

# Allelopathy: A Physiological Process with Ecological Implications

Edited by  
Manuel J. Reigosa,  
Nuria Pedrol and  
Luis González



Springer

# ALLELOPATHY

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## A Physiological Process with Ecological Implications

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## PREFACE

There are many good books in the market dealing with the subject of allelopathy. When we designed the outline of this new book, we thought that it should include as many different points of view as possible, although in an integrated general scheme. Allelopathy can be viewed from different perspectives, ranging from the molecular to the ecosystem level, and including molecular biology, plant biochemistry, plant physiology, plant ecophysiology and ecology, with information coming also from the organic chemistry, soil sciences, microbiology and many other scientific disciplines. This book was designed to include a complete perspective of allelopathic process.

The book is divided into seven major sections. The first chapter explores the international development of allelopathy as a science and next section deals with methodological aspects and it explores potential limitations of actual research. Third section is devoted to physiological aspects of allelopathy. Different specialists wrote about photosynthesis, cell cycle, detoxification processes, abiotic and biotic stress, plant secondary metabolites and respiration related to allelopathy. Chapters 13 through 16 are collectively devoted to various aspects of plant ecophysiology on a variety of levels: microorganisms, soil system and weed germination. Fundamental ecology approaches using both experimental observations and theoretical analysis of allelopathy are described in chapters 16 and 17. Those chapters deal with the possible evolutionary forces that have shaped particular strategies. In the section named “allelopathy in different environments”, authors primarily center on marine, aquatic, forest and agro ecosystems. Last section includes chapters addressing application of the knowledge of allelopathy.

Despite this diversity of topics, the text is plenty of points of contact and it covers a broad spectrum of allelopathy, from molecular to ecological processes including, of course, a physiological point of view. We have tried to include all the key features of allelopathy that are critical to successful allelopathy research and application.

We wish to sincerely thank the contributors of this book for their hard work and their cooperation in achieving our goal and making this volume reality. We also want to give our most sincere thanks to the people in the old Editorial Board who promoted the book.

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# CHAPTER 1

## INTRODUCTION TO ALLELOPATHY

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### INTRODUCTION

Theophrastus (372-285, BC), a disciple of Aristotle, reported an example of the inhibitory effect of pigweed on alfalfa (Jelenic, 1987). Yang and Tang (1988) made an extensive review on plants used for pest control as described in *Shengnong Ben Tsao Jing* (神農本草經) in 25-220A.D. in China. They described 267 plants considered to have pesticidal activity, and many of them also exhibited allelopathic potential. Lee Shi-Jen (1518-1593, AD), a famous Chinese pharmacologist, wrote a book on Chinese medicinal herbs, illustrating the toxic and nutritious natures of chemical constituents to organisms, particularly to humans. He also indicated that the plant constituents might be affected by habitats. In 1832, De Candolle, a Swiss botanist, suggested that the soil sickness problem in agriculture might be due to exudates of crop plants (see Rice, 1984). Later, Hoy and Stickney (1881) reported a deleterious effect of black walnut trees on the growth of plants nearby. Schreiner and Reed (1907,1908) found some soil organic acids, which were originally released by plant roots, suppressed the growth of some crops. Years later, Molisch (1937) coined a term *Allelopathy* from two Greek words of “Allelo” and “pathy” meaning “mutual harm”, expressing that a natural phenomenon of one plant releases inhibitory substance which inhibits the growth of other plant sharing the same habitat. Rice (1984) further defined the allelopathy as both stimulatory and inhibitory effects of one plant upon another including microorganisms. Among other terms, antibiotic refers to a chemical produced by a microorganism and effective against other microorganisms; *marasmin* concerns a chemical produced by a microorganism and active against a higher plant; and *Koline* is for chemicals produced by higher plants and effective against higher plant (Grummer,

1955). On the other hand, autointoxication is one plant produces toxic substance(s) which inhibit the growth of its own (Chou and Lin, 1976; Chou, 1999). In the early 20th century, both allelopathy and autointoxication received a great attention in agricultural productivity, in particular to a continuous monoculture causing yield reduction of crops (Bode, 1940; Börner, 1960; Evenari, 1949; Havis and Gilkason, 1947; Patick, 1955).

Until the late 1960, the allelopathic concept was firstly applied to plant ecology in elucidating the mechanism of plant interference, such as plant dominance, succession, and climax formation (Muller, 1969). *Salvia leucophylla*, a California soft chaparral produced monoterpenes, namely  $\alpha$ -pinine,  $\beta$ -pinine, cineole, camphore, which suppressed the growth of many herbaceous plants nearby, resulting in a unique bare, inhibition, and normal growth zones (Muller, 1966). Muller and his students also contributed significant findings on other chaparral shrubs, such as *Adenostoma fasciculatum* (McPherson and Muller, 1969), *Arctostaphylos glandulosa* var. zacaensis (Chou and Muller, 1972). Muller rather used plant interference, including both competition and allelopathy, and defined competition to mean that one plant takes up necessary substances from a habitat so as to have a harmful effect on the growth of other plant that required the same substances. On the other hand, allelopathy is the process that plant releases phytotoxic compounds into the environment to inhibit the growth of plant sharing the same habitat. Whittaker and Feeny (1971) published a classic paper entitled “Allelochemistry: chemical interaction between species”, and stated that “chemical agents are of major significance in adaptation of species and organization of communities.” Allelopathy thus plays a significant role in plant dominance, succession, formation of plant communities and climax vegetation, and crop productivity (Muller, 1969; Rice 1984; Rizvi and Rizvi, 1992; Chou, 1999). Furthermore, a term “allelochemical” derived from allelochemistry was firstly used by Chou and Waller (1983), and has become popular in agricultural science, dealing with the mechanism of chemical interactions among organisms, such as plant-plant, plant-insect, insect-insect, plant-microorganism, and microorganism-microorganism. Finally, the definition of allelopathy is confirmed by the International Allelopathy Society in 1996 meant that “any process involving secondary metabolites produced by plants, algae, bacteria, and fungi that influences the growth and development of agriculture and biological systems”. In other words, allelopathic compounds may regulate plant growth and development, involving photosynthesis, respiration, transpiration, biochemical metabolism and even in molecular basis of protein and nucleic acid synthesis.

## INTERNATIONAL DEVELOPMENT OF ALLELOPATHY AS A SCIENCE

At least, ten major international conferences on *Allelopathy* have been held since 1968. A list of the conferences is given as follows:

Conference took place		Conference title and (or) proceedings (book) title
Year	place/organizer	
1968	UC, Santa Barbara, USA	Biochemical Interactions Among Plants
*(1971)	**(C. H. Muller)	
1971	Univ. of Reading, UK	Phytochemical Ecology
(1972)	(J. B. Harborne)	
1982	Academia Sinica, Taiwan	Allelochemicals and Pheromones
(1983)	(C. H. Chou and G. R. Waller)	
1984	Univ. of Hawaii, USA	The Science of Allelopathy
(1986)	(A. R. Putnam and C. S. Tang)	
1985	Chicago, USA	Allelochemicals: Role in Agriculture and Forestry
(1987)	(G. R. Waller)	
1988	Academia Sinica, Taiwan	Phytochemical Ecology: Allelochemicals, Mycotoxins, and Insect Pheromones and Allomones
(1989)	(C. H. Chou and G. R. Waller)	
1996	Univ. of Cadiz, Spain	The 1st World Congress of Allelopathy
(1999)	(F. A. Macias et al.)	***Recent Advances in Allelopathy, Volume 1)
1999	Lakehead Univ., Canada (N. A. B. Mallik)	The 2nd World Congress of Allelopathy
2002	Tsukuba, Japan (Y. Fujii)	The 3rd World Congress of Allelopathy
2004	India (G. L. Bensal)	International Workshop on “Protocols and Methodologies in Allelopathy”

\* Data in parenthesis indicates the year of monograph or book published.

\*\* Name of organizer or editors who chair the conference.

\*\*\* Publication title outcome of the conference. The title of publication is the same as conference held

Through the effort of scientific leaders the aforementioned conferences in the field of allelopathy were held and the science of allelopathy has, therefore, been established. In due course of the development, eminent scientists like late Professors C. H. Muller (1966) and E. L. Rice (1984) published the most important classic papers or books, thus, scientists and students are able to follow their steps to work on allelopathy that become well-established. Allelopathy in the field of life science, in

particular to the agriculture, has made significant contribution to the science that benefit to the humans in the past and foreseeing future.

## NATURE OF ALLELOPATHIC COMPOUNDS

Allelopathic compounds are secondary plant products released into environment through volatilization, leaching, root exudation and decomposition of plant residues in soil. These metabolites, such as phenolics, flavonoids, alkaloids, terpenoids and cyanogenic glycosides have often attracted scientists to elucidate their structure and biological function (Cutler and Cutler, 1999; Rice, 1995).

Muller and his associates demonstrated a unique pattern of allelopathy of *Salvia leucophylla* in the semi-desert area of the south California. Because of arid climate in summer time, volatile compounds, such as 1,8 cineole, pinine, and camphor were released and suppressed the growth of herbaceous plants around the dominant *S. leucophylla*. The phenomenon is particularly noticeable in the Mediterranean region. On the contrary, a substantial amount of natural products is leaching out of plants through rain drips that occurs in the tropical and subtropical region. Most of the allelopathic compounds released are hydrophilic, such as phenolic acids, alkaloids, flavonoid glycosides, etc. Many compounds were identified by various workers in different habitats. (Bode, 1940; Chou, 1999; Cutler and Cutler, 1999; Del Moral and Muller, 1969; Kohli et al. 2001; Macias, et al. 1997; Rice, 1984).

It is well known that a certain quantity of unharvestable portions of plant is left on and in the soil, particularly on a farm after crop harvest. During the microbial decomposition and enzymatic degradation of such plant material, a wide variety of chemicals are released into the soil, where many of them have important biological activities, such as inhibition of seed germination, plant growth and yield reduction of many crops (Börner, 1960; Chou and Patrick, 1976; Chou and Lin, 1976; McCalla, 1971; Patrieck, 1971). Soil microorganisms, in particular, *Fusarium*, *Pseudomonas* and *Thielaviopsis* are fully involved during the decomposition of plant residues in soil. The responsible allelopathic compounds through this process are short chain fatty acids, phenolic acids or alkaloids. For example, o-hydroxyphenylacetic acid produced during the decomposition of rice residues in soil exhibited a phytotoxic effect on radicle growth of rice seed at a concentration as low as 25 ppm (Chou and Lin, 1976). Incidentally, the liberation of organic compounds during the decomposition of crop residues is often alternated by environment stress conditions (Chou, 1999). In a poor drainage area, the accumulation of the aforementioned compounds reaches to a certain degree, resulting in a detrimental effect on crop growth and causes yield reduction (Chou and Lin, 1976).

Regarding the allelopathic compounds released by the exudation of root, Rovira (1971) made a comprehensive review on the nature of root exudates, methods of collecting them, factors affecting exudation, mechanisms of such exudation, and root exudates in relation to nutrients and soil microflora. Recently, Tang (1986) made a root exudation device using XAD-4 resin and collected some hydrophilic compounds of root exudates. Young (1986) using that apparatus to collect root exudates from *Asparagus officinalis*, and these suppressed the growth of *Asparagus* seedlings. The responsible compounds are 3,4-dihydroxy benzoic, 3,4-dimethoxy benzoic, 2,5-dihydroxy benzoic, 3,4-dihydroxyphenylacetic and  $\beta$ -(m-hydroxyphenyl)-propionic acid, and 3,4 dimethoxyacetophenone. More than 20 phenolic compounds have been found in the decomposition of crop residues and root exudates (Chou, 1999).

## PROSPECTS OF ALLELOPATHIC RESEARCH

Previous findings on allelopathy have often concentrated on the elucidation of allelopathic mechanism in natural and agricultural ecosystems. Further advanced techniques and instruments, such as HPLC, Mass spectrometry, NMR, are available, and the research becomes more easier than before. In addition, different disciplinary scientists, such as biochemists, molecular biologists, natural product chemists and biotechnologists, are interested in the multidisciplinary or integrative research. Thus, resolutions for the complexity of allelopathy mechanism and action mode of allelopathy has become possible. The future prospective researches are emphasized as follows:

1. *Continuous study on isolation and identification of allelopathic compounds in plants and rhizosphere.* Although many biologically active compounds have been found, we still need to explore new compounds from plants and microorganisms.
2. *Investigating the naturally occurring allelopathic compounds used as agrochemicals.* Two distinguished cases were found in Agrostem which was isolated from the corn cokle of *Agrostemma githago* L. and in Neem plant (*Azadirachia indica*) which constituents a variety of natural products used for herbicide, pesticide, fungicide and nematocide (Chou, 1999). An excellent reference on Biologically Active Natural Products: Agrochemicals was published (Cutler and Cutler, 1999).
3. *Solving the disputable problem of plant interference involving competition and allelopathy.* Many plant ecologists still do not believe the fact of allelopathic interaction in nature- and agro-ecosystem, simply because it is difficult to clarify the interaction in rhizosphere. More and advanced techniques need to be developed for clarification of the complexity in field.

4. *Allelopathy plays an important role in biodiversity.* The allelopathy may regulate the density and production of plant community under the canopy of a dominant species and limits the population of its associated species, but the allelopathic compounds often do not kill the seeds of plant species (Chou, 1999). Therefore, allelopathic compounds are better be used as naturally biological control agent without extinguishing the affected species. In addition, allelopathy also acts as an evolutionary strategy for species survival under a dominant plant.
5. *Mode of action of allelopathic compounds on plant growth.* There are many important action mode for allelopathic compounds in plants (Rice, 1984), such as (1) effect of allelopathic compounds on division, elongation and ultra structure of cells, (2) effect of growth inhibitors on hormon-induced growth, (3) effects of allelopathic compounds on membrane permeability, (4) effects of allelopathic compounds on mineral uptake, (5) effects on photosynthesis, (6) effects on respiration, (7) effects on protein synthesis, lipid and organic acid metabolism, (8) inhibition or stimulation of specific enzyme activity, (9) effects on water relationship, (10) effects on DNA or RNA synthesis. However, the mode of action is not fully understood.
6. *Allelopathy challenge to biotechnologist.* It is believed that allelopathic compounds produced are regulated by certain genes. Using advanced biotechnology techniques, scientists in the foreseeing future may be able to isolate allelopathic genes from one plant and transfer the gene(s) to another plant. Numerous genes, such as trypsin inhibitor gene and ethylene regulatory gene, have been successfully isolated from one plant and transferred to another plant (Yeh et al. 1997; Yang, 1998). It is hope that biotechnologists should work on this line in order to transfer the allelopathic gene(s) to an agronomic plant to avoid using or to lessen use of agrochemicals to prevent environment from deterioration.

## CONCLUSIONS

Allelopathy has increasingly received attention by scientists and has played important roles in plant biodiversity and sustainable agriculture (Chou, 1999). Much information of allelopathy has accumulated, yet, more difficult questions remained to be answered, such as (1) the action mode of allelopathic compounds, (2) allelopathic substances in rhizosphere, (3) develop and enhance allelopathic properties of agronomic species, (4) use molecular biotechnology to transfer allelopathic gene from one plant to another, (5) establish proper methods to agriculture practice in the field, and (6) continuously work on potential naturally occurring allelopathic substances from plants, microorganisms to replace synthetic agrochemical to save our environment.

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## CHAPTER 2

# BASIC PATHWAYS FOR THE ORIGIN OF ALLELOPATHIC COMPOUNDS

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## INTRODUCTION

Allelopathy, originally defined as chemically elicited interactions between plants or fungi, is mediated by many types of compounds (Seigler, 1996). In recent work, however, considerable insight into the actual mechanisms of interactions is becoming available. Not only have many of the compounds involved been isolated and characterized, they have been established to occur *in situ*. The role of root exudates is just now being fully appreciated. Nearly 5-21% of all photosynthetically fixed carbon is transferred through the roots of plants into the rhizosphere (Marschner, 1995). Root-root and root-microbe communication can either be positive (epiphytes, mycorrhizal fungi, nitrogen-fixing bacteria) or negative to the plant (parasitic plants, parasitic bacteria, fungi, and insects) (Walker et al. 2003). In natural systems, roots are in continual communication and quickly recognize and prevent the presence of invading roots.

In other instances, the known effects of compounds on processes related to growth and development of plants suggest that the compounds should have activity. This is true for many hormonal substances such as auxin, gibberellic acids, and ethylene, but also for compounds such as jasmonic acid and salicylic acid. Allelopathic compounds could interact by inhibiting synthesis, accumulation or utilization of energy rich compounds such as fatty acids or triacylglycerols. Numerous steps are involved in the formation, accumulation, movement, and metabolism of these compounds. In some instances, the compounds have specific toxic effects in other organisms, and, in yet other instances, the compounds are involved in modification of the soil matrix or the availability of substances needed for plant growth and development.

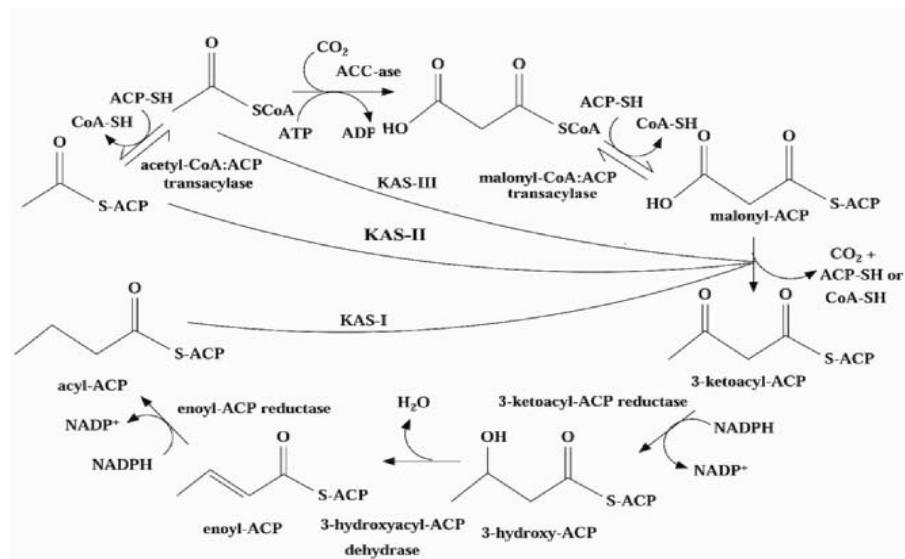
Methodology for distinguishing the effects of competition and allelopathic interference, at least for relatively simple systems, now exist (Thijs et al. 1994, Weidenhamer et al. 1989). These methods are important not only because of their practical utility, but because, they illustrate that effects of chemicals in the soil matrix need not produce obvious toxic effects on all species in the system.

In the following chapter, I have considered the basic paths of origin of most of the major groups of compounds that have been demonstrated to have allelopathic activity, as well as others for which circumstantial or less solid evidence exists at present.

## ACETATE-DERIVED COMPOUNDS

### Fatty Acids

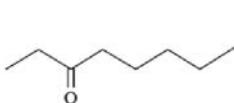
Fatty acids and their derivatives are among the most common of all plant lipids. Fatty acids are derived from acetyl-CoA and malonyl-CoA via the intermediacy of a complex pathway involving three major steps. The first is the union of malonyl-ACP and acetyl-CoA, the second involves a series of reactions leading to palmitoyl-CoA and the final step involves conversion to stearoyl-CoA. These compounds are subsequently converted to triacylglycerols by a complex series of prokaryotic and eukaryotic steps associated with plastids and endoplasmic reticulum in the cytoplasm of plants (Buchanan, 2000).



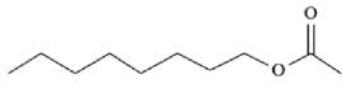
As components of triacylglycerols, fatty acids provide much of the energy reserve of both plants and animals. As important parts of phospholipids and galactolipids, they are major components of plant membranes (Buchanan et al. 2000). In contrast to these combined forms, free fatty acids are toxic to plant cells and to fungi.

Lower molecular weight fatty acids are toxic to fungi. This is in part because they alter pH, but propionic acid inhibits bacterial and fungal growth, both as the free acid and as salts. Lactic acid is important in bacterial cultures and as a food preservative. This compound is responsible for the properties of many fermented food products, e.g., yoghurt and sauerkraut.

Metabolized fatty acids act as insect attractants (Buchanan et al. 2000). Acetate and propionate are known to have potent antifungal effects (Seigler, 1998).



3-octanone



octyl acetate



1-octanol



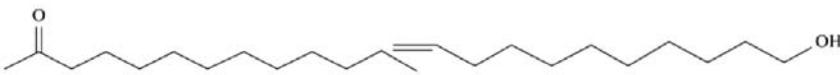
octanal



2-undecanone



1-dodecanol

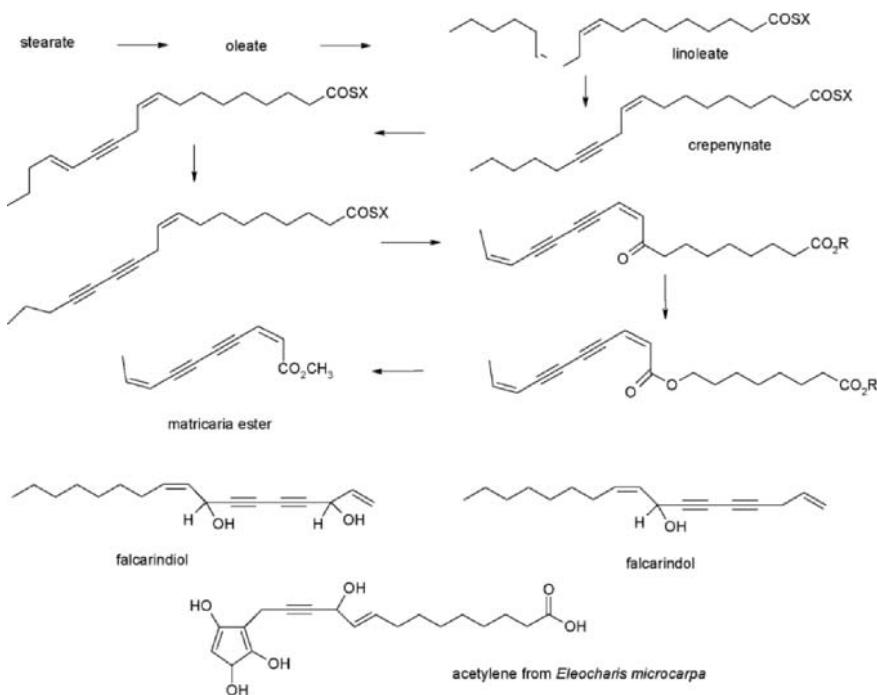


2-tridecanone (53)

10-undecen-1-ol

Fatty acids are converted in plants into many other types of bioactive compounds. Jasmonic acid, a compound involved in plant growth regulation, a secondary messenger within cells, and in signaling between plants and between plants and insects, is one of the most important of these compounds (Parchman et al. 1997;

Watanabe et al. 2001; Weiler, 1997). Phosphatidyl inositol have important roles in signal transduction pathways (Buchanan et al. 2000). Jasmonate also is a key signal in pollen development.

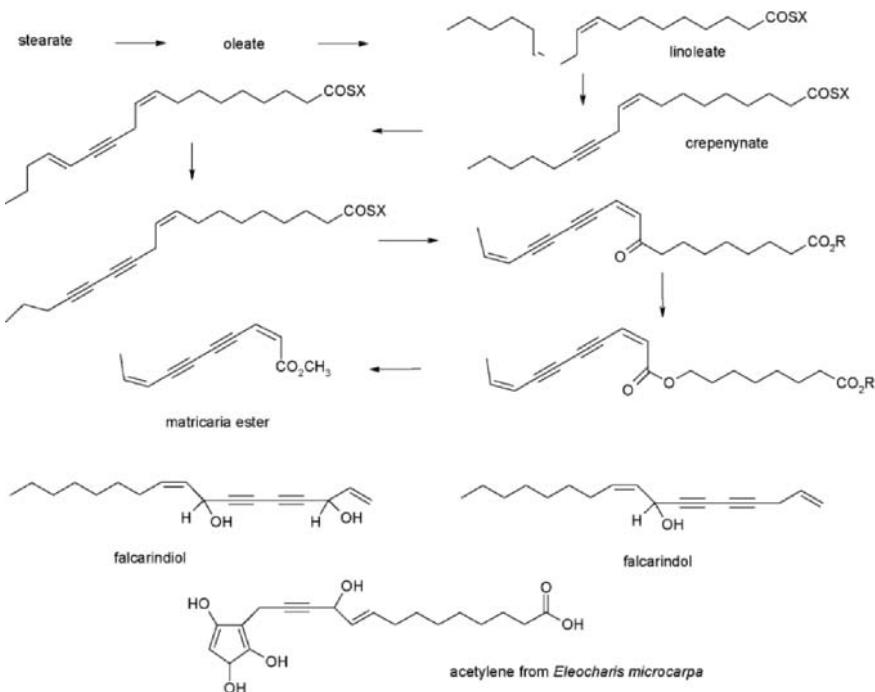


## Compounds with acetylenic linkages

Most acetylenic compounds are derived from fatty acids (Seigler, 1998).

Some acetylenes are active in plant-algal interactions. For example, falcarindiol and falcarindol from *Berula erecta* are algicidal (Gross, 1999). An acetylenic fatty acid from *Eleocharis microcarpa* also has been shown to have algicidal activity (Gross, 1999).

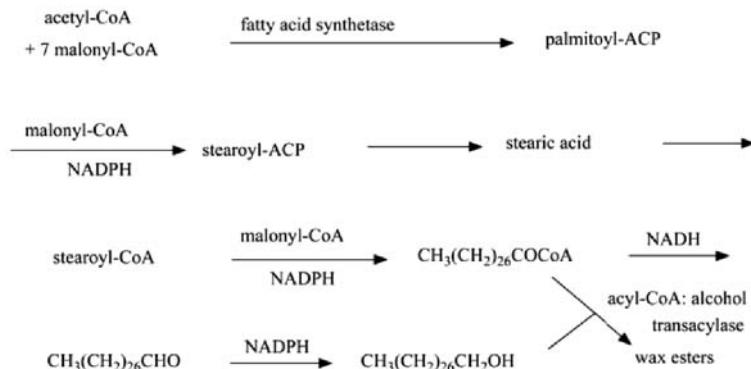
Because they are restricted in distribution to a small number of plant families, they may not be of widespread importance. However, two of these families, the Asteraceae (Compositae) and the Apiaceae (Umbelliferae) are common in many types of vegetation.



## Waxes

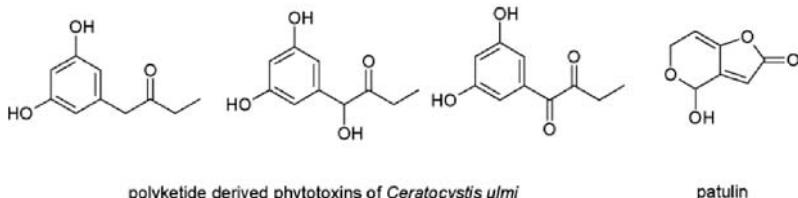
Waxes are complex mixtures of hydrocarbons, aldehydes, alcohols, acids, and esters formed from fatty acids. Complex esters of hydroxylfatty acids, often containing phenolic materials, also are deposited on the epidermal surface of plant cells. They are important for the protection of plants from desiccation and from fungal and bacterial attack. Many insects recognize their hosts by the composition of the waxy epidermal coating. Binding of bacterial and fungal spores to plant surfaces also appears to involve recognition of epidermal surface features. Phenolic substances in this layer also provide screening from deleterious UV radiation.

The enzyme systems involved in elongation of fatty acids bear little sequence similarity to other condensing enzymes (Buchanan et al. 2000).

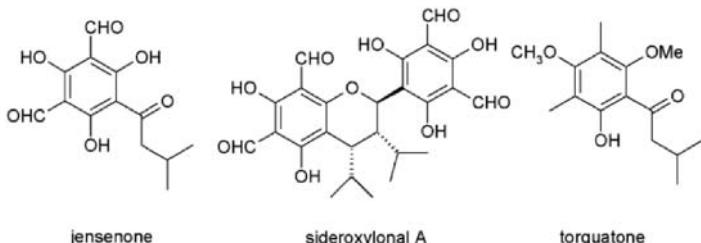


## POLYKETIDES

Polyketides are common products in fungi and lichens, but also are widely produced by higher plants (Cutler, 1992). Many of the polyketides of fungi are mycotoxic, that is, toxic to mammals, but others, such as tetracyclines, are valuable antibiotics for humans. Polyketides are among the phytotoxins used by fungi to attack plants. These compounds often are involved in the breakdown of membranes, permitting the attacking fungus to absorb nutrients of the host plant. Some of these compounds, such as patulin, are powerful carcinogens, mycotoxins, phytotoxins, and allelopathic agents all at the same time (Cutler, 1992; Seigler, 1998). Usnic acid, a component of many lichens, inhibits the enzyme *p*-hydroxyphenylpyruvate dioxygenase, a key enzyme in carotenoid biosynthesis, and is phytotoxic (Romagni et al. 2000, 2004). Both (+)- and (-)-usnic acid inhibit transpiration and oxygen evolving processes in maize and sunflower seedlings. A number of other lichen metabolites have been assayed for activity against higher plants (Cutler, 1992). The relatively simple polyketides of *Ceratocystis ulmi*, a pathogen of *Ulmus* species, are representative of these phytotoxins.



Plant-produced polyketides, such as jensenone, sideroxylnal A, and torquatone (phloroglucinols), are accumulated in *Eucalyptus* species and appear to play a role in limiting herbivory by browsing mammals (McLean et al. 2004). The aldehyde groups of these compounds appear to be responsible for activity; torquatone is generally inactive. Because many *Eucalyptus* species appear to be allelopathic (Willis, 1999), allelopathic activity of these compounds should be investigated.



In one complex situation, seedling survival of *Picea glehnii* seedlings against the pathogen *Pythium vexans* was increased by pretreatment with *Penicillium damascenum* (Yamaji et al. 2001). Production of a polyketide antibiotic agent may be responsible for this activity.

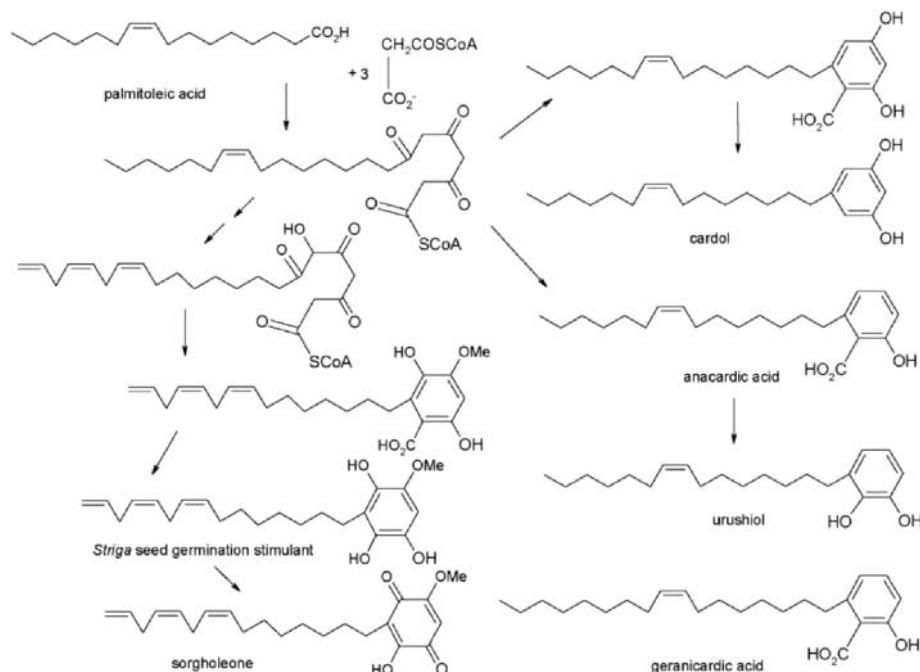
## QUINONES, NAPHTHOQUINONES, AND ANTHRAQUINONES

### Quinones

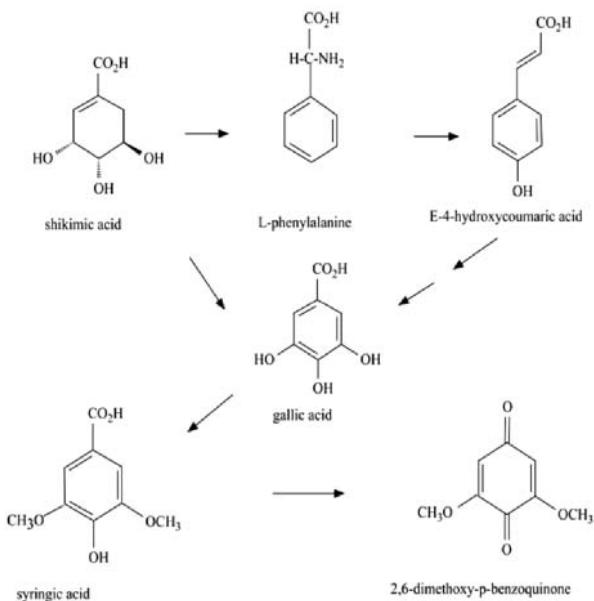
The first germination stimulant isolated from *Sorghum bicolor* was the precursor of sorgoleone (Galineo et al. 2004). This hydroquinone is exuded in droplets from the root hairs of the host plant and readily oxidizes to the stable quinonoid form. The reduced form is localized quite close to the root which assures that the parasite will germinate within a reasonable distance from the host (Galindo et al. 2004).

*Sorghum bicolor* or other related species have the ability to inhibit the growth of a number of competing weedy species (Weston et al. 1999). Sorgoleone, isolated from this source, arises biosynthetically from extension of a fatty acid precursor followed by cyclization. This compound is the major (>85%) component of the exudates of *Sorghum bicolor* and related species. This compound binds to the Q<sub>B</sub>-binding site of the photosystem II complex of higher plants (Czarnota et al. 2001). Soil impregnated with sorgoleone had activity against a number of species. Sorgoleone reduces growth of broadleaf and grass weeds in hydroponic assays at a concentration as low as 10 µM (Rimando et al. 1998).

Urushiol derivatives from *Metopium brownii* (Anacardiaceae) have the ability to inhibit several soil fungi and may be involved in allelopathic activity by this plant (Anaya et al. 1999).



Quinones of many different groups of plant secondary compounds are known (Seigler, 1998). Parasitic plants in the Scrophulariaceae use chemicals released by host plant roots to signal development processes critical for development of haustoria that permit them to attach to the host plant roots. When the roots of the hemiparasitic plant *Triphysaria versicolor* are exposed to extracts of the roots of *Zea mays* or to the known haustorial inducer 2,6-dimethoxybenzoquinone (DMBQ), upregulation of a large number of transcripts occurs (Matvienko et al. 2001). Many of the proteins encoded by the transcripts are predicted to participate in quinone detoxification, whereas others are more likely associated with haustorium formation. DMBQ also occurs in the Mexican legume species *Bowdichia nitida*, where it has been shown to have allelopathic activity (Fisher and Quijano, 1985).



## Naphthoquinones

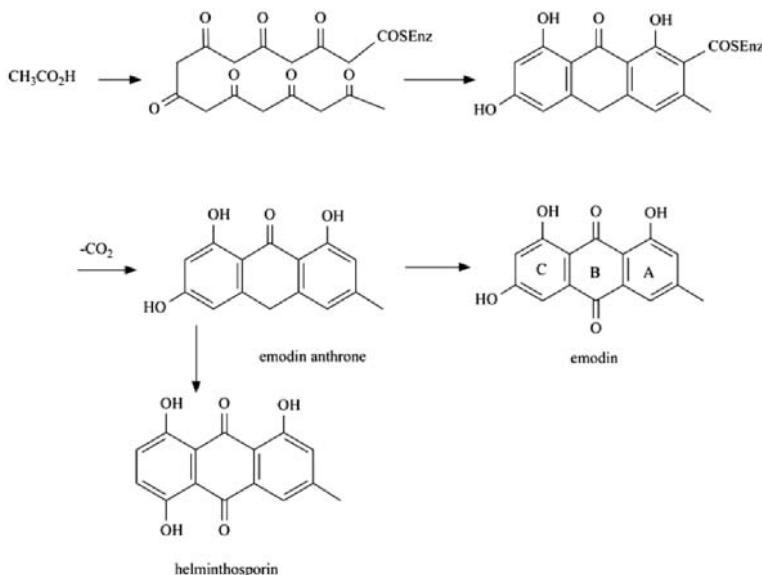
The naphthoquinone juglone is one of the best known examples of allelopathic compounds. This compound, mainly from *Juglans* species (walnuts) has the ability to inhibit growth of many plants and is exuded by the roots, but also liberated by leaves and fruits of the offending plants (Seigler, 1998).

Pigmented derivatives of shikonin are produced in root cells of *Lithospermum erythrorhizon* (Brigham et al. 1999). Contact by hyphae of several species of fungi induced localized production of pigments, but challenge by *Rhizoctonia solani* increased shikonin production more than 30 times. The growth of the mycorrhizal fungus *Glomus intraradices* is not affected (Flores et al. 1999).

## Anthraquinones

Anthraquinones are common fungi, lichens, and higher plants. Emodin, derived from polyketide pathways is among the most common of these compounds (Izhaki, 2002). This compound has been identified from 17 plant families, but probably occurs in many more. Emodin may protect plants against herbivores, pathogens, competitors and factors such as light; it may protect seeds from unripe fruit from dispersal, but help to

disperse mature fully developed seeds (Tsahar et al. 2002). Emodin at 10-100 mg/l inhibits growth of *Helianthus annuus* and *Zea mays*. Emodin from *Polygonum sachalinense* inhibits seedling growth of lettuce, green amaranth (*Amaranthus viridis*), and timothy grass (*Phleum pretense*). The growth of lettuce seedlings was severely inhibited with as little as 50 mg/l emodin (Izhaki, 2002). It is likely that emodin released by plants adversely affects phosphate-requiring species by depleting phosphate from the soil (Inderjit and Nishimura, 1999). A series of emodin analogs has been found to inhibit germination and primary root formation (Romagni et al. 2004).



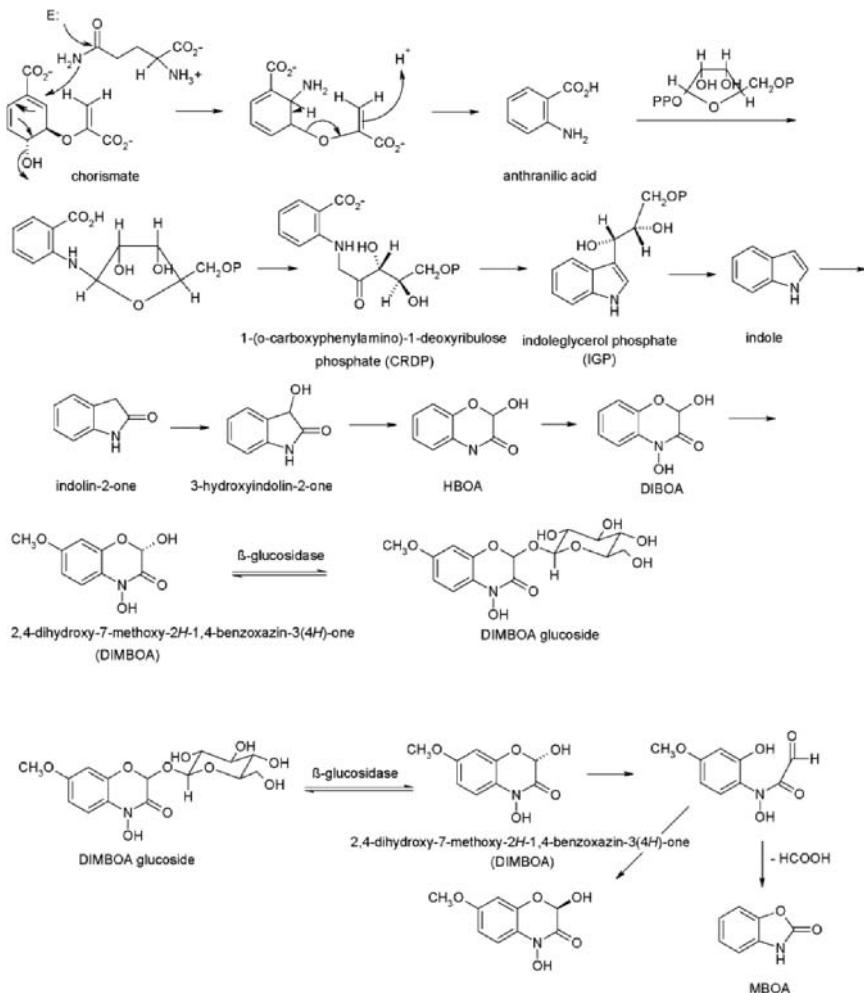
## SHIKIMIC ACID PATHWAY

Shikimic acid is a key intermediate in the formation of numerous types of secondary compounds in bacteria, fungi, and plants (Seigler, 1998). The most numerous of these are phenylpropanoids, but others are derived secondarily from anthranilic acid and tryptophan.

### Benzoxazolinones

DIMBOA and a series of related compounds appear to be biosynthesized from tryptophan. These substances are restricted to a small number of plant families: Among

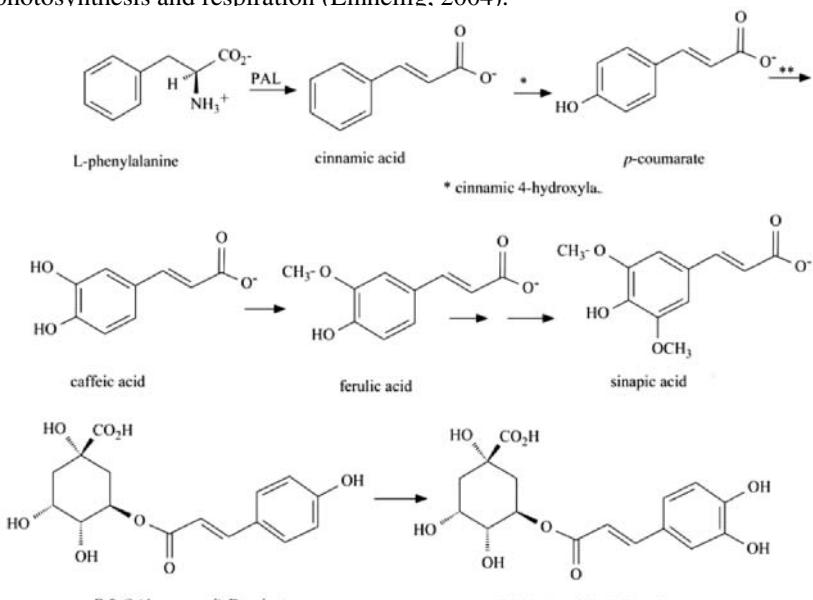
them the Acanthaceae, Poaceae, Ranunculaceae, and Scrophulariaceae, although no systematic search has been made for them (Sicker et al. 2004). These compounds decompose readily; for example, DIMBOA glucoside decomposes to DIMBOA and to MBOA. DIMBOA has been shown to be an active allelopathic compound in corn or maize (*Zea mays*) and in wheat (*Triticum aestivum*). Significantly more of this compound is found in spelt (*T. speltoides*) and allelopathic accessions all contained higher levels of DIMBOA (Quader et al. 2001).



## PHENYLPROPANOIDS

### Cinnamic acids

Many relatively simple cinnamic acid derivatives ( $C_6C_3$ -compounds or phenylpropanoids) are widespread in plants and in soils. For these compounds to be considered functionally allelochemical, they must occur in an active form (normally the protonated acid), they must be involved in chemically mediated plant, microbe, or plant/microbial interactions and the concentrations must be sufficient to modify plant or microbial responses, either in a positive or negative manner. Among the best known of these compounds are caffeic acid, ferulic acid, *p*-coumaric acid, protocatechuic acid, sinapic acid and vanillic acid (Blum, 2004). They appear to exert their effects through a series of generalized pathways. They interact with cell membranes, alter ion fluxes, and hydraulic conductivity of roots (Einhellig, 2004). In general, these compounds reduce hydraulic conductivity and net nutrient uptake by roots. These effects are mostly reversible once the phenolic acids are removed (e.g., by microbial action). For this reason, interactions with microorganisms are important in allelopathic problems involving this group of compounds (Blum, 2004). However, these perturbations also lead to alterations in ion balance, plant-water relationships, stomatal function, and rates of photosynthesis and respiration (Einhellig, 2004).

