune*, 111, 335
- Schizopora flavigipora*, 115
- Sciariidae*, 203
- Sclerotiorina*, 109, 112
- Sclerotinia sclerotiorum*, 189
- Sclerotium rolfsii*, 193  
    *sclerotiorum*, 332
- Sebacina*, 232
- Serpula lacrimans*, 335
- Solanaceae*, 236
- Spartina*, 304–307, 317  
    *alterniflora*, 303, 304–308, 316
- Sporidesmium sclerotivorum*, 129
- Sporobolomyces*, 289
- Sporothrix insectorum*, 170
- Squamanita odorata*, 112
- Stemphylium*, 294
- Stenotrophomonas maltophilia*, 72
- Sterium hirsutum*, 293
- Stigmaeidae*, 203
- Stropharia aurantiaca*, 115  
    *rugosoannulata*, 115
- Suillus*, 234
- variegatus*, 209,
- Talaromyces flavus*, 131
- Tappania*, 4
- Tarsonemidae*, 203
- Taxomyces andreanae*, 224
- Terminalia*, 60
- Thelephora*, 109
- Torubiella*, 171
- Tracipleistophora*, 7
- Trametes*, 71  
    *versicolor*, 337
- Trichoderma*, 7, 74, 128, 153, 206, 294, 326, 330  
    *reesei*, 328  
    *viride*, 296
- Tricholoma matsutake*, 121
- Tsuga mertensiana*, 61
- Tuber*, 238  
    *melanospermum*, 113
- Tulasnella*, 232
- Tulasnellaceae*, 245
- Tulostoma*, 119
- Tydaeidae*, 203
- Tylenchida*, 202
- Tylospora fibrillosa*, 106
- Typha*, 303, 307, 318  
    *angustifolia*, 306, 307  
    *latifolia*, 303
- Tyromyces*, 336
- Ulocladium consortiale*, 296
- Vaccinium*, 75
- Varroa destructor*, 178
- Venturia ditricha*, 220
- Verticillium*, 153, 296  
    *lecanii*, 170, 172
- Vuilleminia comedens*, 293
- Xylaria*, 221  
    *hypoxylon*, 337
- Zonocerus variegatus*, 175
- Zoophthora radicans*, 164, 176
- Zygomycota*, 10, 231  
    *Endogonales*, 232

---

## Subject Index

- acid rain, 58, 72  
actinomycetes, 259, 275, 295  
aggregatusphere, 49, 50  
agriculture, 223, 265  
agrochemicals, 258, 275  
air pollution, 58  
airborne pollutants, 72  
algae, 288, 289  
Allee effect, 179  
alphaproteobacteria, 3, 5, 7, 11–14  
amino acid, 237–239, 241  
    glutamine, 238, 241  
    glycine, 237, 246  
    reduction, 239  
amitochondriate, 7  
ammonium, 237–239  
antibiosis, 128, 192  
    direct antagonism, 192  
    limitation in nutrients, 193  
    low-molecular weight, 192  
antifungal agents, 258, 260, 261, 264  
antifungal volatiles, 194  
    benzothiazole, 194  
    decimate ascospore production, 194  
    good soil amendments, 194  
    nonanal, 194  
    reduce apothecial formation, 194  
    sclerotial control, 194  
aquatic ecosystems, 301  
aquatic hyphomycetes, 309–311, 313–315, 317  
arbuscula mycorrhiza, 74, 75, 229, 230, 231, 233–236, 237–243, 245  
arbuscule, 236, 240  
arbutoid mycorrhiza, 229, 232  
Arthropoda, 202  
ascostromata, 265, 267  
  