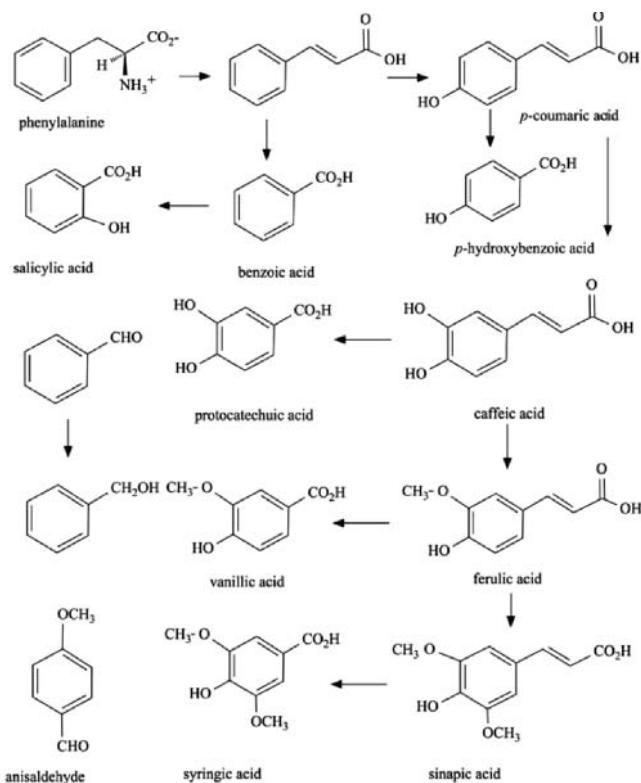


\*\* These oxidations probably occur as quinic and shikimic esters, not as the free acids

Cinnamic acid is known to be autotoxic to plants of guayule, *Parthenium argentatum* (Seigler, 1998). Dihydrocinnamic acid inhibits seed germination and growth of a number of plants of the Florida scrub (Fischer et al. 1988).

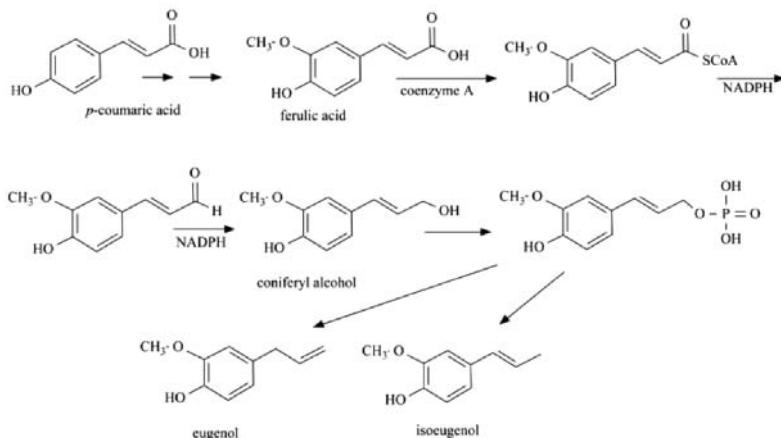
## C<sub>6</sub>C<sub>1</sub>-compounds

C<sub>6</sub>C<sub>1</sub>-compounds are also well known to have allelopathic activity (Seigler, 1998). A series of compounds from both the C<sub>6</sub>C<sub>3</sub>- and C<sub>6</sub>C<sub>1</sub>-series have been isolated from chamise, *Adenostoma fasciculatum* and from madrone, *Arbutus* species. These compounds inhibit the germination and growth of many plants of the California chaparral community. Compounds such as vanillic acid and syringic acid generally have the same properties as C<sub>6</sub>C<sub>3</sub>-compounds (Einhellig, 2004). Salicylic acid is sometimes a component of allelopathic phenolic mixtures (Buchanan et al. 2000; Seigler, 1998). Salicylic acid can retard senescence of petals, induce flowering, and is involved in disease resistance in plants (Raskin, 1995).



## Allylbenzenes

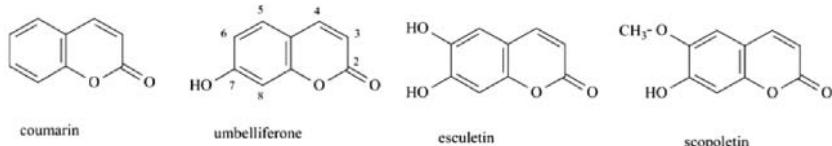
Allylphenols, volatile phenylpropanoid compounds, also have allelopathic activity. A series of these compounds from *Pimpinella* species strongly inhibit ryegrass and velvetleaf seed germination (Cutler, 1992).



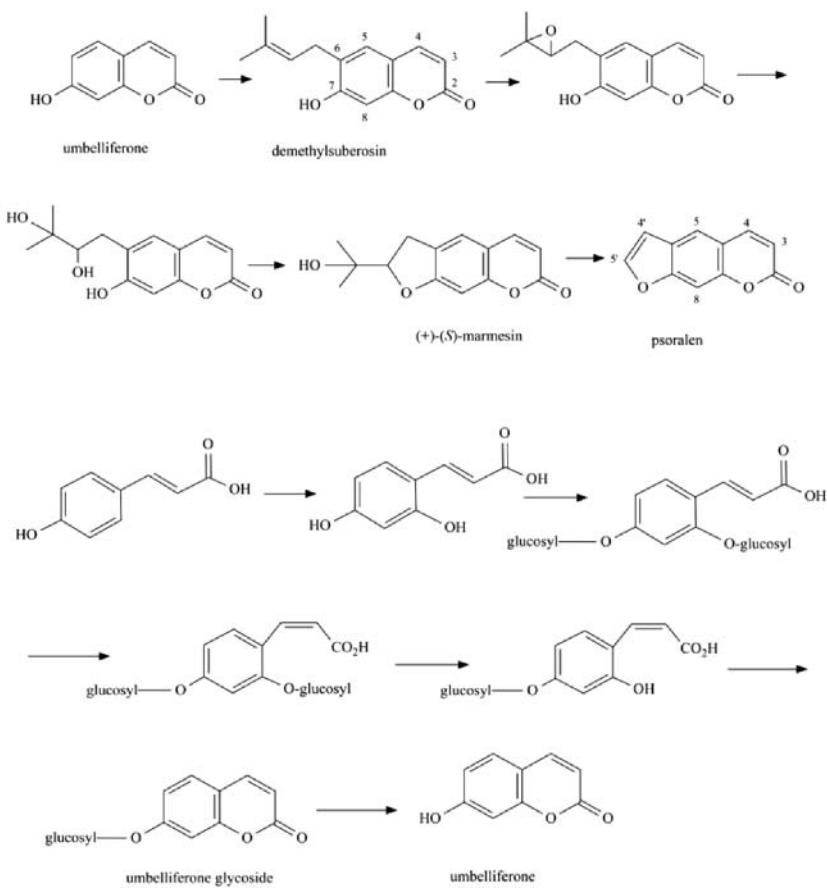
## Coumarins

Simple coumarins are widespread in plants; many of them are highly active. Coumarin itself may be either positively or negatively allelopathic (Abenavoli et al. 2001). In the concentration range of 25  $\mu\text{M}$ -1 mM, coumarin causes developmental changes in wheat (*Triticum durum*) roots and is correlated with increased uptake of nitrate.

In resistant lines of sunflowers (*Helianthus annuus*), 7-hydroxylated coumarins, e.g., scopoletin and its glycoside scopolin, play a defensive role against the parasitic plant *Orobanche cernua* by inhibiting germination of the seeds of the parasite and their attachment to the host (Sergolini et al. 2001). Either root tissue penetration of the host, connection to the vascular system, or tubercle development are prevented.

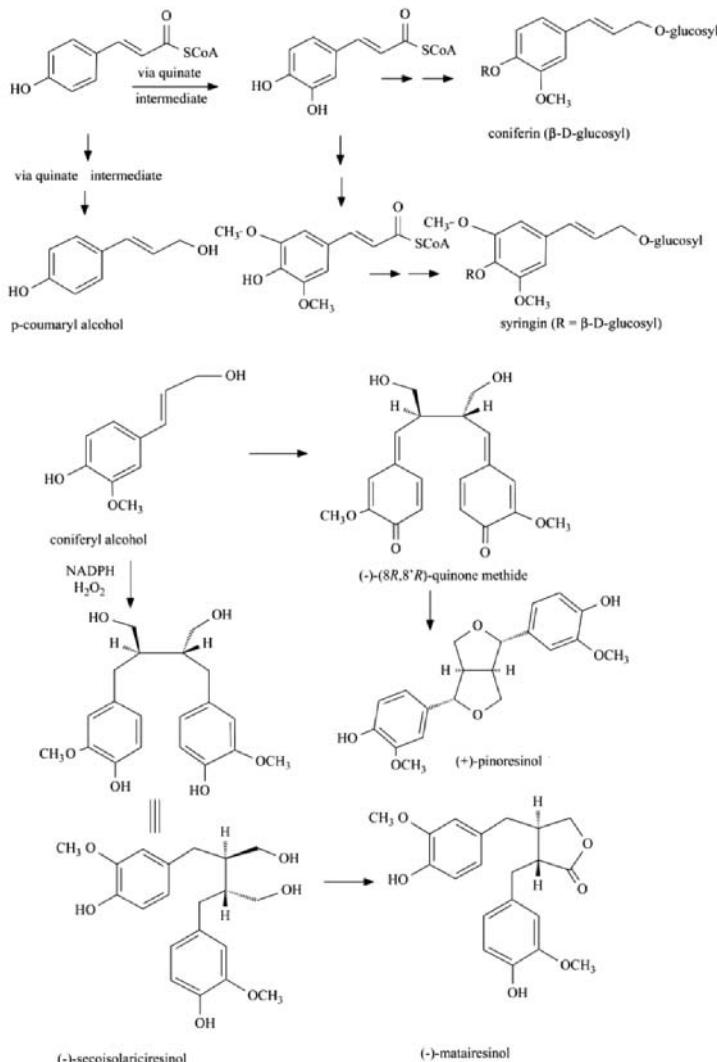


Furocoumarins (furanocoumarins), formed by addition of a unit derived from isopentenyl pyrophosphate, also have allelopathic activity (Aliotta and Cafiero, 1999). Linear types of furocoumarins bind DNA. In plants, these coumarins inhibit water uptake, swelling of the seed coat and endosperm, and cell elongation in the radicle of radish seeds (*Raphanus sativus* L.) (Aliotta and Cafiero, 1999). Application of 5% infusion of rue three times at 5 day intervals checked the growth of the weedy species *Portulaca oleracea*. Psoralen, xanthotoxin, bergapten, and marmesin are among the furocoumarins found in rue. This species also contains coumarin, simple coumarins, and a series of monoterpenes and alkaloids derived from anthranilic acid (Aliotta and Cafiero, 1999).



## Lignans and lignin

Four phytotoxic lignans from cenizo, *Leucophyllum frutescens*, are responsible for the phytotoxic activity exhibited by this species (Rimando et al. 1999). Three of these compounds, diayangambin, epiyangambin, and diasesartemin, inhibited all phases of onion root cell division.

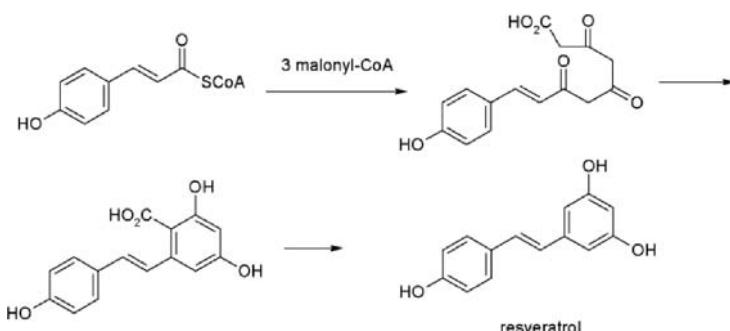


## PHENYLPROPANOIDS WITH ACETATE CHAIN EXTENSION

A series of phenylpropanoids with extension by three malonate groups may be condensed by two different mechanisms. The products formed by an aldol type condensation are known as stilbenes, whereas those that undergo a Claisen type condensation are called flavonoids.

### Stilbenes

Stilbenes are widespread in nature, although they have been inadequately studied from many plant groups. Most of them seem to have pronounced biological activity. Many are antifungal and some are induced as phytoalexins. Lunularic acid and a series of structurally related stilbenes from *Hydrangea* species have been shown to have allelopathic activity (Cutler, 1992). Resveratrol and piceatannol from *Scirpus maritimus* (Cyperaceae) have activity in both plant and animal bioassays. Piceatannol inhibits growth of *Lemna* (Cutler, 1992).



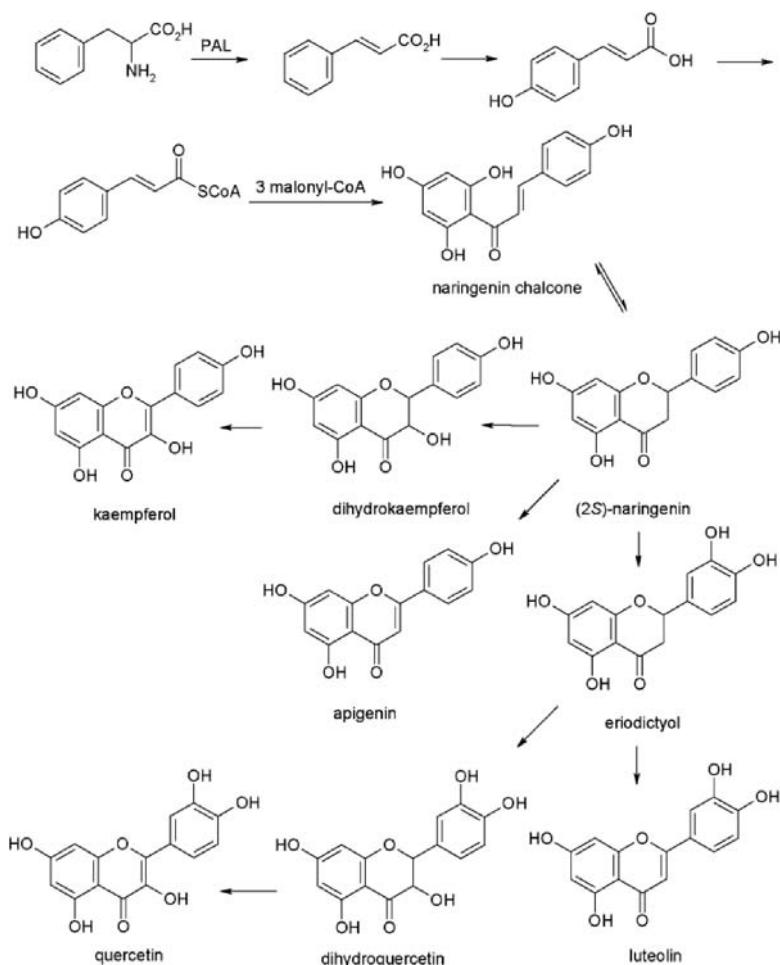
### Flavonoids

Flavonoids are derived from phenylpropanoid metabolism by extension of a *p*-coumaryl CoA precursor with three malonate units. These compounds are found in virtually all plants and their role in pollination is well known. In other instances, flavonoids are important in limitation or selectivity of herbivory. Flavonoids may play a role by screening harmful ultraviolet light from sensitive areas within plants.

Many flavonoids appear to be involved in nitrogen fixation. They are involved in activation of *Rhizobium meliloti* genes responsible for the nodulation process. The flavone luteolin, secreted by alfalfa seedlings and seed coats, provides one of the

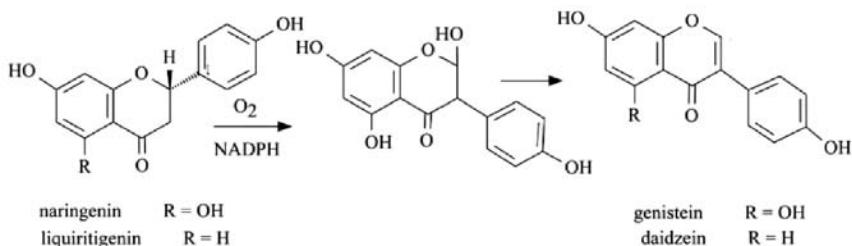
signals that induces the nodulation genes of *R. meliloti*. Similar compounds may be responsible for vesicular-arbuscular mycorrhizal colonization (Walker et al. 2003).

Flavonoids are actively excreted by the roots of many plants and are released from leaf litter. Flavones from *Celaenodendron mexicanum* (Euphorbiaceae) were shown to inhibit the growth of seeds and shoots of *Amaranthus* and *Echinocloa* species. A flavonoid from *Tithonia diversifolia* inhibited germination of radish, cucumber (*Cucumis sativus*), and onion (*Allium cepa*) seeds (Berhow and Vaughn, 1999). Many other examples of allelopathic flavonoids are cited in this article.



## Isoflavonoids

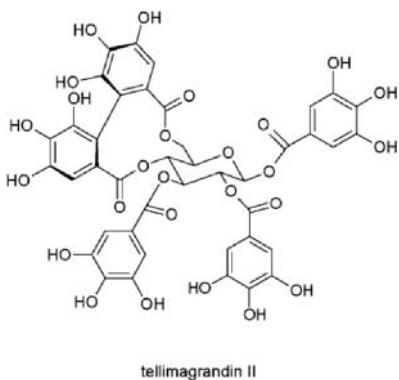
Many isoflavonoids also appear to be allelopathic. These compounds are derived by the same basic pathways as the more common flavones and flavonols, but are found primarily in a small number of families, among them the Fabaceae and Asteraceae. A series of flavanone, flavone and isoflavone glycosides excreted by the weed *Pluchea lanceolata* inhibited mustard root and shoot growth and the growth of asparagus seedlings (Berhow and Vaughn, 1999). Soil accumulation of isoflavonoids released by red clover (*Trifolium pretense*) has been implicated in a disease called “red clover sickness” (Berhow and Vaughn, 1999). Medicarpin and 4-methoxymedicarpin from alfalfa inhibit lettuce seed germination (Cutler, 1992).



## TANNINS

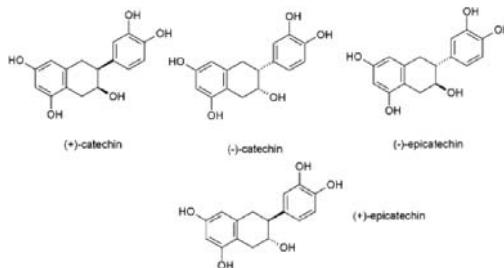
### Hydrolysable tannins

Hydrolysable tannins also have been shown to possess algicidal activity, e.g., tellimagrandin II from *Myriophyllum spicatum*. Another aquatic plant, *Trapa bicornis*, also produces this compound (Gross, 1999). Tellimagrandin II appears to inhibit alkaline phosphatase activity in the algae.

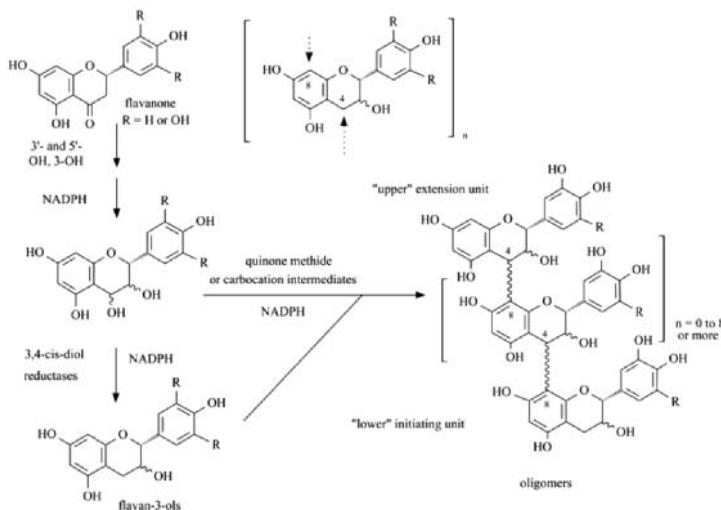


## Condensed tannins

The monomeric units that comprise condensed tannins, flavan-3-ols, are considered innocuous in many systems. Excretion of (-)-catechin by *Centaurea maculosa*, knapweed, exhibits negative root-root communication (Veluri et al. 2004; Walker et al. 2003). However, (+)-catechin was inhibitory to soil-borne bacteria. A mixture of the two compounds is excreted from the plant roots (Veluri et al. 2004). The biosynthetic process leading to formation of (-)-catechin is not known.



The dimer propelargonidin and the corresponding monomer afzelachin inhibit growth of rice seedlings and may be responsible for limited growth of peach seedlings (Ohigashi et al. 1982). Secretion of propelargonidin may explain, in part, the problem with replanting peaches, *Prunus persica* (Ohigashi et al. 1982). Interestingly, (+)-catechin and procyanidins promote growth of callus cultures of other *Prunus* species (Feucht and Treutter, 1999).

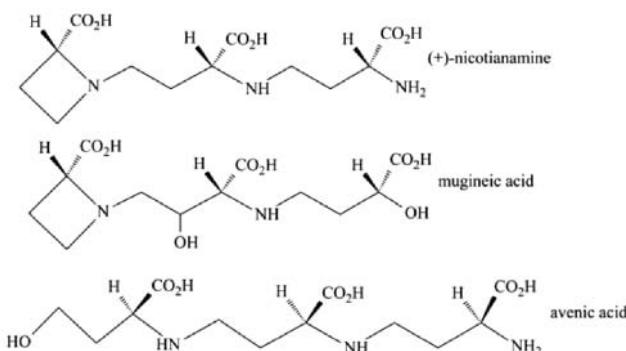


## AMINO ACID-DERIVED NITROGENOUS COMPOUNDS

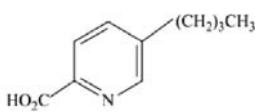
### Non-protein amino acids

About 300 non-protein amino acids occur in plants. These have a broad spectrum of biological activities; many are toxic because they are substituted for protein amino acids in various synthetic processes in plants, animals, and microbes (Seigler, 1998). These compounds are most common in members of the Fabaceae, Hippocastanaceae, Aceraceae, Sapindaceae, Liliaceae and Cucurbitaceae. Compounds such as (+)-nicotianamine are involved in iron transport in plants in plants such as tomatoes (*Solanum esculentum*). Similar compounds such as mugeneic acid and avenic acid, produced by roots of grasses, serve as siderophores. These compounds actively scavenge iron from the environment, and are one factor in explaining the dominance of grasses in many habitats (Buchanan et al. 2000). These phytosiderophores also can bind Zn<sup>++</sup>, Cu<sup>++</sup>, Mn<sup>++</sup>, Ni<sup>+</sup>, and Co<sup>++</sup>.

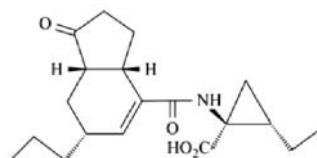
Similar compounds, often called phytochelatins, may be involved in the active uptake of these ions as well. Some plants that are especially effective in uptake of Zn<sup>++</sup> and Cd<sup>++</sup> have been employed in phytoremediation schemes. Siderophores from bacteria are often peptides. They also appear to be involved in allelopathic interactions at the microbial levels. The level of iron and other ions can be reduced to extremely low levels, precluding growth of other organisms.



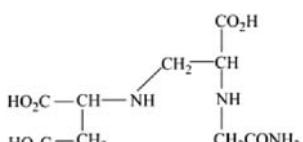
Pathogenic bacteria and fungi produce a series of peptides and other amino acid-derived substances that help to break down tissues and weaken the host plant. These often have molecular weights less than 600. The phytotoxins of bacteria tend to lack great specificity. Attack of the fungus *Fusarium oxysporum* on tomato involves both fusaric acid and lycomarasmin (Seigler, 1998). The former compound is involved in chelation of metal ions and the latter in water-permeability effects.



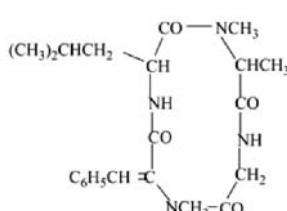
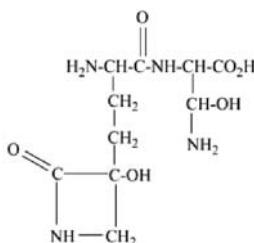
fusaric acid



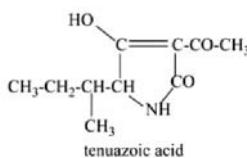
coronatine



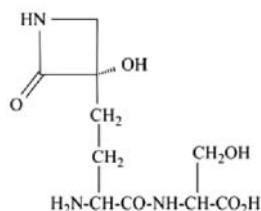
lycomarasmin

tentotoxin  
*Alternaria tenuis*, cotton chlorosis

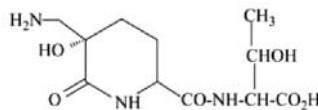
tabtoxin



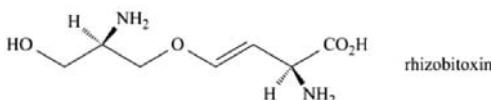
tenuazonic acid



serine tabtoxin



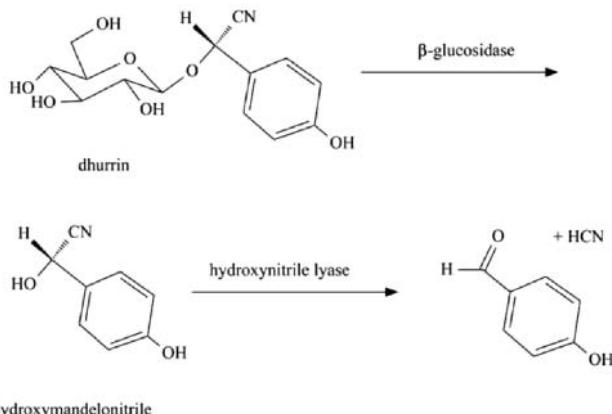
isotabtoxin



rhizobitoxin

## Cyanogenic glycosides

Cyanogenic glycosides are derived from a series of protein and one non-protein amino acid, (2-cyclopentenyl)glycine. On damage to the plants in which they occur, these substances are usually broken down by co-occurring glycosidases to yield glucose, an aldehyde or ketone, and hydrogen cyanide.

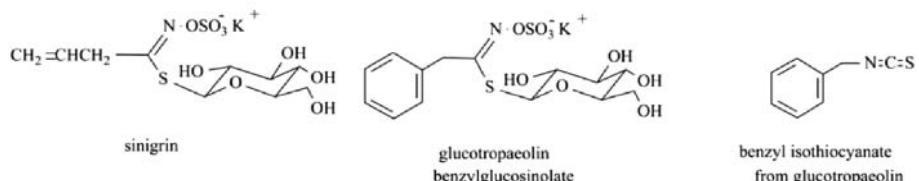


Cyanogenic glycosides or their decomposition products have been thought responsible for replant problems (Barazani and Friedman, 1999). Sorghum is quite strongly inhibitory to many competing species. This plant contains the cyanogenic glycoside dhurrin (Weston et al. 1999). Another compound, prunasin, is common in plants of the genus *Prunus*. Although it has been suggested that cyanide or other bioactive substances from these compounds are responsible for the allelopathic activity of *Sorghum* species and difficulties in re-establishing orchards of *Prunus* and other fruit crops, aldehydes (in this case, either benzaldehyde or *p*-hydroxybenzaldehyde) may actually be the active participant. These problems also may be attributed to the presence of allelopathic bacteria and not to the presence of cyanogenic compounds (Barazani and Friedman, 1999). Others have suggested that these effects are due to the presence of condensed tannins (Ohigashi et al. 1982). In the case of sorghum, it is likely that much of the allelopathic activity is attributable to sorgoleone.

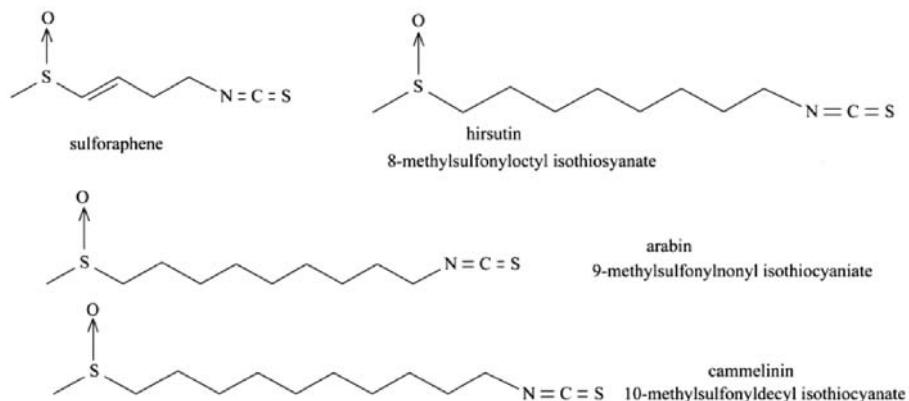
## Glucosinolates

Glucosinolates are derived from several protein amino acids. A number of them have been demonstrated to have inhibitory activity against seed germination and plant growth. In practice, the activity is often attributable to the isothiocyanates, thiocyanates

and nitriles that result from decomposition of glucosides. Because these compounds are common in the Brassicaceae (Cruciferae) and related families, many species of this group of plants probably have allelopathic activity.



The ethyl acetate extracts of *Rorippa indica* contain the isothiocyanates hirsutin, arabin, camelinin, and a series of glucosinolates. These compounds severely inhibit lettuce (*Lactuca sativa*) hypocotyl and root growth at 0.01 mM or more (Yamane et al. 1992).



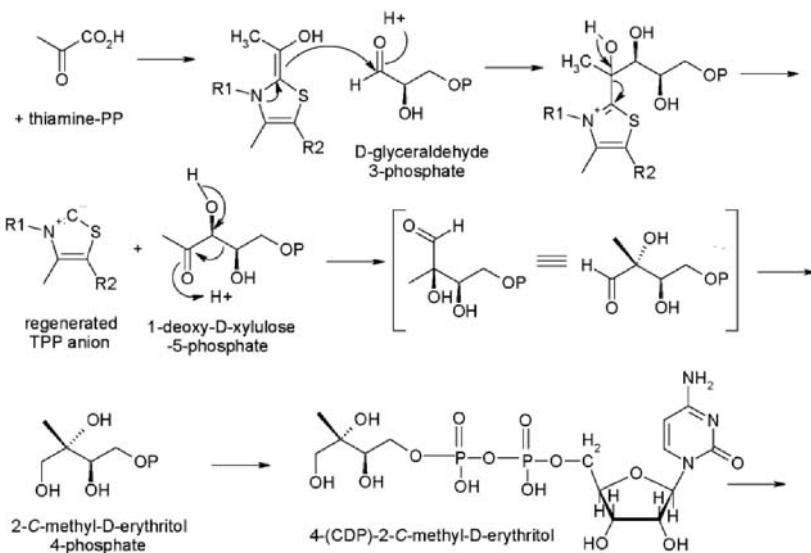
Plants of *Brassica napus*, rapeseed, release glucosinolates that break down to yield allyl isothiocyanate, a compound known to have strong antibiotic properties (Choesin and Boerner, 1991). Rape plants capable of producing allyl glucosinolate suppressed growth of competing alfalfa (*Medicago sativa*) plants. In other studies, the isothiocyanates from the turnip, *Brassica rapa*, inhibited seed germination of a number of weedy species (Petersen et al. 2001).

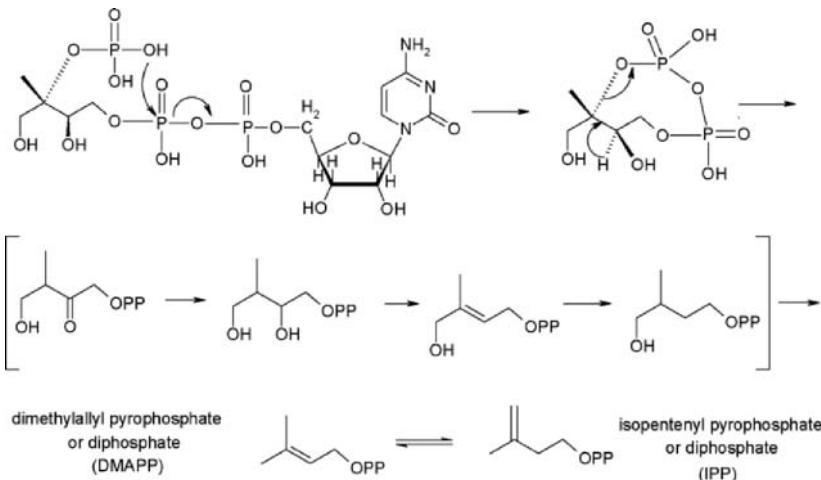
Incorporation of cruciferous plant material into oil suppresses a number of plant pests. When winter rapeseed meal was incorporated, isothiocyanate concentrations increased and this was correlated with a decrease in individuals of wireworms (*Limonius infuscatus*) (Brown et al. 1991).

## TERPENES

Terpenes are the largest of all groups of plant and fungal secondary metabolites. All are derived from two key intermediates, isopentenyl pyrophosphate (or isopentenyl diphosphate, IPP) or dimethylallyl pyrophosphate (or dimethylallyl diphosphate, DMAPP).

Although it was thought that IPP and DMAPP were derived from a single precursor, mevalonic acid (MVA), for many years, it has become apparent that IPP and DMAPP in the biosynthetic systems in plants that lead to monoterpenes, diterpenes, and triterpenes or carotenoids are derived from another series of precursors primarily associated with plastids in the plant cells. This does not appear to be the case in fungi, in which mevalonic acid appears to be involved in the formation of all terpenes.



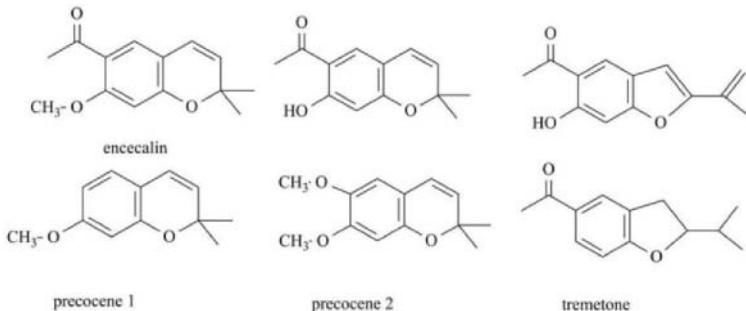


## DOXP-derived terpenes

With the discovery of the DOXP pathway, many observations concerning introduction of labeled precursors and distribution of terpenes within plants and plant groups became clear. IPP and DMAPP from the DOXP pathway associated with plastids are responsible for the formation of monoterpenes, diterpenes, and triterpenes.

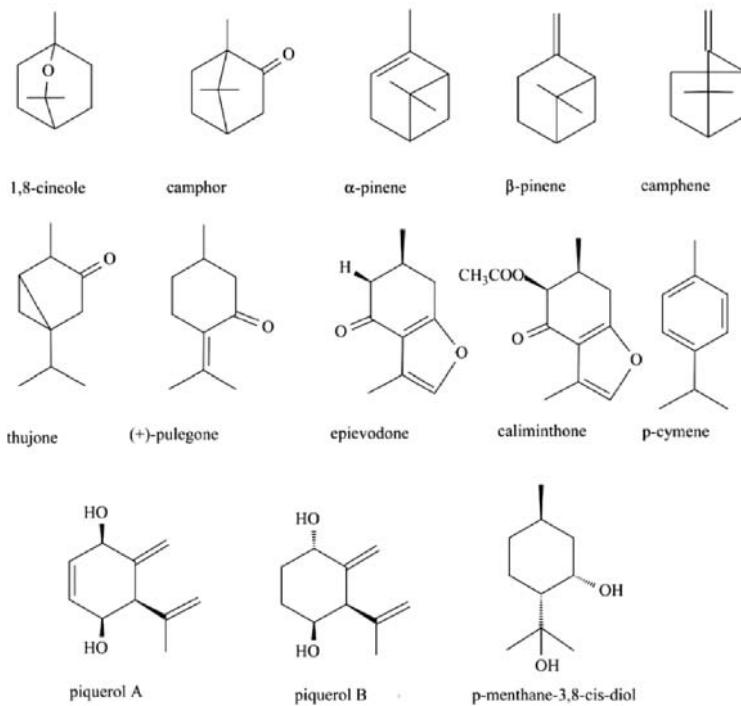
### Hemiterpenes

The origin of a small group of compounds including one unit of DMAPP or, in some instances, IPP, the hemiterpenes, is not well established. Many of these compounds are bioactive; many interfere with normal development of insects (Seigler, 1998).



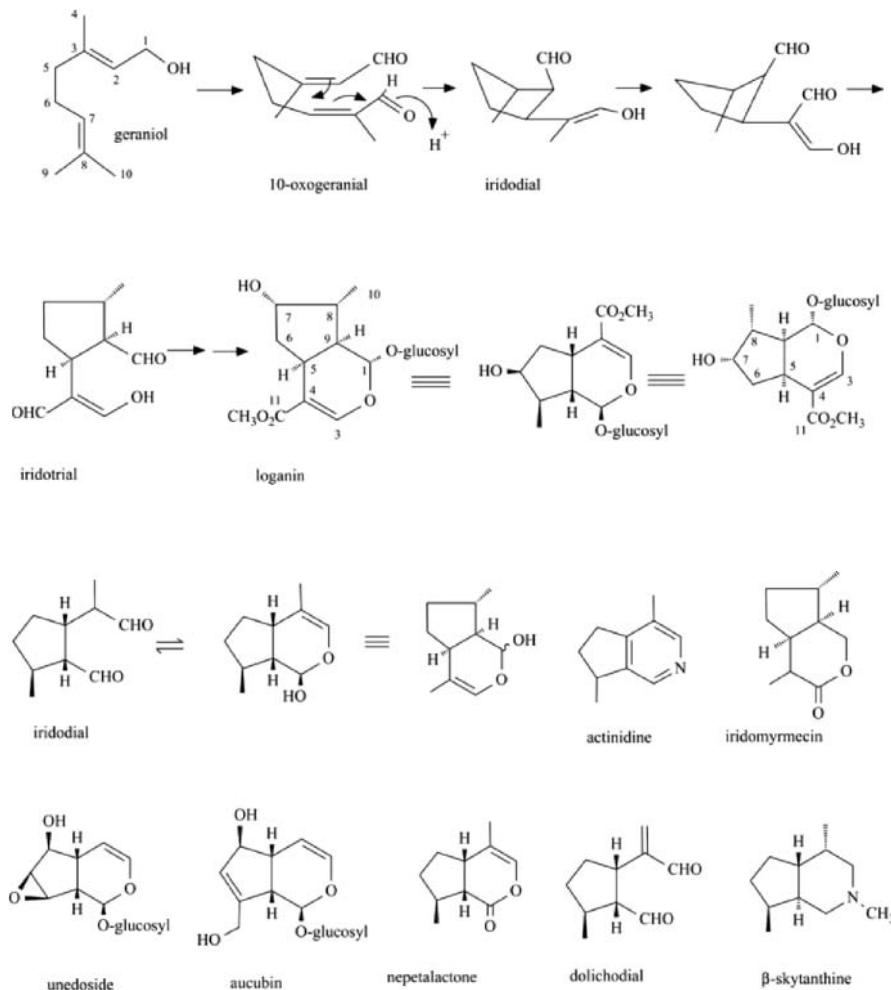
## Monoterpenes

Many monoterpenes are phytotoxic and several have been proposed as starting structures for herbicides (Duke and Oliva, 2004). Relatively high concentrations of 1,8-cineole inhibit mitochondrial respiration of isolated organelles. 1,8-Cineole inhibits all stages of mitosis. Both 1,4- and 1,8-cineole are strong growth inhibitors. The molecular target for 1,4-cineole has been shown to be asparagines synthetase. Camphor also has effects on mitosis and respiration, similar to those of 1,8-cineole (Duke and Oliva, 2004). Many volatile monoterpenes have seed-germination inhibition. These substances also inhibit growth of seedlings and roots (Cutler, 1992; Seigler, 1998). Much work involving monoterpenes has been carried out in the California chaparral community, the Florida scrub, and with species of *Eucalyptus*. Although the compounds involved are volatile, they may also be sufficiently water soluble, especially in the presence of ursolic acid, to be effective (Weidenhamer et al. 1993). The most effective toxins for *Salvia leucophylla* were  $\alpha$ -pinene,  $\beta$ -pinene, and camphene. Those of *Artemisia californica* are 1,8-cineole and camphor. Cineole and  $\alpha$ -pinene proved to be the most effective for *Eucalyptus camaldulensis* (Seigler, 1998).



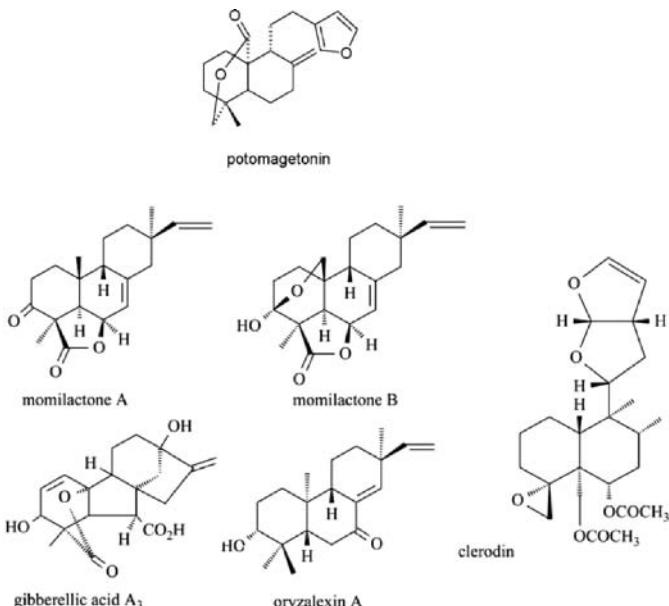
### Iridoid monoterpenes

Further, it has now been demonstrated that secologanin, a precursor of monoterpene-derived indole alkaloids, is derived from the DOXP-pathway (Contin et al. 1998). This finding suggests that most other types of iridoid monoterpenes are also of this origin.



## Diterpenes

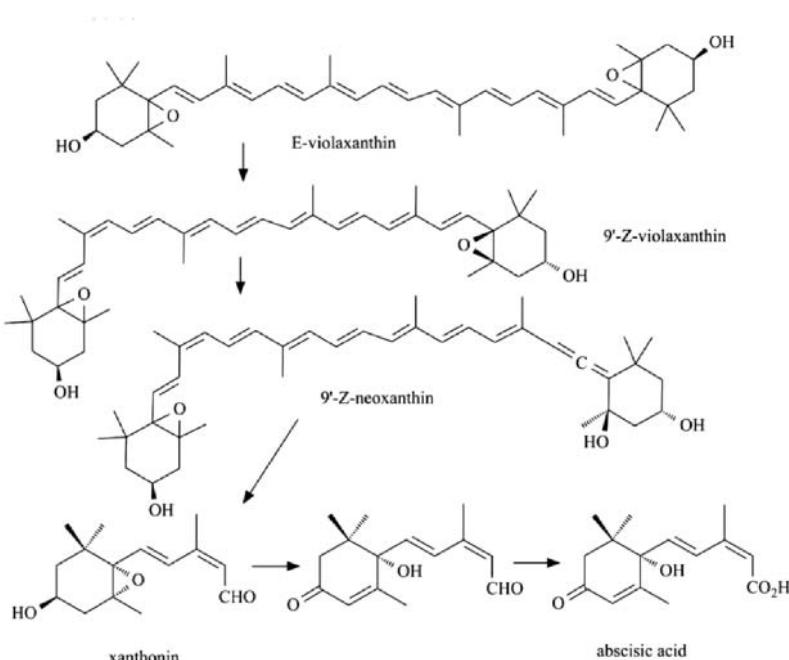
Clerodane, a diterpene from *Viguiera tucumanensis*, inhibits both germination and root growth of *Sorghum halepense* and *Chenopodium album* (Vaccarini et al. 1999). A series of diterpenes, primarily *ent*-labdane derivatives from *Potomageton natans* and *Ruppia maritima* were found to have toxicity to a number of animals, but also toward the alga *Selenastrum capricornutum* (DellaGreca et al. 2004). Potomagetonin proved to be the most active of these. Duvatrienediol from tobacco leaves is phytotoxic to the serious weed *Echinocloa crus-galli* (Duke and Oliva, 2004).



Gibberellins are derived from diterpene pathways. These plant hormones are involved in a number of processes. They retard leaf and fruit senescence and promote seed germination. Several fungi have the ability to produce these compounds as well (Buchanan et al. 2000).

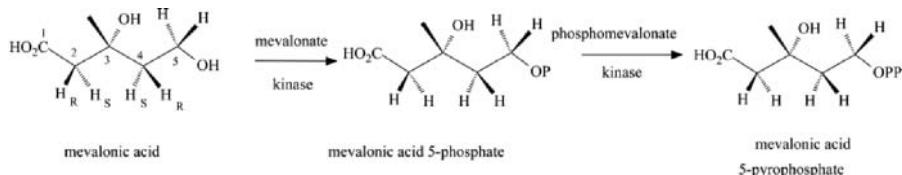
## Tetraterpenes or carotenoids

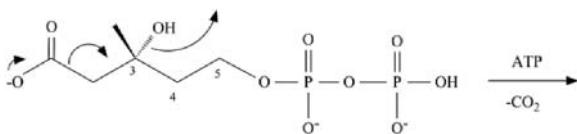
The plant hormone abscisic acid, involved in many plant dormancy interactions, is derived from carotenoid pathways. This compound is produced as a sesquiterpene by several fungi (Seigler, 1998).



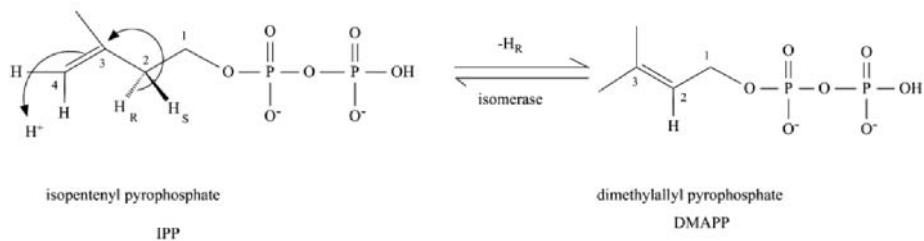
## Mevalonic acid-derived terpenes

The IPP and DMAPP precursors of another large group of terpenes, the sesquiterpenes, triterpenes and steroids, the glycosides of these latter compounds, a series of modified triterpenes found primarily in the Rutaceae, Meliaceae, and Simaroubaceae, and polyterpenes, are derived in plants from mevalonic acid. Further, synthesis is associated with the cytoplasm, in particular with the endoplasmic reticulum.



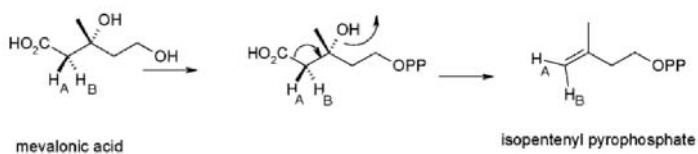


mevalonic acid 5-pyrophosphate



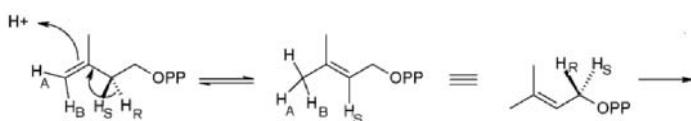
isopentenyl pyrophosphate  
IPP

dimethylallyl pyrophosphate  
DMAPP

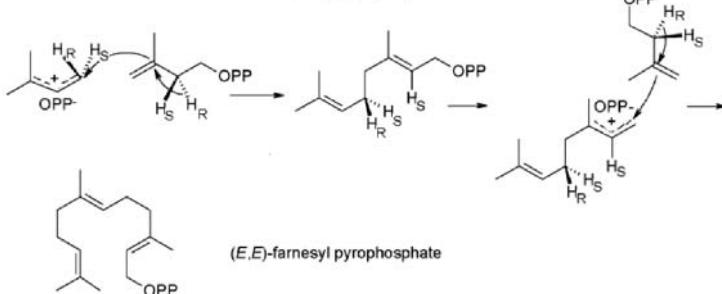


mevalonic acid

isopentenyl pyrophosphate



dimethylallyl pyrophosphate

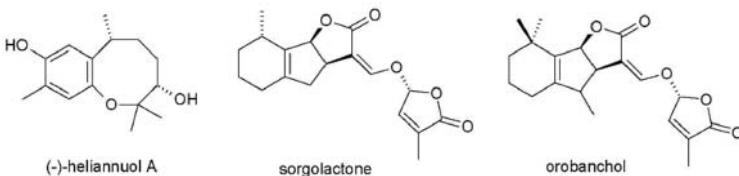


(E,E)-farnesyl pyrophosphate

## Sesquiterpenes

Sesquiterpenes are among the most numerous of all secondary metabolites. They probably occur in all plants, most fungi, and in many animals (Seigler, 1998). These compounds are often biologically active (Duke and Oliva, 2004). The major components of the essential oil of *Callicarpa japonica* are sesquiterpenes. This oil is phytotoxic to bentgrass and to lettuce seeds (Kobaisy et al. 2002). Although the phytotoxic activity cannot be entirely attributed to these compounds, certain sesquiterpenes undoubtedly have growth inhibitory activity. A mixture of  $\beta$ -bisabolene,  $\alpha$ -guaiene,  $\alpha$ -bulnesene,  $\beta$ -patchoulin, and bergamotene from *Ambrosia artemisiifolia* strongly inhibited germination of onion, oat, rye grass, and *Amaranthus palmeri* seeds (Fischer, 1991).

The heliannanes, a group of sesquiterpenes from *Helianthus annuus* and from marine sources have pronounced phytotoxic properties (Macías et al. 2004). Surprisingly, this group of compounds is only known from one terrestrial source, sunflower cultivars. (-)-Heliannuol A is the most active compound of this series in several assays.

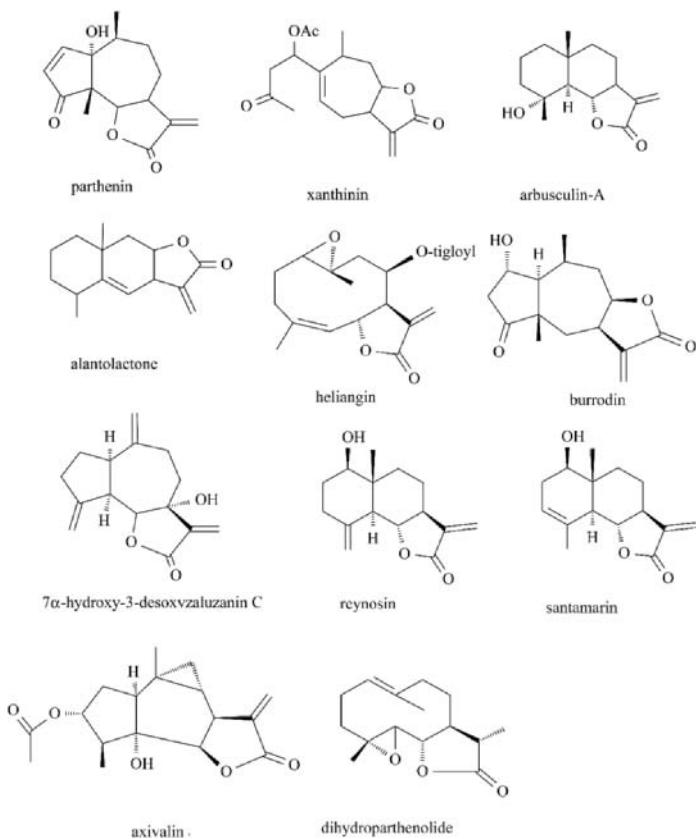


Surprisingly, the first seed germination stimulant for *Striga* and for *Orobanche* species, strigol, was isolated from cotton, *Gossypium hirsutum*. Subsequently, however, similar compounds such as sorgholactone and orobanchol were isolated from plants that can serve as hosts for the parasites (Galindo et al. 2004).

Several terpenoid compounds serve as fungal gamete attractants. The sesquiterpene sirenin is involved in attraction of male gametes to the female gametangia of *Allomyces macrogyrus* (Seigler, 1998). Abscisic acid, although derived from tetraterpenoids in higher plants, is formed from mevalonic acid and is a sesquiterpenoid from fungi. This plant hormonal substance has powerful biological activity and is involved in many plant growth and developmental processes (Seigler, 1998). A number of sesquiterpene lactones also are capable of inducing germination of *Striga asiatica* seeds. The germacraneolide dihydroparthenolide and the eudesmanolides reynosin and santamarin were especially active (Galindo et al. 2004). Artemisinin, from *Artemisia annua*, is a highly active hydroperoxide (Duke and Oliva, 2004).

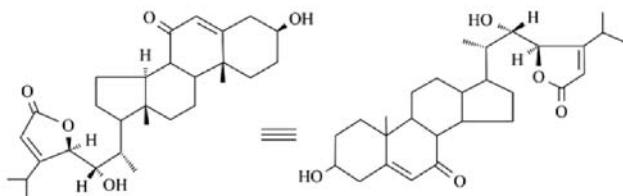
Although best known as an antimalarial drug, this compound inhibits respiration in *Lenna minor* and all mitotic phases in onion root tips.

Many plants that produce sesquiterpene lactones appear to have allelopathic properties. These compounds are most common in the family Asteraceae (Seigler, 1998). Parthenin and coronopolin from *Parthenium hysterophorus* appear to affect numerous crop plants. Arbusculin-A from *Artemisia* spp. is responsible for many of the allelopathic effects of that plant. Alantolactone, from many members of the Asteraceae, inhibits seed germination and growth of the common weedy species *Amaranthus retroflexus* and *Chenopodium strictum* var. *glaucomphyllum* (Picman, 1986). The sesquiterpene lactones burrodin, confertiflorin, desacetylconfertiflorin, dihydroparthenolide, parthenin, and 7 $\alpha$ -hydroxy-3-desoxyzaluzanin C inhibited or promoted germination of seeds of 16 dicot and 9 monocot species (Fischer et al. 1989).

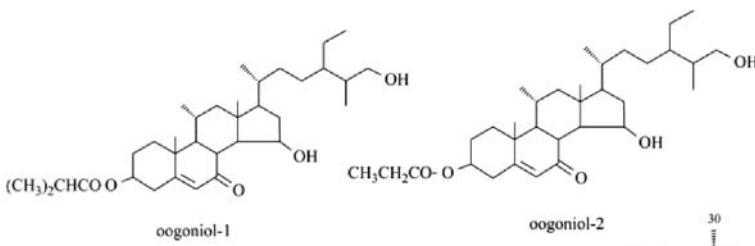


## Triterpenes and steroids

Most triterpenes do not appear to be highly phytotoxic, although they may serve in some instances to co-solubilize other bioactive compounds. Allelopathic monoterpenes are volatile, but they may also be sufficiently water soluble, especially in the presence of ursolic acid, to be effective as allelopathic agents (Weidenhamer et al. 1993).

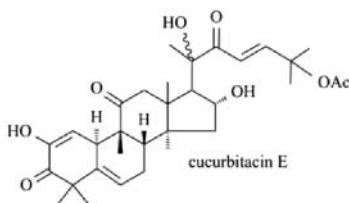


antheridiol

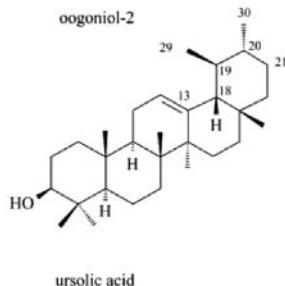


oogoniol-1

oogoniol-2

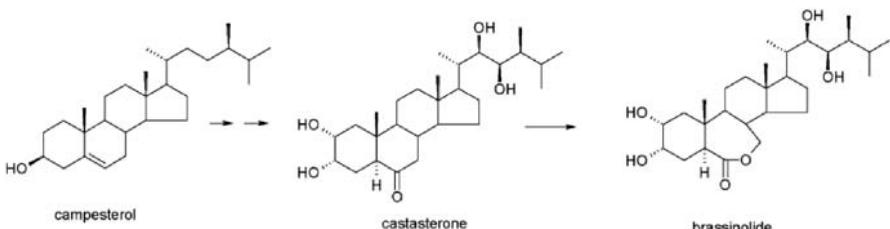


cucurbitacin E



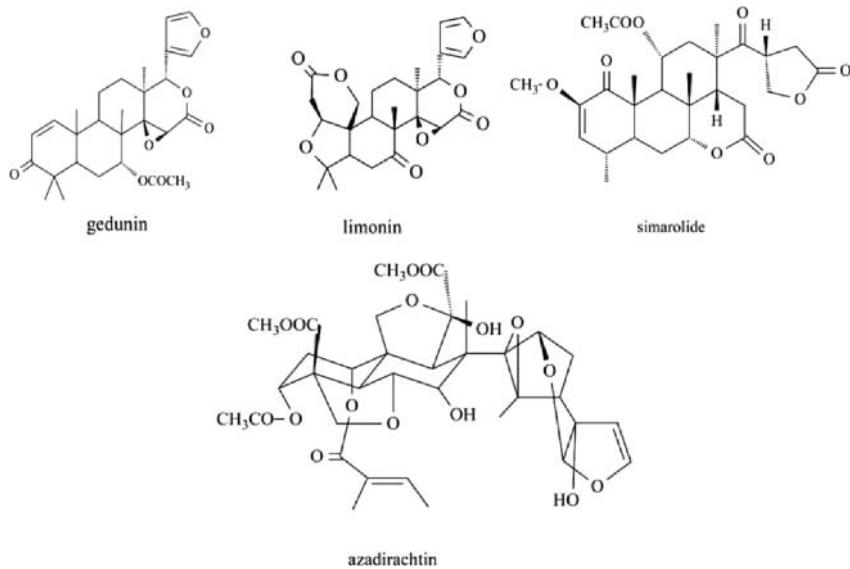
ursolic acid

Sterols are common components of plant membranes (Buchanan et al. 2000). Brassinosteroids are found in all plants, but have not been found in microorganisms. More than 40 are known. These compounds are involved in growth of pollen tubes, internode elongation, unrolling of grass leaves, bending of grass leaves at the joing, and are considered plant growth regulators (Buchanan et al. 2000). They are derived from campesterol. Antheridiol and oogoniols are components of gamete attractants in fungi of the genus *Achlya* (a coenocytic water mold (Seigler, 1998).



Tetranortriterpenoids and decanortriterpenoids

A series of tetranortriterpenoids and decanortriterpenoids occur in the related plant families Rutaceae, Cneoraceae, Meliaceae, and Simaroubaceae. A number of these compounds have insect antifeedant activity and several have antimalarial activity. A number of species of these families exhibit allelopathic activity. One of these, *Ailanthus altissima*, Simaroubaceae, is a common weedy plant in many parts of the world. The most active phytotoxic compound in this species is the quassinoïd ailanthone (Lin et al. 1995).



## Saponins

Saponins, steroidal or triterpenoid compounds substituted with various carboxyl, carbonyl, or hydroxyl groups and usually bearing sugar chains, are often responsible for changes in membrane permeability, leakage and hemolysis. These compounds are widespread among plants. Certain saponins either promote or inhibit seed germination and development of roots and shoots (Oleszek et al. 1999).

The aglycones soyasapogenols B and E are capable of inducing haustorium formation in the parasitic plant *Agalinus purpurea* (Oleszek et al. 1999). Saponins based on soyasapogenols promote growth of wheat. These compounds are common in many plants of the Fabaceae: subfamily Papilionoideae.

The most widely studied saponins for allelopathic interactions are those of alfalfa. The saponins of alfalfa (*Medicago sativa*) consist of at least 30 glycosides based on a variety of triterpenoid nuclei (Bialy et al. 1999, Oleszek et al. 1999). Alfalfa roots often contain as much as 4-5% saponins. These saponins consist of mono-, bi-, and tridesmosides and have from 2-7 sugar moieties attached. These saponins are responsible for many of the antinutritional aspects of alfalfa, and may also be responsible for the autotoxic effects and allelopathy attributed to this species (Oleszek et al. 1999). When cotton is grown in the same fields following alfalfa crops, the yields are considerably reduced. In other studies, alfalfa root saponins have been shown to be toxic to cotton. As little as 10 ppm alfalfa saponins is toxic to barnyard grass (*Echinochloa crus-galli*) and to cheat (*Bromus secalinus*). However, soil microflora can decompose alfalfa saponins quickly (Oleszek et al. 1999).

## ALKALOIDS

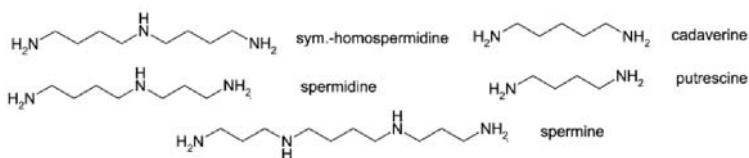
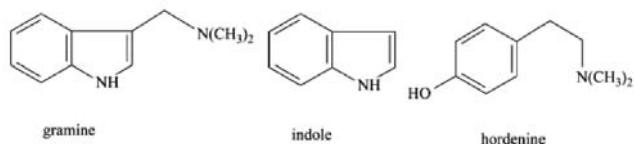
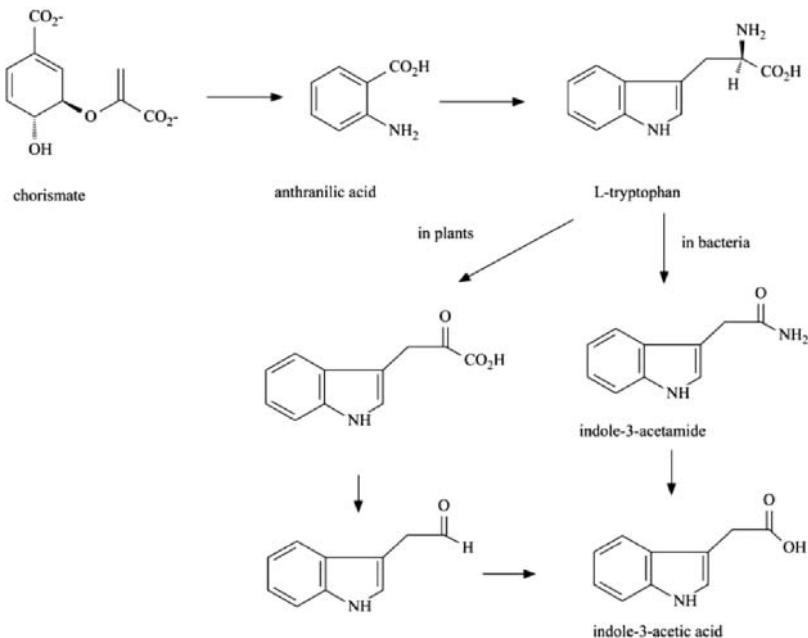
Alkaloids, as a group, have not been investigated thoroughly for allelopathic activity. Many alkaloids have been evaluated in a series of bioassays that may be applicable for these studies, however (Wink et al. 1999).

### Amines and simple alkaloids, polyamines and polyamine alkaloids

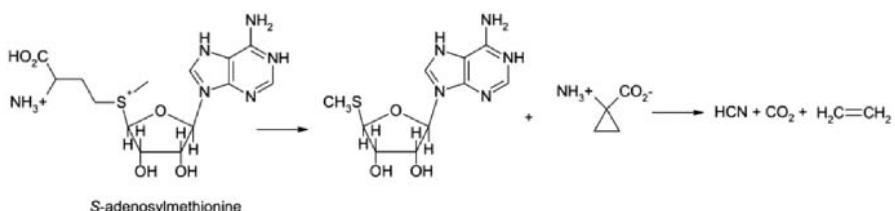
Among the amines and simple alkaloids, indole 3-acetic acid, is important. This plant hormonal substance is produced by both plants and fungi, albeit by different pathways.

Hordenine and gramine from barley plants have generally been considered to be allelopathic as barley fields are usually quite free from weeds (Seigler, 1998). Gramine inhibits radicle growth of several plants (Wink, 1993).

Polyamines are of relatively simple biosynthetic origin. These compounds appear to play a number of fundamental roles in plant cells. Among these is the production of ethylene. A series of polyamine alkaloids is derived from precursors of this type.

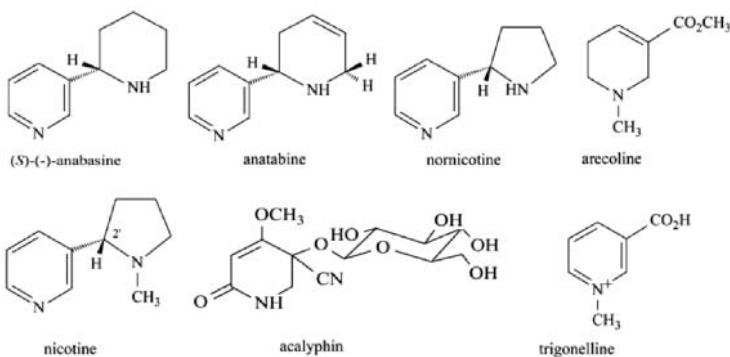


Ethylene is liberated from the non-protein amino acid 1-aminocyclopropane-1-carboxylic acid (ACC), which is formed from the same series of pathways that leads to synthesis of many of the polyamines above (Seigler, 1998).



## Pyridine alkaloids

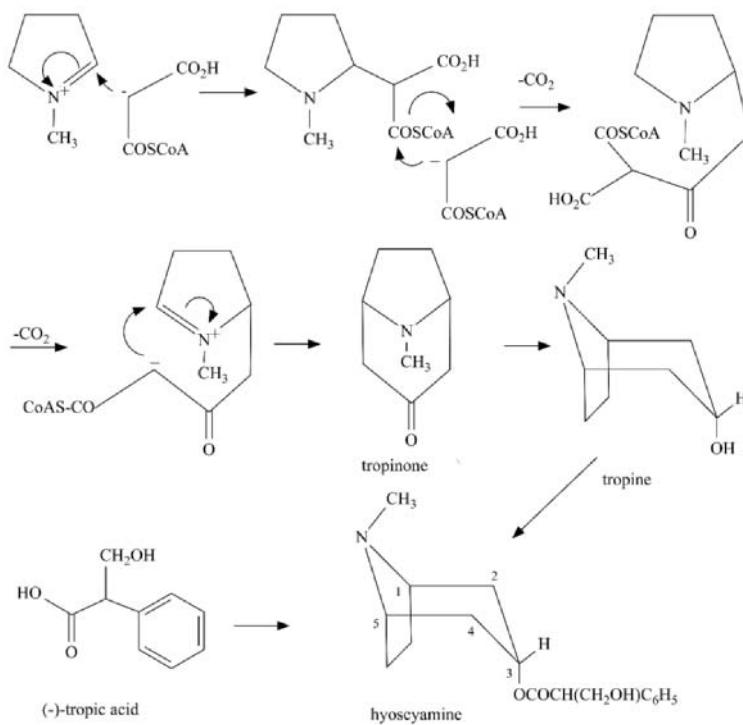
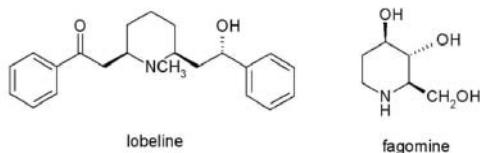
Nicotine, a pyridine alkaloid, inhibits radicle growth of *Lepidium*, and is toxic to *Lemna* plants (Wink, 1993). Trigonelline, now known to be widespread in plants, promotes cell arrest in the G<sub>2</sub>-stage of the cell cycle (Seigler, 1998). Salsoline weakly inhibits protein synthesis (Wink et al. 1999).



## Pyrrolidine and piperidine alkaloids, tropane and pelletierine alkaloids

Pyrrolidine and piperidine alkaloids, tropane and pelletierine alkaloids are all formed from the diamines putrescine or cadaverine. Fagomine, a piperidine alkaloid, was one of the allelopathic alkaloids isolated from *Fagopyrum esculentum*, buckwheat (Iqbal et al. 2002). This type of alkaloid is a sugar mimic. Many of these alkaloids have biological activity greater than might be expected (Seigler, 1998). Lobeline, a

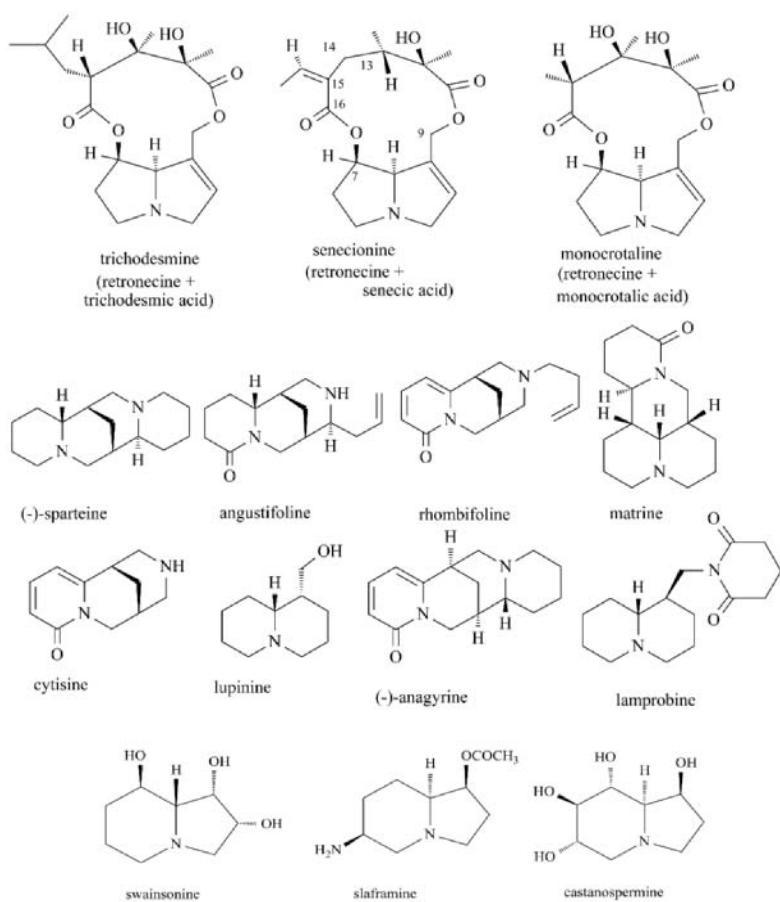
piperidine alkaloid, inhibits DNA polymerase I, reverse transcriptase activity, and protein synthesis (Wink et al. 1999).



## Pyrrolizidine, quinolizidine and indolizidine alkaloids

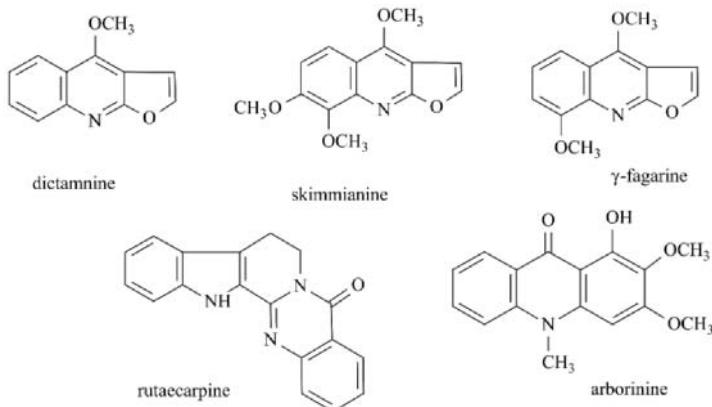
Pyrrolizidine, quinolizidine and indolizidine alkaloids are of restricted distribution in plants. Pyrrolizidine alkaloids are hepatotoxic in mammals, but are involved in many

plant-insect interactions. Derivatives of these alkaloids serve a role as apodisiacs in certain insects. There are few, if any, reports of the allelopathic activity of this group of compounds. Indolizidine alkaloids are also found in only a few plants; they are most common among legumes, especially the genera *Astragalus* and *Swainsona*. Although highly active biologically, these alkaloids also have not been implicated to date in allelopathic interactions. However, field data with alkaloid-rich and alkaloid-free lupines clearly show that quinolizidine alkaloids are the crucial factor controlling herbivory under natural conditions (Wink, 2004). Quinolizidine alkaloids are especially rich in seeds where they may make up as much as 8% of the total seed weight. These compounds also can inhibit germination and growth of other plants (Wink, 2004).



## Anthranoilic acid-derived alkaloids

A series of alkaloids derived from anthranilic acid are important in rue (*Ruta graveolens*) and other members of the family Rutaceae, but are not common in other families. Among the compounds that may be associated with allelopathy are: Arborine, rutacridone, skimmianine, 6-methoxydictamnine,  $\gamma$ -fagarine, and graveoline.

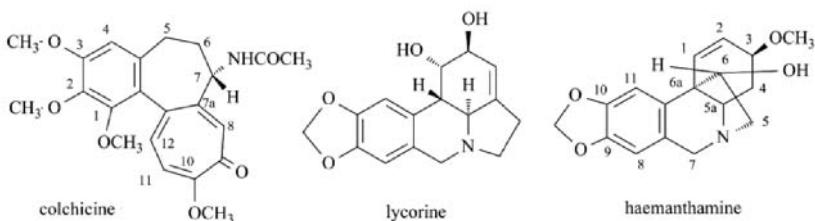
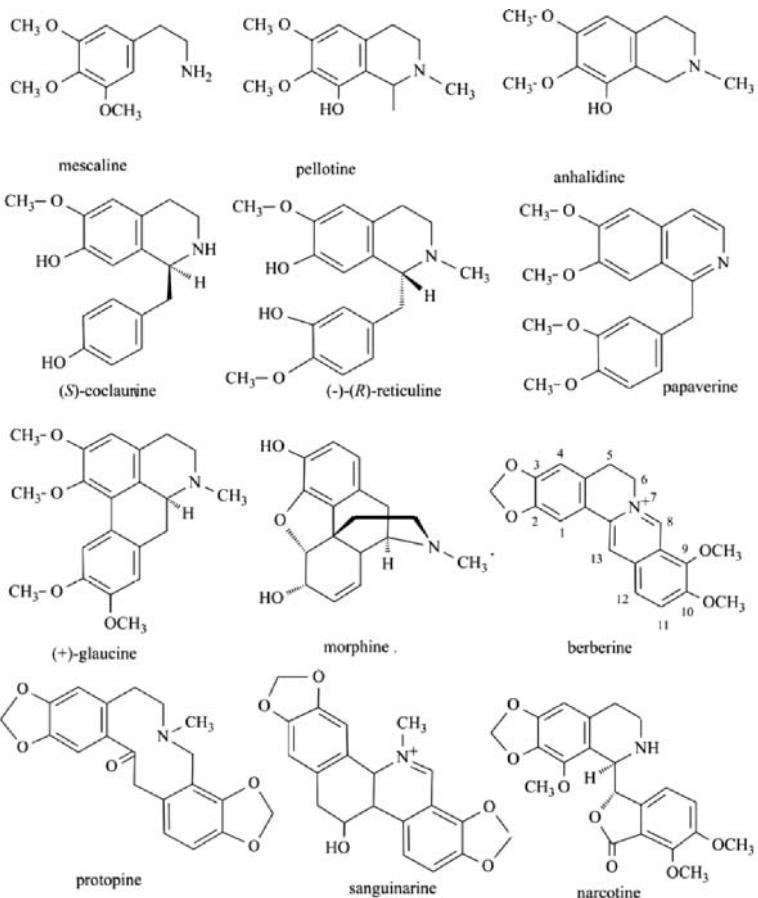


## Isoquinoline and benzylisoquinoline alkaloids

The isoquinoline and benzylisoquinoline alkaloids are among the most important and largest of alkaloid groups. Several alkaloids of this group were found to interact at basic molecular targets by Wink and coworkers (1999). Boldine, berbamine, berberine, and sanguinarine intercalate with DNA. Sanguinarine, berberine, berbamine, and boldine inhibit DNA polymerase I. Sanguinarine, berbamine, berberine, papaverine, and boldine inhibit RNA reverse transcriptase. Papaverine, boldine, and berberine inhibit protein synthesis.

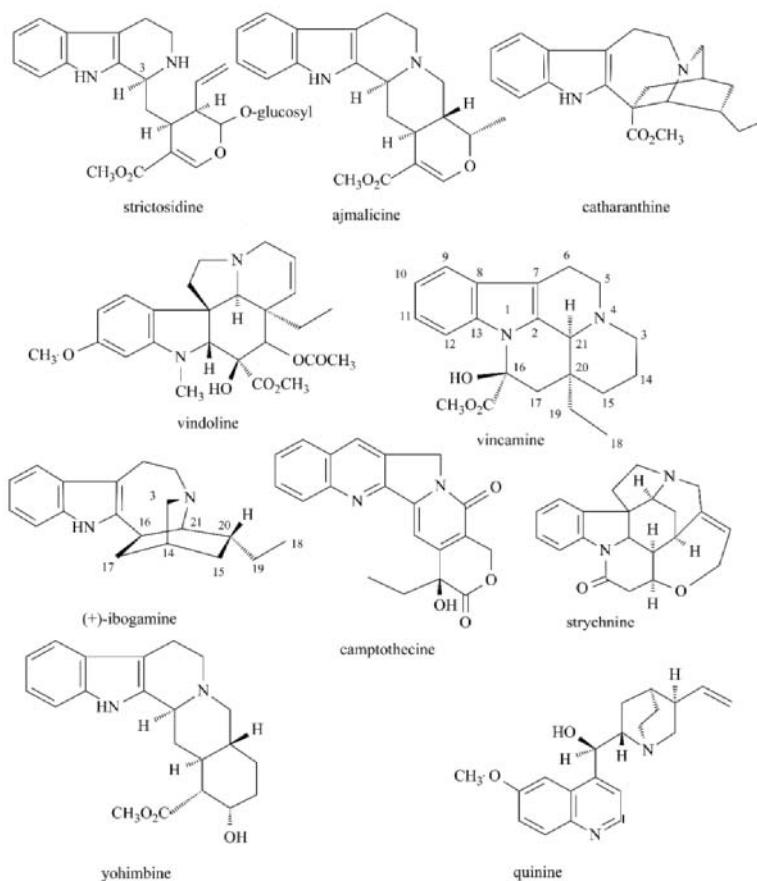
## Phenylalanine and tyrosine-derived alkaloids

Another series of alkaloids is derived from both phenylalanine and tyrosine. One representative of this group, colchicines, inhibits cell division. The amaryllidaceous alkaloids lycoricidinol and lycoricididine from *Lycoris radiata* showed growth-inhibiting activity on *Avena* coleoptile sections. Narciclassine and narciprimine from daffodils have antimitotic activity in plants (Seigler, 1998).



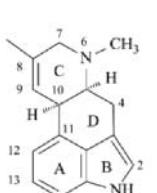
## Monoterpene-derived indole alkaloids

Secologanin, a precursor of monoterpene-derived indole alkaloids has been shown to be isolated from the DOXP-pathway (Contin et al. 1998). Ajmaline, cinchonine, cinchonidine, quinidine and quinine intercalate with DNA. Cinchonine, cinchonidine, quinidine, and quinine inhibit DNA polymerase I. Ajmalicine, cinchonine, cinchonidine, quinidine, quinine, and strychnine inhibit RNA reverse transcriptase. Ajmaline, cinchonidine, quinidine, quinine, and yohimbine inhibit protein synthesis (Wink et al. 1999). These alkaloids are mainly restricted to the families Apocynaceae, Loganiaceae, and Rubiaceae, but occur in several others in isolated instances (Seigler, 1998).

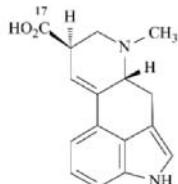


## Other indole alkaloids

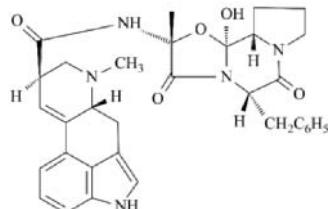
Although monoterpane-derived indole alkaloids are the most common type in nature, other alkaloids with indole-ring structures are known. The most common ones are ergot alkaloids from the fungal family Clavicipitaceae and the morning glory family, Convolvulaceae (Seigler, 1998). Ergometrine, harmalin, harmin, and norharman intercalate with DNA. Harmaline, harmine, and norharman inhibit DNA polymerase I. Ergometrine, harmalin, harmin, and norharman inhibit RNA reverse transcriptase. Harmaline, harmine, and norharmane inhibit protein synthesis (Wink et al. 1999).



agroclavine

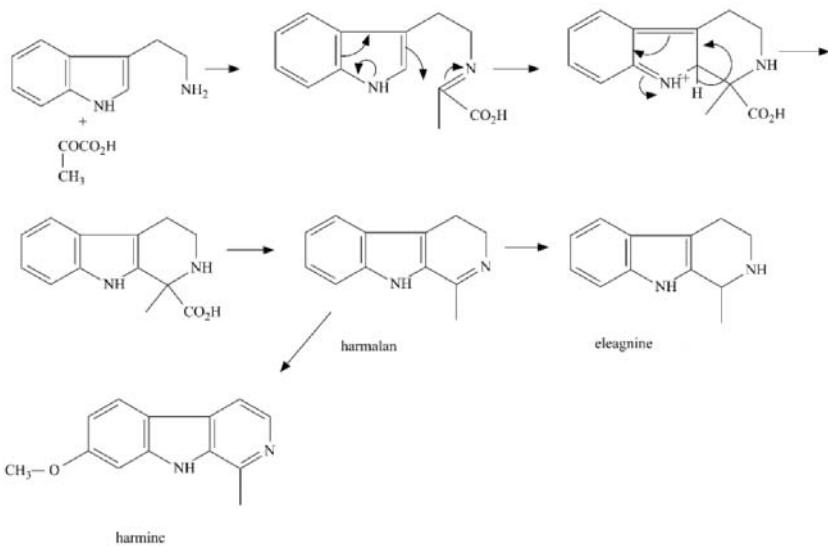


lysergic acid



ergotamine

Harmine is an insect inhibitory compound produced by the roots of oca, *Oxalis*, *tuberosa*, for the insect *Trichoplusia ni* (Walker et al. 2003). In this instance, the compound may be photoactivated.

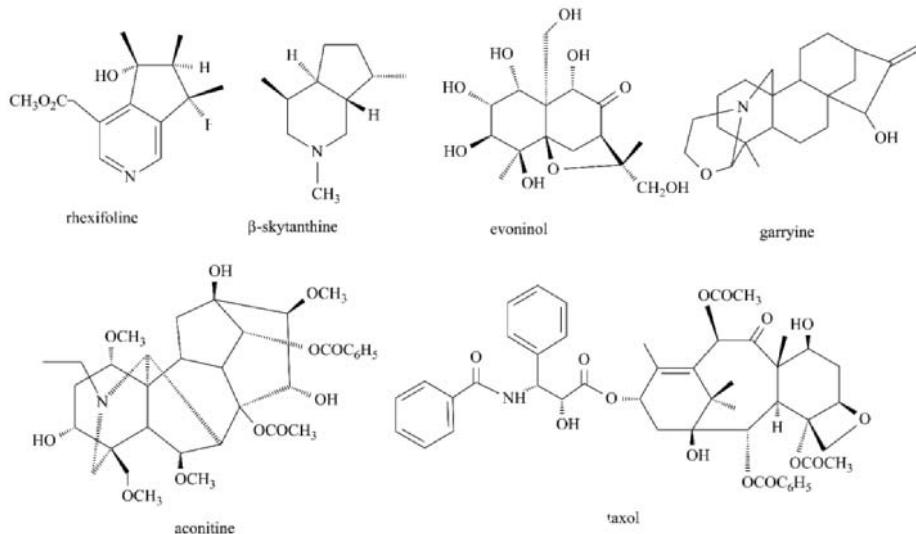


## Terpene-derived alkaloids

A large number of alkaloids are derived from terpenoid starting units. These include most major groups of terpenes.

The *Solanum* alkaloid solanine inhibits RNA reverse transcriptase, protein synthesis and causes hemolysis of membranes (Wink et al. 1999).

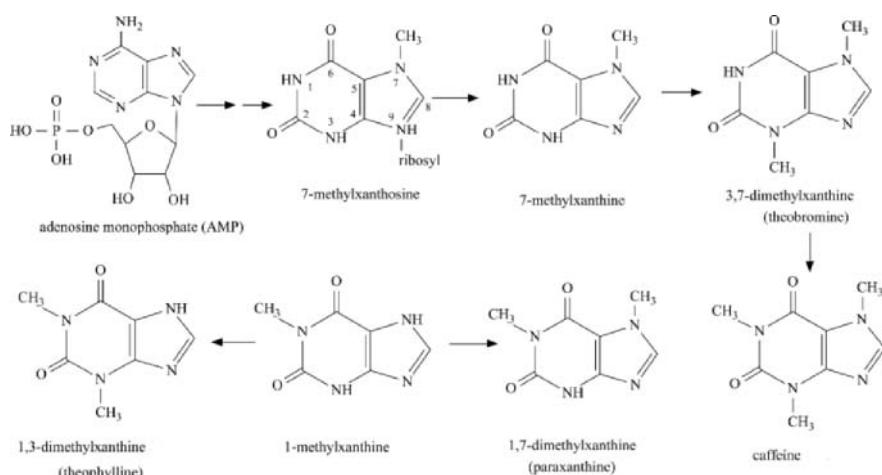
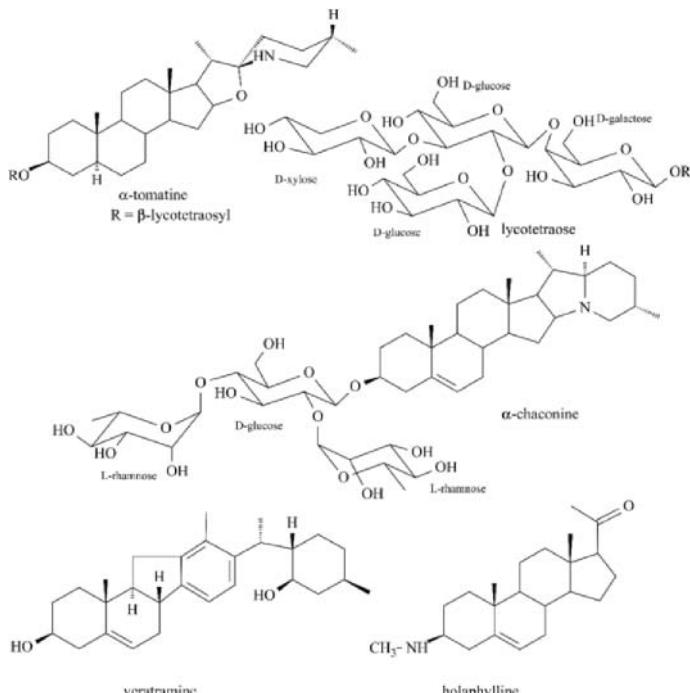
Caffeine and related xanthine alkaloids are known to have allelopathic properties. Caffeine is autotoxic to *Coffea arabica*, seedlings. Caffeine, theobromine, and theophylline inhibit growth of lettuce seedlings and certain other species of plants (Wink, 1993).

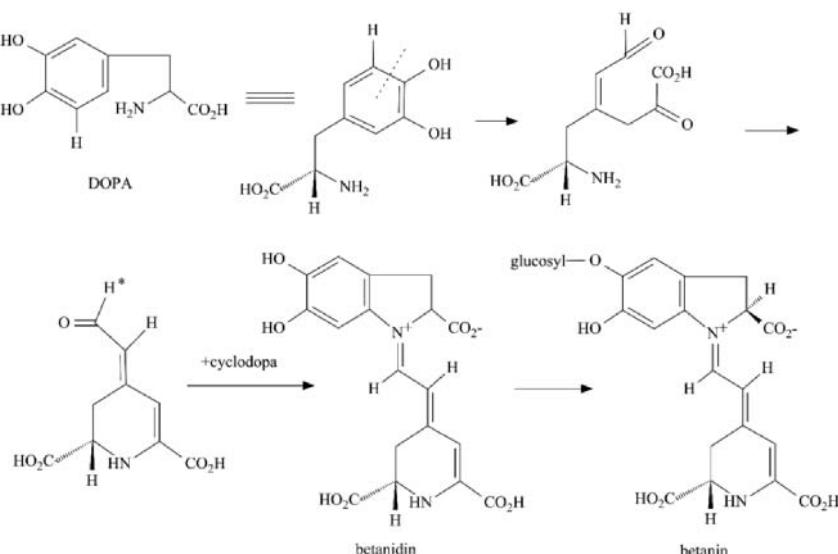


## Betalains

Betalain pigments, derived from tyrosine and other amino acids, are restricted to a group of about 10 families in the order Caryophyllales. These pigments occur in all plant parts.

Approximately 50 compounds of this type are known (Seigler, 1998). Although relatively innocuous to humans, these compounds may have greater activity than usually recognized. Their role as allelopathic agents should be examined.





## MISCELLANEOUS

Many plants have the ability to exude organic acids that alter the soil pH. This often is linked to aluminum tolerance (Buchanan et al. 2000; Ma et al. 2001). Citrate, malate, and oxalate are commonly released by aluminum tolerant species. Some plants, e.g., buckwheat leaves, *Hydrangea*, and *Melastoma malabathricum* are capable of accumulating aluminum. The ability to detoxify aluminum in the soil or to inhibit other plants from doing so may contribute to patterns of vegetation and allelopathic problems.

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## CHAPTER 3

# CLUES IN THE SEARCH FOR NEW HERBICIDES

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## INTRODUCTION

Modern agricultural weed management practices rely significantly on the use of synthetic herbicides. The ability to control weeds efficiently has been one of the key components, along with the use of fertilizers, of the ‘green’ revolution that resulted in formidable increases in crop yield over the past 50 years. While there is a large number of commercial active ingredients, these represent relatively few chemical classes that affect an even fewer number of target sites. In spite of the successful weed control achieved with herbicides, certain weed species ultimately evolved resistance to some of these compounds. In some instances, evolution of resistance to a specific herbicide occurred very rapidly (2-3 years) and has led to cross resistance to entire chemical classes (e.g., ALS inhibitors), underlining the constant need for new chemistries and new target sites.

The plant protection industry is undergoing a rapid transformation, as new paradigms driven by biotechnology-based, herbicide-resistant transgenic crops have destabilized the traditional herbicide market. The technological advantages of these techniques usually encompass broader weed spectra, lower crop injury, and simpler and comparatively less expensive agricultural practices. Therefore, efforts to discover new, selective herbicides have been dramatically reduced in most companies, and few new chemical classes that target new molecular target sites are being developed. Furthermore, public concern over the safety of pesticides has been a powerful force that led to the Food Quality Protection Act in the USA, and similar legislation in other countries that have introduced more stringent registration guidelines for chemical pest management tools. These new guidelines have placed additional pressures on the

agrochemical industry and indirectly decreased the incentive to invest in the development of traditional herbicides.