bacteria, 7, 49, 56, 71, 74, 287, 289, 295, 296, 316  
bacterial, 289  
    aggregates, 191  
    endophytic mode, 191  
    extracellular polysaccharide production, 191  
    flagellar motility, 191  
    pigmentation, 191  
    production of biosurfactants, 191  
baculoviruses, 180  
balsam fir, 220  
barcode DNA, 20  
barley, 236  
barrier, 113  
BCA
- application, 192  
application at the cotyledon stage, 192  
appropriate place and time, 192  
bacteria onto the petals, 192  
host is susceptible, 192  
pathogen life cycle, 192  
bikont, 7  
bioassay, 171, 260  
bioavailability, 70  
biodegradation, 71  
biodiversity, 31, 32, 221, 259  
    neutral theory, 221  
bioenergetic electron flow, 11, 13  
    diagram, 8, 14  
biogenic minerals, 76  
biogeochemical cycles, 75  
biogeography, 105, 221  
bioindicator, 78, 79  
biological control, 223, 127, 190  
    antagonist, 190  
    antifungal antibiotics, 190  
    bacteria, 190  
    enzymes, 190  
    mechanism, 190  
    siderophores, 190  
    systemic resistance, 190  
    volatiles, 190  
biomarkers, 69  
biomass, 49, 304, 311, 315, 316  
    bacterial, 56, 71, 316  
    fungal, 49–51, 56, 61, 304, 311, 315, 316  
    microbial, 49–51, 56, 59, 61  
biomimetic system, 139  
biopesticide, 165, 177, 178  
bioprospecting, 223  
bioremediation, 71  
biosorption, 74, 76  
biosynthesis, 258, 275  
biotechnological application, 325  
    enzyme, 325  
    fungi, 325  
biotechnology, 223  
biotrophic, 128  
birch, 220  
BLAST, 221  
blastospores, 173, 174  
brown-rot fungi, 72, 335  
  
C:N ratio, 290, 291  
C:nutrient ratio, 294  
canola, 189

- blackleg, 189  
 stem rot, 189  
 vegetable oil, 189
- carbohydrate binding module, 328
- carbohydrates, 235, 239–241  
     fructose, 240  
     glucose, 240, 243  
     hexose, 240, 241  
     monosaccharide, 240, 241  
     sucrose, 240
- carbon 234, 235, 238, 239, 241–245
- CAZy database, 326
- cellulose, 203, 291, 326, 327  
     -binding domain, 328  
     to lignin ratio, 291
- chelators, 236  
     defense, 260, 265, 266  
     ecology, 259, 266, 274
- chitin, 202
- chitinases, 89, 131–135, 140–142, 147, 150, 153, 154, 172, 195–197
- Choanozoa, 7, 8
- coevolution, 217
- commercial producers, 165, 170
- common mycelial networks, 242, 243, 246
- community, 32, 218, 304  
     structure, 204, 311
- competition, 31, 128
- compost, 19, 87, 135–136, 173, 175
- conservation, 105  
     of fungi, 117
- convergence, 4
- copper, 70
- coprophilous, 271  
     fungi, 261–264
- cottonwood, 220
- Cryptostigmata, 202
- cultivation, 172  
     diphasic fermentation, 173  
     submerged liquid fermentation, 172  
     surface cultivation, 173
- dark septate endophytes, 233, 242
- decline and extinction of fungi, 116
- decomposers, 31, 301, 303, 309
- decomposition, 218, 301, 317  
     wood, 313
- delignification, 336, 337  
     selective, 337  
     simultaneous, 336
- dereplication, 260
- destruxin, 177
- detritusphere, 49, 50
- DGGE, 17, 18, 21, 25, 136, 215, 311
- diel periodicity, 308, 316
- disease, 54, 61  
     laminated root rot, 61  
     take-all, 54
- disjunct distribution, 110
- distribution  
     atlas, 113  
     maps, 105, 113  
     patterns, 105, 107, 109, 110, 304  
     changes in pattern, 115
- continental patterns, 106, 112  
 expansion areas, 115  
 global patterns, 106, 109  
 local patterns, 107, 114  
 national patterns, 113  
 regional patterns, 106, 113
- distributional data, 106
- DOM, 318
- dot maps, 107
- Douglas fir, 60, 214, 220, 221, 294
- drench, 175
- drilosphere, 49, 50
- earthworm, 48, 50, 59  
     invasive, 48, 59
- ectoendomycorrhiza, 229
- ectomycorrhiza, 72, 229, 231–246
- ectomycorrhizal fungi, 74, 75
- electrostatic sprayers, 175
- elicitors, 134
- endemics, 111
- endophyte, 175, 213, 222, 260, 268, 269  
     bacteria, 223  
     evolution, 216  
     infection, 220  
     inoculum, 220  
     life cycles, 216  
     transmission, 220
- endospores of *Bacillus*, 192
- endosymbiont, 3, 7, 13
- environmental pollution  
     acidification, 120  
     forests on acidic soils, 120  
     grassland, 119  
     nitrogen accumulation, 120  
     old-growth forests, 119  
     peat bogs, 119  
     pristine, 119  
     sand dunes, 119
- enzyme, 150, 154, 287, 291, 293, 296, 307, 314, 327  
      $\alpha$ -L-arabinofuranosidase, 334, 335  
      $\alpha$ -galactosidase, 334  
      $\alpha$ -glucuronidase, 334  
      $\beta$ -N-acetylglucosaminidase, 295  
      $\beta$ -galactosidase, 335  
      $\beta$ -glucosidases, 328  
      $\beta$ -mannosidases, 331  
      $\beta$ -xylosidases, 330  
     acetyl xylan esterase, 334  
     acetylglucomannan esterase, 334  
     acid phosphatase, 293  
     arabinoxylan arabinofuranohydrolase, 334  
     aryl-alcohol oxidase, 338  
     cellobiohydrolases, 327  
     cellobiose dehydrogenase, 336, 338  
     collagenase, 154  
     endo- $\beta$ -1,4-galactanase, 331, 335  
     endo- $\beta$ -1,6-galactanase, 335  
     endo-acting, 327  
     endo-cellulase, 295  
     endoarabinase, 335  
     endoglucanases, 328, 330  
     endopolysaccharide hydrolase, 332  
     endorhamnogalacturonase, 333

- endoxylanases, 330  
 exo-acting, 327  
 exo-cellulase, 295  
 exoarabinase, 335  
 exogalactanase, 335  
 exopolygalacturonase, 332, 333  
 exorhamnogalacturonase, 334  
 feruloyl esterase, 334  
 glucose 1-oxidase, 338  
 glyoxal oxidase, 338  
 invertases, 240  
 keratinase, 154  
 laccase, 337  
 lignin peroxidase, 337  
 lignocellulase, 293  
 manganese peroxidase, 337  
 methanol-oxidase, 338  
*N*-acetylglucosaminase, 293  
 pectate lyase, 332, 333  
 pectin acetyl esterase, 334  
 pectin lyase, 332, 333  
 pectin methyl esterase, 333, 334  
 pectinase, 332  
 peroxidase, 337  
 phenol oxidase, 337  
 phosphatase, 235, 236  
 phosphoesterase, 236  
 phytase, 236  
 protease, 151  
 rhamnogalacturonan acetyl esterase, 334  
 rhamnogalacturonan lyase, 332, 334  
 rhamnogalacturonase, 332  
 serine protease, 150, 151  
 subtilisin, 151  
 xylan- $\alpha$ -1,2-glucuronosidase, 334  
 xyloglucan hydrolases, 330
- epiparasites, 245  
 epiphyte, 216, 221  
 ergosterol, 233, 304, 305  
 ergot, 265, 266  
 ericoid mycorrhiza, 229, 242  
 eukaryotes, 3-5, 7, 11-14, 86
  - amitochondriate eukaryotes, 5
- european beech, 220  
 European Council for Conservation of Fungi, 118  
 European Union, 177  
 extinction, 116  
 extraradical mycorrhizal mycelium, 230, 233-235, 237-239, 241
- Fenton reaction, 336  
 fermentation, 265, 275  
 ferns, 245  
 fingerprint methods, 22-24
  - AFLP, 17, 18, 23, 25
  - DGGE, 17, 18, 21, 25, 136, 215, 311
  - RAPD, 17, 18, 23, 25, 215
  - RFLP, 17, 18, 23, 25, 176, 215, 304, 311
- foliar application, 190
  - biocontrol agents, 190
  - cotyledon, 190
  - petal, 190
  - six-leaf growth, 190
- food preference tests, 204
- food-web, 53  
 formulation, 174  
 fossil, 4, 5, 11, 71, 110, 230, 232, 325, 326  
 FPOM, 302, 318, 319  
 freshwater
  - aquatic fungi, 263
  - marsh, 303
- funal
  - populations, 70, 72, 74
  - to-bacterial ratio, 48, 49, 58
- fungi as
  - biotrophs, 325
  - endoparasites, 149
  - entomopathogens, 150
  - necrotrophs, 325
  - nematophags, 149, 150
  - saprotrophs, 325
- fungicolous, 270, 273
- genet, 115, 242, 243  
 genetic
  - manipulation, 141
  - modification, 179
- geographic barriers, 111  
 geological timescale, 3
  - Archaean, 3, 4
  - Hadean, 3
  - Phanerozoic, 3
  - Proterozoic, 3, 4
- gliovirin, 128, 133  
 glomalin, 52  
 glycogen, 241  
 grass, 216
  - maize, 219
  - ryegrass, 219
  - tall fescue, 219
- grazing intensity, 207  
 green fluorescent protein, 177, 180  
 Green Muscle, 169, 170, 178  
 grid maps, 107  
 gut contents, 204
- Hartig net, 240  
 heavy metals, 48, 296  
 hemicellulose, 96, 307, 314, 326, 329, 331, 334-338
  - arabinogalactan, 329
  - galactoglucomannans, 331
  - galactomannans, 331
  - glucomannans, 331
  - mannan, 331
  - xylans, 330
- gypsy moth, 297  
 herbivores, 218, 222  
 heterokaryosis, 231  
 heterologous isolate, 171  
 homokaryosis, 231  
 homologous isolate, 171, 172  
 homoplasy, 4  
 hot particles, 296  
 hybridization, 18, 24, 25, 217
  - AFLP, 17, 18, 23, 25
  - DGGE, 17, 18, 21, 25, 136, 215, 311
  - FISH, 18, 24, 97, 311
  - RAPD, 17, 18, 23, 25, 215