Nonetheless, herbicides account for more than 60% of all pesticides used in agriculture in the USA, and they will continue to play an important role in the foreseeable future. While the industry is recovering from the challenge imposed by biotechnology, the need for new molecular target sites and new chemistries has not abated. The computer-aided design and combinatorial approaches touted as the most promising venues have failed to fulfill expectations. While some companies are returning to their traditional synthesis and screen approaches, other groups are focusing on compounds of natural origins.

Natural products have historically been a valuable source of many pesticides, used either directly as crude preparations, as pure compounds, or as structural leads for the discovery and development of natural product-based pesticides. The impact of natural products have historically been greater on the development of fungicides and insecticides than on herbicides (Dayan, 2002), but the potential benefits of natural product-based herbicides remain underestimated. There is a myriad of plant, microbe, and animal natural products with an enormous range of structural diversity (Henkel et al. 1999) that arose from co-evolution between competing organisms. Thus, most secondary metabolites are biologically active. Their natural functions may be as phytotoxins, as with compounds like phosphinothricin produced by *Streptomyces hygroscopicus* (Lydon and Duke, 1999) or the allelochemical sorgoleone produced *Sorghum bicolor* (Netzly and Butler, 1986; Einhellig and Souza, 1992). However, secondary metabolites can also be used for purposes quite different from their natural function, as with many natural product-based pharmaceuticals.

There has been less interest in plants than in microbes as sources of lead compounds for herbicides, although many plant-derived compounds are highly phytotoxic. Some examples include cinmethylin (Cinch®), a herbicide with limited success, which mimics plant cineoles, and the triketones, a new class of herbicides from Zeneca, that were derived from the plant secondary metabolite leptospermone (Lee et al. 1997). Pelargonic acid (nonanoic acid), a plant-produced fatty acid, is sold for weed management in turf (Bradley and Hagood, 2002; Irzyk et al. 1997; Pline et al. 2000). Renewed interest in bioactive compounds of natural origin may, in part, be attributed to recent technological advances facilitating the isolation, characterization and testing of natural products. Other factors, including the realization that nature has already selected for very specific biological activities, that many natural compounds have yet to be discovered, and that the biological activities of relatively few of the known natural products have been characterized, have also stimulated interest in the pesticidal uses of natural products.

As mentioned above, the modes of action of commercial herbicides are limited to a few (Chapter 24) target sites. As more and more weed species evolve resistance to

specific herbicides, primarily via single mutations at the target site, potentially rendering them cross-resistant to entire chemical classes, the need for new molecular target sites is pressing. Mode of action studies are difficult because of the thousands of putative molecular targets sites that exist in plants. Some of the advantages of studying the mode of action of natural products is that these phytotoxins tend to inhibit target sites that are different from those affected by synthetic herbicides (Duke et al. 1996, 1997, 2000a). Therefore, studying the mechanisms of action of natural products enhances the likelihood of discovering new mechanisms of action.

In this chapter we describe some of the methods that can be used to search for novel phytotoxic compounds and to identify new molecular target sites for herbicides. This is an update of a previous review of the topic (Dayan et al. 2000). The methods presented are by no means exhaustive, but are intended to help design preliminary studies yielding important information from which more detailed experiments can be devised to completely elucidate the structures and their modes of action.

## **BIOASSAY-DIRECTED ISOLATION**

Bioassay-directed isolation is a simple, iterative process in which the compounds of an organism are removed in different fractions, and the fractions are bioassayed for biological activity of interest. Those fractions with activity are further fractionated and the new fractions bioassayed until, eventually, a pure compound with the desirable activity is found. With luck, a new, highly potent compound is discovered. However, the activity of the crude extract could be due to a high concentration of a compound with low activity, or the active compound could be a known compound. In some cases, activity is lost during fractionation, either by chemical modification or degradation of the active compound or due to the synergistic requirement for two or more of the compounds in the crude extract for activity. Nevertheless, bioassay-guided isolation of active compounds is a standard procedure that has become much simpler with the advent of modern analytic equipment and high-throughput, miniaturized bioassays (Duke et al. 2000b).

## **FRACTIONATION, ISOLATION, AND IDENTIFICATION**

Typically, for discovery purposes, plants are extracted with a graded series of solvents in decreasing or increasing polarity in order to remove all soluble natural products. Extraction can be accelerated by heating and/or refluxing (e.g., Soxhlet), however, even when extracting at room temperature, chemical artifacts can be created. Critical fluid extraction with CO<sub>2</sub> under pressure may give less artifactual extraction. Active fractions can be further fractionated with other chromatographic methods. Modern tandem instrumentation in which separation and analytical steps are coupled,

such as GC/MS, LC/MS and LC/MS/NMR, have streamlined bioassay-directed isolation and identification of natural products from plants. Using this technology, fractions can be analyzed for their constituents after bioassay. These techniques can often provide a putative identification of the likely bioactive constituent before it is purified and tested. If so, the process can be streamlined to purify this compound without having to generate subfractions that must be tested and further fractionated.

## BIOASSAYS

### Selection of bioassay

Herbicide discovery programs are dependent on suitable bioassays that must be standardized and reproducible. Miniaturization of the bioassays is highly desirable since, by nature, bioassay-guided isolation of natural products normally yields small amounts of the compounds to be tested (see previous section). The species selected should germinate easily and uniformly, and grow relatively rapidly in order to obtain data without undue delays. Lettuce (*Lactuca sativa* L.) and bentgrass (*Agrostis stolonifera* L.) are used on a regular basis in our laboratory as model dicotyledonous and monocotyledonous species, respectively, to determine potential selectivity of the substance tested (Dayan et al. 2000). However, many other species are suitable for bioassays. For example, duckweed species (*Lemna* spp.) are small aquatic plants well suited for bioassays due to their rapid growth and relative sensitivity to xenobiotics (e.g., Amagasa et al. 1994; Michel et al. 2004). The extensive knowledge of the genome of *Arabidopsis thaliana* and its small size make it well suited for bioassays, however, it is naturally resistant to some phytotoxins to which other test species are susceptible. As well, known allelopathic effects of one species over another may direct the selection of the target species when testing compounds produced by the allelopathic plant. This approach is often considered in our laboratory. Thus, barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.), gooseweed (*Sphenoclea zeylandica* Gaertn.), and ducksalad (*Heteranthera limosa* (Sw.) Willd.) are often selected as target species when investigating phytotoxins isolated from allelopathic rice varieties (Rimando et al. 2001). Using weed species in bioassays presents some difficulties. Many of these species have low germination rates and require some form of stimulation such as scarification (either mechanical or acid), light, osmotic shock or temperature treatments (Buhler and Hoffman, 1999).

Bioassays can be performed on filter papers, inert substrates (e.g., sand or vermiculite), hydroponics and soils. The last two forms are not often used because the amount of compound to be tested is often limited. There are, however, many reports where aqueous extracts of allelopathic plants (leaf or root extracts) were used in hydroponic systems (e.g., Nimal and Weston, 1997), and plant tissues or extracts

were incorporated into soils used to grow target weed species (e.g., Lydon et al. 1997; Dayan and Tellez, 1999).

There are many other factors to consider in developing bioassays. The ratio between chemical to total seed weight ratio is an important consideration in assessing the potency of phytotoxins (Weidenhamer et al. 1987). A particular concentration of a phytotoxin may seem more active on species with small seeds than one with large seeds. As well, the same concentration of a compound may show different levels of activity if the number of seeds is not constant between assays. Testing volatile compounds requires special modifications to the assays in order to contain the test compounds within their compartments, making the use of 24-well plates undesirable for these compounds (Romagni et al. 2000). One must also check that the osmotic potential or pH of the test solution is not affected by the compounds tested (e.g., Duke et al. 1983).

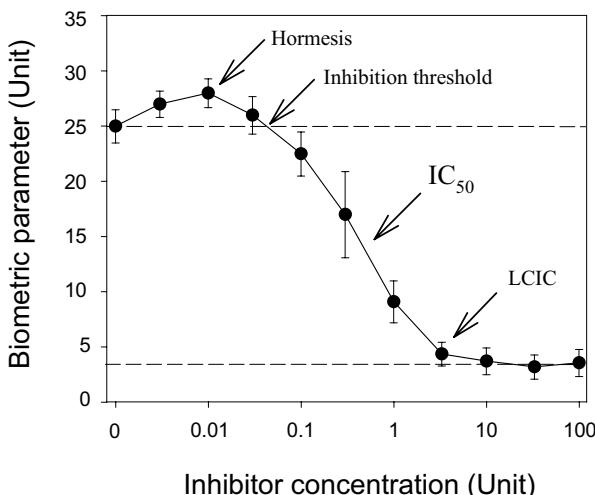
Many natural toxins have limited water solubility, so stock solutions are often prepared in a transfer solvent. Acetone and dimethylsulfoxide tend to be the best transfer solvents (other than water), with no physiological effect detected when levels stay below 1% v/v or less in the assay. Many natural products can be tested with one of these solvents (Weidenhamer et al. 1993). However, highly non-polar compounds, such as essential oils, are mostly water insoluble. It is possible to apply these compounds at the bottom of the wells by using non-polar transfer solvents such as chloroform, pentane or hexane (Dornbos and Spencer, 1990). The solvent is allowed to evaporate prior to adding the seeds and water. This system permits the testing of non polar compounds, but it is less than optimal because the actual concentration of the compounds is undetermined.

## Dose-response curves

Initial experiments typically consist of compounds tested at a single concentration, either 1 mg/ml for mixtures or 100  $\mu\text{M}$  for pure compounds, but the amount of information generated by such tests is limited to active/not active or subjective scales (e.g., 1 = no effect to 5 = death). Dose-response curve experiments are much more meaningful and provide several key parameters related to the potency of the compounds tested. Complete replicated dose-response curves can be obtained in 24-well plates with 200  $\mu\text{g}$  to one milligram of a compound, using half-log response curve from  $1 \cdot 10^{-9}$  (or lower) to  $1 \cdot 10^{-4}$  M. Concentrations greater than  $1 \cdot 10^{-4}$  M are not often tested because of insufficient amount of sample.

Important parameters determined by a dose-response curve include the inhibition threshold (lowest concentration required to initiate inhibition), the  $\text{IC}_{50}$  (inhibitor concentration causing 50% inhibition), and the  $\text{LCIC}$  (lowest complete-inhibition concentration) (Figure 1). These parameters are important because they establish a

frame of reference to which subsequent tests can be compared. Two sets of controls should always be included in the assays. One is conducted with water alone, and the other with an equivalent volume of the transfer solvent without the test compound to evaluate the potential solvent effects.



*Figure 1.* Typical dose-response curve. Hormesis consists of a stimulation of the dependent parameter at low concentration of a phytotoxin. Inhibition threshold is the highest concentration at which no inhibition is observed.  $IC_{50}$  is the concentration at which 50% inhibition is observed. This part of the curve often has the most variations in the replicates. LCIC is the lowest-complete inhibition concentration.

Herbicidal compounds can stimulate growth at very low concentrations (Figure 1). This phenomenon (called hormesis) is not unusual and, while it can occur quite reliably, its causes are not well understood. Hormesis can complicate calculation of the  $IC_{50}$  since the values of the dependent variable (labeled Biometric parameter on Figure 1) increase, relative to the control. Finally, it is also common for the standard deviation to be greatest around the  $IC_{50}$  value because the response varies most at that concentration.

## **Selection of the biometric parameter**

A bioassay is based on the ability to measure a parameter that responds to the presence of the test compound. Typically, the parameter selected is the one that is most sensitive to the phytotoxin, but this is not necessarily so. Root and shoot lengths are commonly used. Biomass (either fresh weight or dry weight) is also used often, but with seedlings it is not as sensitive as longitudinal growth because a plant must grow several days before photosynthetic carbon assimilation contributes biomass to the plant.

## **MODE OF ACTION STUDIES**

As mentioned in the introduction, the discovery of new molecular target sites for herbicides is particularly important to address ever increasing problems associated with the development of herbicide-resistant weed biotypes. One of the limitations of traditional (industrial) pesticide discovery is that chemistry programs tend to favor the synthesis of inexpensive compounds that are easy to synthesize and to crystallize. The resulting libraries tend to have a limited molecular diversity. Also, industry tends to exhibit a ‘me too’ mentality that leads to the synthesis of many derivatives of compounds already developed by other companies, further reducing the likelihood of discovering a new target site. Therefore, relatively few number of target sites are currently being exploited by herbicides. On the other hand, natural products tend to inhibit target sites not exploited by synthetic compounds (Duke et al. 1996, 1997, 2000b; Dayan et al. 1999).

While approaching mode of action studies by focusing on natural phytotoxins may increase the chances of discovery of new target sites, the determination of the mode of action of herbicidal compounds is difficult because of the multitude of potential molecular targets. In our laboratory, we devised a systematic approach to mode of action studies, aiming at simplifying the initial steps. All of our studies are filtered through a stepwise process considering structural, biological and physiological clues.

### **Structural clues**

At the inception of every mode of action study, the structural features of the molecule being investigated are carefully examined. The structural backbone or any substructural pharmacophore may be similar to known phytotoxins and suggest potential molecular target sites. Many tools are available for structural search. The compound of interest can rapidly be screened through databases such as the Dictionary of Natural Products, Bioactive Natural Products Database, and Available Chemical

Directory. This initial step is very inexpensive, as it does not require any experiment, and may greatly reduce the amount of work required later on.

While bioactive natural products tend to be structurally more complex than synthetic herbicides, there are sometimes strong structural similarities between some natural phytotoxins and some commercial products (Figure 2). These molecules have unusually simple structural backbones, and are not representative of the structural complexity normally associated with natural products. Instead, it highlights the relatively low degree of complexity of synthetic herbicides resulting from the economic restrictions controlling their development.

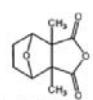
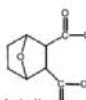
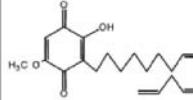
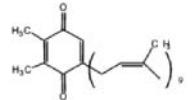
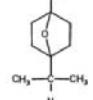
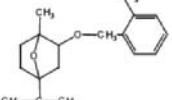
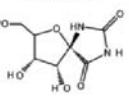
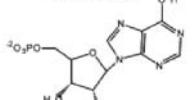
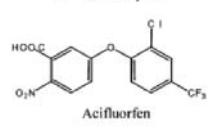
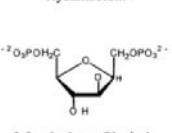
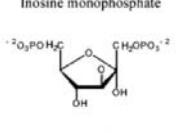
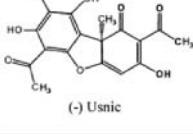
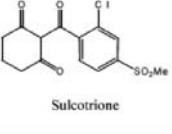
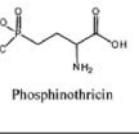
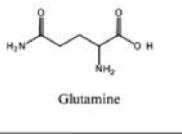
Natural Phytotoxins	Synthetic Herbicides	Natural Phytotoxins	Substrate or Reaction intermediates
 Cantharidin	 Endothall	 Sorgoleone	 Plastoquinone
 1,4-cineole	 Cinmethylin	 Hydantocidin	 Inosine monophosphate
 Cyperine	 Acifluorfen	 2,5-anhydro-D-Glucitol	 Fructose-1,6-bisphosphate
 (-) Usnic	 Sulcotriione	 Phosphinothricin	 Glutamine

Figure 2. Structures of the compounds mentioned in the text.

When the mode of action of the synthetic herbicide is known, one may be tempted to assume that the natural structural analog has a similar mechanism of action. However, this is not necessarily true. For example, the natural phytotoxin cyperine is structurally similar to the commercial diphenyl ethers that inhibit the enzyme protoporphyrinogen oxidase. In fact, quantitative structure-activity relationship analysis can predict its inhibitory activity on protoporphyrinogen oxidase (Dayan et al. 2000), but the level of inhibition of this target site is not sufficient to explain its overall

phytotoxicity (Harrington et al. 1995), and its mechanism of action still remains unexplained.

The benefit of a comprehensive analysis of the structural features of a phytotoxin extends beyond the identification of structural homology to known inhibitors. This first analysis may also identify homology to known biological substrates. This may be significant since the toxin of interest may act as a competitive inhibitor by mimicking natural substrates or reaction intermediates (Figure 2). Bialaphos, a natural herbicide produced by the microorganism *Streptomyces hygroscopicus*, is metabolically converted *in planta* into phosphinothricin. This phytotoxin, which is also available commercially as the herbicide glufosinate, resembles a reaction intermediate in glutamine synthesis (Lydon and Duke, 1999).

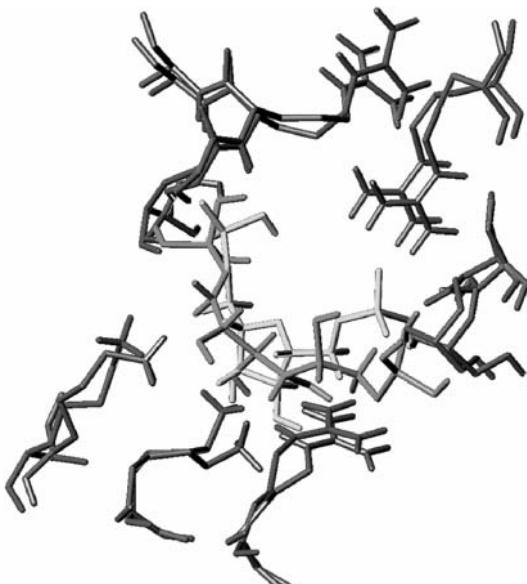


Figure 3. Homology model of spinach cytosolic Fru-1,6-bisP aldolase and the binding of AhG-BP to the catalytic site. Binding of the bioactivated fungal toxin anhydro-D-glucitol bisphosphate (light gray) in the catalytic site of the plant aldolase (medium gray), compared to the binding of on the modeled structure of plant fructose-1,6-bisphosphate based on the crystal structure of human aldolase (dark gray). From Dayan et al. 2002.

Since bioactivation of natural products is not unusual, it is important to consider possible metabolic variations of the compound during the structural analysis. Typical

bioactivating transformations include hydroxylation, phosphorylation, decarboxylation, and conjugation. For example, 2,5-anhydro-D-glucitol, a natural phytotoxin produced by an isolate of *Fusarium solani*, is a structural analog of fructose. The mechanism of action requires the bioactivation of the compound by plant glycolytic kinases to form a bisphosphate metabolite that inhibits fructose-1,6-bisphosphate aldolase by competing for the binding site of fructose-1,6-bisphosphate (Figure 3) (Dayan et al. 2002). In a similar manner, hydantocidin, a natural phytotoxin produced by *Streptomyces hygroscopicus* is phosphorylated by plants, to form an analog of inosine monophosphate. This bioactivated phytotoxin becomes a potent inhibitor of adenylosuccinate synthetase (Siehl et al. 1996).

## Biological clues

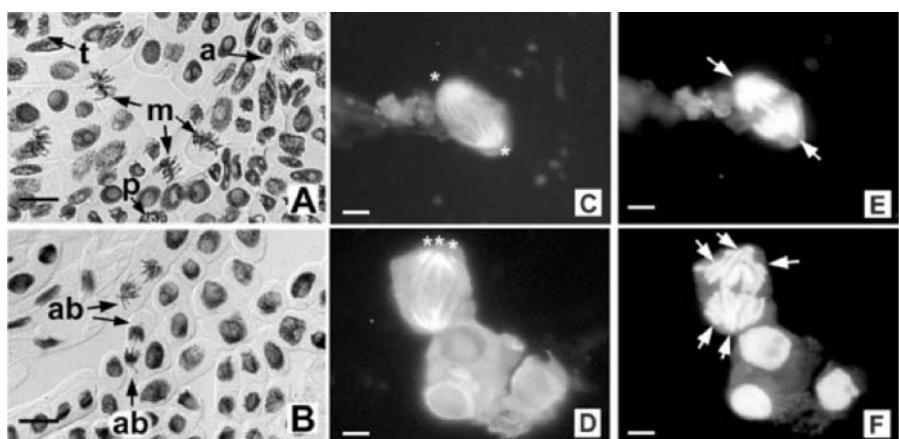
Unlike most current pharmaceutical discovery programs that rely primarily on high-throughput screenings of large numbers of compounds on target specific assays, our approach often starts with dose-response experiments on whole-organisms. These experiments are crucial and can provide important qualitative and quantitative information in evaluating the effect of a phytotoxin. Careful examination of the seedlings may offer some hints as to the possible sites targeted by the compound. However, the data should not be over-interpreted, because the symptoms and appearance of the plant may not be directly related to the physiological process first affected by the test compound. Often, a phenotypic response is the result of secondary effects, rather than the primary mechanism of action of the compound. Distinguishing primary from secondary effects can be difficult when the mode of action of the test compound is unknown. While most of our studies begin at the organismal level, there are some exceptions to this. In particular, when the structural analysis of the compound tested identifies toxins with similar structural features for which their mode of action is characterized. Under these circumstances, it is possible that the compound may be tested directly in an enzyme assay to determine if it inhibits that target site. In the case of usnic acid, analysis of the structure highlighted the similarity between one-half of the structure and well known triketone inhibitors of the enzyme p-hydroxyphenylpyruvate dioxygenase. Experiments showed that usnic acid was a potent inhibitor of this enzyme (Romagni et al. 2000). Subjective observations obtained from these experiments include root and shoot morphology, and visual rating of the phytotoxic effect. However, objective quantitative measurements, including measurement of the length and weight of the plant parts, or chlorophyll concentration, can also be obtained. Whenever root growth inhibition is observed, a more detailed investigation of the putative effect on root cell division can be conducted easily by measuring the mitotic index with onion roots.

### ***Morphological clues***

The morphology of seedlings grown in the presence of a phytotoxin may also yield important information. While reduction in root length is often observed in the presence of phytotoxins, it is often the result of secondary effects. However, abnormal root formation such as the condition called 'clubbing' may indicate that the phytotoxin affect microtubule assembly. Similar symptoms are observed with dinitroaniline herbicides, which are known inhibitors of microtubule assembly (Vaughn and Lehn, 1991). For example, podophyllotoxin inhibits root growth by affecting the formation of mitotic microtubular organizing centers (Oliva et al. 2002). As mentioned above, one must never over-interpret the data since the symptoms observed are not always due to the expected mechanism of action. Other symptoms associated with root development, such as stimulation of lateral root growth to the detriment of the primary root, can indicate disruption of hormonal balance. Sometimes, the seedlings appear chlorotic, which may be the results of any of a large number of known target sites, and an even larger number of unknown sites. Indeed, chlorosis can be the result of direct inhibition of chlorophyll synthesis, or indirectly by inhibition of the carotenoid pathway, or even more remotely, by inhibition of plastoquinone synthesis, which is required for sustaining the carotenoid pathway. Finally, it can simply be the result of the inhibition of an unrelated target site, like amino acid synthesis or even occur in response to loss of membrane integrity.

### ***Mitotic index***

In the event that root growth or development is affected, the effect of a phytotoxin on mitosis should be checked. This can be done relatively easily by following the onion root tip squash techniques of Armbruster et al. (1991). The concentration of inhibitor to use should be decided from the initial dose-response curves mentioned previously. The mitotic stages of the onion root tip cells are observed and the cells in various stages of mitosis are counted. This method permits a quantitative evaluation of an initial visual observation (Figure 4).



*Figure 4.* Light micrographs of A) control and B) podophyllotoxin-treated ( $100 \mu\text{M}$ ) onion root tips. Cells at various stages of mitosis are indicated in A as follows: p = prophase, m = metaphase, a = anaphase, and t = telophase. Abnormal mitotic stages (ab) are indicated in podophyllotoxin-treated root tips. Double fluorescence labelling with anti-tubulin (C and D) and DAPI (E and F) of onion root cells in anaphase. In untreated cells, microtubules radiated toward the cell equator (arrow) from single spindle poles (asterisks) (C) and chromosomes are pulled toward the poles in a symmetrical manner and their kinetochores are converging at the spindle pole (arrow) (E). In cells treated with  $100 \mu\text{M}$  podophyllotoxin, microtubules radiated from multiple spindle poles (asterisks) in tripolar (D) star anaphase conformations. Abnormal chromosomal configurations (F) are the results of the contraction of the spindle apparatus towards multiple poles. Bars represent  $30 \mu\text{m}$ . Modified from Oliva et al. 2002.

Furthermore, this experiment also detects abnormal mitotic arrangement or atypical cell wall formation that would suggest either a disruption of the microtubule organizing centers (Lehnert et al. 1990; Lehnert and Vaughn, 1992; Sherman and Vaughn, 1992), or alteration of processes involved in cell wall biosynthesis (Vaughn and Vaughn, 1990; Vaughn et al. 1996), respectively (Figure 4).

## Physiological Clues

Many physiological clues can also be collected during the initial experiments. In particular, measurement of chlorophyll, carotenoids, and other pigment concentrations are used routinely in our laboratory. The methods of Hiscox and Israelstam (1979) for measurement of chlorophyll concentration and that of Sandmann (1993) for carotenoids can be adapted to use 5-10 mg tissue samples.

### **Loss of membrane integrity**

The effect of phytotoxins that destabilize membranes can be quantified easily by measuring cellular leakage from leaves and cotyledons (Duke and Kenyon, 1993). One of the advantages of this protocol is that it distinguishes light-dependent from light-independent mechanisms of action (Figure 5).

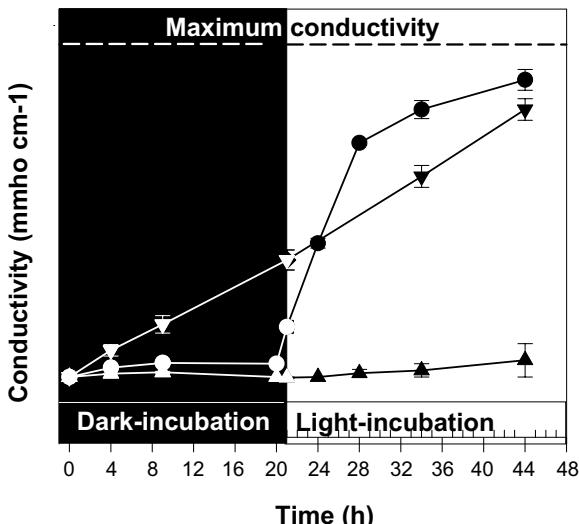


Figure 5. Typical electrolyte leakage study demonstrating the differences between light-dependent (●) and light-independent (▼) loss of membrane integrity, compared to a control (▲) sample where no significant leakage is observed.

Light-dependent loss of membrane integrity may be associated with compounds that divert photosynthetic electrons from photosystem I (such as the bipyridiliums) or cause the accumulation of photodynamic pigment (such as with inhibitors of protoporphyrinogen oxidase). On the other hand, light-independent loss of membrane integrity may be associated with phytotoxins that target the plasma membrane or cellular respiration or that cause oxidative stresses independent of light.

### **Reversion or complementation studies**

Reversion studies are based on the premise that the inhibitory effect of a phytotoxin can, in some cases, be alleviated by providing a biological substrate that

complements a physiological deficiency due to a phytotoxin. While this concept makes sense on a purely physiological basis, reversion does not necessarily occur. We have found that optimum reversion, if it occurs, is achieved when the inhibitor is applied at the LCIC level. This is attributed to the stoichiometry involved in attempting to reverse the effect of a phytotoxin. Reversion of growth inhibition may not be detectable when extremely high concentrations of an inhibitor are used because the compound tested may be acting on secondary sites.

One must be careful not to misinterpret the result of a reversal study. While the goal of the experiment is to identify biological molecules that complement a physiological imbalance caused by the presence of an inhibitor, it is possible that reversal may not be a direct correction of the deficiency or imbalance. In some cases, the substrate selected may react directly with the inhibitor. For example, we found that glutathione can react with natural phytotoxins that possess  $\alpha$ ,  $\beta$ -unsaturated ketone functionalities (Galindo et al. 1999).

### ***Photosynthetic efficiency***

Photosynthetic efficiency can be assessed non-destructively by monitoring chlorophyll fluorescence in order to detect various plant stresses. The use of a modulated chlorophyll fluorometer can distinguish conveniently between compounds directly affecting electron flow from those indirectly decreasing photosynthetic efficiency (Krause and Weis, 1991).

Photochemical efficiency of PSII, measured on dark-adapted leaves, provides an indication of the capacity of the photosystem apparatus to return to ground state after being exposed to a short burst of saturating light. Compounds competing for the same binding site as plastoquinone on the D1 protein generally cause dramatic decreases in photochemical efficiency, because the inhibition of electron flow prevents chlorophyll molecules from rapidly returning to ground state energy levels.

### ***Carbon dioxide exchange***

Another non-invasive method of evaluating physiological effect of inhibitors on plants consists of monitoring carbon dioxide exchange by using an infrared gas analyzer (IRGA). This method can measure both net photosynthesis and dark respiration with high sensitivity. The basic concept behind the system is that CO<sub>2</sub> is a strong absorber of infrared radiation in three bandwidths. Most modern IRGAs utilize a nondispersive source of infrared light and a detector sensitive only to the CO<sub>2</sub> absorption. The IRGA is commonly used in either the differential (in which the instrument measures the difference between the CO<sub>2</sub> concentration in two cells) or the absolute (where the reference cell is filled with CO<sub>2</sub>-free gas) mode.

The system may be used with either whole plants or individual leaves. Carbon dioxide flux may be measured continuously by recording data on a datalogger, or it may be measured by using the depletion technique. In general, the CO<sub>2</sub> analyzer is accurate with a resolution of approximately 1 μmol mol<sup>-1</sup> (1 ppm). Thus, herbicides that inhibit photosynthetic electron transport (atrazine, bentazon, diuron), ribulose bisphosphate carboxylase oxygenase, or mitochondrial electron transfer (e.g., 2, 4-dinitrophenol) can easily be identified by using this method. Uptake of CO<sub>2</sub> is decreased in plants that have been treated with compounds having the first two modes of action. This is measured easily by the IRGA in the differential mode. Compounds affecting mitochondrial electron transport exhibit decreased CO<sub>2</sub> production in the dark. IRGA can be miniaturized to accommodate bioassays with small amounts of tissues (e.g., Amagasa et al. 1994)

### ***Radiolabeled incorporation and pulse-chase studies***

The use of radioactivity for either total incorporation or pulse-chase studies is often very useful in elucidation of modes of action. The easy detection of low levels of radioactivity by liquid scintillation of plant extracts or of samples that were oxidized to release <sup>14</sup>CO<sub>2</sub> provide important quantitative data on the fate of the numerous labeled substrates/precursors commercially available. Analysis of extract by HPLC coupled to an in-line radioactivity detector may also be used to provide more qualitative information as to the fate of the radiolabeled substrates.

Such experiments can eliminate many physiological pathways not involved in the mechanism of action of the phytotoxin, and can point to disruption of specific physiological processes such as amino acids/protein synthesis, nucleic acids/DNA and RNA synthesis, or lipid biosynthesis (Backman and DeVay 1971; Deal et al. 1980; Tranel et al. 1993; Turner 1985).

### **Metabolic profiling**

Singh and Shaner (1995) developed a method of fingerprinting modes of actions by analysis of pools of free amino acids associated with inhibition of different molecular target sites. Free amino acids pool levels of inhibited pathways often decreased only slightly, while there were stronger effects on levels of amino acids of other pathways. For example, inhibitors of acetolactate synthase (key enzyme in branched chain amino acids) caused small decreases levels of valine and leucine, but caused relatively large increases in threonine, serine and alanine. Glyphosate, an

inhibitor of aromatic amino acid biosynthesis, and amitrole, an inhibitor of histidine biosynthesis, also caused large increases in the pools of these amino acids. This method is further complicated in that herbicides that have no direct effect on amino acids biosynthesis, such as photosynthetic inhibitors or fatty acid synthesis inhibitors, also eventually have pronounced effects on pool levels of amino acids. Interpretation of secondary or tertiary effects of a phytotoxin, no matter how pronounced, can be challenging. This type of metabolic profiling might be good for recognizing known modes of action, but it is unlikely to be useful for determination of unknown modes of action that are unrelated to amino acid metabolism.

A more global method of metabolic profiling, involving all primary metabolites would theoretically be more robust. This has recently been attempted by <sup>1</sup>H NMR analysis of crude plant extracts from plants treated with various herbicides (Ott et al. 2003). The <sup>1</sup>H NMR spectra of plants treated with herbicides representing 19 modes of action were classified by artificial neural network analysis to discriminate the herbicide modes of action. This analysis also recognized new modes of action. A problem with this specific approach is that it does a poor job of identifying key, signature metabolic intermediates and quantifying them.

## Genetic mutations

Mutants can be used to determine mode of action by finding or generating a mutant that is resistant to the phytotoxin and then determining the biochemical basis of the resistance or by comparing biochemically defined mutant with a similar phenotype to that caused by the herbicide. In the first case, the mutation is not always at the molecular target site. Weeds can also evolve resistance through enhanced metabolic degradation, reduced bioactivation, or by limiting access to the molecular target site by reducing uptake or translocation, or by compartmentalization. The big success story with this approach was the discovery of the target site of PSII inhibitor herbicides. Pfister et al. (1981) found that weeds that had evolved resistance to atrazine had an altered 32 kD protein that eventually was named D1. In other cases of evolved resistance at the molecular target site, the target site was known before the weeds evolved resistance. Otherwise, the resistant weeds would have been valuable tools in determination of the mode of action. The other approach was used by Vaughn and Duke (1981), who noted that phenotypes of mutants missing polyphenol oxidase were similar to plants treated with tentoxin, a highly phytotoxic cyclic tetrapeptide produced by the plant pathogen, *Alternaria alternata*. Among other effects, tentoxin prevents the posttranslational processing of polyphenol oxidase needed to activate the enzyme (Vaughn and Duke, 1984). There is also no polyphenol oxidase activity in the tissues of some *Hosta* spp. mutants with a similar phenotype. The complete mechanism of this phytotoxin is still not clear; there is strong evidence that loss of polyphenol oxidase activity causes most, if not all, of the symptoms (Duke et al. 1996).

Many well-characterized *Arabidopsis* mutants are now available. These could be useful in comparing phenotypes similar to those caused by a phytotoxin. However, the ultimate phenotype of a herbicide-treated plant is a dead plant, so the available mutant might better represent the phenotypes caused by unsuccessful herbicides.

## Gene transcription profiling

Theoretically, gene transcription profiles can help in determination of a molecular target site of a phytotoxin, as well as providing information on its mode of action. To do this, gene chips with microarrays of the entire genome or from a cDNA library of all of the genes expressed by an organism under normal conditions and under a variety of stresses are used to determine the gene transcription responses of a test plant species to various herbicides with different molecular target sites. From this information, a gene transcription profile library can be generated for known target sites. Then, the gene transcription profile of a phytotoxin with an unknown molecular target can be compared with those of the library to determine if it has a known mode of action. If not, analysis of up- and down-regulated genes might provide clues to the mode of action. This strategy is being employed with fungicides, herbicides, and insecticides in the pesticide industry, but little has been published yet. Our laboratory is also taking this approach to determine mode of action of natural product fungicides and phytotoxins.

Although the concept of using gene transcription profiles to determine mode of action is simple, implementation is complicated. For good quality information, one must have well-designed DNA chips with various internal controls and carefully thought-out experiments with adequate replication (Beneš and Muckenthaler, 2003). All of the experiments must be done by examining gene transcription profiles affected by a dose of the phytotoxin that causes a similar effect (e.g., the IC<sub>50</sub>) and at a time before there are too many secondary and tertiary effects that confound the results. Things are further complicated by the fact that not all phytotoxins affect the same tissues and work at the same rate.

A phytotoxin can be expected to affect genes that encode three types of proteins: 1) stress proteins and enzymes; 2) xenobiotic detoxification enzymes; and 3) those associated directly with the molecular target site. At lethal doses, many other types of genes should be eventually affected, but early in the time course of symptom development, genes of these three categories might be more clearly recognized. The first two categories might be common to phytotoxins with many different molecular targets.

If a phytotoxin inhibits a particular enzyme of a metabolic pathway, several enzymes of the pathway may be up-regulated. Thus, this method can only be used to suggest a particular pathway is involved in the mode of action. Other studies (e.g., mutants with genetic lesions in the pathway, complementation studies, or direct enzymology) will be needed to determine the target enzyme(s) of the pathway.

## CONCLUSIONS

Phytochemicals are still a good source of new compounds and leads for new chemical classes of herbicides. Modern separation and analytical instrumentation, coupled with miniaturized bioassays, have simplified and reduced the time required for bioassay-directed isolation and discovery of these compounds. Knowledge of the molecular target site is highly desirable in making a decision to develop a phytotoxin as a herbicide. New strategies and techniques for determination of molecular target sites are also simplifying this process, although it is still not as simple as discovery of the phytotoxin.

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## CHAPTER 4

# DISTINGUISHING ALLELOPATHY FROM RESOURCE COMPETITION: THE ROLE OF DENSITY

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## INTRODUCTION

### The potential significance of allelopathy in plant communities

The broadest definition of allelopathy encompasses both stimulatory and inhibitory biochemical interactions among plants at all levels of complexity, including microorganisms (Molisch 1937). Over the years, it has been proposed that beyond the direct or indirect chemical effects of a plant on its neighbors, allelopathy may also affect community-level processes such as succession, nitrogen cycling and community dynamics (Muller, 1966; Rice, 1984; White, 1994; Wardle et al. 1998). Muller (1966, p. 332) summarizes allelopathy's potential importance to plant communities:

The significance of allelopathy to ecological theory is very great. Small quantities of toxins may be responsible for massive reductions in plant growth and in water or mineral absorptions and thus strongly influence microclimate. Traditional theories of competition, reaction, biomass proportions, energy flow, mineral cycling, and ecosystem organization are all liable to reevaluation where allelopathy is demonstrable.

Elucidating allelopathic interactions has also been of interest for intensely practical reasons. Understanding this phenomenon could lead to the development of new herbicide chemistries, the use of allelopathic mulches, cover crops and cropping rotations to control weeds, the development of allelopathic crop varieties, and the control of soil sickness and replant problems resulting from autotoxicity (Gliessman,

1983; Duke and Lydon, 1988; Weston, 1996; Olofsdotter, 1998; Anaya, 1999; Birkett et al. 2001).

## The reality of ecological complexity: methodological consequences

Despite the obvious benefits to the understanding of both natural and agroecosystems, allelopathy has proven to be difficult to demonstrate under field conditions. Like other ecological interactions, allelopathy is a complex phenomenon. These complexities have been explored in several reviews (Einhellig, 1987; Williamson, 1990; Weidenhamer, 1996; Inderjit and del Moral, 1997; Blum et al. 1999), and have important ramifications for experimental design (Romeo and Weidenhamer, 1998; Blum, 1999). Unfortunately, the scientific literature is replete with examples of work that has failed to take this ecological complexity into account (Romeo, 2000).

Plants interact with their neighbors in a variety of ways (Harper, 1977; Fuerst and Putnam, 1983; Bazzaz, 1996). Plants may compete with one another for resources such as nutrients, water, light, and pollinators (resource competition). They may produce chemical substances that inhibit or stimulate germination or growth, or indirectly affect the growth of neighbors by inhibiting or stimulating mycorrhizal or *Rhizobium* symbioses (allelopathy). Volatile chemical emissions in response to herbivory can be detected by neighboring plants, inducing changes in their defensive chemistry (Baldwin and Schultz, 1983). Plants may indirectly affect their neighbors by harboring pathogens or herbivores (Sandfaer, 1970), or by modifying soil properties in some way (Inderjit and Mallik, 1997; Jain and Singh, 1998). Other effects can also be envisioned.

The focus of this review is specifically the problem of distinguishing allelopathy from other mechanisms of plant-plant interaction, as well as from abiotic factors (e.g., soil pH) which may affect plant growth. Such efforts are complicated by the fact that plants experience their physical, chemical and biological environment all at once, and various mechanisms of plant-plant interaction may and do occur simultaneously, sequentially, or interact with one another. Trenbath (1974) noted that slight allelopathic effects at the seedling stage might be exaggerated or reversed through competition. Furthermore, it is known that various stresses, including nutrient limitation, can increase plant production of phytotoxic allelochemicals. Nutrient limitation can increase the toxicity of some allelochemicals (Einhellig, 1987; Williamson et al. 1992). Blum et al. (1999) point out that one can thus envision a situation in which competition for nutrients might result in allelopathic interactions. It is obvious that the allelopathic inhibition of a plant will likely reduce its effectiveness in competing for resources (Humphry et al. 2001). Many other such interactions are

theoretically possible. The results of Nilsson (1994) are noteworthy in providing evidence of the magnitude of interaction between resource competition and allelopathy in one system.

The emphasis here is on “distinguishing” these mechanisms of plant-plant interaction rather than “separating” them, though both terms are used in the literature (Fuerst and Putnam, 1983; Weidenhamer et al. 1989; Nilsson, 1994; Inderjit and del Moral, 1997). While it may be argued that for the reasons outlined above, “separating allelopathy from resource competition is essentially impossible in natural systems” (Inderjit and del Moral, 1997), investigators *must* be able to identify positive evidence that differentiates allelopathy from other mechanisms of plant-plant interaction if allelopathy is to be a scientifically viable hypothesis. “Separating” allelopathy from competition in the latter sense is not impossible – it is essential.

## **DENSITY-DEPENDENCE OF PLANT GROWTH**

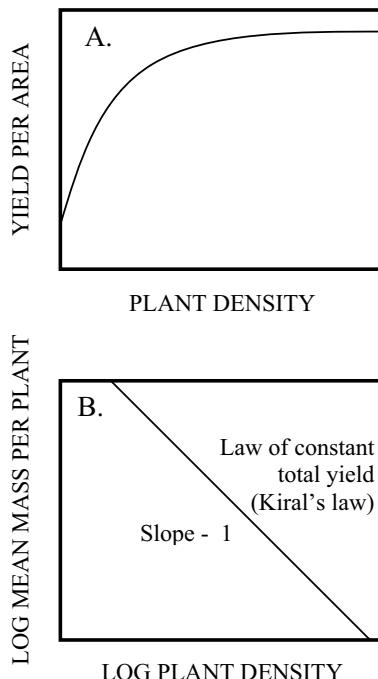
### **Density and plant growth**

When plants are grown together, total yield (measured as production of fruit or seed, or total biomass) increases linearly with density up to the point at which neighboring plants begin to compete with one another for resources (Figure 1a). Above a certain density, individual plant growth is reduced but yield per unit area remains constant across a wide range of densities (Kira et al. 1953; see Bell and Wright, 1998 and Bednarz et al. 2000 for representative examples of studies with crop plants). In the range of densities where yield is constant, the relationship of log mean plant weight and log density is linear with a slope of  $-1$  (Figure 1b). Factors such as resource availability may alter the maximum yield achieved under given conditions, and thus the intercept of the log weight–log density line, but do not alter the predicted slope. Because the  $-1$  law of constant final yield is universal (White and Harper, 1970; Harper, 1977; Gorham, 1979; White, 1980), it has been considered one of only two major ‘laws’ in plant ecology (White, 1980; Harper, 1982).

### **Density-dependent phytotoxicity**

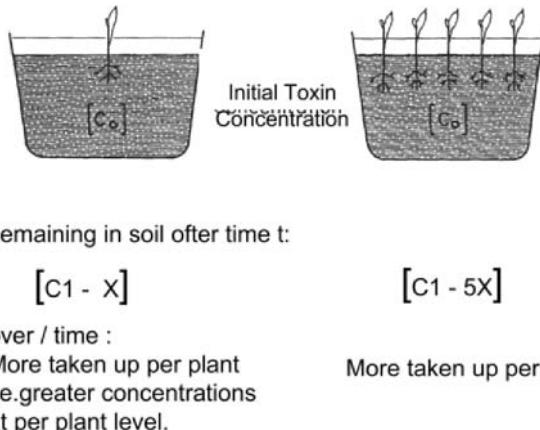
Density-dependent phytotoxic effects may be defined as the differences in the magnitude of inhibition observed when plants grow at varying densities in soil containing a phytotoxic substance. In a given soil volume containing a finite amount of phytotoxin, plants growing at low densities have a larger amount of the toxin available per plant and therefore should suffer greater growth reductions than those in high densities, where the toxin is shared (‘diluted’) among many plants and each

receives a proportionately smaller dose. This is in contrast to resource competition, where plants growing at low densities have a larger amount of the resource available per plant and therefore have increased growth as a result.



*Figure 1.* Effects of phytotoxins on yield-density relationships: (A) expected response of yield to increasing plant densities; (B) expected relationship of log mean mass per plant and log plant density in the range of plant densities where total yield is constant. (Original figures appeared in Weidenhamer et al. 1989; used with permission of Blackwell Science, Ltd.)

An alternate way to understand the situation is to realize that plants “compete” for phytotoxins in the same way that they do for resources. While the consequences of winning competition for resources are positive, the consequences of winning competition for phytotoxins are negative. This differential in response thus provides a means to distinguish resource competition from allelopathy. Hoffman and Lavy (1978) supported their assertion that plants compete for herbicides with experiments using  $^{14}\text{C}$ -labelled atrazine, in which atrazine uptake per plant decreased by 50% when soybean populations increased from 1 to 6 plants per pot. This is illustrated schematically in Figure 2.



*Figure 2.* Illustration of the basis for density-dependent phytotoxic effects. Over time, more toxin is available for uptake per plant at low plant densities. (Original figure appeared in Weidenhamer, 1996; used with permission of American Society of Agronomy)

Density-dependent phytotoxicity was first reported in work with herbicides (Skipper, 1966; Hoffman and Lavy, 1978; Andersen, 1981; Winkle et al. 1981). Weidenhamer et al. (1987, 1989) reported the first investigations of the density-dependent effects of natural allelochemicals. Weidenhamer et al. (1989) proposed that demonstration of either of the following results would be contrary to the expected results of resource competition and would suggest the presence of toxic substances in soil: (a) Compared to a control soil, growth reductions occur at low but are diminished at high density; (b) Maximum individual plant weight occurs at an intermediate density, due to a reversal in slope of the predicted log yield-log density line. A growing literature now supports the usefulness of experiments varying plant density as a tool to distinguish allelopathy from resource competition.

## Experiments in monoculture: laboratory and greenhouse

Weidenhamer et al. (1989) grew tomato (*Lycopersicon esculentum* Mill.) in varying densities in flats of soil collected from beneath and around black walnut, *Juglans nigra*. In a low density treatment, growth reductions of tomato grown in black walnut soil were 62% compared to those grown in soil from adjacent fields. In the highest density treatment, growth reductions were only 16%. In another experiment, bahiagrass was grown in soil treated with hydroquinone and gallic acid, the suspected inhibitors produced by the Florida scrub perennial *Polygonella myriophylla* (Weidenhamer and Romeo, 1989). The eight-week biomass of bahiagrass grown in

soil treated with 400 :g/g each of hydroquinone and gallic acid was 63% of control at 2 plants/pot, but no effect was seen at 16 plants/pot. Stimulatory effects were observed at lower concentrations, and these were also density-dependent. The five-week biomass of bahiagrass grown in soil treated with 100 :g/g of each compound was 233% of control at 2 plants/pot, but only 139% of control at 16 plants/pot.

Choesin and Boerner (1991) examined the response of *Medicago sativa* to allyl isothiocyanate added to soil. Five densities (2, 4, 8, 16 or 32 plants per pot) of *M. sativa* were treated with different concentrations of allyl isothiocyanate, selected to be representative of those found under *Brassica napus*. No inhibition of *M. sativa* growth was observed at any density. The absence of density-dependent phytotoxic effects was used as evidence against the allelopathic potential of *B. napus*.

El-Khatib (2000) sowed seeds of *Chenopodium murale*, *Glinus lotoides* and *Malva parviflora* in trays of rhizosphere soil of *Alhagi graecorum*, a species that forms monospecific stands on fallow land. Seeds were planted at densities of 50, 100 and 150 per tray in trays that were 1480 cm<sup>2</sup> in area. A significant decrease in emergence value was found for each of the species at low density, but the effect was not significant at high density.

Humphry et al. (2001) investigated the effect of plant density on the response of *Agrostemma githago* to spraying of 2,4-D amine. The ED<sub>50</sub> (defined here as the dose at which plants show a 50% response to herbicide based on the fresh weight) values were significantly higher at the highest plant density. The difference between high and low density treatments was 15-fold – the ED<sub>50</sub> was 616 g active ingredient ha<sup>-1</sup> at high density (64 plants per 10-cm diameter pot) and 42 g ha<sup>-1</sup> at low density (2 plants per pot). A mathematical relationship was derived for density and dose received. Experimental measurement of the amount of herbicide captured by plants at high and low density was accomplished by substituting the dye tartrazine for 2,4-D. After spraying, the dye was washed from the leaves and analyzed spectrophotometrically. It was found that plants at high density received 2.5 times less herbicide per unit leaf area, and four times less herbicide per plant. Humphry et al. suggested that it would be interesting to examine which of these measures of dose received better predicted response to the herbicide. Allelopathic effects are typically mediated by root rather than absorption, but similar differences in the dose received based on root mass or surface area as compared to dose per plant might be expected – though being much harder to measure.

Tseng et al. (2003) evaluated the allelopathic potential of *Macaranga tanarius*, an early succession tree that can invade grasslands and has relatively few species at lower than expected abundance in its understory. *Macaranga* leaf powder was incorporated into soil and assayed with lettuce (*Lactuca sativa* L.) and *Alocasia macrorrhiza*, each grown at three densities. Lettuce was used as a sensitive test species, while *Alocasia*

*macrorrhiza* grows abundantly in the *Macaranga* understory. Lettuce growth reductions were density-dependent, with reductions of 68%, 55% and 23% for the low, medium and high density plantings, respectively. Growth of *Alocasia* was reduced by the addition of leaf powder, but the reductions in growth were not density-dependent for this species. While such experiments do not “prove”, in the most rigorous sense, that an allelopathic interaction is occurring in the field, they do answer the complaint of Harper (1977), who criticized similar experiments by Rice as being open to the interpretation. Harper argued that a common result of incorporating a readily decomposed organic material, such as leaf powder, is nitrogen deficiency resulting from a burst of microbial activity. However, any nitrogen deficiency in the pots with incorporated *Macaranga* powder would be the same for all density treatments (as is seen when *Alocasia* was used as the test species). The disappearance of growth reductions at high density is not consistent with a hypothesis of nitrogen deficiency, but is consistent with a hypothesis that toxins from the leaf powder are the cause.

### Experiments in monoculture: field

Density-dependent phytotoxic effects have also been observed in a number of field studies. Hoffman and Lavy (1978) found that high plant populations were not as sensitive to the presence of atrazine. In a field treated with atrazine at  $1.1 \text{ kg ha}^{-1}$ , soybean dry weight was 97% of control with 6 plants per  $100 \text{ cm}^2$ , but only 26% of control with 1 plant per  $100 \text{ cm}^2$ . In a two year field study, Andersen (1981) reported density-dependent yields of soybean planted at four densities in fields treated with different rates of metribuzin. The data clearly show density-dependent phytotoxic effects. For the second year of the study, soybean yields at the highest rate of metribuzin and highest seeding density were  $2280 \text{ kg ha}^{-1}$  (compared to  $2820 \text{ kg ha}^{-1}$  in the high density control), while yields in the corresponding low density plots were  $1070 \text{ kg ha}^{-1}$  (compared to  $2570 \text{ kg ha}^{-1}$  in the low density control).

Gentle and Duggin (1997) carried out field experiments to investigate the allelopathic effects of *Lantana camara* L. on two indigenous tree species, *Alectryon subcinereus* and *Cryptocarya rigida*. *Lantana* was either physically removed, burned, or left in place. Seeds of the assay species were germinated and grown in the thickets, and thinned to densities of 10, 20 and 30 seedlings  $\text{m}^{-2}$ . Plants were harvested at 14 months after planting. Where *Lantana* was left in place, both species showed an increase in individual seedling biomass with an increase in density. In the plots where *Lantana* was left in place, the biomass of individual seedlings increased from 1.75 g for 10 seedlings of *Cryptocarya rigida*  $\text{m}^{-2}$  to 2.58 g for 30 seedlings  $\text{m}^{-2}$ . For

*Alectryon subcinereus*, the biomass increased from 1.95 g to 2.53 g for the 10 and 30 seedlings  $\text{m}^{-2}$  treatments, respectively. Where *Lantana* was removed, seedling

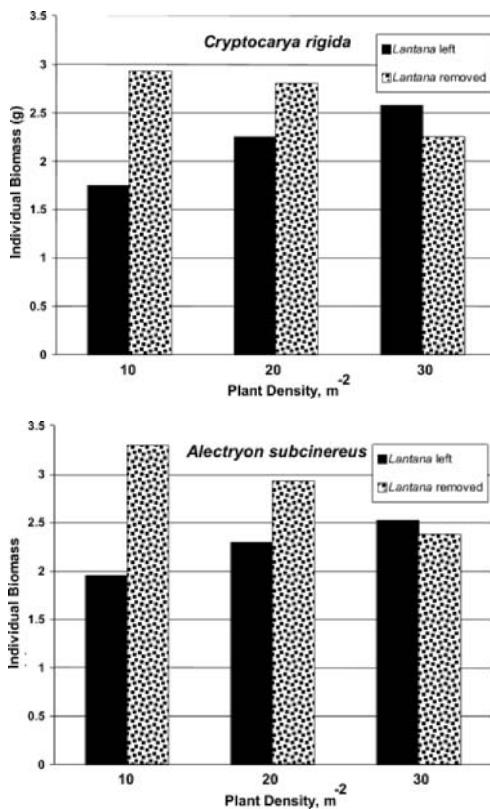


Figure 3. Individual seedling biomass of (A) *Cryptocarya rigida* and (B) *Alectryon subcinereus* when planted at varying densities with *Lantana camara* either left in the plots or removed from them. Data from Gentle and Duggin, 1997.

biomass decreased with increasing seedling density, which is the expected response when resource competition is the mechanism of interaction (see Figure 3). The occurrence of the opposite trends in the presence and absence of *Lantana* — of increasing plant size with increasing density when *Lantana* is present, but decreasing plant size with increasing density when it is absent — supports the hypothesis that *Lantana* is exerting allelopathic effects in this field situation.

## Effects on yield-density relationships

Weidenhamer et al. (1989) predicted that the presence of active concentrations of a phytotoxin in the soil would cause deviations from the expected yield-density relationships. Depending on toxin concentration, maximum plant size may occur at an intermediate density, with reduced size at both low density (the result of phytotoxicity) and high density (due to intense resource competition) (Figure. 4a).

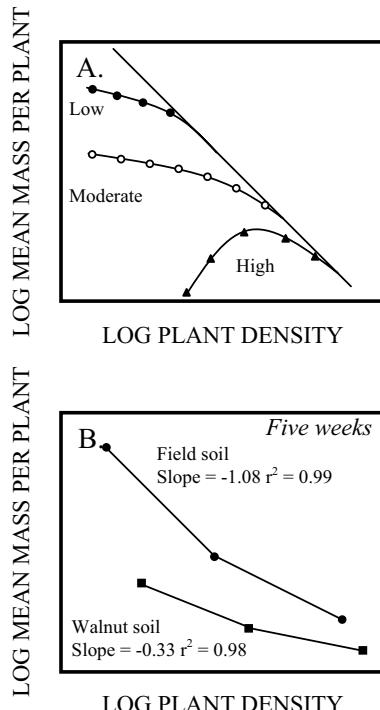


Figure 4. Effects of phytotoxins on yield-density relationships: (A) predicted deviations in the log mean mass-log density relationship in the presence of low, moderate and high concentrations of phytotoxins; (B) relationship of log mean mass per plant and log plant density for tomatoes grown in black walnut and normal field soil for 5 weeks in the greenhouse. (Original figures appeared in Weidenhamer et al. 1989; used with permission of Blackwell Science, Ltd.)

Such a result is contrary to the predicted effects of resource competition, and therefore provides strong evidence for a hypothesis of chemical interference. In the

experiments with tomato grown at three different densities in soils collected beneath and around black walnut, the slope of the log yield-log density line was reduced in walnut soil compared to adjacent field soils (Weidenhamer et al. 1989; Figure. 4b). Reanalysis of data from the weed science literature (Weidenhamer et al. 1989) showed similar reductions in slope for soybean seed yield at different levels of metribuzin residue (Andersen, 1981), and a reversal of the slope of the log yield-log density line in a study of soybean response to atrazine (Hoffman and Lavy, 1978).

In the study of Choesin and Boerner (1991), yield-density analysis (comparison of the slopes of the log mean plant mass – log plant density line) showed no effects of allyl isothiocyanate on the growth of *Medicago sativa*, which is to be expected given that no phytotoxicity was observed under the conditions of the study.

Gentle and Duggin (1997) did not analyze their data on tree seedling growth in the presence and absence of *Lantana camara* in terms of yield density relationships. These data are plotted in Figures 5a (for *Cryptocarya rigida*) and 5b (for *Alectryon subcinereus*). In the absence of *Lantana*, the slope of the log mass – log density line is negative. In the presence of *Lantana*, the slope of the log mass – log density line is reversed. Such a result is inconsistent with resource competition, but does support the presence of toxins from *Lantana*.

In the study by Tseng et al. (2003) of the allelopathic potential of *Macaranga tanarius*, analysis of the relationship of log mean weight per plant and log density showed a significant reduction of slope for lettuce grown with incorporated *Macaranga* leaf powder compared to the no incorporation control, from -0.53 to 0.004. However, the slope of this line for *Alocasia macrorrhiza*, which is not inhibited by *Macaranga*, showed no difference between regular soil and soil with incorporated *Macaranga* leaf powder.

## Experiments in mixed culture

Density-dependent phytotoxic effects have also been used in the study of plants grown in interspecific competition (Thijs et al. 1994). Corn and soybean were grown together in the target-neighbor design proposed by Goldberg and Werner (1983) for the study of plant competition. In this design, varying densities of one species are planted around a target plant. Because looking at competitive effects over a range of densities is an integral part of the design, it seemed suitable for examining density-dependent effects such as those from phytotoxins. Atrazine, an herbicide used to control broadleaf weeds, was applied to corn and soybean as a model of natural situations in which two species differing in their sensitivity to a toxin grow together in the presence of a third species or leaf litter which is the source of the chemical.

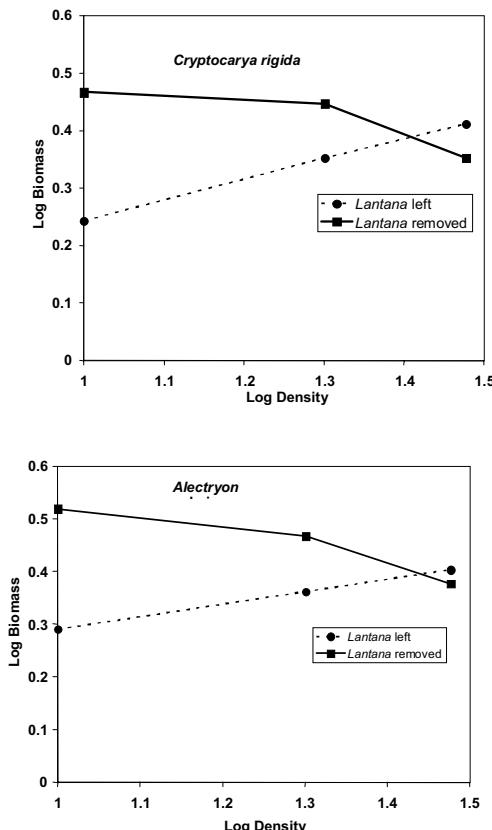


Figure 5. Data from Fig 3 replotted to show relationship of log mean mass per plant and log plant density for (A) *Cryptocarya rigida* and (B) *Alectryon subcinereus* planted at varying densities with *Lantana camara* either left in the field plots or removed from them. Plants were harvested at 14 months. Data from Gentle and Duggin, 1997.

Examples would include the understory beneath allelopathic trees and shrubs, or the release of inhibitors from cover crop residues. With atrazine applications of 3.0 mg/L, the dry weight of soybean target plants increased from 0.2 g with no neighbors to 0.5 g with 9-12 neighboring corn plants (Thijs et al. 1994). Increased growth of the soybean target at higher densities of competitors is contrary to the expected results of resource competition. This is due to uptake of atrazine by the corn seedlings, which

are not sensitive to this toxin, thereby decreasing the amount available to the soybean target.

Hegazy et al. (2001) studied the effects of *Nymphaea lotus* on rice growth. An experiment was conducted using a target-neighbor design in which a constant density of rice plants (three seedlings per pot) served as the target, and varying densities of lotus rhizomes (0, 3, 6, 9, and 12 per pot) were planted as the neighbors. Field data were collected from rice stands of constant density, but varying densities of lotus (0, 5, 8 and 12 rhizomes per m<sup>2</sup>). In both cases, growth of rice was reduced as lotus density increased. In this case, however, there is not a compelling differentiation of allelopathic and competitive effects. While the chemical effects of an allelopathic neighbor would be expected to increase as its density increased, so would the resource competitive effects be expected to increase. There is no way to sort out the relative contributions of allelopathy and competition, or to infer the necessary contribution of allelopathy to explain the results of such an experiment. An alternative would be to use the susceptible species as the neighbor, and the suspected allelopathic species at a constant density as the target. In such an arrangement, the allelopathic effects would be expected to diminish as neighbor density increased, while competitive effects would increase. Depending on the toxicity of the allelopathic target, mean plant size of the neighbor could be reduced at its lowest density, and increase as neighbor density increased due to the sharing of the available pool of toxin among more neighbors. Such a result would be contrary to the expected results of resource competition and would therefore be compelling evidence for allelopathy. Similar results were observed by Thijs et al. (1994) when corn (non-susceptible target) and soybean (susceptible neighbor) were grown in pots treated with atrazine (Figure 6).

## Density and dose-response relationships

Suman et al. (2002) examined the effects of *Vigna mungo* (black gram) seeds on the germination and radical growth of several crop plants. Ten seeds of wheat, maize, gram or lentil were sown in petri dishes with 0, 6, 12 or 24 seeds of black gram. Water was added as necessary to the dishes in order to prevent competition. The germination of all four crop seeds was inhibited by black gram, and this germination was density-dependent. Inhibition increased as the density of black gram seeds increased. Such an experiment does not measure differences in phytotoxicity in response to the density of the target species as originally proposed by Weidenhamer et al. (1989). Rather, the varying numbers of the allelopathic source allow an investigation of dose-response relationships to the presumed toxins. If steps are taken to eliminate resource competition, the finding of a dose-response relationship supports the hypothesis of toxin release. If resource competition is not eliminated, any allelopathic effects of the

increased numbers of the source plant will be confounded by increased resource competition.

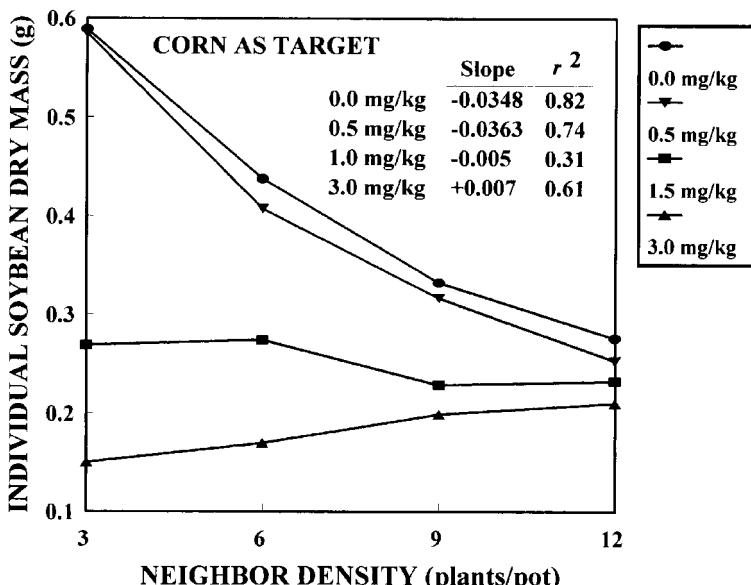


Figure 6. Relationship of individual soybean dry mass and soybean density in pots where corn was planted as target and atrazine was applied at varying rates. (Original figure appeared in Thijs et al. 1994; used with permission of the Ecological Society of America).

Belz and Hurle (2001) developed a hydroponic bioassay of wheat cultivars in which the dose of suspected allelochemicals was varied by varying the density of wheat plants in the pot, while the test plant (*Sinapis alba*) was maintained at a constant density. No nutrients were added to the hydroponic culture, so this strengthens the argument that growth reductions of the test species were due to chemicals exuded by the wheat roots. Belz and Hurle used their results to determine the ED<sub>50</sub> (plant density with a 50% inhibition of root length) for each of the 130 cultivars of wheat studied. Inderjit et al. (2001) studied the interaction between wheat and perennial ryegrass (*Lolium perenne*) seedlings under controlled conditions. Five densities (0-20 seeds) of perennial ryegrass were sown with five densities (0-20 seeds) of wheat in petri dishes containing vermiculite. Perennial ryegrass root length was suppressed by wheat. Inhibition increased with increasing density of wheat seedlings. Such a result

could be consistent with increased concentrations of allelochemicals exuded from wheat roots, similar to the experiments of Belz and Hurle (2001).

## Mathematical modeling of density-dependent phytotoxicity

Sinkkonen (2001) has extended the biological response model of An et al. (1993) to incorporate the density-dependence model of Weidenhamer et al. (1989). This model was applied successfully to data from the literature, indicating the potential of the model to estimate density-dependent phytotoxicity mathematically. Sinkkonen (2003) extended this work further by combining this model with a residue allelopathy model proposed by An et al. (1996) to take into account density-dependent responses to decomposing residues. The resulting model predicts that the balance between inhibitory and stimulatory effects of allelochemicals will depend on the time of observation, plant density, chemical concentration, as well as environmental conditions. Sinkkonen suggested that this might explain why conflicting results have plagued previous studies of chemical interference in plants.

## Other practical consequences

Hoffman and Lavy (1978) found that pot volume is a significant consideration in studies of phytotoxicity because of its impact on the amount of phytotoxin available to plants. At a constant density, plants in larger pots will have a larger dose of toxin available to them. With one soybean plant per pot grown in Keith silt loam soil, no reduction in growth was seen for a 0.1 ppm atrazine treatment in the smallest pots (225 g), while reductions of 25 and 70% were seen in the 450 g and 900 g pots, respectively. The practical consequences of these effects is that in studies of allelopathy, the greatest inhibitory effects will be seen with the lowest density of the assay species and largest pot volumes (Romeo and Weidenhamer, 1998). The existence of such effects may provide one explanation for the sometimes conflicting results of investigations of allelopathic effects.

## A caution

It should be noted that allelopathy is not the only phenomenon which can cause density-dependent growth responses contrary to the expected effects of resource competition. Jain and Singh (1998) planted *Terminalia arjuna* at three densities (10 000, 20 000 and 30 000 plants ha<sup>-1</sup>) on degraded land. They found that plant height, diameter and biomass production increased significantly as density increased. The increased growth at higher plant densities was attributed to the improvement of

soil properties. Higher plant densities were found to decrease soil bulk density and increase porosity and water holding capacity. Soil pH and exchangeable sodium were also reduced as density increased. Careful observation of the particular field situation under study, as well as careful experiments to eliminate other explanations, are necessary to provide insight into whether allelopathy or another factor is responsible for density-dependent growth responses suspected to be due to the presence of a toxin in the soil. Alternative hypotheses should always be considered.

## SUMMARY

### **Methodological implications of density-dependent phytotoxicity**

Allelopathy is a complex ecological phenomenon. A realization of the complexity of plant communities and mechanisms of ecological interaction leads to important practical consequences for the conduct of bioassays. Despite the difficulties, experimental approaches do exist which can provide evidence of the operation of allelopathy in the field. Density-dependent phytotoxicity is a result of plant competition for phytotoxic allelochemicals, but because the substance being competed for is not beneficial for the plant, a plant which takes up a larger quantity suffers reduced growth as a result. Plant size can actually increase as plant density increases, a result which is contrary to the expected effects of resource competition. These effects have been demonstrated in both greenhouse and field experiments, and in mono- and mixed cultures. Demonstration of either of the following results is contrary to the expected results of resource competition and supports the hypothesis of the presence of a toxin(s) in soil: (a) Compared to a control soil, growth reductions occur at low but are diminished at high density; (b) Maximum individual plant weight occurs at an intermediate density, due to a reversal in slope of the predicted log yield–log density line. The use of assays in which plant density is varied thus appears to be a useful tool for the study of allelopathy. For mixed culture of plants, the target-neighbor design (Thijs et al. 1994; Gibson et al. 1999) may prove to be useful for the study of allelopathic interactions because the variation of plant density is an integral part of the design. As pointed out above, density-dependent phytotoxicity will be manifested by differences in growth of the target plant at different densities. While varying the density of the source plant may be useful for experiments looking at dose-response relationships (Belz and Hurle, 2001), this can also blur competitive and allelopathic interactions (e.g., Suman et al. 2002).

## Ecological implications

As significant as the phenomenon of density-dependent phytotoxicity may prove to be for methodological reasons, the ecological implications may be equally important (Weidenhamer et al. 1989). For example, the fact that the greatest phytotoxic effects occur at low seedling densities could result in selective pressure on susceptible species to increase seed production and seedling establishment. In agricultural settings, practices which reduce weed seedling densities could also contribute to enhanced control of remaining seedlings by allelopathic interactions from cover crops or the crop plant itself. Yet another question is whether allelopathic interactions are more important ecologically in communities where overall plant densities are inherently lower due to environmental and other constraints? In the Florida scrub, there is evidence that allelopathic interference by fire sensitive shrubs of the scrub community deter the invasion of fire-prone grasses and pines of the sandhill community (Richardson and Williamson, 1988; reviewed in Williamson et al. 1992b and Fischer et al. 1994). Along with low plant densities, the scrub is typified by harsh environmental conditions and nutrient limitation which can intensify allelopathic effects (Stowe and Osborn, 1980; Hall et al. 1982; Einhellig and Eckrich, 1984; Williamson et al. 1992b). Low plant densities and harsh environmental conditions could contribute to the increased effectiveness of phytotoxins in such communities.

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## CHAPTER 5

# TOXICITY IN ALLELOPATHY: *IN SILICO* APPROACH

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## INTRODUCTION

The modern life style massively relies on chemistry. Diverse chemical compounds assist us in food and energy production, health care, etc... In spite of their wide use, not everything is known about the properties of chemicals that surround us, especially about their side effects and hazards. Moreover, the poor knowledge about chemicals is much worse for natural compounds, such as allelopathic compounds, where regulators can not force chemical industries to produce experimental data as happens for synthetic chemicals, which will be introduced to the market.

The toxic characterization of chemicals is done experimentally using testing animals, but, due to the cost and the time needed for testing, for most of chemicals introduced in our life there is insufficient information about toxicological properties. The costs of *in vivo* testing, i.e. tests involving living animals, is prohibitive and massively affects the final price of chemicals. In a recently published white paper (Commission of the European Communities, 2001), the European Commission estimated that “*the testing of the approximately 30,000 existing substances would result in total costs of about € 2.1 billion, over the next 11 years until 2012.*” Besides economical constraints, ethical consideration and public pressure push to reduce tests on animals (Omen, 1995). Thus, more convenient and efficient methods to predict biological activity from structural information is demanded. Moreover, the use of validate methods is encouraged by the European Union and USA (Walker, 2003).

In this framework, *in silico* approaches are challenging methods to cover many knowledge gaps. These approaches differ from laboratory experiment, *in vivo* and *in vitro*, because they do not involve the use of any biological system. They are based on the theoretical knowledge gained in different fields of the science and aided by the powerful computational capabilities of modern computers. *In silico* approach includes the design of biomaterials, and the understanding and prediction of physicochemical and biological properties such as solubility, biotransformation, receptor affinity and toxicity. Nowadays, using *in silico* approach together with the molecular biology it is possible to accelerate the identification of biological processes and better understand the mode of action, and guide, focus and limit laboratory experimentation.

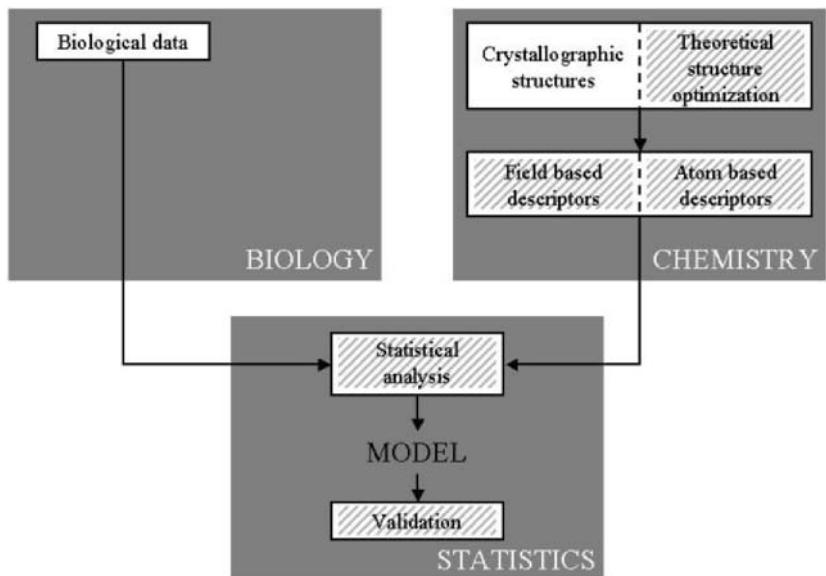
## METHODS

### QSAR

Quantitative structure-activity relationships (QSARs) lead to finding a relationship (model) between the chemical structures of compounds and a given property. In computer chemistry, such a mathematical model is then responsible for representing and explaining the underlying mechanisms of the property, for predicting property values for other chemicals, and for designing new chemicals with a given property value. Especially in the field of toxicity, QSAR are used for deriving predictive models of the impact of chemicals to human health, wildlife and environment.

The basic assumption of QSAR is, of course, that a quantitative relationship between the molecular structure of compounds and their biological, chemical and physical properties does exist. Then the development of QSARs should be through the interaction of a group of multidisciplinary experts from biology, chemistry and mathematics. In Figure 1 a schematic representation of the main building blocks used in development of a toxicity-based QSAR is outlined:

- A dataset that provides a measure of toxicity for a group of compounds.
- Evaluation of minimum energy conformation for each compound and calculation of descriptors related to each chemical structure.
- These two parallel data collections are then treated by some statistical technique, that gives a variable selection to ascertain the best predictive regression model for the activity. At the end the resultant QSAR should be validated and only used within the descriptor space and chemical domain of the model, to ensure that it is capable of providing sufficiently accurate predictions of toxicity for new compounds not used yet to generate the models.



*Figure 1.* Building blocks used in development of a toxicity-based QSAR. Hatched boxes are computer-assisted steps done by QSAR modeller.

### Toxicity data

The first step in formulating QSARs is to build-up a data set that must reflect one or more well-defined toxic endpoints. Data should be reliable because high quality toxicity data will have lower experimental error associated with them. All toxicity measurements are subject to experimental error. The reality of toxicity testing is that even with standardized protocol, it is not possible to obtain unbiased data, because different laboratory conditions such as individual characteristics, age, health of testing animals influence the results. Therefore, toxicity values are often reported as the mean for a series of replicates. A perfect statistical fit will never be achieved with a QSAR, and some degree of uncertainty in the model is expected and acceptable. The dataset should be made to represent molecular diversity, but typically a low number of compounds tested are available.

When the database of toxicity used in developing a QSAR becomes of sufficient molecular complexity, outliers will appear. They are chemicals that do not fit the model or are poorly predicted by it (Egan and Morgan, 1998). There are several

potential reasons for a chemical being an outlier, generally such compounds can be recognized as acting by a different mode of action from the other chemicals, which are well modeled. Outliers are useful in QSAR development because they assist in establishing the chemical domain of the model.

### ***Crystal structure, conformational search and geometry optimization***

Chemical are commonly thought of as a two-dimensional structure, but their toxic effects are an expression of their three-dimensional structure. In the living organisms the majority of biochemical processes follow the lowest-energy reaction pathways. The large biomacromolecules like proteins, nucleic acids or polysaccharides, as well as small molecules like peptides and hormones, normally exist in the most stable conformational state - the lowest energy conformation. In order to correctly describe the 3D structural and electronic properties of the molecule under the study in QSAR one has to consider it in a stable (optimized) conformation.

Finding a global energetic minimum of a flexible molecule is not always an easy task. Even a small molecule composed of few tens of the heavy atoms (non-hydrogen atoms) containing a few rotatable bonds has a high number of degrees of freedom, which include bond stretching, bond angle bending, and torsion angle rotations.

Throughout the years of the development of QSAR and modelling methods, various techniques to obtain stable conformers of a compound have been found and applied in the research.

### ***Experimental methods***

The most reliable molecular conformations can be found using the experimental X-ray diffraction methods, which produce the real picture of the 3D arrangement of the heavy atoms of molecules in the analyzed crystal. These methods are quite demanding due to the efforts necessary to grow the high quality crystals suitable for structure determination. The molecules in the crystals are packed and are subjected to intermolecular forces produced by surrounding molecules of the same compound or the solvent that may influence their conformation. However, the intermolecular forces are usually not too large and the conformation in the crystal is close to global minimum. As the X-ray crystallography is an experimental technique, the resolved structure can be in certain cases distorted due to presence of multiple energetically accessible conformations of the same compound or the impurities in the crystal. In this case the molecular structure can be “tuned” by optimization techniques of computational chemistry. Crystal structures of biomacromolecules (e.g., proteins, nucleic acids) are stored within the Research Collaboratory for Structural

Bioinformatics (RCSB) database (<http://www.rcsb.org/>) and Cambridge Crystal Database Centre (CCDC) holds the structures of small organic molecules (<http://www.ccdc.cam.ac.uk/>).

### *Theoretical methods*

Theoretical-computational approaches in conformational searching are based on the variation of the degrees of freedom in the molecule. The variations of torsion angles have the biggest impact on the energy of the molecule and determine the overall molecular shape. The values of the torsion angles can be varied either systematically or randomly.

The most reliable results can be obtained with systematic variation of torsion angles also called systematic conformational hyperspace sampling, in which several local minima as well as global minimum can be identified. The quality of the results directly depends on the capacity of the computational resources available, because the total number of evaluated conformations is determined by the number of searched torsion angles (rotatable bonds) in the molecule and the torsion angle variation step size. Due to this limitation the systematic conformational searching is usually used for small molecules with few rotatable bonds.

With random searching methods the degrees of freedom of the molecule are varied randomly. A typical searching iteration consists of the random generation of the torsion angle values and geometry optimization to the nearest local minimum. If the optimized geometry is unique, it is saved and used as a starting point for next iteration. The conformational search can finish if no new conformation can be found in a series of consecutive iterations. Random searching methods are suitable for conformational analysis of large molecules, typically peptides and saccharides.

### *Geometry optimization*

The conformations obtained by X-ray crystallography or conformational search are not absolute minima and in most cases need to be fully optimized. The main goal of the optimization procedure is to minimize the internal strain of the molecule and find the nearest local, possibly global, minimum. The optimization procedure is driven by a gradient normal, which reflects the energy changes with respect to structural changes. When the gradient value becomes low, the structure optimization finishes in a minimum.

The energy of the optimized molecule can be calculated by various methods of computational chemistry (Cramer, 2004). Molecular mechanics methods (e.g., MM2, MM+, AMBER) are based on the classical Newton laws, when atoms are

approximated by balls and bonds by springs with a given force constant. The set of all force constants for all atoms is called “force field” and can be derived either from experiment (IR spectroscopy) or from a high level *ab initio* calculation. Molecular mechanics calculations are very fast and thus may be applied to study of molecular systems composed of thousands of atoms. In the semiempirical methods (e.g., CNDO, AM1, PM3, ZINDO) the computationally most expensive part of quantum chemical calculation (evaluation of two electron integrals) is either completely neglected or replaced by empirical parameters, which leads to increased performance in comparison to full calculation. These methods provide good results for compounds that are similar to those used for parameterization. Due to their good performance they have been widely applied in QSAR. *Ab initio* quantum chemical methods, that are based on the first principles of physics and chemistry, are computationally the most expensive, but can provide structures and thermodynamic properties with excellent agreement with the experimental values.

### ***Chemical Descriptors***

Once the structure is in the minimum energy state it is possible to calculate the descriptors that mathematically characterize the molecule. A variety of properties have been used in structure-toxicity modelling, but more often the chemical descriptors used in structure-property correlations are based on the lipophilic, electronic and steric nature of substituents. The influence of molecular shape has always been difficult to describe, and a large variety of descriptors, from simple molecular weight to complex topological indices, have been employed to model steric properties. Of these, physicochemical descriptors of hydrophobic, electronic and steric parameters and quantum chemical properties (including charge and orbital energies) have been shown consistently to be important in modelling toxicity. Although some of the steric descriptors, such as molecular volume, encode some 3D information, molecular conformation has not been considered.

Nowadays, commercial software packages have become available that possess the capability of calculating many hundreds of molecular descriptors from simple structural inputs.

The descriptors can be divided according to the type into two general classes:

- *atom-based descriptors* that only describe the magnitude of particular physical properties but no directional preferences that these properties may have. These descriptors may include the atoms themselves, molecular fragments or substructures (functional groups), molecular indices derived by topological methods (molecular connectivity indices, related to the degree of branching in the compounds), atomic properties (electrotopological indices or atomic

polarizability), geometrical properties (molecular surface area and volume, moment of inertia, shadow area, projections and gravitational indices), physicochemical properties ( $\log P$ ), electrostatic properties (partial atomic charges and others depending on the possibility to form hydrogen bonds).

- *field-based descriptors* that describe the micro-environment surrounding the molecules (molecular electrostatic and steric potential and Van der Waals volume). This approach, which is called Comparative Molecular Field Analysis (CoMFA), looks at the molecules in 3-dimensions, and describes the magnitude and directions of electronic and steric interaction (Cramer et al. 1988 and Cramer et al. 1989). This technique measures the steric and electrostatic interaction energies between a small probe at a series of regular grid positions around the molecules. At each grid point, the steric and electrostatic interaction energies between the probe and each molecule are evaluated and recorded, producing a 3D box of interaction energies. These values become new steric and electrostatic descriptors (many hundreds or even thousands) for a QSAR analysis. It is important to underline that the structures have to be aligned (superimposed) to occupy the same position in space.

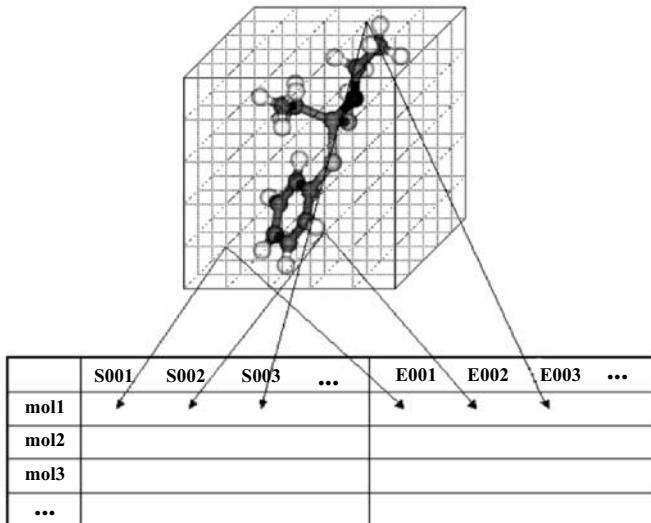


Figure 2. At each grid point, the steric and electrostatic interaction energies between the probe and each molecule are evaluated and recorded and these values become new steric and electrostatic descriptors in a QSAR analysis.

Typically, the models for toxicity prediction use algorithms involving atom based descriptors (Gini et al. 1999 and Villemin et al. 1994), but in some cases this method has certain limitations and it is necessary to split compounds in individual chemical classes Benfenati and Gini, 1997 and Ashby, 1994). The difficulties in constructing more general models using this kind of descriptors lies in the generic characterization of them. This divides the compounds and means that success and interpretation of the models is possible within well-represented and defined classes. A helpful way to avoid these difficulties can be the use of 3D-QSAR methodologies such as CoMFA that use descriptors distributed in various regions around the chemicals, presenting the residues related with their contribution to various biological processes. This is possible because the “*in vivo*” data contain information about various physico-chemical and biological processes, and the different chemical groups may involve different biological ligand-complex interactions. It is difficult to assess the contribution of these interactions, but the experimental data can highlight the possible modes of action. CoMFA allows to ascertain predictive relationship between molecular fields and biological activity, that is expressed as the sum of contributions from every variable in the model and the size of the coefficient for each variable that underlies the importance in describing activity. As each variable represents a variation in interaction energies at a defined point in 3D space, the regression coefficient can be mapped back onto the initial  $x$ ,  $y$ ,  $z$  coordinates of the variables, generating a 3D regression map. Therefore the advantages of CoMFA methods are that describing properties in terms of 3D fields, the results can be mapped into 3D space and one can localize points within the spatial distribution of properties which are strongly related to the activity. However the major problem of CoMFA models stems from the alignment of compounds and for this reason it is difficult to study highly heterogeneous datasets.

Moreover, while it is generally accepted that toxicological assessments are subject to error, it should also be accepted that descriptor values used in QSARs are also subject to variability, and when possible the descriptors used in formulating the QSAR should allow for a mechanistic interpretation of the model.

### ***Mathematical approach***

Once biological data have been collected and chemicals have been associated with a proper set of descriptors, mathematics takes care to extract the information hidden in the numbers. Several techniques are nowadays used and developed. Here we reviewed the most popular and promising ones.

### Multiple Linear Regression

Multiple Linear Regression (MLR) is amongst the first and most diffuse techniques in chemometrics, and especially in QSAR studies. The purpose of MLR is to fit a linear model of the form:

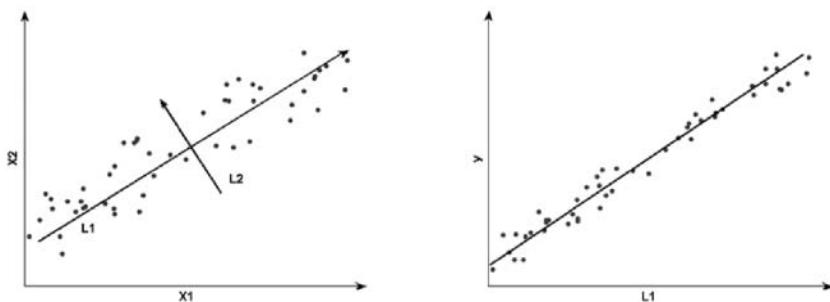
$$y = b_0 + b_1 X_1 + b_2 X_2 + \dots + b_k X_k + \varepsilon \quad (1)$$

where  $y$  is the dependent variable (activity) and  $X_1, X_2, \dots, X_k$  are the independent variables (descriptors), and  $\varepsilon$  is random disturbance (error).  $b_0, b_1, b_2, \dots, b_k$  are the regression coefficients, which are estimated from the data finding the least square solution of (1), i.e. regression coefficients are chosen so as to minimize the difference, error, between predicted values and actual values. MLR is performed for: a) understanding which descriptors have the greatest effects; b) knowing the direction of the effect (i.e. increasing  $x$  increases/decreases  $y$ ); c) Using the model to predict future values of the activity when only the descriptors are known.

### Partial Least Square

Partial Least Square (PLS) was originated in social science (especially economy) by Herman Wold (Herman, 1985) and became popular in chemometrics partially due to Herman's son Svante (Kohonen, 1987 and Hawkins, 2004). PLS is especially useful where the number of independent variables is comparable to or greater than the number of compounds (data points) and/or there exist other factors responsible for correlations between variables, because it leads to stable, correct and highly predictive models even for correlated descriptors (Martens and Naes, 1989 and Erikson et al. 2001).

PLS is based on a linear transformation of the descriptors space, producing a new variable space based on small number of orthogonal factors (latent variables), so that there is no correlation. In other words, factors are mutually independent (orthogonal) linear combinations of original descriptors (Figure 3a). Latent variables are chosen in such a way as to provide maximum correlation with dependent variable; thus, PLS model contains the smallest necessary number of factors (Höskuldsson, 1988) (Figure 3b). With increasing number of factors, PLS model converges to ordinary MLR.



- (a) Transformation of original descriptors to latent variables (L<sub>1</sub>, L<sub>2</sub>).      (b) Construction of activity model containing one PLS factor.

Figure 3. PLS analysis. (a) Descriptor space transformation. (b) Model construction.

Strictly, if  $y$  (in matrix form) is the dependent variable and  $X$  (in matrix form) are the predictor variables, a PLS regression using factor extraction for this type of data computes the factor score matrix  $T = XW$  for an appropriate weight matrix  $W$ , and then considers the linear regression model  $y = TQ + \varepsilon$ , where  $Q$  is a matrix of regression coefficients (loadings) for  $T$ , and  $\varepsilon$  is an error (noise) term. Once the loadings  $Q$  are computed, the above regression model is equivalent to  $y = XB + \varepsilon$ , where  $B = WQ$ , which can be used as a predictive regression model.

### Neural Network

The first step toward artificial neural networks (NNs) came in the early 1940's when scientists looked at neurons, fundamental, active cells in all nervous systems as devices for manipulating binary numbers (McCulloch and Pitts, 1943). As computers advanced, it became possible to begin to model the rudiments of these theories concerning human thought.

NNs mimics biological nervous systems, and, as in nature, are composed of simple processing elements (neurons) operating in parallel (layers). The network function is determined largely by the connections between elements (weights), which are responsible for the network's intelligence. The inter- and intra-layer connection define the architecture of the NN. NNs can be trained to fit a particular function by adjusting the values of the weights between neurons.

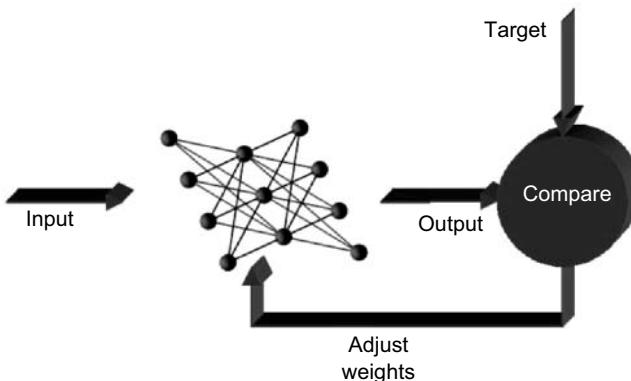


Figure 4. Supervised learning for neural networks.