- RFLP, 17, 18, 23, 25, 176, 215, 304, 311  
 hydrogen hypothesis, 13  
 hydrogenosomes, 11, 12  
 hyperthermophilic bacteria, 5
- immobilization, 293, 294, 313  
 nitrogen lock-up, 294  
 immunological methods, 17, 18, 25  
 ELISA, 18, 19, 305, 311  
 indole diterpenoids, 266  
 induced systemic resistance, 134, 195  
 enhancement of lignification, 195  
 stimulation of host-defence enzymes, 195  
 synthesis of pathogenesis-related (PR) proteins, 195
- infection  
 cotyledons, 189  
 petals, 189  
 stem, 189
- interaction, 214  
 antagonism, 224
- International Union for Conservation of Nature, 118
- introductions of fungi, 116
- invasion, 160  
 appressoria, 162  
 cuticle, 160  
 enzymes, 160  
 germination, 160  
 haemocyte, 162  
 proteases, 162
- ITS, 19, 20, 22-24, 221, 273, 304, 310
- lateral gene transfer, 8, 11, 13  
 lead, 70  
 leaf surface, 191  
 lectin, 138  
 legal protection, 120  
 lichens, 77, 223, 288-290  
 lignin, 57, 58, 291, 307, 312, 314, 335  
 lignin:N ratio, 290, 291  
 lignocellulose, 238  
 lipids, 241  
 fatty acids, 241  
 phospholipid fatty acids, 233  
 triacylglycerides, 241
- litter, 49, 301  
 quality, 49, 50  
 litter/substrate quality, 57
- liverwort, 245  
 LUBILOSA, 178  
 lycophytes, 245  
 lytic enzyme, 140
- maceration, 314  
 macrofungi, 77, 78  
 maize, 236  
 management of habitats, 120  
 mapping, 106  
 of fungi, 105  
 programmes, 106, 113
- melanin, 76, 162, 296
- metal  
 immobilization, 76  
 mobilization, 76  
 pollution, 74
- metalloid  
 transformations, 75
- metalloids, 75, 77, 79  
 arsenic, 75, 77  
 selenium, 75, 76, 77  
 tellurium, 75, 77
- metals, 69, 70, 72-79  
 silver, 76, 77  
 aluminium 76, 77  
 calcium, 76  
 cadmium, 73-78  
 cobalt, 76, 77  
 copper, 73, 74, 75, 77, 78  
 chromium, 74  
 cesium, 73, 76, 77  
 iron 76  
 mercury 74-78  
 potassium, 76  
 magnesium 76  
 manganese 76, 77  
 sodium, 76  
 nickel, 74, 76, 77  
 lead, 74, 76-78  
 strontium, 77  
 zinc, 74-78
- microbial insecticides, 178
- microclimate, 191  
 humidity, 191  
 leaf wetness, 191  
 temperature, 191  
 ultraviolet radiation, 191
- microscopy, 214
- microsporidia, 8
- mineralization, 290, 294, 296, 309
- mitochondria, 3, 5, 8, 11-14,  
 mitosomes, 11
- molecular clock, 4, 11  
 molecular markers, 176  
 monotropoid mycorrhiza, 229, 232  
 morphospecies, 223  
 mosquitoes, 180  
 mutualists, 31, 214  
 mycelial preparations, 174  
 mycoheterotrophic, 244-246  
 cheaters, 245, 246  
 mixotrophy, 245, 246  
 mycoheterotrophy, 244, 245
- mycoinsecticides, 165, 170 179  
 Green MuscleTM, 169, 170, 178  
 LUBILOSA, 170
- mycoparasites, 269, 270, 271  
 mycoparasitism, 128  
 mycophilic, 270
- mycorrhiza(e), 51, 59, 62, 214, 287, 289  
 arbuscular, 51, 52, 55, 56, 60, 289  
 ectomycorrhiza, 51, 52, 53, 58-60, 289, 297  
 extraradical hyphae, 51, 55, 56  
 extramatrical hyphae, 56  
 intraradical hyphae, 51
- mycorrhizal fungi, 78, 289, 297  
 mycorrhizosphere, 230  
 mycotoxins, 177, 257, 268
- natural product, 257, 262, 263, 265, 266, 271, 275

- antiinsectan, 266, 267, 273  
 nature reserves, 120  
 necrotrophic, 128  
 necrotrophs, 287  
 nematodes, 49, 53, 147-155, 164, 202-209, 236, 296  
 nitrate, 238, 239  
 nitrogen, 48, 50, 56-58, 60, 61, 234, 235, 237-239, 241, 243  
 non-target invertebrates, 176  
 nutrient, 47, 48, 50, 51, 53, 55-57, 59-62, 313
  - dissolved, 313
  - enrichment, 313
  - limiting, 313
 Opisthokonta, 7  
 orchid, 245, 246
  - mycorrhiza, , 232, 229
 Oregon white oak, 220  
 organic acids, 288, 289
  - citric, 288, 289
  - formate, 289
  - malate, 289
  - oxalic, 288, 289
  - succinic, 289
  - tartaric, 288
 organometals, 77
  - organomercury compounds, 77
  - organotins, 77
 oribatid mites, 202  
 outline maps, 107  
 PA23 induced resistance, 195  
 palm, 222  
 parasexual, 217  
 pathogen suppressiveness, 135  
 pathogenesis, 180  
 pathogens, 222  
 pectin, 307, 314, 326, 331
  - homogalacturonan, 331
  - rhamnogalacturonan, 331
  - xylogalacturonan, 331
 peptides, 237  
 perithecia, 216  
 pest control, 163
  - classical, 163
  - combinations, 164
  - imidacloprid, 164
  - inoculative augmentation, 164
  - inundative augmentation, 164
  - mycoinsecticide, 164
 pesticide, 275  
 pharmaceuticals, 257  
 phosphate, 235, 236, 239, 240
  - phosphate transporters, 236
  - polyphosphate, 236
 phosphorus, 57-59, 61, 234, 235, 237, 243  
 photosynthesis, 222, 239-241, 244, 246
  - achlorophyllous plants, 244, 246
 phylloplane, 290, 291  
 phyllosphere biocontrol, 191
  - antibiotics, 192
 phylogenetic tree, 3, 5
  - eukaryote tree, 7
  - long-branch attraction, 5
 phylogenies, 3  
 phyogeography, 221  
 phytochemicals, 171  
 phytotoxins, 269, 270  
 plant cell wall, 326
  - composition of, 326
  - degradation of, 327
 plant disease
  - brown rust of wheat, 224
  - citrus variegated chlorosis, 223
  - tan spot of wheat, 224
 plant litter, 301  
 plant pathogenic fungi, 269, 270  
 plant pathogens, 127, 148  
 pollutants, 69, 70, 296
  - enhanced carbon dioxide, 296
  - ozone, 296
  - sulfur dioxide, 296
  - UV-B light, 296
 polycyclic aromatic hydrocarbons, 70, 71  
 polygalacturonase-inhibiting protein, 332  
 poplar, 240  
 porosphere, 49, 50  
 potato, 236  
 prairie, 56  
 pre-emptive colonization, 194
  - competence, 194
  - niche exclusion, 194
  - successful colonization, 194
  - synchronization, 194
 products
  - BotaniGard® ES, 169
  - Green Muscle, 169, 170, 178
  - Vertalec, 168
 production
  - bacterial, 316
  - fungal, 304, 311, 315, 316
  - plant, 316
 prokaryotes, 7  
 proteins, 237, 238  
 protists, 4, 12  
 PSD, 177  
 pyromorphite, 76  
 radiocaesium, 77, 78, 294-296  
 radionuclides, 73, 76, 77, 79, 296, 297  
 ratios
  - C:N, 290, 291, 306, 313, 314
  - C:nutrient, 294
  - C:P, 306, 313, 314
  - cellulose:lignin, 291
  - lignin:N, 290, 291
 rDNA, 230, 231  
 reactive oxygen species, 336  
 recombination, 217  
 Red Data List, 118  
 reproduction, 304, 317  
 respiration, 308, 309, 317, 318  
 rhizomorphs, 234, 242, 290
  - cords, 293, 295
 rhizosphere, 49, 289  
 rice, 236  
 risk assessment, 176  
 rock, 287-289
  - amphibolite, 288