Commonly, NNs are trained through a specific learning rule so that a particular input leads to a specific target output. Figure 4 shows a recursive learning process called supervised learning. In this case inputs fed in the NN and are mapped in the output. The output is then compared with the target and network weights are adjusted accordingly until the network output matches the target. On the other hand unsupervised learning do not need target values, and unsupervised NNs learn to recognize similarity among inputs.

Among the most popular NNs used in QSAR studies we can list: multi-layer perceptron (Rosenblatt, 1961), back-propagation (Rumelhart et al. 1986), self-organizing maps (Kohonen, 1987), and radial basis (Chen et al. 1991). They consist of different architectures and therefore have different characteristics and features but they all share the useful ability of dealing with complicated or imprecise data, especially when non-linear relationships are involved.

#### *Genetic Algorithm*

Genetic algorithms (GAs) are inspired by Darwin's theory of evolution and are part of evolutionary computing, which is a rapidly growing area of artificial intelligence. They were invented by John Holland, from the University of Michigan at the beginning of the 60s. A first achievement was the publication of *Adaptation in Natural and Artificial System* by Holland in 1975.

GA is a stochastic global search method that mimics the natural biological evolution (Holland, 1975 and Kinnear, 1994). GAs operate on a population of potential solutions applying the principle of survival of the fittest to produce approximations to a solution. A flow-chart of the procedure is shown in Figure 5.

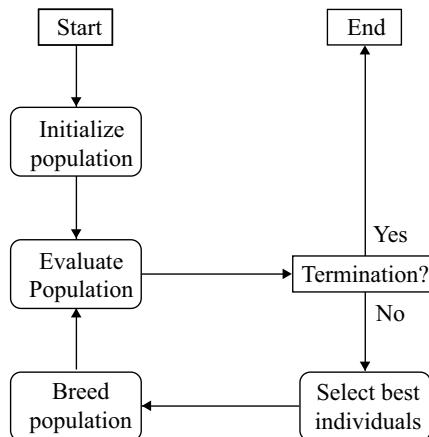


Figure 5. Flow-chart of the genetic algorithm procedure.

Algorithm begins with a set of solutions called population. At each generation, a new set of approximation is created by the process of selecting individuals according to their level of fitness in the problem domain and breeding them together using operators from natural genetics. This process leads to the evolution of populations of individuals from a seed population, just as in natural adaptation, converging to the solution. This is repeated until some condition (for example number of populations or improvement of the best solution) is satisfied, and the result is a population that better fit the problem.

Generally speaking, GAs are used for finding the solution of a given problem, therefore they can be used in several steps of a QSAR analysis. However, they showed to be very useful especially in the selection of relevant variables (Hasegawa et al. 1997 and Mazzatorta et al. 2003).

### **Validation**

The model validation is the process of assessing its ability to correctly predict the value for an unknown object of the same type. This is particularly true with fitting

complex models, when we may run in overfitting problems (Hawkins, 2004), i.e. the model fits well training data but can not be generalized to future data.

An optimal validation procedure should include tests on an external set of objects never used in any steps of the QSAR development. Unfortunately, often researchers deal with too few data to afford to reduce the information, leaving out from the analysis some data for testing. Moreover, even in the lucky case of data set large enough, there is still the problem of splitting data into training and test sets, which raises doubts about interpolation and extrapolation of the derived model.

It is common practice in QSAR studies to use internal cross-validation. In  $k$ -fold cross-validation, data are divided into  $k$  subsets of (approximately) equal size. QSAR models are developed, each time leaving out one of the subsets from training, but using only the omitted subset to compute the model performance. If  $k$  equals the sample size, this is called leave-one-out cross-validation. Leave- $v$ -out is a more elaborate and expensive version of cross-validation that involves leaving out all possible subsets of  $v$  cases.

A test widely used to ensure the robustness of a QSAR is the y-randomization test. In this test, the dependent variable is re-assigned randomly to different objects and a new model is developed. The process is repeated several times. It is expected that the resulting models should have low performances. If the random models' activity prediction is comparable to the original QSAR model, the set of observations is not sufficient to support the model due to a chance correlation or structural redundancy of the training set (Topliss and Costello, 1972 and Topniss and Edwards, 1979).

Validation is still an open issue in the research community (Hawkins, 2004; Golbraikh et al. 2002 and Tropsha et al. 2003). It is generally believed that when the size of the data set do not allow for a clear representative identification of training and test sets, it is better to use all data for the deriving the model and check it by cross-validation, making sure that the cross-validation is carried out correctly.

## Biochemical analysis

Structural information about biological system can be obtained with the experimental methods (X-ray crystallography and multidimensional NMR) or using a theoretical approach that, utilizing computational simulations of molecular systems, can provide essential alternatives and have become a major factor in modern research. The role of biochemical analysis is to understand biological processes and try to analyze and model the interaction of the ligand with the binding site and the formation of the ligand-macromolecule complex. Therefore, the search for relationships between biological activity and molecular structure can be seen as the search for stereo-electronic properties required for the recognition process to occur. This approach relies

on the hypothesis that the recognition process between the ligand and the receptor is based on the spatial distribution of certain properties of the active site being complementary to those of the interacting ligands. Moreover the properties common to the ligands would provide the information about the stereo-electronic requirements of the receptor active site. If the 3D structure of biological target is known the degree of similarity of the ligands can be defined in absolute terms like its structural complementarity in size, shape and electronic properties to the binding site. The situation is much more difficult if such structure is not known. In this case sets of structurally different molecules must be investigated to derive a hypothesis and rules which help to explain the activity or lack of activity of the different compounds.

### ***Docking Methodology***

Docking (Morris et al. 1998) is a computational method used to find the best matching between a biological macromolecule and a ligand. In molecular docking the ligand is placed inside the receptor pocket and the free energy of binding of the molecular complex is estimated computationally.

Today crystallography and NMR contribute significantly to biostructural research and give many details about the structure and function of macromolecules, such as nucleic acids, polysaccharides and proteins. Thus when the 3D structure of the protein to be studied is available, 3D-coordinates of the atoms in the receptor are known; otherwise homology model (HM) has to be constructed. The generally accepted procedure when the crystallographic structure of the target protein is not available involves sequence homology alignment with those isozymes for which crystallographic data are known (an appropriate template is a protein of known structure that shares a minimum of 20-25% homology in amino-acid sequence), followed by aminoacid residue replacement, insertion and deletion, as required by the alignment and, finally, energy minimization of the raw structure.

Then the task is to generate a set of energetically favorable configurations of the receptor-ligand complex. Stabilizing molecular interactions should be identified for each of them, which should give an estimate of the free energy of binding of this complex (Lengauer and Rarey, 1996).

### ***GRID Methodology***

GRID (Goodford, 1985) is a computational procedure for determining possible binding sites and it is used to predict the position of ligands in proteins. This methodology detect the interaction between the target molecules and different probes (alkyl hydroxyl, phenolic hydroxyl, ether oxygen, ketone oxygen, carboxy, phosphate groups, ....) over a 3D grid around the molecules. The procedure is similar to CoMFA,

but in GRID one can analyze the interaction with different probes that mimic the functional group of the ligands studied.

The graphic representation of the energetic terms allows simple interpretation and visualization of the regions where the probe interacts more strongly with the target either by attraction or repulsion. Therefore using GRID maps it is possible to identify the possible binding sites for known 3D enzyme structures.

## APPLICATION

Allelochemicals are natural chemicals which are present in common crops such as wheat, rye and maize, and are toxic to most terrestrial animals and plants (Yeung et al. 2002). As the toxicological evaluation of synthetic pesticides is well documented, while the toxicological effects of allelochemical compounds are not known at all, our efforts were aimed on designing a model to assess the toxicity values of wheat allelochemicals, in particular cyclic hydroxamic acid derivatives and lactams in comparison to synthetic pesticides using rules-based prediction of toxicity.

### Rat toxicity prediction model

#### *Method*

The dataset was built by selecting 73 synthetic pesticides with structures similar to those of the considered allelochemicals (Figure 6). In particular, all compounds contained an aromatic and/or heterocycle ring with nitrogen and/or oxygen atoms. The set was randomly split into the training (52 compounds) and test sets (21 compounds). The toxicity values were collected from different sources: The Pesticide Manual (Tomlin, 1997), the U.S. EPA Ecotox database (<http://www.epa.gov/ecotox/>), and the CIRCA website (<http://forum.europa.eu.int>). The end-point was LD<sub>50</sub> (50% lethal dose, i.e. dose leading to death of 50% of the exposed organisms) for the rat expressed as follows: output = log (MW/LD<sub>50</sub>) (MW = molecular weight) to refer to the moles of the chemical and not the weight.

Molecular optimization was done using semi-empirical methods (PM3) and molecular dynamics simulations were used for conformational analysis using Hyperchem software module (HyperChem®, Hypercube, inc.).

*Field based* descriptors (steric and electrostatic interaction energy) were calculated using ChemX software (Chem-X®, Chemical Design Ltd.). Common phenyl

ring was used as a template to align the compounds, and for compounds with two or more phenyl rings, the phenyl rings attached to the heterocycle ones were selected. The ligands were superimposed by flexible fitting option in Chem-X and PLS analysis was applied in order to extract the underlying information. The results of the CoMFA models were represented by regression contour maps indicating how the different regions of the ligands contributed to the variations in toxicity.

GRID and docking methods were also used to compare and improve the information obtained from CoMFA descriptors.

In rat the cytochrome P450 subtype CYP1A2 has an important role in metabolism of the examined chemicals and its homology modeling was done using crystallographic structure of rabbit cytochrome CYP2C5 (Williams et al. 2000), available from the RCSB protein databank (reference code: 1DT6) (Berman et al. 2000), as a template. To investigate the potential interaction of the enzyme and chemical structure, a series of GRID calculations was performed using the generated structure of rat cytochrome CYP1A2. The calculations were done in order to search for binding sites complementary to the functional groups of the pesticides. Hydrophobic and water molecule,  $Csp^3$  and aromatic  $Csp^2$  atoms were used as probes. By means of GRID maps we identified the favored interaction and the possible binding area of the enzyme investigated.

The AutoDock 3.0 program was used for docking study (Goodsell et al. 1996) of the cytochrome CYP1A2 structure mentioned above. A Larmarckian genetic algorithm (LGA) was used to find the best ligand-protein complex (Morris et al. 1998).

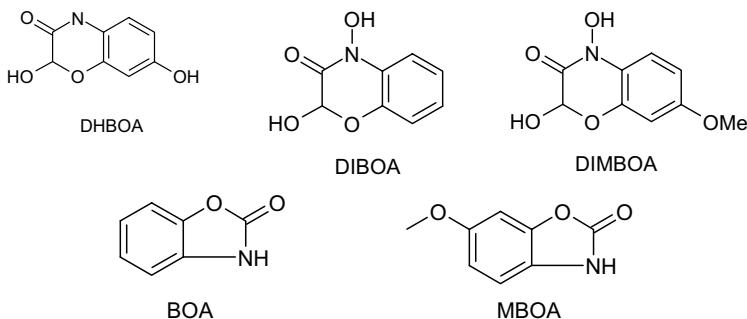


Figure 6. Cyclic hydroxamic acids and lactams.

## Results

QSAR procedure using CoMFA descriptors (steric and electrostatic field) led to a model with high predictive ability on the test set containing 21 compounds ( $R_{pred}^2 = 0.92$ ). Using six components, the value of the cross-validated coefficient (leave-one-out) was  $q^2 = 0.94$  indicating a high predictive ability of this model. Therefore predicted values are very close to the experimentally observed ones (Figure 7-8). Comparison of the electrostatic and steric models showed better predictive ability on the electrostatic fields reflecting the higher contribution of this type of interaction. Moreover the model obtained by means of only steric or electrostatic field gave good predictive results, allowing a confident assessment of contributions on these interactions at the different parts of the compounds.

Regression squared contour maps showed the regions related to toxicity obtained by CoMFA model. These maps allowed identification of the areas and substituents that are important for the toxicity variations in terms of electrostatic and steric contribution. The results are summarized:

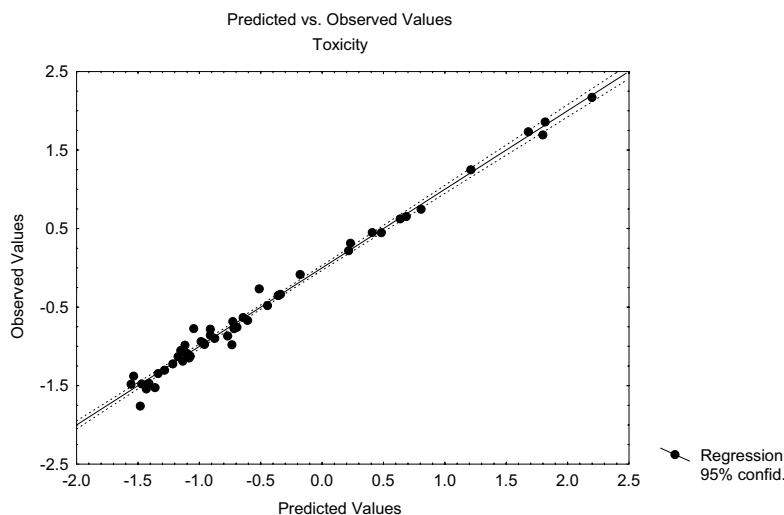


Figure 7. Conventional Co MFA model results for training set compounds: experimental versus predicted- $\log(\text{LD}_{50})$  toxicity values.

*Electrostatic contribution:* since the compounds were overlapped to common phenyl rings, the high  $R^2$  values in this region means big contribution of this area to the toxicity values, which can be explained with  $\pi-\pi$  ligand-receptors interactions of this part of the molecules. High regression values were observed near to the substituents of the heterocycle ring.

*Steric contribution:* bigger regression values are concentrated around the substituents of the heterocycle ring, so differences in the occupation of this region can lead to wide toxicity variations. The differences in the molecular structures and in toxicity values of the compounds analyzed led to conclusion that the toxicity values increase with the Van der Waals volume and the steric interaction in this part of the chemicals. Comparing the two maps there is also an overlap between steric and electrostatic areas in this part of the molecules.

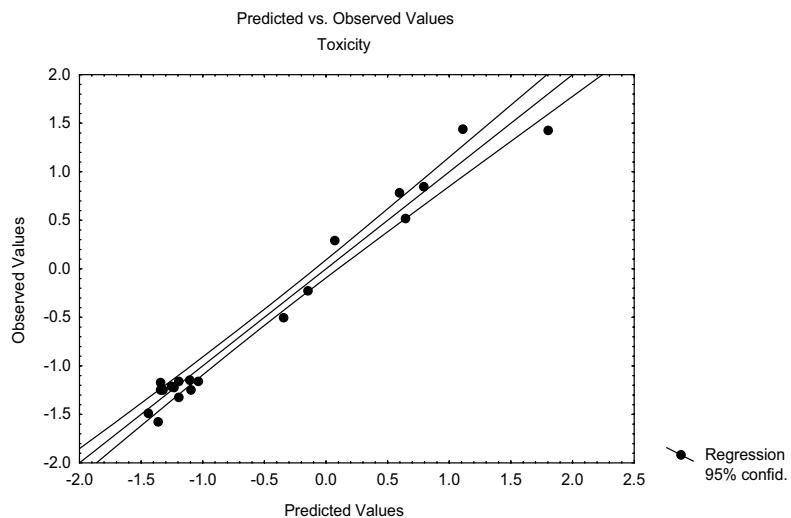


Figure 8. Conventional CoMFA model results for test set compounds: experimental versus predicted- $\log(\text{LD}_{50})$  toxicity values.

The CoMFA maps allowed studying the possible mechanisms that contribute to the final toxicity. The starting point was to describe the processes related to the chemical transport (LogP values prediction). A CoMFA model was built to predict LogP for the same dataset using the same descriptors obtained for toxicity prediction. There is no similarity between toxicity and LogP maps indicating differences in the

process modeled. Thus, the conclusion is that the transport mechanism is not the key process and we studied the possible metabolic contribution of phase I enzymes and especially cytochrome P450 family member CYP1A2. Therefore the docking of some compounds contained in our dataset in the active site of rat CYP1A2 enzyme was performed to find the significant residues and positions of oxidation in the binding site. The results are in agreement with CoMFA because the most important interaction are located around the heterocycle ring.

GRID map calculated using aromatic  $Csp^2$  atom as a probe showed that the favorable areas for the ligand-enzyme interaction is close to the heme, around the common phenyl ring. Moreover the use of hydrophobic probe produced big contribution to hydrophobic interactions on the heteroaromatic ring.

The results showed that the favored GRID binding areas overlap the region of the CoMFA map with a high regression coefficient, indicating that the cytochrome CYP450 contributes to the final toxicity.

## Conclusion

3D QSAR model based on CoMFA method showed highly predictive abilities with results in good agreement with those obtained by GRID and docking approaches. CoMFA, GRID and docking approaches can provide important information for understanding toxicity and for describing the mechanism of action. Each of this methods independently can produce useful information looking the molecules from different perspectives. The CoMFA models presented a good capability for toxicity prediction, the docking technique gave important information about the metabolic pathway, and GRID procedure suggested information about possible binding sites. There are several examples such as structure-based 3D-QSAR where the combinations of these techniques sufficiently increase the quality of the models and also clearly explain the mechanism of action (Sippl, 2002 and Martin et al. 1996).

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## CHAPTER 6

# ALLELOCHEMICALS AND PHOTOSYNTHESIS

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## INTRODUCTION

Allelopathy in natural and agricultural ecosystems is receiving increasing attention because allelochemicals significantly reduce the growth of other plants and the yields of crop plants (Rice 1984; Korner and Nicklisch 2002; Leu et al. 2002; Inderjit and Duke 2003). Allelochemicals are found to be released to environment in appreciable quantities via root exudates, leaf leachates, roots and other degrading plant residues, which include a wide range of phenolic acids such as benzoic and cinnamic acids, alkaloids, terpenoids and others (Rice 1984). These compounds are known to modify growth, development of plants, including germination and early seedling growth.

Allelochemicals appear to alter a variety of physiological processes and it is difficult to separate the primary from secondary effects. There are increasing evidences that allelochemicals have significant effects on cell division, cell differentiation, ion and water uptake, water status, phytohormone metabolism, respiration, photosynthesis, enzyme function, signal transduction as well as gene expression (Singh and Thapar 2003; Inderjit and Duke 2003; Belz and Hurle 2004, Science 2003). It is quite possible that allelochemicals may produce more than one effect on the cellular processes responsible for reduced plant growth. However, the details of the biochemical mechanism through which a particular compound exerts a toxic effect on the growth of plants are not well known.

Photosynthesis is the basic physico-chemical process for plant growth, by which plants, algae and photosynthetic bacteria use light energy to drive the synthesis of organic compounds. The overall equation for photosynthesis is deceptively simple, in fact, a complex set of physical and chemical reactions must occur in a coordinated manner for the synthesis of carbohydrates (Figure1). The photosynthetic process in plants and algae occurs in small organelles known as chloroplasts that are located inside cells. The photosynthetic reactions are traditionally divided into two stages - the “light reactions,” which consist of electron and proton transfer reactions and the “dark reactions,” which consist of the biosynthesis of carbohydrates from CO<sub>2</sub>. The light reactions occur in a complex membrane system (the photosynthetic membrane) that is made up of protein complexes, electron carriers, antenna pigment (chlorophyll) and lipid molecules. In the light reactions, two different reaction centers, known as photosystem II and photosystem I, work concurrently but in series. As a result, electrons were transferred from a water molecule to NADP<sup>+</sup>, producing the reduced form, NADPH. The NADPH together with ATP formed by the light reactions provides the energy for the dark reactions of photosynthesis, known as the Calvin cycle or the photosynthetic carbon reduction cycle. Calvin cycle occurs in the aqueous phase of the chloroplast and involves a series of enzymatic reactions. It is firstly catalyzed by the protein Rubisco (Ribulose 1,5-bisphosphate carboxylase/oxygenase), which attaches CO<sub>2</sub> absorbed through stomata to a five-carbon compound and at last produce two molecules of a three-carbon compound. Subsequent biochemical reactions involve several enzymes such as Fapase and at last triose phosphates is produced (Raghavendra 1998).

Photosynthesis is greatly influenced by environmental factors such light, temperature, CO<sub>2</sub> concentration, water condition and microbes. Recent studies showed that allelochemicals also significantly influenced photosynthesis. A reduction in CO<sub>2</sub> assimilation has been widely observed in many plants after treatment with allelochemicals. It is evident that allelochemicals can potentially impair the performance of the three main processes of photosynthesis, the stomatal control of CO<sub>2</sub> supply, the thylakoid electron transport (light reaction), and the carbon reduction cycle (dark reaction). The detailed mechanism for the reduced assimilation induced by allelochemicals in most studies, however, remains largely unclear.

## CHLOROPHYLL CONTENT

Chlorophylls are the core component of pigment-protein complexes embedded in the photosynthetic membranes and play a major role in the photosynthesis. Any changes in Chl content are expected to bring about change in photosynthesis. Reduced chlorophyll content in allelochemical-treated plants has been frequently reported. Einhellig and Rasmussen (1979) and Patterson (1981) all found that treatment of

soybean plants with phenolic acids such as ferulic, p-coumaric, and vanillic acids greatly decreased the biomass associated with reduced chlorophyll content in leaves. Similar results are also found in species such as *Parthenium hysterophorus*, (Kanchan and Jayachandra, 1980), *Cucumis sativus* (Pramanik et al. 2001; Yu et al. unpublished). In contrast, growth inhibition by allelopathic agents in grain sorghum seedling was not followed by decreased chlorophyll content in some cases (Einhellig, 1986).

Allelochemicals may reduce Chl accumulation in three ways: the inhibition of Chl synthesis, the stimulation of Chl degradation, and both. Rice (1984) suggested that some allelochemicals may interfere with the synthesis of porphyrin, precursors of Chl biosynthesis. Recently, Yang et al. (2002) investigated the effects of three phenolic acids on the concentration of Chl and its three biosynthetic porphyrin precursors (Proto, Mg-Proto and pchlido) and found that those of Proto and Pchlido showed quite different patterns of changes in concentration while the mole percent of Mg-Proto affected exhibited the same pattern. They argued that Mg-chelatase, the enzyme responsible for the conversion of Proto into Mg-Proto, may be the major target of the test phenolic acids. In contrast, the reduced Chl content by allelochemicals from the trichomes of *Parthenium hysterophorus* were thought to be caused by enhanced Chl degradation (Kanchan and Jayachandra, 1980).

## STOMATAL RESPONSES

### Allelochemical-induced water loss

Plant root is often the first tissue to contact an allelochemical, thus, it is logical to study the effects of allelochemicals on ion uptake and water uptake. Inhibited water and ion uptakes have been well documented (Glass, 1974; Yu and Matsui, 1997). Inhibition of ion uptake is directly related to membrane perturbation. The inhibited ion uptake in many studies would directly lead to disruption of plant water balance, or the resulting mineral deficiency would indirectly change the water relation. Some suggested that these observations are due to interference with normal membrane function and disruption of active transport (Glass, 1974; Barkosky et al. 1999, 2000). Our unpublished results showed that cinnamic acid, a compound found in root exudates of cucumber, significantly inhibited the activity of plasma H<sup>+</sup>-ATPase, PPase and . Similar results were also observed in juglone treated corn and soybean seedlings by Hejl and Koster (2004). The plant cell plasma H<sup>+</sup>-ATPase and associated membrane proteins play an essential role in the maintenance of cell turgor and uptake of mineral essential for plant growth. Any changes in plasma H<sup>+</sup>-ATPase together with reductions in ion and water uptake by roots would lead to stomata closure, decreased

cell turgor of leaves and have indirect effects on other essential plants functions such as photosynthesis.

## Impact of allelochemical on stomata

Allelochemical treatment frequently resulted in a decrease in stomatal conductance together with loss of leaf turgor. In cucumber, allelopathic agents would result in a reduction in stomatal conductance in several hours after the treatment (Yu et al. 2003). In tobacco and sunflower, the effects lasted as long as several days (Einhellig and Kuan, 1971). The precise mechanism involved is complex since many factors are related in the regulation of stomatal opening.

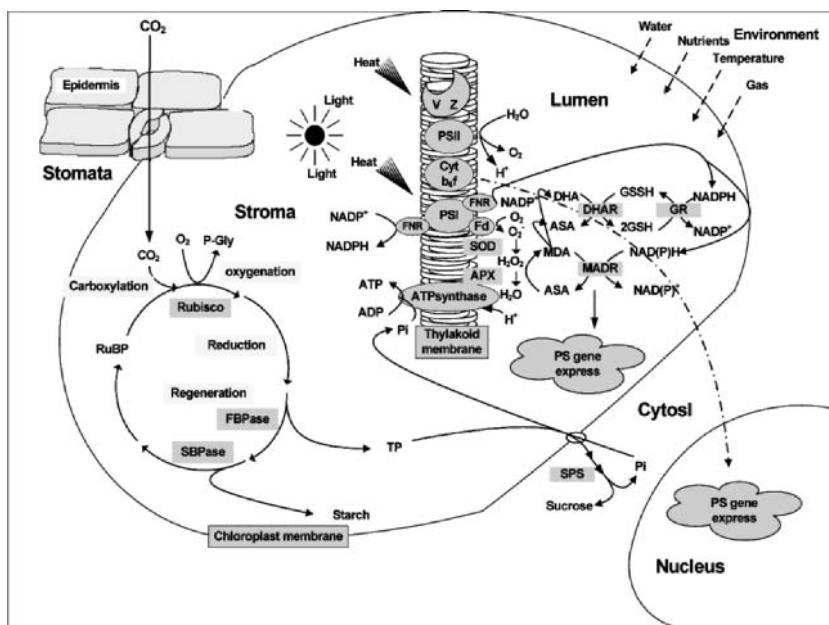


Figure 1. Photosynthetic process and possible impact site by allelochemicals.

At present, it is tempting to ascribe the reduced CO<sub>2</sub> assimilation to the reduction in stomatal conductance since both stomatal and non-stomatal factors could result in a reduction in CO<sub>2</sub> assimilation. In the case of stomatal limitation, reduced stomatal conductance is generally accompanied by decreased intracellular CO<sub>2</sub> concentration.

On the contrast, non-stomatal limitation is characterized by a reduced stomatal conductance and an increased intracellular CO<sub>2</sub> concentration (Farquhar and Sharkey, 1982). From the response of CO<sub>2</sub> assimilation rate to intracellular CO<sub>2</sub> concentration, it is also possible to distinguish the stomatal limitation from non stomatal limitation. Unfortunately, results in most experiments about the allelopathic effects on photosynthesis do not allow us to distinguish the stomata limitation from the non-stomata limitation since fewer people have examined the response of CO<sub>2</sub> assimilation rate to intracellular CO<sub>2</sub> concentration. Recently, we found that a decrease in stomatal conductance coincided with the decline in CO<sub>2</sub> assimilation rate and a decrease in intracellular CO<sub>2</sub> concentration, suggesting the decrease in photosynthetic rate induced by allelochemicals was at least partly due to stomata closure.

Stomata function is influenced by a lot of factors such as water status, K concentration and ABA signals. There are no enough evidences that allelochemical is involved in the regulation of stomatal aperture. One possibility is that allelochemicals influenced stomatal aperture indirectly by modifying water status, hormone balances and ion uptake. Since root is the first organ that come into contact with allelochemicals in most cases, impaired water and ion uptake and increased ABA accumulation are the most possible mechanism involved. On the other hand, Rai et al. (2003) found that inclusion of volatile essential oils from *Prinsepia utilis* L or its leaves greatly inhibited the stomatal opening and reduced the stomatal conductance. Furthermore, they also found that the K concentration in guard cell was significantly reduced by the oils and they concluded that the stomatal closure was directly related to their ability to inhibit the influx of K into the guard cell.

## **IMPAIRED PSII FUNCTION (PHOTOINHIBITION) AND ELECTRON TRANSPORT**

Photosystem (PS) uses light energy to drive two chemical reactions - the oxidation of water and the reduction of plastoquinone. Many environmental stresses reduce the capacities of photosynthetic system to utilize incident light, leading to a photoinhibition process. Photoinhibition of photosynthesis is typically characterized as a reduction in quantum yield of photosystem (PS) II photochemistry and a decrease in Chl a fluorescence. Recent advance in Chl fluorescence analysis make it easy to detect photoinhibition by measuring the maximal Chl fluorescence (Fv/Fm).

Barkosky et al. (2000) reported that exposure to cafferic acid resulted in a significant increase in leaf diffusive resistance and transpiration rate on 12 d of the treatment and a significant decrease in Fv/Fm on 28 d. Similar results were also observed in leaf spurge after treatment with hydroxyquinone (Barkosky et al. 1999). They concluded that disruption of plant water relations is the primary mechanism of the growth inhibition and the chronic reduction in available CO<sub>2</sub> and water stress are

the possible causes for the reduction in Fv/Fm. In our experiments, we found that cinnamic acid treatment resulted in a slight reduction in Fv/Fm at the later stage of cucumber plant growth, however, reductions in photosynthetic rate and plant growth were observed as soon as the plants were subjected to CA treatment, suggesting that photoinhibition was not responsible for the reduced CO<sub>2</sub> assimilation. Regardless of the photoinhibition, lowered photosynthetic electron transport ( $\Phi_{PSII}$ ) were frequently observed in allelochemical-treated plants such as cucumber seedlings. There are two possibilities that may result in a reduction in  $\Phi_{PSII}$ , one is the impaired PSII function due to the photoinhibition and another is the decreased requirement for the NADPH and ATP, a down-regulation mechanism. Since there are not enough evidences that showed allelochemicals induced photoinhibition, the observed reduction in  $\Phi_{PSII}$  may be attributed to a reduction of CO<sub>2</sub> fixation capacity which will be discussed later.

A few studies have showed that allelochemicals or phytochemicals from higher plants, cyanobacteria and algae exhibited inhibition to the ATP synthesis, uncoupled electron transport and phosphorylating electron flow. Hernandez-Terrones et al. (2003) found that trachyoban-19-oic acid, a compounds isolated form *Iostephane heterophylla*, acts as Hill reaction inhibitor and inhibited uncoupled PS II electron from H<sub>2</sub>O to DCPP and they concluded that a perturbation in the thylakoids at the level of LHC II occurred. Xanthorrhizol (Gonzalez et al. 2003) and trachyoban-19-oic acid from *Iostephane heterophylla* (Hernandez-Terrones, 2003), sorgoleone (Gonzalez, 1997) and resorcinolic lipids (Rimando et al. 2003) from *Sorghum bicolor*, polyphenolic allelochemicals from aquatic angiosperm *Myriophyllum spicatum* (Leu et al. 2002), all significantly inhibited PSII. Almost all these experiments, however, were done with isolated chloroplast or thylakoid membranes and these studies did not provide convincing evidence for the role of these compounds in intact plants. Accordingly, it is difficult to relate the effects on thylakoid membranes to that of intact plants. In fact, little information is available about the accumulation of the tested allelochemicals in chloroplast or even in leaves. For higher plants, allelochemical must be absorbed in roots and transported through xylem to chloroplast before it works as inhibitor of PSII electron transport. Showing disruption of PSII electron transport *in vivo* does not necessarily mean that this is a primary or direct mechanism of the allelochemical-mediated inhibition of photosynthesis. Since we still lack data to show that allelochemicals reach chloroplast and accumulate in significant amounts, we argue that allelochemicals may have low activity beyond the rhizosphere. Such evidence is urgently needed before we ascertain the role of allelochemicals on PSII electron transport. However, it needs to be noted that some allelochemicals in aquatic ecosystems may have direct effects on electron transport since they readily reach and enter the photosynthetic apparatus of algae.

## CARBOHYDRATE METABOLISM

Recently, it has become increasingly apparent that CO<sub>2</sub> assimilation is regulated in a sophisticated manner. In this regard, carbohydrate metabolism is also thought to be related to the CO<sub>2</sub> assimilation. End-product inhibition, impaired stromal bisphosphatases and Rubisco activities are three potential components involved in the regulation of photosynthesis in many studies, unfortunately, very little information is available about the impact of allelochemical on these components.

A significant increase in CO<sub>2</sub> assimilation rate is well observed in plants in response to a switch from ambient to non-photorespiratory conditions. In some cases, CO<sub>2</sub> assimilation rate do not increase with this switch due to the limitation of thylakoid ATPase synthase activity arising from insufficient return of inorganic phosphate (Pi) to the chloroplast caused by the accumulation of triose phosphates. It has been well reported that many allelochemicals have detrimental effects on phosphate uptake, but it is not clear whether the inhibited phosphate uptake would result in Pi limitation in photosynthesis. Moreover, stunted growth with increased carbohydrate content was frequently observed in plants receiving allelochemical treatment. An increase in carbohydrate metabolite such as sugars would lead to a reduction in CO<sub>2</sub> assimilation as reported in other stressed plants. Accordingly, more definitive and systematic experiments need to be conducted to ascertain whether end-product limitation is involved in the reduction of CO<sub>2</sub> assimilation by allelochemicals.

There are almost no evidences about the impacts of allelochemicals on Rubisco and stromal bisphosphatases. Recently, our laboratory found that cinnamic acid treatment followed by pathogenic *Fusarium* inoculation significantly reduced the photosynthetic capacity of cucumber leaves. This reduction was accompanied by reductions in both carboxylation efficiency and regeneration of RuBP. However, it is not clear weather the reduced carboxylation efficiency arose from the reduction in Rubisco content or in activity or both.. Reduction in both content and activity, of Rubisco induced by biotic and abiotic stresses have been well reported in plants (Allen and Ort, 2001).

## EFFECTS ON PHOTOSYNTHETIC PRODUCTIVITY

Plant growth and productivity are usually correlated to both the total leaf area and the photosynthetic rate per unit of leaf. It has been well documented that allelochemical treatment significantly decreased plant biomass together with reduced leaf area and stunt plant growth. The factors associated with the decreases in the photosynthetic capacity per unit leaf area induced by allelochemicals have been discussed above. Moreover, allelochemicals also have detrimental effects on cell

division and enlargement, eventually induce a reduction in leaf area. Accordingly, decreases in the capacity to capture photosynthetically active radiation are also a main factor in determining the reduction in the photosynthetic productivity of the allelochemical exposed plants.

## CONCLUSION

The major components of photosynthesis that are typically affected by allelochemicals in plants are discussed above and some of them are listed in Table 1. It is difficult to answer which mechanism of the action is predominant in a given situation from the present knowledge. Generally, allelochemicals in the rhizosphere usually change the plant-water relation by disturbing the membrane of root cell and the water stress-induced changes is one of the candidates for the reduced CO<sub>2</sub> assimilation rate. However, it is also likely that some volatile components directly inhibit CO<sub>2</sub> assimilation rate by modifying the stomata function although the detailed mechanism remains to be studied. There is not enough proof to show that allelochemicals in the rhizosphere have a direct impact on photosynthetic electron transport or the activity of carbohydrate metabolism since we do not have evidence to prove that allelochemicals are accumulated in the chloroplast. In the aquatic ecosystem, however, allelochemicals may directly depress photosynthesis by inhibiting electron transport or carbohydrate metabolism since they could readily enter into the photosynthetic apparatus.

Table 1. Effects of allelochemicals on components in photosynthesis

Function	Allelopathic agents or allelochemical	Concent. mM	Species and material	Action	References
Chlorophyll content	Ferulic acid	0.5	Soybean	Decreased	Einhellig and Rasmussen (1979)
	Monoterpenes	0.1	<i>Cassia occidentalis</i>	Decreased	Singh et al. (2002)
	Secalonic acid	0.3	Sorghum	Decreased	Zeng et al. (2001)
	Microcystin-LR		<i>Ceratophyllum demersum</i>	Decreased	Pflugmacher (2002)
	Phenolic acids	0.2	Rice	Decreased	Yang et al. (2002)

Root membrane function					
	Juglone	0.01-1	Corn soybean	Decreased	Hejl & Koster (2004)
Stomatal conductance					
	Phenolic acids	0.25	Cucumber	Decreased	Yu et al. (2003)
	Juglone	0.01-0.1	Soybean Corn	Decreased	Jose & Gillespie (1998)
	<i>Prinsepia utilis</i>	volatile oil	<i>Vicia faba</i>	Decreased	Rai et al. (2003)
	Hydroxybenzoic acid	0.50	Soybean	Decreased	Barkosky (2003)
	Hydroquinone	0.25	<i>Leafy spurge</i>	Decreased	Barkosky (1999)
Carbohydrate metabolism					
	Cinnamic acid	0.25	Cucumber	Decreased	Yu et al. unpublished
Water potential					
	Hydroxybenzoic acid	0.75	Soybean	Lowered	Barkosky (2003)
	Ferulic acid	0.25	Sorghum	Lowered	Einhellig (1985)
Electron transport					
	<i>Prymneshium parvum</i>		Baltic sea plankton	Inhibited	Fistarol et al. (2003)
	Caffeic acid	0.1-0.25	<i>Euphorbia esula</i>	Inhibited	Barkosky (2000)
			Sorgoleone	Inhibited	Gonzalez et al. (1997)
					Czarnota et al. (2001)
	Trachyloban-19-oic acid		Spinach	Inhibited	Hernandez-Terrones et al. (2003)
	Resorcinolic lipids				Rimando et al. (2003)
	Polyphenols		Spinach	Inhibited	Leu et al. (2002)
	<i>Myriophyllum spicatum</i>		Phytoplankton	Inhibited	Korner & Nicklisch (2002)

	Hydroxybenzoic acid	0.75	Soybean	Inhibited	Barkosky (2003)
	Fischerellin A			Inhibited	Srivastava et al. (1998)
	Tricolorin A	20 µM	Spinach	Inhibited	Achinine et al. (1999)
	Acetogenius		Spinach	Inhibited	Chavez et al. (2001)
	Xanthorrhizol		Spinach	Inhibited	Gonzalez et al. (1997)
	Hydroquinone	0.25	<i>Leafy spurge</i>	Inhibited	Barkosky (1999)

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## CHAPTER 7

# CELL CYCLE ANALYSES FOR UNDERSTANDING GROWTH INHIBITION

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## INTRODUCTION

Cell division appears in the organisms to be an answer to the necessity of cell growth, and this uniform division of the all cell components allows an equilibrated growth of the organism. This process underlies growth and development and is central to the heredity and evolution of all life forms. Therefore, information about the mode of action to alter growth is central in the research of stress conditions and, of course, in allelopathy (Einhellig, 1994). At present, it is well known that allelochemical compounds can interfere with many vital processes of organisms. Some works have been performed using the light and electron microscopy to study the effects of allelochemicals on plant growth (Aliotta et al. 1993; Burgos et al. 2004), but direct measurements on cell cycle dynamics and mitotic index have not been usually recorded after allelopathic exposition.

However, kinetic, dynamic, and rate characteristics of cell cycle in plant cell cultures or tissues are very important parameters involved in multiple physiological events. Therefore, the study of the basic mechanisms of the cell cycle (rates of proliferating and quiescent cells, characterization of cell subsets and states upon cell

cycle length and progression), as well as the study of the effects of different putative modulators and inhibitors (hormones, growth factors, toxins, maybe allelochemicals, etc.) and environmental conditions (including stress) on the cell cycle is a key knowledge in several research works (Galbraith, 1989; Gray et al. 1990).

In plant research, the role of cell division in the stress-response of plants should be evaluated by quantitative analyses of cell cycle. This can give us esteem about the plant behavior under temporal or continuous environmental conditions. For example, a reduction in leaf expansion rate is, in most species, the primary reaction to environmental stresses (Boyer, 1982) such as water, light, or nutrient deficits. Cell cycle is not the only determinant process of final leaf growth, but the correlation between tissue expansion and cell division determines a final leaf area that gives us information about plant's response to stress. In this way, Tardieu and Granier (2000) observed that water or light deficits caused a partial blockage of nuclei in G1 increasing cell cycle duration and decreasing final cell number with a concomitant reduction of the final leaf area. This effect was very fast detected in the plant after the application of the stress and, sometimes, it was independent of the carbon metabolism without altering the photosynthetic rate.

Something similar happens with the root development, where each time more works are focusing the interest by the study of auxin-regulated gene expression, the role of protein kinases as key regulators in plant growth and development and, of course, the cell cycle rate and dynamic measurements in these stressed tissues.

The most interesting use for the present work is the knowledge of the way on which factors affect cell cycle activity. In this way, a lot of factors such as developmental stage (O'Really and Owens, 1987; Fielder and Owens, 1989); stress conditions, as frost hardiness studied by Colombo et al. (1988), water stress by Chiatante et al. (1997), low temperatures by Harrison et al. (1998), or microgravity by F-Yu et al. (1999) were studied. Some chemical substances were studied too, as contaminants (Wonisch et al. 1999), or insecticides (Chauhan et al. 1999).

Some works were also performed measuring mitotic activity in plants exposed to secondary metabolites. That is the case of Wisniewska and Chelkowski (1994), and Packa (1997), who studied the potential genotoxicity of *Fusarium* mycotoxins on cells from wheat. In their works, strong effects from these toxins on mitosis were shown. These secondary metabolites decreased mitotic index, produced excessive condensation of prophase and metaphase chromosomes, caused accumulation of metaphases and rose significantly the percentage of cells with chromosomal aberrations in the treated seeds and seedlings with respect to the control.

One year later, in 1998, Packa could observe similar effects of these mycotoxins on nuclei and chromosomes from root tip cells of *Vicia faba* and *Pisum sativum*. In the same year (1998) Hemmerlin and Bach used mitotic index to study the importance of mevanolic acid for cell cycle progression in tobacco cells, and in 1999 Fernandez et al. used it also to know the origin of somatic embryos from an *in vitro* cellular culture of *Triticum durum* Desf.

Rimando et al. (1999) studied the mitotic index of some lignans from *Leucophyllum frutescens* (diayangambin, epiyangambin, diasesartemin) on onion root tips germinated in presence of these compounds, and found a significant decrease in the total cells observed in the different mitotic phases. Artemisinin, a highly phytotoxic compound produced by *Artemisia annua* (annual wormwood), and broadly known as an anti-malarial drug, was also found by Dayan et al. (1999) to show abnormal metaphase and anaphase configurations.

Continuing with the study of natural compounds, Romagni et al. (2000) compared also the effects of 1,4- and 1,8-cineole (natural product from which the herbicide cinmethylin was derived) on two weedy plant species. They monitored germination, mitosis, root and shoot growth, chlorophyll content, and photosynthetic efficiency and found that both volatile cineoles severely inhibited root growth. However, mitotic index data showed that 1,8-cineole severely decreased ( $p < 0.001$ ) all stages of mitosis when compared with controls, while 1,4-cineole only decreased prophase ( $p < 0.05$ ). In a recent work (Oliva et al. 2002), aryltetralin plant lignans were tested for phytotoxicity, showing that all phases of mitosis were inhibited by nearly 50%, relative to the controls. Some of these lignans induced also abnormal star anaphase chromosomal configurations. The exact mechanisms of action of these effects are still unknown, but a primary effect seems to be the alteration of the formation of the spindle microtubular organization centers, resulting in the formation of multiple spindle poles and an asymmetrical convergence of the chromosomes.

A recent work (Burgos et al. 2004) focusing on the effects of 2(3H)-benzoxazolinone (BOA) on the growth and root ultrastructure of cucumber root tips was published. The data presented in this paper, which suggest that BOA affects root growth by inhibiting lateral growth and altering root ultrastructure are the only published until now with a more detailed description of BOA effects on root growth inhibition. Authors concluded that BOA ‘reduced root growth by disrupting lipid metabolism, protein synthesis, and transport or secretory capabilities’. In the same way, our previous studies about BOA (2-benzoxazolinone; Aldrich Chem. Co., 15,705-8) effects on cell cycle progression in lettuce root meristems using Petri-dish bioassays showed a stop on root growth and root hair formation in *Lactuca sativa* due to the addition of 1 mM BOA after 6 h exposition. Changes in root morphology (thicker and yellow root and absence of root hairs) were detected after 24 h BOA-exposition by light and electron microscopy. This suggested that BOA exerts an effect on the meristematic cells, altering its conformation and difficulting perhaps the cell division.

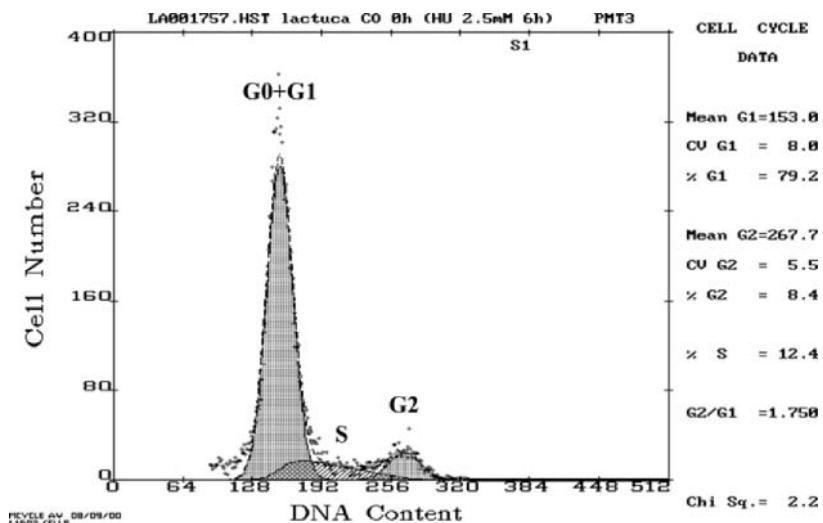


Figure 1. Cell cycle analyses by flow cytometry of meristematic cells in 20 h roots of lettuce.

With the goal of doing a detailed characterization of these and previous BOA effects, flow cytometry and mitotic index were performed in our group to measure cell cycle parameters in *Lactuca sativa* seedlings, developing a methodology for detecting the putative BOA-effects on root meristems.

Flow cytometry, consisting on the measurement of particles suspended within a high-velocity fluid stream (Haynes, 1988), allows to carry out different studies on cell cycle in connection with the effects of drugs and radiations, DNA amount and ploidy determination, different cellular parameters, the detection of a wide variety of antigens, and it even allows the physical sorting of particles like organelles and chromosomes. So, this technique is very useful in physiology, cytology and immunology (Tiersch, 1989; Steen, 1990; Jayat and Ratinaud, 1993).

Asynchronous cell populations from different tissues, meristems, and cell suspensions can be analyzed, so the percentage of cells in each cell cycle phase can be estimated (Figure 1).

In this simple case (Figure 1), three nuclear populations are shown: the first one ( $G_0+G_1$ ) corresponds to 2C nuclei ( $G_0$ ,  $G_{1Q}$ ,  $G_1$  undifferentiated cells, and 2C differentiated cells). The second peak ( $G_2$ ) corresponds to 4C nuclei ( $G_2$  cycling nuclei after DNA replication,  $G_{2Q}$  and differentiated 4C cells, not involved in the proliferating cell cycle); the mean fluorescence intensity of this 4C peak is approximately double than that of 2C peak. And, finally, the third population (S, between both peaks) constituted by S-phase nuclei in different stages of DNA replication (cycling S and non-cycling  $S_Q$  cells). This type of monoparametric analysis by flow cytometry allows simple and useful cell cycle analysis.

But if we are interested in testing the effect of putative cell cycle modulators, a previous synchronization is required. There are some commercially available inhibitors that block or stop the cell cycle in a specific phase, which is the case of aphidicolin and hydroxyurea. Aphidicolin causes a specific and reversible inhibition of the DNA polymerase  $\alpha$ , leading to a removable cell cycle block at the  $G_1/S$  boundary (Cuq et al. 1995 and references therein). Hydroxyurea (HU) reversibly inhibits the enzyme ribonucleotide reductase, and therefore the production of deoxyribonucleotides. Treatment with this inhibitor induces the accumulation of cells in  $G_1$  and early S phase (Doležel et al. 1999 and references therein). Starvation and physical methods have also been used for inducing partial cell cycle synchronization, principally in cell suspensions, but chemicals are more specific tool. Once the commercial inhibitor is added, cycling cells continue the cell cycle progression up to the cycle phase point where that inhibitor has a specific effect, and all the cells will arrest the cell cycle at that phase after an incubation time. After some time, inhibitor is removed from the medium by washing and whole cycling cell population re-start and goes on the cycle simultaneously, and this synchronous cell population progression can be acutely analyzed. In the same way, the specific effect of a putative cell cycle modulator under study can be finely analyzed. By adding the tested substance at different times after inhibitor removing, cell cycle phase-and subphase-specific effects can be detected. Lee et al. (1996) used hydroxyurea for root tip synchronization and subsequent metaphase chromosome isolation from maize.

In the present work, *Lactuca sativa* root meristems were synchronized using the inhibitor hydroxyurea (HU), blocking the cell cycle in  $G_1$  phase. *Lactuca sativa* cv. Great Lakes, California (Phyto) seeds were placed on moistened filter paper, into plastic trays and germinated at 27°C in dark for 20 h. After this time, 1-3 mm-root length seedlings were transferred to Petri dishes containing filter paper moistened with 5 mL of 2.5 mM Hydroxyurea (pH 6.0). Twenty seedlings were placed in each Petri dish and incubated for 6 h at 27°C in dark.

After removing HU by washing twice with distilled water (pH 6.0), a synchronous cell population of about 30% of total recorded nuclei was detected in progression through S and G<sub>2</sub> phases. Analyzing these data in detail, it seems clear that synchronization is favoring the accumulation of cells in a determined phase of the cell cycle making easier its analyses. Like so, immediately after HU inhibitor removal, 79% of detected nuclei is at G<sub>0</sub>+G<sub>1</sub> phase, 8.4% in G<sub>2</sub> phase, and 12.4% in S phase. The exposition to HU induced blockage and accumulation of nuclei in G<sub>1</sub> phase. Start and advance of synchronized nuclei in S phase (29.6% of detected nuclei) was observed 30 min after. One hour after HU release, synchronized nuclei population began to incorporate into G<sub>2</sub> phase. Percentage of synchronized nuclei diminished along time after inhibitor release. Finally, all synchronized nuclei were incorporated in G<sub>2</sub> phase of the cell cycle two hours after HU release. G<sub>2</sub> population, normally about 10% in non-synchronized meristems, reached amounts near 25% in this situation. After this step, mitosis started and the samples presented abundant metaphasic chromosomes. In this particular experimental system, a new synchronized G<sub>1</sub> phase was not observed.

With this synchronized cell population, which allows the detection of weak as well as strong effects of BOA on cell cycle progression, the present work continued after conducting hydroxyurea synchronization. Immediately after HU-washing, synchronized seedlings were transferred to other Petri dishes for starting the treatment. The fact that 1 mM BOA appeared to be the IC<sub>50</sub> in the lettuce seedling growth bioassays (Chiapuso et al. 1997) was the reason to select it for this experiment.

Therefore, seedlings were placed on filter papers moistened with 4 mL of either 1 mM BOA (treated seedlings) or distilled water (control seedlings). These seedlings were incubated at 27°C in dark. From this moment, samples of BOA-treated seedlings and corresponding controls were processed simultaneously for flow cytometry analysis. The 1 mm-apical tips of root meristems from forty BOA-treated or control seedlings were chopping in 700 µL of Galbraith nuclear buffer (45 mM MgCl<sub>2</sub>, 30 mM sodium citrate, 20 mM MOPS pH 7.0, 0.1% (w/v) Triton X-100), supplemented with 100% Tween 20 and 100% beta-mercaptoethanol. The obtained suspension was filtered twice through 30 µm-nylon filters, and 500 µL of filtered nuclei suspension were obtained into eppendorfs. Once obtained the nuclei suspension, 5 µL of 1% RNase solution and, immediately after, 30 µL of 10 mg/mL Evidium Bromide (EtBr), a nucleic acid-specific dye, were added for nuclei labeling. After an incubation of 30 min, labeled plant nuclei suspension was analyzed in a flow cytometer Coulter Elite with visible excitation source and the laser on 488 nm excitation wavelength. Cell cycle histograms were recorded for BOA-treated and control seedlings every 2 h, up to 12 or 14 h after BOA addition. At least 10,000 nuclei from each sample were analyzed in the flow cytometer. Histogram profiles were analyzed using the computer program

Multicycle (Phoenix Flow Systems, San Diego), and G0+G1, S and G2 populations were estimated comparatively in control and BOA-treated seedlings.

But, when mitosis takes place, nuclear envelope disappears, and the dispersed chromosomes (of different sizes and weak fluorescence intensities) cannot be detected or distinguished from debris in this experimental approach. Thereby, mitotic cells are lost and not detected by flow cytometry in these conditions, and this population (M) is not recorded in the histograms. In fact, for a correct evaluation of G2, M and G1 lengths, mitotic indices (percent of mitosis) must be evaluated complementarily to flow cytometry. This technique has been used for several studies and for different research teams (Quastler and Sherman, 1959; Wimber and Quastler, 1963; Lavania, 1996). Cuq et al. in 1995 used also this technique to confirm cytometric data in maize root meristems. Applying flow cytometry and mitotic index the knowing of the accurate stage of every cell cycle phase is possible.

Therefore, lettuce roots were fixed every 2 h (at the same times that for cell cycle analyses by flow cytometry) with a mixture (6:3:1) of acetic acid:chloroform:ethanol and iron traces for 24 h. Samples were stored for at least three days in fresh fixative at 20°C. Once finished this 3-days storage, samples were hydrolysed with 1N HCl at 60°C for 25 min. Staining started with the exposition to Schiff's reagent for 10 min, which stains chromosomes with a characterized pink color that can be distinguished by light microscopy. After Schiff's reagent, meristems were cut, covered with a drop of acetic carmin, and heated over a flame. The meristems were then squashed by pressure (for allowing the separation of nuclei) and scored with light microscopy. Mitotic index was so calculated by counting 1000 cells per slide in three replicates of each treatment.

## **RESULTS AND DISCUSSION**

Figure 2 shows the cell cycle progression in treated- (1 mM BOA) and control- (distilled water) cells in synchronized lettuce meristems.

In these graphics, a predominant effect of BOA on lettuce root meristems is detected before 10 h after allelochemical exposure. Cells progressed from G0+G1, (where HU blocked cell cycle impairing the G1/S transition) to S phase and from S phase to G2 in the first 4 h treatment. After these 4 h, the number of cells in G2 phase was significatively higher in treated meristems than in controls. In the next 6 h, control meristems normally continued the cell cycle, and more and more cells got going from G2 to G0+G1 phase starting a new division in an asynchronous way. But in BOA-treated meristems, in these same 6 h, a high number of cells in division seem to be blocked at G2 phase. The cell cycle went so slowly in these meristems that the number of cells in G2 phase was double of the control after 10 h BOA treatment.

Flow cytometry analysis showed an each time more significant arrest in the cell cycle progression delaying the entry of cells in mitosis by blocking the cell cycle in the G2/M transition. This effect in the cell cycle progression of treated-root meristems appeared very clear after 10 h BOA exposition (Figure 2).

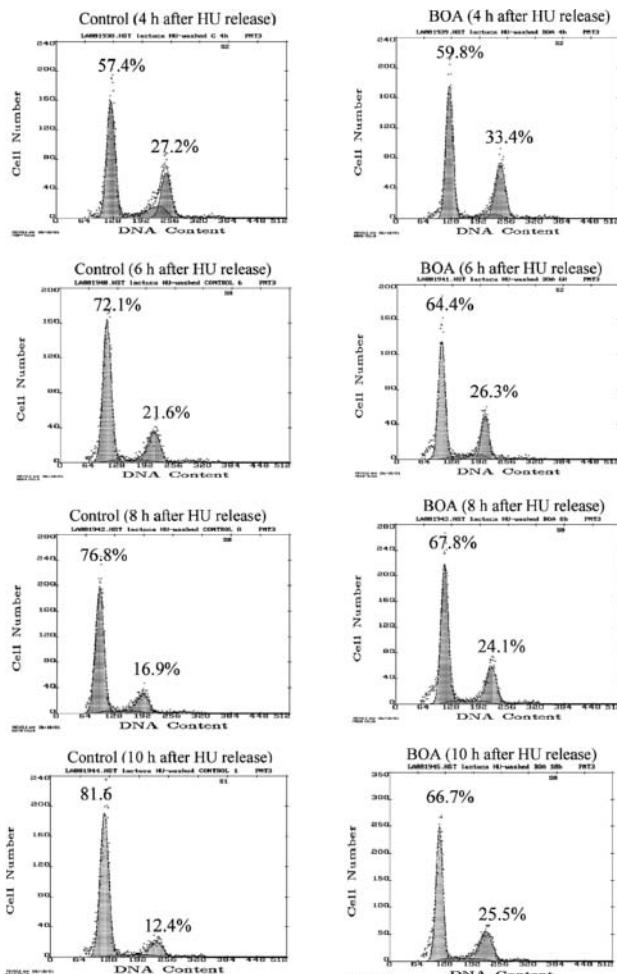


Figure 2. Comparative cell cycle analysis of 6 h HU-blocked root meristematic cells from control and 1 mM BOA-treated lettuce meristems.

These effects showed in the flow cytometry analysis were also clearly detected by the mitotic index technique, which appears to be an excellent complementary technique in cell cycle studies with flow cytometry. The mitotic index (Figure 3) confirmed the flow cytometry results and revealed that the cell cycle progression went slower in BOA-treated meristems than in control meristems.

But made known also that the maximum number of cells suffering cell division was fewer and appeared later in treated meristems (18% after 6 h BOA exposure time) than in control meristems (36% after 4 h distilled water exposure time).

With flow cytometry results we only detected an impasse in treated-meristems before mitosis, but with mitotic index we can see that, after 14 h treatment, cell division restarted in control as well as in BOA-treated cells. Control of cell cycle in response to BOA resulted in a modulation of cell division activity followed by an adaptive growth response.

So, we can conclude that a clear effect of BOA on lettuce cell cycle process can be considered as an important mechanism of action of this compound on the before detected inhibition of seedling growth in this plant species.

The previously obtained light microscopic images (Burgos et al. 2004; Sánchez-Moreiras et al., unpublished data) showed that meristematic cells were altered in presence of BOA, suggesting a possible effect on cell cycle process. This putative effect has been evidently demonstrated with these analyses.

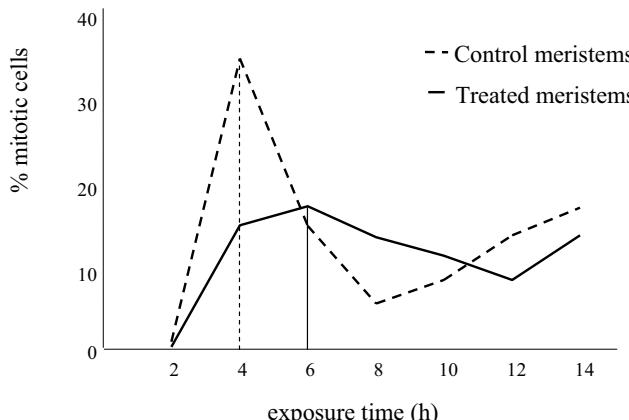
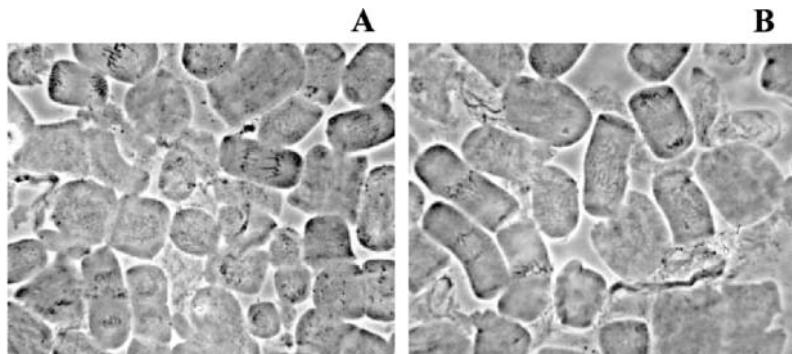


Figure 3. Mitotic index in root tip cells from lettuce meristems treated for 2-14 h with 1 mM BOA (treated meristems) or distilled water (control meristems) after 6 h HU synchronization.

Mitotic index data showed that 1 mM BOA severely decreased all stages of mitosis with a high number of cells in prophase when compared with control. The observation of stained chromosomes obtained from the mitotic index study revealed that no chromosomal aberrations appeared in the meristems treated with BOA. Metaphase and anaphase configurations, previously found to be affected by secondary compounds (Dayan et al. 1999), were totally normal, and also normal condensations were detected in prophase and metaphase (Figure 4). It seems like no direct effects are taking place in the mitotic machinery.

The control on cell cycle progression and cell number will be crucial for plant growth and morphogenesis, and any alteration on the precise times and order of these events will be critical on the plant development. Cell division control in plants can be related with the presence of regulators of the cell cycle (cyclin-dependent kinases, cyclins, CDK inhibitor genes, etc.) but can also show interactions with plant hormones and developmental regulators as well as with plant-specific processes such as cell wall metabolism (Menges et al. 2002).

A large number of plant genes show cell-cycle-dependent regulation of their expression, and are involved in cellular processes such as cell cycle control, cytoskeleton, transcription, signal transduction, hormone response, etc. (Menges et al. 2002). Cell cycle arrest by retarding the DNA replication and delaying also the start of mitosis has been associated with an inhibition of the activity of cyclin-dependent kinases, cell cycle gene expression, and a concomitant activation of stress genes (Reichheld et al. 1999). This variety of interactions at cell cycle level makes that a combined effect of delay and inhibition in cell division at G2/M transition could be related with multiple mechanisms of action.

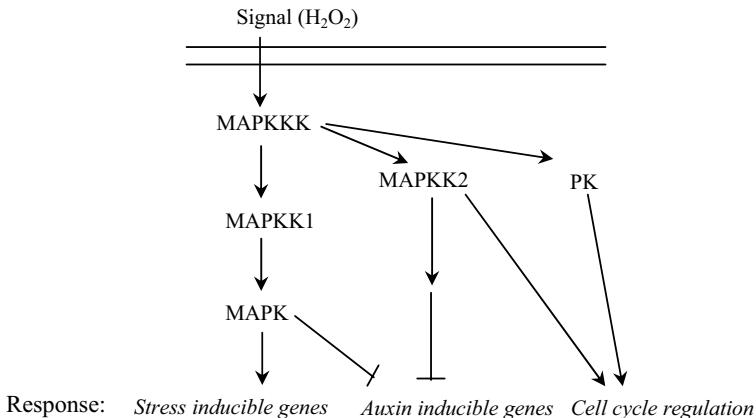


*Figure 4.* Mitotic cells from control (A) and BOA-treated (B) meristems of lettuce. Control meristems were 4 h in distilled water and treated meristems were 6 h in 1 mM BOA after HU synchronization. No aberrations were detected in these samples.

Some results defined an oxidative stress checkpoint pathway modulating the expression of cell cycle genes and also oxidative defense genes. This relation suggests that redox status could play an important role in the regulation of cell cycle progression under stress conditions. In this respect, Reichheld et al. (1999) suggested the existence of an oxidative stress checkpoint pathway that controls cell cycle progression in environmental stress conditions and is seemingly responsive to one or more redox-sensing systems.

In this way, the broadly-reported plant growth inhibition under stress conditions due to the stop on the shoot and root meristematic mitotic activity can be the consequence of the derivation of the metabolic energy from growth processes to defense reactions in the protective response. Has been demonstrated that the reactive oxygen species are the second messengers in this pathway, activating resistance genes which block cell cycle in the passage from G1 to S, retard the DNA replication, and also delay the start of mitosis in some plant cell cultures. So, these oxygen species (hydrogen peroxide, peroxide radical, etc.) have been found to play crucial roles acting on cell cycle to orchestrate the correct defense response in front of stress. Naderi et al. published recently (2003) the first report describing the relationship between the quiescence state and the antioxidative defense. Their findings indicated that there was a correlation between the cell cycle arrest by contact inhibition and resistance to oxidative stress-induced apoptosis. In 1990, Pérez suggested the interference of natural compounds, in particular BOA-related hydroxamic acids, with auxins at cell cycle level. In 1996, Anai et al. reported also the 4-Cl-6,7-dimethoxy-2-benzoxazolinone like a new auxin-inhibiting substance. But, how could BOA affect cell cycle events at this level?

It is well known that several natural products alter redox status with the concomitant ‘oxidative damage’ or even ‘oxidative stress’ by accumulation of free radicals, which can help to induce cell death or act as a signal in the activation of defense response. There are evidences that the mitogen-activated protein kinases (MAPKs), which mediate signal transduction of stress, cell cycle, and growth control in all eukaryotes, are involved in reactive oxygen species (ROS) production in most systems. With an increased accumulation of free radicals like ROS, some of them like  $H_2O_2$  can activate a MAPK pathway, involved in mitotic regulation, resulting in the induction of stress-inducible genes but also in the inhibition of auxin-inducible genes (Figure 5).



*Figure 5.* The connection between oxidative stress, auxin, and cell cycle regulation through a plant mitogen-activated protein kinase pathway has been previously described by Hirt in 2000. The oxidative stress signal results in a positive regulation ( $\downarrow$ ) of stress inducible genes and the called division genes and in a negative regulation ( $\perp$ ) of auxin-inducible genes, difficulting the plant development.

The multiple functions that auxins exert on plant metabolism are largely known, like cell elongation, phototropism and gravitropism, which results in the regulation of apical dominance, promotion of lateral root formation, delaying of leaf abscission, or promotion of vascular differentiation and fruit development.

In the cell elongation plays an important role the increase of cell wall extensibility reached by the auxin activity, by way of a biochemical event cascade that finishes in the cell wall weakness by acidification (acid growth hypothesis), and the production of new proteins implicated in the plant growth. In this way, auxin can play a 'key role in the control of the division and elongation of plant cells via altering the gene expression pattern' (Györgyey et al. 1997).

Even when different factors can regulate cell cycle, there are two essential checkpoints in the division: G1-S and G2-M. At G2-M transition cell assesses two important conditions before entering in mitosis, which are ‘has DNA duplication completed?’ and ‘is the cell large enough to undergo mitosis?’ (Nilsen and Orcutt, 1996). If someone of these questions can not be answered correctly, the cell will not divide. It is so, that an inhibition of auxinic function and the concomitant inhibition of cell elongation could cause the observed seedling growth inhibition. In conclusion, the delay and inhibition in cell division at G2/M transition found in this study after BOA exposition make sense into the scheme proposed by Hirt (2000) for explaining the connection between oxidative stress, auxin, and cell cycle regulation.

The knowledge of the existence of an altered redox status in presence of BOA (Sánchez-Moreiras and Reigosa, unpublished data) better support the hypothesis of auxin-interference, which was raised by Pérez in 1990, and supported later by Anai et al. in 1996 and Friebe et al. in 1997.

But the control by phytohormones of the plant meristem activity makes that auxins as well as gibberellins or cytokinins play an important role in cell division and the differentiation of the newly formed cells. Like so, cytokinins play also a decisive role in the G2/M transition of the cell cycle by activation of a Cdc-phosphatase that dephosphorylates and thereby activates a cyclin-dependent kinase (Frank and Schmülling, 1999). Therefore, different factors must be considered in the interpretation of the altered cell cycle progression observed in lettuce root meristems treated with 1 mM BOA. Even, some of them can be taking place simultaneously.

Concluding, after having detected previously a strong inhibition of root growth, a more detailed investigation of the putative effects on root cell division should be conducted by measuring effects on cell cycle rate and evolution (Duke et al. 2002).

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## CHAPTER 8

# DETOXIFICATION OF ALLELOCHEMICALS - THE CASE OF BENZOXAZOLIN-2(3H)-ONE (BOA)

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## INTRODUCTION

Why studying detoxification processes in allelopathy? This question seems to be important, as in the relevant literature possibly existing mechanisms of detoxification that allow plants to diminish or to escape from harmful effects caused by allelochemicals are seldom, if ever mentioned. An objection might be that allelopathic interactions themselves are not unequivocally proven under field conditions, detoxification events not all the more. Others may oppose that detoxification eliminate allelopathic interactions and one can question whether those interactions are still allelopathic ones or not. However, the idea of using allelochemicals as natural herbicides in sustainable agriculture that can replace or reduce the application of synthetic compounds must be regarded in a much more detailed way when detoxification can appear under certain circumstances and perhaps just with the most noxious weeds. It is well known that many synthetic herbicides underlay effective detoxification in numerous crops and weeds (Coleman et al. 1997; Schulz and Friebe, 1999). Why should allelochemicals be excluded from those mechanisms?

Detoxification may be an explanation for the well known phenomenon of species dependent sensitivity to allelochemicals (Einhellig, 1995). The higher sensitivity of

dicotyledonous species in comparison to monocots was often observed, but the molecular reasons were unclear. Different sensitivity of species were found, for instance, with juglone and benzoxazinoids, such as DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one), DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one), BOA (benzoxazolin-2(3H)-one), and MBOA (6-methoxybenzoxazolin-2(3H)-one) (Massey, 1925, Barnes et al. 1997; De Scisciolo et al. 1990;) (see figure of ecochemical interactions at the end of this paper for formula of all allelochemicals and detoxification products mentioned in the text). We assume a considerable participation of detoxification processes in the resistance of species against allelochemicals and the development of those pathways might be regarded as an aspect of co-evolution. A still theoretical consequence of sensitive and resistant species can be a shift in the composition of species in given plant communities when aggressive neophytes with a high allelopathic potential invade, or, on the other hand, support of the establishment of balanced plant communities where members are also physiologically adapted to each other. It is not a new idea that beside all other factors physiological, biochemical and genetic adaptation of plants to each other may play a role in plant communities (Boas, 1935).

Studying detoxification mechanisms under field conditions is at least difficult as no clear experimental concepts can be realized, e.g., several, perhaps unknown allelochemicals can be absorbed and may interact in the plant cell, involvement of microorganisms stays obscure, the plant material is not homogeneous and other imponderables have to be taken in account. Working with model systems has the advantage of working under defined conditions, e.g., controlled application of one allelochemical, use of identical developmental stages of the plant material, reduced or none contamination with soil particles etc. This allows an easier isolation of detoxification products and performance of biochemical studies. With model systems, the plasticity of plant detoxification capacities can be elucidated. Whether they will occur or what for reactions will be realized under natural conditions is another question, the potency, however, is fixed in the genomes of the species.

## BENZOXAZINOIDES

Benzoxazinones (DIBOA, DIMBOA) are secondary compounds of a number of Poaceae, Acanthaceae and Scrophulariaceae. DIBOA was also found in one Ranunculaceae, *Consolida orientalis* (Baumeier et al. 2000; Özden et al. 1992; Wolf et al. 1985; Kanchanapoo et al. 2001; Nagao et al. 1985; Hofman and Masojidkova, 1973; Schulz et al. 1994; Kumarasinghe and Wratten, 1998; Pratt et al. 1995; Alipieva et al. 2004). The reason for the scattered appearance of the compounds in plant kingdom is hitherto unknown. Benzoxazinones are stored in the vacuoles as glucosides until the cells are destroyed e.g., by herbivore attack. Then specific glucosidases are

released that hydrolyze the sugar moiety. The aglycones are also produced during plant rotting. Whereas the glucosides are not toxic, the aglycones are highly bioactive compounds. Recently, several articles have been published considering the biosynthesis and the broad bioactivity of benzoxazinones (Niemeyer 1995, Frey et al. 1997; Glawischnig et al. 1997; Gierl and Frey 2001; Sicker et al. 2000; Sicker and Schulz 2002; Spiteller et al. 2001).

The aglycones DIBOA and DIMBOA are only short living since they are not stable in aqueous solution but undergo spontaneously ring contraction resulting in benzoxazolinones and formic acid release (Smissman et al. 1972). The reaction products MBOA and BOA are still bioactive compounds but they are less toxic than the original molecules. Contrarily to those, benzoxazolinones are more stable. They are known to suppress certain weeds, such as barnyardgrass, crabgrass, or redroot pigweed. Phytotoxic properties of rye were investigated in several studies (Barnes et al. 1986; Barnes and Putnam, 1987; Yenish et al. 1995). It was calculated that three week old rye has the potential to release 14-16 kg/ha of DIBOA. The phytotoxicity of rye mulch, mainly due to the degradation product BOA, is reduced over time and it is abolished after about 3 months. Micro-organisms are thought to be responsible for the loss of the phytotoxic properties. *Acinetobacter* species have been described to degrade the compound yielding 2-aminophenol, which is oxidatively dimerized to phenoxazinones (Gerber and LeChevalier 1964). Those actinomycin analogues were also found as degradation products resulting from HBOA (2-hydroxy-2H-1, 4-benzoxazin-3(4H)-one, Blepharigenin). The biotransformation was performed by the endophytic fungus *Chaetosphaeria* sp. from *Aphelandra tetragona* (Zikmundova et al. 2002). Phenoxazinones may be adsorbed to soil particles or biodegraded.

In European grain field communities characteristic dicotyledonous weeds co-exist with rye or wheat. These weeds seem not to be sensitive to benzoxazinones or benzoxazolinones in concentrations that may appear under natural conditions, neither are numerous gramineous weeds. They may have developed strategies to compensate inhibitory effects due to BOA or MBOA uptake or to reduce them, at least. There are several possibilities how plants can develop a reduced sensitivity. The major ones might be: 1. Avoidance of uptake, 2. Detoxification after uptake, 3. Degradation in cooperation with microorganisms. Alterations in cellular targets such as defined enzymes or receptors due to mutations are believed to be less important as benzoxazinoids interact with proteins and nucleic acids not specifically. Thus, the development of detoxification pathways was assumed to be most likely in case the compounds are absorbed. For the purpose of studying possible detoxification mechanisms the use of model systems was most suitable. In a first approach, not weeds but crops were used to elucidate the mode of uptake and the appearance of possible detoxification products.

## DETOXIFICATION OF BOA IN WEEDS OF EUROPEAN PLANT COMMUNITIES, FURTHER WEEDS AND CROPS

*Avena sativa* and *Vicia faba* were hydroponically cultivated. Six day old oat seedlings and two week old beans were used for incubation studies with 100 and 500 µM BOA for 24 hours. Uptake kinetics were monitored over a period up to 72 h. The roots were harvested and extracted with 50% methanol. The methanolic extracts were analyzed by HPLC (Wieland et al. 1999).

Analysis of the extracts from BOA incubated oat roots (100 and 500 mM) revealed the appearance of three new compounds not existing in the controls. By means of MS, <sup>1</sup>H-NMR, and <sup>13</sup>C-NMR they were determined as BOA-6-OH (6-hydroxybenzoxazolin-2(3H)-one), BOA-6-O-glucoside (6-β-D-glucopyranosyl-O-benzoxazolin-2(3H)-one) and glucoside carbamate (1-(2-hydroxyphenylamino)-1-deoxy-β-D-glucoside 1,2-carbamate) (Wieland et al. 1998; Sicker et al. 2001). Whereas detoxification via BOA-6-OH and BOA-6-O-glucoside is catalyzed by constitutive enzymes, synthesis of glucoside carbamate is inducible. Accumulation of the carbamate can be observed 6-8 hours after start of incubation. The products BOA-6-OH and glucoside carbamate appear in the incubation medium after 24 to 48 h, thus they seem to be exuded, at least a part of them. Contrarily, broad bean var. Alfred was not able to synthesize glucoside carbamate, BOA-6-O-glucoside was found in the roots, when the incubation was performed with 100 µM BOA. Higher concentration led to a break down of the detoxification going along with the accumulation of free BOA and high amounts of BOA-6-OH. Furthermore, the root tips blackened after 48 h, indicating cell death. Interestingly, detoxification capacity seems to depend on varieties. We tested other cultivars, e.g., *Vicia faba* var. Dreifach Weiße, which were able to produce small amounts of glucoside carbamate after 24 hours of incubation (Sicker et al. 2003).

In maize, BOA-6-O-glucoside can be found in very low amounts in roots of the control plants. In roots of BOA-incubated plants BOA-6-O-glucoside presents only the major detoxification product during the first 6 hours. Later on glucoside carbamate becomes the dominant compound, whereas accumulation of BOA-6-O-glucoside does not further appear. After 14-16 h an additional product was found. The purified compound was identified as gentiobioside carbamate (1-(2-hydroxyphenylamino)-1-deoxy-β-gentiobioside 1,2-carbamate), which was hitherto not found in other species. Plant age and physiological fitness seem to influence detoxification processes. In 14-day old seedlings synthesis of BOA-6-O-glucoside is preferred, but in older ones detoxification in favor of glucoside carbamate was found. Supplementation of the incubation medium with salicylhydroxamic acid, an inhibitor of peroxidase activities, stimulated synthesis of glucoside carbamate. The detoxification products are obviously released into the environment via root exudation. They are detectable in the tap water,

where plants were transferred after BOA incubation. Appearance of the compounds in the tap water went along with a decrease of the BOA metabolites in the roots.

When methoxylated BOA, MBOA was applied, BOA-6-O-glucoside was the major detoxification product, thus demethylation is the first step of MBOA detoxification, followed by glucosylation of the resulting BOA-6-OH. BOA-6-O-glucoside is not the final product, but is further converted. MBOA-incubation led to a fourth detoxification product (Schulz unpublished).

Carbamates were not detectable in the extracts after 24 h. Detoxification of MBOA via demethylation and following glucosylation of resulting BOA-6-OH is also possible in other species, as far as tested (Table 1).

Table 1. Tested species for MBOA detoxification.

Family	Species	Major product of MBOA detoxification	Further products
Brassicaceae	<i>Arabidopsis thaliana</i>	BOA-6-O-glucoside	Not detectable
	<i>Brassica oleracea</i>	BOA-6-O-glucoside	n.d.
Asteraceae	<i>Galinsoga ciliata</i>	BOA-6-O-glucoside	n.d.
	<i>Lactuca sativa</i>	BOA-6-O-glucoside	n.d.
Poaceae	<i>Zea mays</i>	BOA-6-O-glucoside	Yes
	<i>Hordeum vulgare</i>	BOA-6-O-glucoside	Yes
	<i>Avena sativa</i>	BOA-6-O-glucoside	Yes
Caryophyllaceae	<i>Agrostemma githago</i>	BOA-6-O-glucoside	n.d.

We tested species belonging to the former Secalietea, Chenopodietea, Artemisietae vulgaris, Agrostietea stoloniferae and Amarantho-Chenopodion communities for their detoxification capacities (Table 2). All the species were able to absorb benzoxazolinone and to detoxify the compound via BOA-6-OH resulting in BOA-6-O-glucoside. Glucose carbamate production was possible in the most species as well, but with the exceptions *Plantago major* (Agrostietea stolonifera) only small amounts in comparison to BOA-6-O-glucoside were accumulated (Schulz and Wieland, 1999). Differences in the velocity of BOA-6-O-glucoside and especially in the glucose carbamate accumulation were observed. Dicotyledonous species of the former Secalietea communities exhibit a higher detoxification capacity than those of the former Chenopodietea communities, because they start detoxification earlier and accumulation of the detoxification products appears faster. *Consolida orientalis* is able

to perform N-glucosylation, but surprisingly, the major detoxification product appearing after 24 h of incubation is BOA-6-O-glucoside and not glucoside carbamate. A few other species belonging to the Amarantho-Chenopodion communities were tested, but except for *Digitaria sanguinalis* their detoxification capacity was moderate to low. In contrast to species of Secalietea communities, they are not under the same allelopathic pressure as the character species *Consolida orientalis* produces only a low biomass due to the scattered distribution. A possible release of benzoxazinones and resulting benzoxazolinones is therefore low in amount. Thus, allelopathic potential of *C. orientalis* is regarded to be inconsiderable (Weidenhamer et al. 1989).

Poaceae, cereals and wild ones, detoxify mainly by glucoside carbamate production. According to bioassays with cress glucoside carbamate is not toxic up to concentration of 1 mM whereas BOA-6-O-glucoside has still some inhibitory influence on cress radicle growth. BOA-6-OH has a much more higher toxicity than BOA. Thus, detoxification by glucoside carbamate production is more effective than synthesis of BOA-6-O-glucoside.

According to our studies, all species tested possess glucosyltransferases able to accept BOA-6-OH as a substrate for glucosylation. The enzyme activity was checked in some of the species. It was found to be constitutive in the roots of *Arabidopsis thaliana*, *Brassica oleracea*, *Raphanus sativus*, *Agrostemma githago*, *Zea mays*, *Hordeum vulgare*, and *Avena sativa*. In some of the species, the enzyme activity seems to be up regulated when the plants were previously incubated with BOA. *Galinsoga ciliata* has no constitutive glucosyltransferase for BOA-6-OH glucosylation, but an BOA and MBOA inducible one. The presence of the transferase activities, constitutive with high activity, constitutive with low but up regulated activity after BOA incubation and BOA inducible activity is in agreement with the detoxification capacities of the different plant groups found in defined vegetation classes (Schulz unpublished).

Thus effective detoxification and the capacity of detoxification depend not only from the ability to produce glucoside carbamate but also from the fast disposition of suitable transferases for BOA-6-OH glucosylation, at least during the early steps of detoxification. Especially in case of MBOA attacks these glucosyltransferases are highly important as with BOA-6-OH, carbamate production is obviously not possible within 24 h.

It is an interesting question which glucosyltransferases are recruited for BOA-6-OH glucosylation in species that do not contain benzoxazinones and benzoxazolinones naturally, how up regulations and induction of the enzymes are controlled and which differences exist among them on the genetic and biochemical level.

Table 2. Appearance of BOA-6-O-glucoside, glucoside carbamate and further products in the roots after 24 h of BOA-incubation performed with seedlings or fully developed plants (fdp). □: Amarantho-Chenopodiion communities; \* Secalitea communities; • Artemisietea vulgaris communities; ★ neophyte; ○ Chenopodietea communities; ♣ Agrostiectea stoloniferae communities; † extinguished in western parts of Germany; ① endangered species.

Family	Species	BOA-6-O-glucoside	Carbamate	Further products
Amaranthaceae	<i>Amaranthus albus</i> □	Major product	-	Not identified
Apiaceae	<i>Coriandrum sativum</i> *	Major product	Minor product	n.a.
	<i>Daucus carota</i> •	Major product	Minor product	n.a.
Asteraceae	<i>Centaurea cyanus</i> *	Major product	Minor product	n.i.
	<i>Galinsoga ciliata</i> *	Major product	-	-
	<i>Matricaria chamomilla</i> *	Major product	Minor product	n.a.
	<i>Helianthus annuus</i>	Major product	-	-
	<i>Carduus nutans</i> •	Major product	Minor product	n.a.
	<i>Lactuca sativa</i>	Major product	Traces	n.a.
Brassicaceae	<i>Arabidopsis thaliana</i> ○	Major product	Traces	-
	<i>Brassica oleracea</i>	Major product	-	-
	<i>Raphanus sativus</i>	Major product	Minor product (depends on cultivar)	n.a.
	<i>Capsella bursa-pastoris</i> ○	Major product	Traces	n.a.
	<i>Diplotaxis tenuifolia</i> □	Minor product	-	Major product: BOA-6-OH
Caryophyllaceae	<i>Agrostemma githago</i> *†	Major product	Minor product	n.a.
Campanulaceae	<i>Legousia speculum-veneris</i> *○	Major product	Traces	-
Chenopodiaceae	<i>Chenopodium album</i> ○	Major product	Traces	-
Fabaceae	<i>Vicia faba</i>	Major product	Traces (depends on cultivar)	-
Papaveraceae	<i>Papaver rhoeas</i> *	Major product	Minor product	-

Family	Species	BOA-6-O-glucoside	Carbamate	Further products
Plantaginaceae	<i>Plantago major</i> <sup>◊</sup>	Minor product	Major product	n.a.
Portulacaceae	<i>Portulaca oleracea</i> <sup>□</sup>	Major product	traces	Further product
Poaceae	<i>Avena sativa</i>	Major product	Major product	n.a.
	<i>Avena fatua</i>	Major product	Major product	n.a.
	<i>Hordeum vulgare</i>	Major product	Major product	n.a.
	<i>Lolium perenne</i>	Minor product	Major product	n.a.
	<i>Digitaria sanguinalis</i> <sup>□</sup>	traces	Major product	n.a.
	<i>Zea mays</i>	traces	Major product	Gentiobioside Carbamate
	<i>Triticum aestivum</i>	Minor product	Major product	n.a.
Polygonaceae	<i>Polygonum aviculare</i> <sup>○</sup>	Major product	-	-
Ranunculaceae	<i>Consolida orientalis</i> <sup>□</sup>	Major product	Minor product	n.a.
	<i>Consolida regalis</i> <sup>*◎</sup>	Major product	Minor product	-
Urticaceae	<i>Urtica urens</i> <sup>○</sup>	Major product	-	-

It cannot be excluded that the benzoxazinone containing species have no special glucosyltransferase as well and the original function of the enzyme involved in BOA detoxification is the glucosylation of other compounds. Another question is directed to the fine tuning between the glucoside carbamate production (if possible) and BOA-6-OH glucosylation, how shifts in the detoxification pathways are regulated. Moreover, it is clear that BOA-6-O-glucoside presents not the final detoxification product in several species, but it is further modified. At present the chemical identification of some of the (partly unstable) products is not yet completed, but it is clear that additional enzymes take part in the detoxification pathway via BOA-6-OH.

## FURTHER FATE OF DETOXIFICATION PRODUCTS

There is some evidence that in several species at least a part of major products accumulate only temporarily and then undergo additional modifications. At present it

is not clear whether the modified products are finally converted in non-extractable components or whether they are exuded as it was found for low amounts of BOA-6-OH and considerable amounts of glucoside and gentiobioside carbamates from maize roots. However, what happens to the exuded compounds? Are they bound to soil particles, absorbed again by other species or converted/degraded by micro-organisms? As already mentioned a number of bacteria is able to cleave the heterocycle of BOA resulting in 2-aminophenol and phenoxazinones. However, a strain of *Acinetobacter* was not able to cleave BOA-6-OH (Burdziak et al. 2001)

In a first approach to elucidate the further fate of the compounds we checked a number of soil fungi belonging to the mycobiota of the cereal rhizosphere, among others *Doratomyces stemonitis*, a saprotroph fungus which can be isolated also from the soil and rotting plant material. Cultures of the fungus were incubated, for instance with BOA, BOA-6-OH and glucoside carbamate (Voloshchuk et al. submitted). After a lag phase neither BOA nor highly toxic BOA-6-OH in concentrations up to 100 µM inhibited the growth of the fungus further on. The longest lag phase was stated with glucoside carbamate, which was not expected. BOA-6-OH was consumed without considerable accumulation of further intermediates. Interestingly, BOA was consumed via BOA-6-OH production which was first deposited in the medium. To our surprise glucoside carbamate was not metabolized directly as well. Instead, BOA was regenerated and could be isolated from the medium in high amounts. Obviously the fungus has the possibility to excise the glucose moiety and starts BOA metabolism at the moment where no carbamate is available any more. Of course, the idea is hypothetical, but there may be the possibility, that BOA concentrations can be increased in the rhizosphere due to the activity of fungi that regenerate the original phytotoxic compound from the non toxic metabolite glucoside carbamate. Another speculation is that BOA acts somehow as “sugar pump” for a fungus living within the rhizosphere, when regenerated BOA is absorbed again by a higher plant root able to synthesize glucoside carbamate and to exude it again.

Although we are just at the beginning to get some insights into a fascinating, but still hypothetical journey of a molecule through organisms and ecosystems it is clear, that BOA detoxification goes along with the involvement of numerous, partly induced enzymes and transport mechanisms, use of primary metabolites like sugars, and energy input, until the compounds can be finally consumed by micro-organisms. The following figure may illustrate the network of ecochemical interactions between plants, bacteria and fungi participating in BOA detoxification, conversion and consumption:

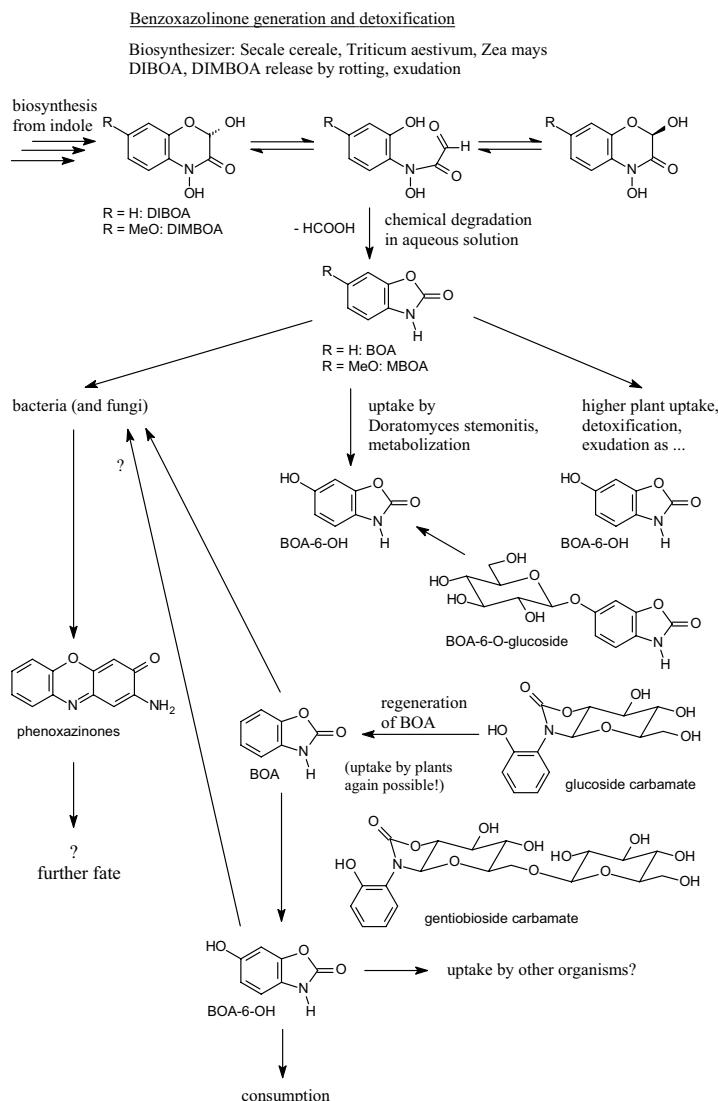


Figure 1. Ecochemical network of BOA detoxification, conversion and consumption.

## SYNTHESIS OF DETOXIFICATION PRODUCTS

Attempts to synthesize detoxification products have been undertaken which yielded not only three main detoxification products but also a chemical surprise as will be discussed below.