- biotite, 288, 289  
 biotites, 289  
 feldspar, 288  
 feldspars, 289  
 fluorapatite, 289  
 granite, 289  
 hornblende, 289  
 limestone, 289  
 marble, 289  
 microcline, 288, 289  
 muscovite, 289  
 orthoclase, 288  
 phlogopite, 289  
 sandstone, 289  
 shales, 289  
 strontianite, 289  
  
 salt marsh, 303  
 sclerotia, 265–267, 270  
 secondary chemicals, 291  
     polyphenols, 291  
     tannins, 291  
 secondary metabolites, 217, 257, 259–261, 268, 271  
     alkaloid, 217  
     ergot, 217  
     ergovaline, 218  
     loline, 218  
     lolitrem, 218  
     lysergic, 218  
     peramine, 218  
     taxol, 224  
     toxic syndromes, 219  
     toxicity, 219  
 seed treatment, 134  
 seedlings, 244  
 selective grazing, 206  
 senescence, 222  
 soft-rot, 58  
 soil, 47, 50, 56  
     aggregate, 47, 49–56, 62  
     erosion, 51, 53, 57, 59  
     organic matter, 47–50, 52–57, 61  
     pH, 59  
     structure, 47, 51–54, 56, 62  
 spruce, 220  
  
 stable isotope analyses, 230, 234, 237, 239, 241, 244–246  
     isotopic signatures, 235  
 sterile dark fungi, 207  
 stoichiometry, 306, 313, 314  
 stromata, 216, 273  
 stromatolites, 4  
 succession, 32, 291, 303  
 suppressive environment, 135  
 swollenin, 329  
 symbiotic (mycorrhizal) interface, 232, 233, 235, 238, 240  
 synergism, 128, 164  
     imidacloprid, 164  
 synergistic effects, 261  
  
 T-RFLP, 304, 311  
 tannins, 238  
 trampling of the soil, 121  
 trehalose, 203, 241  
 trophic properties, 7  
 trophic traits, 3  
     chemotrophs, 14  
     heterotrophs, 8, 14  
     litho-organotrophs, 14  
     organotrophs, 8, 14  
     osmo/phagotrophically, 8  
     osmotrophs, 8, 14  
     phagotrophy, 8  
  
 unikont, 7  
  
 water availability, 308  
 WCS374r, 195  
 weathering, 236, 238  
 western white pine, 221  
 wetland, 301, 303  
 white-rot, 58  
     fungi, 71, 336  
 wood, 305, 309, 311–314, 316  
     decay fungi, 271, 273  
     wood-wide web, *see* common mycelial networks  
  
 xenobiotics, 70  
  
 yew, 224