First, BOA-6-OH has been synthesized by ether cleavage of MBOA with boron triiodide. Then, BOA-6-OH has been enzymatically glycosylated with UDP-glucose and a protein extract from BOA incubated oat roots to form BOA-6-O-glucoside identical in its properties with the natural detoxification product (Wieland et al. 1999), (Scheme 1).

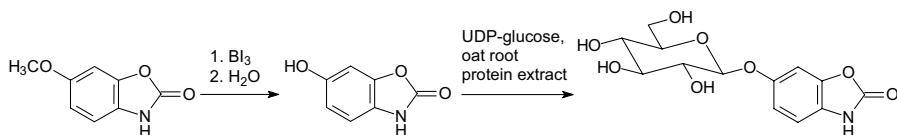


Figure 2. Chemical synthesis of BOA-6-O-glucoside.

Only, during the synthesis of glucoside carbamate (1-(2-hydroxyphenylamino)-1-deoxy- $\beta$ -D-glucoside 1,2-carbamate) it became obvious that our first structural assignation of this detoxification product as BOA-N-glucoside (3- $\beta$ -D-glucopyranosyl-benzoxazolin-2(3H)-one) (Wieland et al. 1998) was not true. Surprisingly, our synthetic attempts directed on BOA-N-glucoside delivered a mixture of compounds rich in glucoside carbamate which contained traces of BOA-N-glucoside, only. In fact, glucoside carbamate is a regioisomer of BOA-N-glucoside. It turned out, that this carbamate arises from the chemical rearrangement as soon as BOA-N-glucoside is available in solution (Scheme 2).

The rearrangement is driven by the high nucleophilicity of the 2-OH-group of the glucose moiety in BOA-N-glucoside, which opens the oxazoline ring of BOA to form another one attached to the sugar unit. Nevertheless, the statement is reasonable, that BOA-N-glucoside is the initial enzymatic plant detoxification product. This makes it belong to the rare plant N-glycosylations products. To find out what enzyme(s) are able to accomplish this transformation of BOA is another interesting goal for the plant biochemist.

Our chemical synthesis led to the acetyl protected form of BOA-N-glucoside. However, even during mild deprotection procedures a solution of mainly glucoside carbamate was obtained, which was obviously formed by the same isomerization of the original BOA-N-glucoside as in the natural product's case. Finally, the elucidation

of this complex matter was only possible by careful NMR analysis of the synthetic mixture mentioned above containing traces of BOA-N-glucoside together with main glucoside carbamate. The  $^1\text{H}$ -NMR spectra of both regioisomers are very similar but could be distinguished by a spectroscopic peculiarity (Sicker et al. 2001).

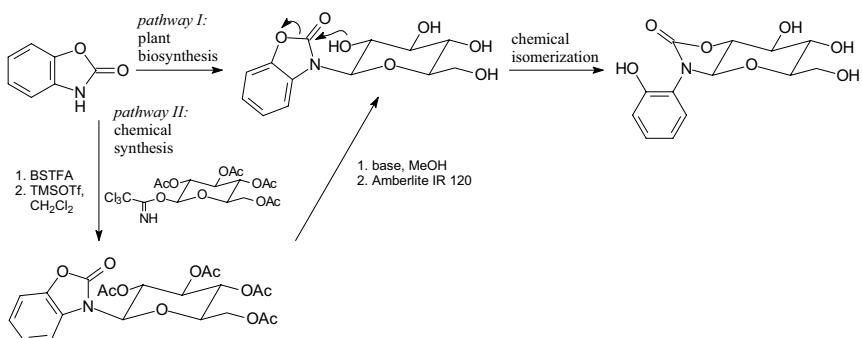


Figure 3. Plant biosynthesis and chemical synthesis of glucoside carbamate.

Eventually, this chapter of interdisciplinary research on the rise and fall of allelochemicals offers an interesting challenge for the organic chemist, too, that is to establish a synthetic procedure leading to BOA-N-glucoside under conditions that prevent its isomerization.

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## CHAPTER 9

### ALLELOPATHY AND ABIOTIC STRESS

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### INTRODUCTION

We intuitively say that *a plant is stressed* when there is a reduction in some physiological rate (water or nutrient absorption, photosynthesis, respiration, growth, development, reproduction or others) below the maximum possible rate expressed under optimal conditions (Salisbury and Ross, 1992; Lambers et al. 1998), thus not attaining its genotypic potential. There are many *environmental stress factors* that can limit growth and development; Levitt (1980) proposed to classify them in biotic and abiotic factors (Table 1), causing biotic or abiotic stress in plants, respectively.

Plant species are *constantly subjected to adverse environmental conditions* such as drought, flooding, extreme temperatures, excessive salts, heavy metals, high-intensity irradiation, allelochemicals, or infection by pathogenic agents, among others. For that reason, plants in their ecological niches usually live far from their physiological optima (at least in some part of the life cycle) (Vrba and Gould, 1986; Osmond et al. 1987).

In this chapter, we review synthetically the state of the art in stress physiology and stress molecular biology as multifaceted branches of Plant Ecophysiology. Then, we focus on allelopathy as stress factor, and bring out many examples of interaction between allelopathy and abiotic stresses. Allelopathic phenomena and environmental stresses are shown as interacting engines of evolution and actual sources of biodiversity, far from representing just a threat for plant fitness.

Table 1. Natural (biotic and abiotic) and man-related factors that can produce stress in terrestrial plants, besides the position of Allelopathy in such context. According to Larcher (1995), Lichtenhaller (1998) and Reigosa et al. (1999, 2002).

## STRESS FACTORS

<b>Environmental factors</b>		<b>Man-related factors</b>
<b>Abiotic factors</b>	<b>Biotic factors</b>	
<ul style="list-style-type: none"> <li>• TEMPERATURE:           <ul style="list-style-type: none"> <li>– Low temperature (cold, freezing – long or short duration)</li> <li>– High temperature</li> </ul> </li> <li>• WATER:           <ul style="list-style-type: none"> <li>– Water deficit (drought, low water potential)</li> <li>– Flooding (long rainy periods, waterlogging, anoxia)</li> </ul> </li> <li>• RADIATION (excess or deficiency):           <ul style="list-style-type: none"> <li>– Infrared</li> <li>– Visible (photoinhibition, photooxidation)</li> <li>– UV-A, UV-B</li> <li>– Ionizing</li> </ul> </li> <li>• CHEMICALS:           <ul style="list-style-type: none"> <li>– Ions</li> <li>– Salt</li> <li>– Mineral deficiencies /excesses</li> <li>– Inadequate pH</li> <li>– Ozone, oxygen excess</li> </ul> </li> <li>• OTHERS:           <ul style="list-style-type: none"> <li>– Wounding, wind, pressure, sound, magnetic fields, electric fields, etc.</li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>• PATHOGENS:           <ul style="list-style-type: none"> <li>– Virus</li> <li>– Fungi</li> <li>– Bacteria</li> </ul> </li> <li>• ANIMALS:           <ul style="list-style-type: none"> <li>– Phytophagia</li> <li>– Footing and grazing</li> <li>– Effects of insects</li> </ul> </li> <li>• OTHER plants:           <ul style="list-style-type: none"> <li>– Parasitism</li> <li>– <b>ALLELOPATHY</b></li> <li>– Competition</li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>– Herbicides, fungicides, pesticides</li> <li>– Pollution</li> <li>– O<sub>3</sub> and photochemical smog</li> <li>– ROS formation</li> <li>– Photooxidants</li> <li>– Acid rain and mist</li> <li>– Acid water and soils</li> <li>– Mineral deficiencies caused by lixiviates due to soil loss or acid rain</li> <li>– Heavy metals contamination</li> <li>– Nitrogen excess</li> <li>– Eutrophication</li> <li>– Increase of UV radiation</li> <li>– Global climatic change, increase in CO<sub>2</sub> concentration</li> <li>– Increase of saline and dry soils</li> <li>– Noise</li> <li>– Fires</li> <li>– Compacted soils</li> </ul>

### The ‘stress’ concept

The current most accepted and general plant stress concept (Larcher, 1995; Lichtenhaller, 1998) was developed with two main objectives: (i) to distinguish between plant homeostatic changes in response to daily small environmental fluctuations, and the real responses to stress, as well *plastic* (permanent in plant life span) or *adaptative* (heritable); and (ii) establishing a clear distinction between the *stress factor* (the external signal) and the *response to stress* (the effects and restrictions induced by the stress conditions to the plant).

Plant stress has been defined as ‘*a state in which increasing external demands lead to the destabilization of plant functions, followed by a phase of normalization and improving of the resistance. If the plant is forced out of its tolerance limits and its acclimation capacity is over passed, the result can be a permanent damage or even plant death*’ (Levitt, 1980). Lichtenhaler (1996) included the possibility of regeneration, which can happen once the stressing factor is removed.

Stress is, according to the previous explanations, a dynamic process. Figure 1 shows the effects of stress in the long term. The first phase, previous to the beginning of the stressing factor, shows a variable environment (producing, for example, oscillations in photosynthesis) that is not considered as stressing. When stress begins, there is a more or less fast and deep response, which may stay for minutes, days or even weeks. After that, if the stress factors continue acting, acclimation begins. *Acclimation* is a medium term process that allows the plants to endure and improve their performance under stress conditions. It has a genetic basis (meaning that genotype includes several possible alternatives to perform physiological processes and some of them only are functional when stress has acted), but it is environmentally mediated. Of course, if stress factors continue acting along generations, *adaptation* takes place, meaning that there is a change in the genetic composition of the adapted populations.

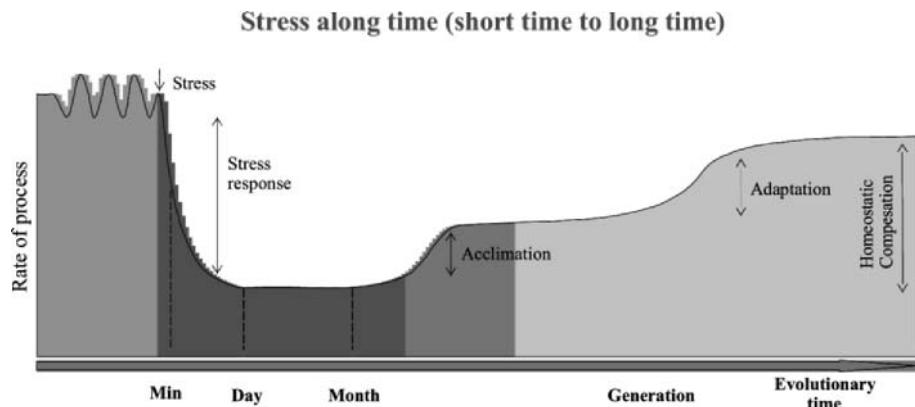


Figure 1. Phases of response to a partially recoverable stressing condition in the long term. After Lambers et al. (1998).

Short- to medium-term *plant stress response* comprises: (1) an *alarm phase*, characterized by a physiological change, with increase of catabolism and diminution of anabolism, and structural destabilization; (2) a *resistance or acclimation phase*: when acute damage does not cause death, plants can endure and acclimate, fixing the

problems created by the stress; (3) a *exhaustion phase*: if the stressing factor continues (long duration stress), senescence processes begin leading to chronic cellular damage and plant death. Finally, if the damage is recoverable and stressing agent finishes its action, a fourth phase can be found, with total or partial regeneration. Sometimes the final physiological functions after this phase can be improved if compared to the non-stressed plant.

Anyhow, *stress effects are dose-dependent* (Lichtenthaler, 1996) so it is depending on the intensity and the time in which the stressing agent acts, but it also depends on the species, variety and individual and previous life conditions (*plants can endure*). Lichtenthaler (1996) even distinguishes *eu-stress* from *dis-stress*, being the first a stimulant and activator stress, this is, a positive element to development, whereas the second is a severe stress that affects plant negatively and causes a deep damage.

### Stress alters patterns of gene expression

Because of their immobility, plants have to make necessary metabolic and structural adjustments to cope with the stress conditions. Stress-induced changes in plant metabolism and development can often be attributed to altered patterns of gene expression. In response to stress, some genes are expressed more intensively, whereas others are repressed. The protein products of stress-induced genes often accumulate in response to unfavourable conditions. The functions of these proteins, named *stress proteins* or *stress-related transcription factors*, and the mechanisms that regulate their expression are currently a central topic of research in stress physiology (see Bray et al. 2000 as a review).

A large number of the stress-responsive genes have been identified and isolated by the differential screening of cDNA libraries and protein identification of electrophotograms (see Ingram and Bartels, 1996; Grover, 2000, for water-stress induced genes and products). The precise identity of the isolated genes has been looked into through search for homology of the corresponding nucleotides/amino acids. From these analyses, most genes have appeared to be novel as no corresponding sequences are found in the databases. Clearly, more efforts are needed to find out the functional role of the genes which are up regulated in response to stress (Grover, 2000). Furthermore, the intricate signalling pathways that are assumed to participate in alterations of plant gene expression in response to stress are also yet to be elucidated.

Under an ecophysiological point of view, most of the stress transcription factors have a role either in (1) helping the plants survive, or (2) minimising the effectiveness of the stress agent (Ho and Sachs, 1989):

- 1) In *helping plants survive under stress conditions*, the stress proteins perform the following functions: (i) maintenance of the basic metabolism in the stressed cell, (ii) protection of cellular components from being damaged by the stressful condition, and (iii) removal of damaged cellular components. Although the stressed cells are usually not metabolically active, the induction of stress proteins can keep the cells from being killed and the cells can recover once the stress condition is relieved.
- 2) In *minimising the effectiveness of the stress agents*, the stress transcription factors take up a more active set of functions: (i) the physical blockage of entry of stress agents; (ii) the sequestration of stressful agents, and (iii) the impairment of the biological stress agents (see Chapter 10).

Under a more strictly physiological perspective, Grover (2000) and Hasegawa et al. (2000), focussing mainly on water stress (Bray, 1997; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997), classify water stress-related genes regarding their physiological roles in stress tolerance:

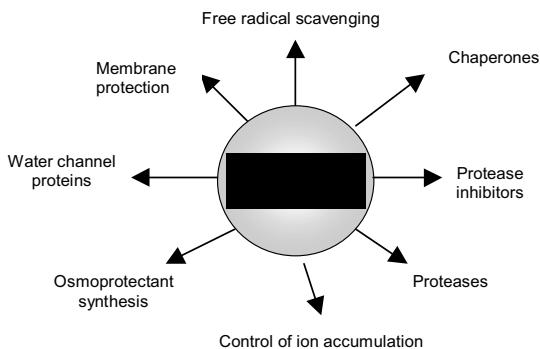
- 1) *Stress adaptation effectors or metabolic proteins*, such as water channel proteins and transmembrane ion-transport proteins; enzymes required for the biosynthesis of various osmoprotectants; proteins which may protect macromolecules and membranes, including LEA (late embryogenesis abundant) proteins, HSPs (heat shock proteins), osmotin, chaperones, and proteases for protein turnover; and the detoxification enzymes such as glutathione S-transferases, catalases, superoxide dismutases, and ascorbate peroxidases.
- 2) *Regulatory molecules* or gene products with regulatory functions in the *stress signal transduction*, including various protein kinases and transcription factors, which regulate the stress adaptation effectors enumerated above.

## GAS AND CO-STRESS

Many different stressing factors produce, along with some specific effects, some similar physiological and molecular responses in the plants (Figure 2). Current knowledge about stress-related transcription factors firmly supports the actual theories of stress in plants: *GAS* and *co-stress* (Larcher, 1995; Lichthenthaler, 1996; Prasad, 1997). As Leshem et al. (1998) summarised, ‘converging data indicate the possible existence of a *general adaptation syndrome* (GAS) in which different types of stress induce identical coping mechanisms. Consequently, this implies a *co-stress* response whereby one type of stress resistance may impart co-resistance to others.’

There are several responses that can be considered common in response to stress in plants: Antioxidant effects to prevent the damage induced by ROS (Reactive Oxygen Species) by means of polyamines synthesis or molecular scavengers;

osmoregulation, ABA increase, molybdenum cofactor, jasmonate synthesis, synthesis of HSPs, phenols and flavonoids synthesis, and often ethylene, increase their concentrations when plant is submitted to stress. They all have been shown to be significantly involved in stress coping mechanisms when plants are challenged by the following environmental stress factors: high light intensity, ozone and SO<sub>2</sub>, heat, hypoxia and anoxia, flooding, chilling, freezing, drought and/or salinity, herbicide exposure, heavy metal toxicity, deficiency of essential elements, wounding, and pathogen infection (Lessem and Kuiper, 1996; Prasad, 1997; Heiser and Elstner, 1998; Tomashow, 1999; Bray et al. 2000; Grover, 2000; Jin et al. 2000; Chen et al. 2002).



*Figure 2.* Some stress-coping mechanisms induced by water stress (After Bray, 1993). Stress proteins involved in such mechanisms have been reported to play a co-stress adaptative role in plants subjected to drought, salinity, chilling, osmotic stress, or other stresses involving cellular dehydration. A notable number of these stress-induced transcription factors known to participate in cell rescue and defence, and potentially associated with chemical detoxification pathways, have been also recently reported to be overexpressed in response to allelochemicals (Bais et al. 2003; Sánchez-Moreiras, 2004; Baerson et al. 2005).

*Osmoregulation* through the synthesis of low molecular weight organic compounds (betaines, polyols, sugars, proline, putrescine) in the cytoplasm is one major manifestation of GAS and co-stress. Besides their osmoregulatory function, compatible solutes have osmoprotective roles such as protein and membrane stabilisation, and ROS scavenging. Many authors have reported the overexpression of several genes that codify enzymes of the biosynthetic routes of different osmolytes, besides other proteins involved in transport mechanisms that occur during osmotic adjustment (see Bray et al. 2000).

Another common factor of co-stress manifestations is the *prevention of oxidative damage*. The oxidative stress results from deleterious effects of reduced oxygen

species such as superoxide and hydrogen peroxide. Hydroxyl radicals cause lipid peroxidation, protein denaturation, DNA mutation, photosynthesis inhibition, etc. The central role in the plant antioxidative mechanism is played by superoxide dismutase, catalase and various peroxidases (Allen, 1995). ROS scavenging is associated with every type of plant stress mentioned here (including allelochemicals and herbicide application), apparently without exception, and this indicates a clear-cut general adaptation function of this mode of stress coping (Sen Raychaudhuri and Wang Deng, 2000).

Also, under the point of view of co-stress, most if not all environmental stress-coping factors involve HSPs. The mode of HSP-mediated stress coping is pleiotropic, and involves mRNA protection, prevention of enzyme—especially photosynthesising—denaturation and/or their stress-induced aggregation and post-stress ubiquitin and chaperonin-aided repair (Heldt, 1997; Leshem et al. 1998).

It appears that an *inherent multiple stress resistance mechanism* is developmentally advantageous, and may be pleiotropically encoded (i.e. controlled by few genes) by evolutionary selection (Leshem et al. 1998). Possibly, during evolution direct responses of plants to each environmental change were gradually replaced by *environmental signal perception/transduction pathways*, which enable plants to cope with stress in a ‘cheaper’ and more efficient way, thus increasing the plant fitness (Kuiper, 1998).

## Stress signalling

The origin and development of signal perception/transduction pathways in green plants is a basic question to understand the functioning of plants in nature, where environmental conditions are continually changing (Kuiper, 1998). It has been proved the existence of either positive or negative interactions among different stress factors in which concerns to gene expression (Xiong et al. 1999; Chen et al. 2002). Signalling is nowadays matter of active research in many plant biology laboratories.

Lichtenthaler (1998) summarises stress signalling as follows (see Figure 3): ‘All biotic and abiotic stressors, natural and anthropogenic, represent external signals. There are many different forms of signal perception and transduction in the plant and its organs (leaves, root, stem, flowers), which will lead (i) to *direct metabolic responses*, and (ii) to the *activation of gene expression*: enzyme formation, synthesis of stress proteins, stress metabolites, stress hormones, etc. The latter further modify the plants’ metabolic responses under stress; i.e., there are fluent transients and feedback controls between gene expression on the one hand and metabolic responses on the other hand’.

Little is known about *how plants recognise stresses* at the cellular level. The best clues come from yeast and bacterial proteins that initiate signal transduction in response to abiotic stresses such as low osmotic potential. However, the study of protein localisation and function in plants cannot rely solely on the results obtained with yeast DNA complementation, because 'plant cells are not just green yeast' (Bassham and Raikhel, 2000). Plants probably contain similar proteins, but functions have not yet been demonstrated (Bray et al. 2000). There is already evidence of signalling cascades in plants that are not known to exist in the unicellular eukaryote (Hasegawa et al. 2000; Knight and Knight, 2001).

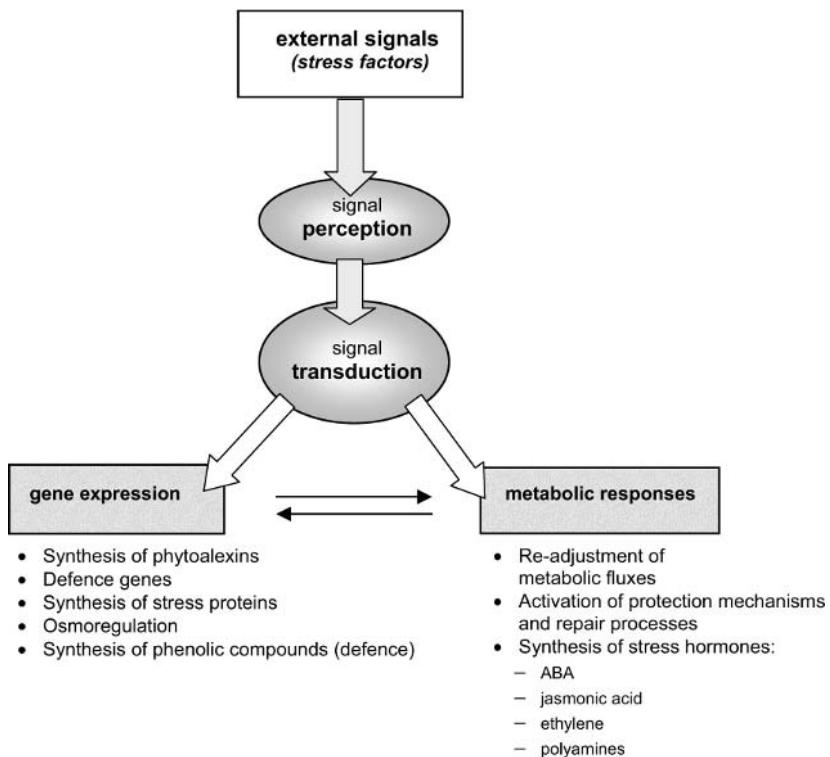


Figure 3. Scheme of the stress signal perception and transduction leading to metabolic responses and gene expression (usually mediated by ABA) as well as stress-induced plant responses. Drawn after Lichtenhaller (1998).

Despite the evidences of 'co-stress' at the level of gene expression, specially for those agents causing cellular dehydration, other recent experiments indicate that plants can sense the differences between different primary potential causes of stress. Stress

triggers the co-ordinated induction of mRNAs involved in different aspects of the adaptative response of the plants. In this sense, varied stressors, like drought or salinity, cause different transcription levels of several genes; thus, the molecular stress response of plants should be triggered by multiple signals (Jin et al. 2000; Tabaei-Aghdaei *et al.* 2000). Having the entire genomic DNA sequence of *Arabidopsis* is allowing plant scientists to approach successfully to plant stress signalling (e.g., Glazebrook, 2001; Chen et al. 2002; Bais et al. 2003; Pickett et al. 2003).

Considerable evidence indicates that the regulation of plant stress responses involves hormones and plant growth regulators, especially *abscisic acid* (ABA), jasmonic acid, ethylene and polyamines, besides secondary messengers such as  $\text{Ca}^{2+}$  (Bray et al. 2000; Tiburcio et al. 2001). Recent findings assign stress-induced ROS a central role in stress signalling (Laloi et al. 2004; Weir et al. 2004).

The accumulation of ABA as an early response to stress, leading to stomatal closure, is a general feature in plants (Hsiao, 1973); in fact, many (but not all) of the genes induced by different stress factors are also induced by exogenous application of ABA (Shinozaki and Yamaguchi-Shinozaki, 1997). ABA plays undoubtedly a central role in stress manifestations by *converting multiple environmental stress signals in gene expression* (Bray, 1993; Chandler and Roberston, 1994; Leung and Giraudat, 1998; Jin et al. 2000). It transmits the environmental adverse stimuli from the contact zone (e.g., roots in a dry soil) to the whole plant, and then acclimation mechanisms are induced (see Figure 3). Intermediate points of signal transduction mediated by ABA (reversible protein phosphorylation, modification of cytosolic pH and calcium levels) besides cascade signals at the cellular level are relatively well known (Leung and Giraudat, 1998), but the nature of ABA receptor/receptors has not yet been completely elucidated. Netting (2000) describes an initial signal consisting on a rise in the levels of cytoplasmic  $\text{Ca}^{2+}$ , which initiates the liberation of an ABA precursor. A rise of pH due to the decompensation of  $\text{K}^+ \text{-H}^+$  fluxes induces ABA liberation to xylem stream, which promotes stomatal closure and the initiation of reparation processes, including stress-proteins. The gene expression associated is primarily controlled at the transcriptional level (Ingram and Bartels, 1996, Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu, 2000). The up-regulation of stress-responsive genes involves a concomitant increase in the levels of the corresponding transcripts. However, as several stress-responsive genes are not triggered in response to ABA, existence of both *ABA-dependent* and *ABA-independent signal transduction pathways* for expression of specific genes has been implied (Shinozaki and Yamaguchi-Shinozaki, 2000; Bray et al. 2000; Chen et al. 2002). Moreover, synthesis pathways that are induced by ABA under lab conditions (where studied until now) maybe are not successfully induced in field conditions (Bray, 1993; Thompson et al. 1997) or under multiple stress (Xiong et al. 1999). Involvement of ABA in plant co-stress manifestations is nowadays a priority area of research.

## ALLELOPATHY AS STRESS FACTOR

### Does allelopathy follow the general plant stress response?

Similarly to stress, allelopathy has been assimilated to the process and the effect. The ‘visible’ physiological effects from allelopathy interactions are frequently observed as inhibited or delayed seed germination or reduced seedling growth, which are secondary expressions of primary effects on metabolic processes as photosynthesis, respiration, cell division, pigment synthesis, production of plant hormones and their balance, membrane stability and permeability, mineral uptake, movement of stomata, amino acid synthesis, nitrogen fixation, and specific enzyme activities. The physiology of allelochemicals action is nowadays one of the most challenging issues in Allelopathy, and the clues and views about sites and modes of action are widely and deeply reviewed and discussed in different chapters of this book.

The physiological responses of plants to allelopathy are particularly complex since they do not reflect an adaptation to a biotic stress alone but the result of generations of co-evolution of many diverse types of environmental relations. Nonetheless, allelopathic chemicals alter plant growth and development by a multiplicity of effects on physiological processes because there are hundreds of different structures (see Chapter 2) and many of the compounds have several phytotoxic effects (Einhellig, 2002). Due to specificity of many secondary metabolites, effects of some allelochemicals are expected to be also specific. Einhellig (2002) suggested that *membrane perturbations* are a common starting point for effects of the phenolic acids, but the current evidence does not allow narrowing to a primary site of action for most plant allelochemicals (Inderjit and Keating, 1999). Many, perhaps most, of the compounds appear to impact growth by effects on more than one target site. Future expansion of biotechnology and functional genomics should provide physiological information relevant to determine the specific mechanisms of action of many allelochemicals, but also differences in responses among species.

Regarding current knowledge, the primary response to the presence of allelochemicals and other xenobiotic compounds in both prokaryotic and eukaryotic organisms involves the induction of detoxifying enzymes and transporters which facilitate the inactivation and elimination of toxins, and the associated metabolic processes including (i) oxidative modification of functional groups, (ii) the formation of glucosyl, glutathione, or malonyl conjugates, among others, and (iii) detoxification via specific membrane transport, leading to vacuolar sequestration or exocytose, and further enzymatic modification in vacuoles or deposition in cell walls (see Chapter 8).

Enzymes associated with detoxification processes primarily perform oxidative modifications. The large families of plant cytochrome P450s proteins (located in the endoplasmic reticulum) catalyze a wide array of oxidative reactions to detoxify

endogenous or foreign compounds. Many aldo-keto reductases and peroxidases are also implicated in detoxifying oxidative reactions. Secondly, conjugation reactions are catalyzed by glutathione S-transferases, glucosyltransferases, NADP-dependent oxidoreductases and malonyl-transferases, among others, thus converting toxins in substrates to be removed by membrane transporters. Many of these transporters have been shown to be induced by cadmium, cold, or salt stress treatments, pathogen infection, salicylic acid, ethylene, and methyl jasmonates, whereas others have been reported to play specific roles in the elimination of phytotoxins unrelated to pathogen defence (Baerson et al. 2005).

14-3-3 proteins have been shown to be implicated in abiotic stress responses (Roberts et al. 2002) and in some cases were correlated with the increase in stress tolerance that occurs during the acclimation process to other different stress factors (Jarillo et al. 1994). The function of these proteins as well as their role in allelopathic plant responses remains still unknown, whereas Zhang et al. (1997) found interactions between 14-3-3 and proteins involved in the phenyl-propanoid metabolism. Products of this biosynthetic pathway are implicated in allelopathy interactions (Rama Devi et al. 1997). The role of these protein-protein interactions is argued to be the maintenance of enzymes in an active state in healthy cells. They should co-ordinate cell metabolic responses to stress by regulating the activity of key enzymes (Roberts et al. 2002). Disturbs of the plasma membrane by allelochemicals are well documented (Einhellig, 1993; Reigosa et al. 2002; Sánchez-Moreiras, 2004) and regulation of the plasme membrane H<sup>+</sup>-ATPase seems to be a target for regulation by 14-3-3 proteins during stress (Roberts et al. 2002). Furthermore, it was suggested that the H<sup>+</sup>-ATPase might be an important early component of stress signalling (Schaller and Frasson, 2001). In the allelochemical response as in other stress responses, many targets for 14-3-3 proteins remain to be identified and the physiological significance of those interactions remains to be determined in the immense majority of cases (Roberts, 2002).

Using nearly full-genome transcriptional profiling studies and quantitative real-time RT-PCR, Baerson et al. (2005) identified specific genes which may represent integrated components of a coordinately-regulated, diverse broad-specificity chemical defence network. The authors stated: 'These data significantly expand upon previous studies examining plant stress transcriptional responses to environmental toxins, and provide a foundation for further biochemical and genetic experiments to more fully elucidate plant xenobiotic detoxification pathways, and the chemo-sensory mechanisms critical to plant survival in the presence of allelochemicals and other environmental toxins', this is, under natural or man-related chemical stress. Some observed up-regulated gene families have been previously reported to be over expressed under mechanical wounding, insect feeding, pathogen infection, cold treatment, and dehydration (Hegedus et al. 2003), indicating an important role for

these factors in mediating transcriptional responses to diverse biotic and abiotic stresses.

Thus, we can argue that allelopathy as stress factor follows the current theory of GAS and co-stress in plants, so that primary and secondary general effects of many allelochemicals have been reported. Under the exposed framework, the classic tools for Allelopathy research: seed germination bioassays, gas exchange techniques, *in vivo* chlorophyll fluorescence, HPLC, TLC, radiolabelling, etc. (Reigosa, 2001), are nowadays invaluabley enriched by functional genomics and proteomics<sup>1</sup> (Berenbaum, 2002; Wullschleger and Difazio, 2003; Edmeades et al. 2004), ‘the hot’ key molecular tools to discover allelochemicals mode/s of action (e.g., Cruz-Ortega et al. 2002; Sánchez-Moreiras, 2004; Baerson et al. 2005).

### **Allelopathic stress signalling**

Plants growing in nature constantly sense their environment and adapt to changes by using a range of biochemical and molecular mechanisms (Roberts et al. 2002). Plant could signal to a neighbouring plant and thus elicit a physiological response (Pickett et al. 2003; Bais et al. 2004). The suitable response is the result of the perception of external information and the transmission of this information between plant cells.

From the emanation of a wide variety of allelocompounds (exudation of soluble chemicals or releasing volatile organic compounds) plants can regulate the soil microbial community in their immediate vicinity (terrestrial plants), endure herbivores, encourage beneficial symbiosis, change the chemical and physical properties of the surrounded environment, and directly inhibit the growth of competing plant species (see Chapter 10). Allelopathy demonstrates how plants use secondary metabolites to stress neighbouring plants. Despite most experiments are not carried out under completely natural conditions (Bais et al. 2004) certain aspects of the general argument for plant interactions via soil solution are strong (Callaway and Aschehoug, 2000; Sánchez-Moreiras et al. 2003). These results provide a case that root-secreted allelochemicals play a role in plant-plant communication outside the laboratory.

Great effort has been dedicated to know the molecular and genetic basis for abiotic stress or herbivores and pathogen infection signalling in plants but less effort has been devoted to allelopathy responses. As said before, it has been demonstrated

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<sup>1</sup> Proteome analysis, the ‘analysis of the entire PROTEin complement expressed by a genOME, or by a cell or tissue’. Proteomics includes high-resolution 2D-electrophoresis, image analysis, microanalytical protein characterisation with multidimensional liquid chromatography/mass spectrometry, and bioinformatics. Functional proteomics technologies include yeast two-hybrid system for studying protein-protein interactions (Sánchez-Moreiras and Pedrol, 2001, and references therein).

that despite stress factors have a tendency to elicit a specific final response, many of the signalling intermediates (ABA, ROS, calcium...) are common to many pathways. Thus plant must integrate its response to an allelopathic interaction within the context of other environmental pressures, more likely by sharing signalling intermediates or response metabolites with overlapping physiological effects (Knight and Knight, 2001, Chen et al. 2002).

Presumably, the signal transduction pathway(s) involved in the expression of the battery of defence genes involved in the detoxification of xenobiotic compounds would not be highly specific, given the structural diversity of environmental and endogenous toxins typically encountered during the life cycle of a plant (Sandermann, 2004). Alternatively, the expression levels for these genes seems to be co-ordinately increased in response to a wide variety of chemical agents through the action of broad-specificity xenobiotic sensing mechanisms (Bais et al. 2003; Baerson et al. 2005).

Bais et al. (2003) found that the phytotoxin (-)-catechin triggers in *Arabidopsis* a wave of ROS initiated at the root meristem, which leads to a  $\text{Ca}^{2+}$  signalling cascade triggering genome-wide changes in gene expression and, ultimately, death of the root system. The elucidation of this mode of action led to explain the invasive strategy of *Centaurea maculosa*, which displaces native species by exuding (-)-catechin from its roots.

Some recently elucidated factors involved in signal transduction pathways of allelochemical defence response in *Arabidopsis* (Baerson et al. 2005) had been previously reported to play roles in mediating transcriptional responses to both abiotic and biotic stresses (Chen et al. 2002), as well as physiological processes such as leaf senescence (Eulgem et al. 2000; Buchanan-Wollaston et al. 2003). Future molecular genetic and bioinformatics-based approaches will throw light on regulation of gene expression changes associated with stress imposed by exposure to allelochemicals.

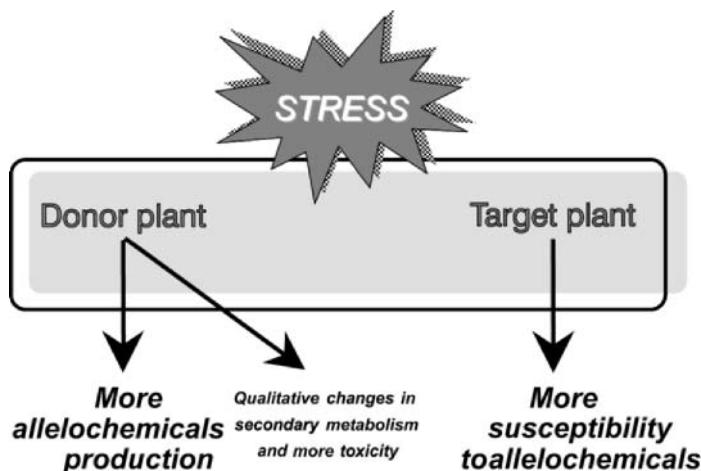
## ALLELOPATHY × ENVIRONMENT INTERACTIONS

The relative importance of abiotic vs. biotic influences on distributions of species, and species coexistence has been argued long ago (Darwin, 1859; Clements, 1916; Gleason, 1917; 1926). The intensity and direction of allelopathic processes and, in general plant interactions, may vary through time, and are in part determined by the abiotic context (Callaway and Walker, 1997; Goldberg and Novoplansky, 1997; Schenk et al. 2003).

The way in which external factors regulate secondary metabolite production is still unknown. Production of phenolic compounds varies depending on the type and/or intensity of stress, and on the species suffering stress; moreover, multiple stress exerts an additive effect on phenolic compounds accumulation by plants (Einhellig, 1999).

Resource competition, allelopathy, nutrient immobilisation and microbial influence all operate in parallel (Inderjit and del Moral, 1997; Inderjit, 2001; Bhowmik and Indejit, 2003). In perennial species, there are wide temporal fluctuations in phenolic compounds production, probably due to the complex interaction among internal factors, like tissue age, and external factors, as well biotic as abiotic. These variations may partly determine changes in competitive relationships with other organisms, with the consequent impact on ecosystem functioning (Nilsson et al. 1998). Recent critical reviews underline the need of facing the study of plant allelopathy by considering the combined effects of all these factors; this is, under an ecophysiological perspective (Inderjit and del Moral, 1997; Reigosa et al. 1999, 2002).

Interactions with different environmental factors play an important role in the expression of allelopathy (Gershenson, 1984; Tang et al. 1995; Kobayashi, 2004). As summarised in Figure 4, stress factors can influence both the production of allelochemicals by the *donor* species and modify the effect of an allelochemical on the *target* plant (Einhellig, 1996).



*Figure 4.* A summary of the general expected effects of plant biotic and abiotic stress (including other allelopathic effects) on the allelopathic relationships between two plants. Stress can enhance the importance of allelopathy in natural stressing conditions.

### Effects of abiotic stresses on the donor plant

Concentration of allelochemicals in the donor plant can be influenced by environmental conditions, such as temperature, light, soil structure or nutrient status. Regarding the *stress hypothesis of Allelopathy* (Reigosa et al. 1999, 2002), allelopathy

can appear and disappear in a place according to environmental changes, so that allelopathy becomes more important when and where plants are under stress.

The effect of some abiotic stressing conditions on secondary metabolites production is well known since a long time (Levitt, 1980; Timmermann et al. 1984). A general view of some classical works can be seen in the book of Rice (1984). More recently Einhellig (1996, 1999) has reviewed some abiotic and biotic stresses that can lead to strong allelopathic effects.

Regarding that stress has an inhibitory effect on growth, the excess of carbon supplied by photosynthesis can be derived to carbon based compounds that accumulate in tissues (Bryant et al. 1983; Estiarte et al. 1994). One typical response is hardening or cell wall lignification as stress response; lignin (phenolic polymers) confers resistance to deformation besides impermeability (Artlip and Funkhouser, 1995; Whetten et al. 1998); moreover, it represents a physical barrier against patogens.

The contents of allelochemicals are usually higher when the allelopathic species grow under low or moderate nutrient availability compared to high availability (Gershzon, 1984; Dustin and Cooper-Driver, 1992; Mwaja et al. 1995; Bhowmik and Inderjit, 2003). Higher concentration of allelochemicals was found to correlate with enhanced toxicity of plant residues or/and an increased release of natural compounds from the donor plant.

Increases in tissue concentration of phenolic compounds have been described due to changes in soil chemical properties such as pH and conductivity (Batish et al. 2002), UV-radiation (Ormond and Beverley, 1995; Zobel et al. 2002; Schmitz-Hoerner and Weissenböck, 2003), light quality (Dudt and Shure, 1994; Johnson et al. 1997), Cd treatment (Zheng and Wu, 2004), and even herbicide or allelochemical application (Gubbiga et al. 1996; Santosh et al. 1999). All these responses have been related to stress tolerance (Einhellig, 1989, 1999) besides inter- and intraspecific allelopathic interference (Inderjit and del Moral, 1997; Einhellig, 1999).

Water stress, caused by drought or osmotic stress, can stimulate accumulation of monoterpenes (Gilmore 1977, Kainulainen, 1992), chlorogenic acid (del Moral, 1972), hydroxamic acids (Richardson and Bacon, 1993), camptothecin (Liu, 2000), or phenolic acids in general (Kumar et al. 1991; Estiarte et al. 1994; Tang et al. 1995; Karageourgou et al. 2002). Allelopathy activity from plants grown in dry soils was greater than allelopathic interactions provoked from plants grown in well watered soils. In the field, under water stress conditions, the growth of target plants was reduced but the donor plants contained a greater amount of allelochemicals per dry weight than in absence of water stress. This fact could cause autotoxicity problems in natural conditions (Tongma et al. 2001).

Temperature influences the concentration of some allelochemicals (Hanson et al. 1983; Lovett et al. 1994) but it could be fully explained by the increase in plant

growth rate (Gianoli and Niemeyer, 1997). Despite this fact, the connection between temperature and growth is dependent on the species and more specific on the variety or cultivar. Probably, the level of allelochemicals is constitutive in some allelopathic plants (Gianoli and Niemeyer, 1997) and inducible for temperature in other species (Corcuera, 1993).

Irradiance and related physiological processes may influence allelochemical production and release on a short time scale of minutes and hours, especially on species which do not store volatile allelochemicals (Peñuelas and Llusià, 1999). The light dependence is usually attributed to the need of photosynthetic products for allelochemical biosynthesis.

UV radiation can increase the concentration of allelochemicals both in the cells and in the surface of the plant (Zobel and Lynch, 1997; Zobel and Clarke, 1999). Increased levels of both ozone and UV-B radiation, the typical combination of stresses for high-altitude sites, showed an obvious interaction at the level of gene expression on Scots pine seedlings, by affecting the mRNA transcript levels of cinnamyl alcohol dehydrogenase and stilbene synthase, both enzymes involved in the synthesis of protective phenolic compounds (Zinser et al. 2000).

Physical damage usually induces the production of volatiles that could help to induce defence responses in the damaged plants or others, attract parasitoids to attack herbivores or simply repel insects (Takabayashi and Dicke, 1996). Kato-Noguchi et al. (2002) suggested that momilactone B may play an important role in rice allelopathy, and the stress caused by the husk-treatment may increase the amount of momilactone B released by root exudation, thus increasing weed control efficacy.

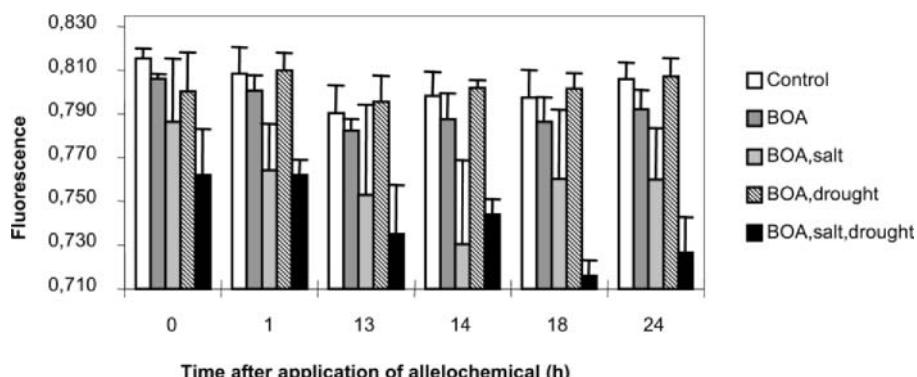
By the way, although they are not natural stress factors, some agronomic practices could help to induce allelopathy (Inderjit et al. 1996; Miller, 1996; Weston, 1996).

## Effects of abiotic stresses on the target plant

Sometimes previous exposure of the target plant to weak stress can result in an increased sensibility, and this could change the post-stress responses of that plant (for example, increasing allelopathic effects). By the contrary, different stress episodes prior to allelopathic stress can induce acclimation (i.e. tolerance to allelochemicals). Lehman and Blum (1999) proved that cucumber seedling acclimation occurred when plants were drought or nutrient pretreated. This authors suggest that acclimation to phenolic acids may be a general stress response since acclimation was achieved by a variety of pretreatment stresses. Since plants in nature are continuously confronted with multiple sources of stress these findings may help to explain why plants grown under field conditions are less sensitive to allelochemicals compared to plants grown in laboratory.

Addition of nutrients can sometimes overcome allelopathy (Kalburjhi and Gagianas, 1997; Kalburjhi et al. 2001), while the decrease of nutrients has been proved to increase allelopathic effects (Xu et al. 1999).

Environmental effects also mediate the effect of allelochemicals (Lehman and Blum, 1997, 1999). The impact of several combined simultaneous stress is very difficult to predict (Chapin et al. 1997). Figure 5 shows the measurements of fluorescence ( $F_v/F_m$ ), (considered as a good instantaneous measure of stress) in a plant submitted to two types of stress + allelopathy (applying BOA) (Sánchez-Moreiras, 2004). It is very interesting to note that tested allelochemicals did not produce a marked effect, nor without other stress nor combined with water stress or salt stress. Nevertheless, in plants submitted to salt and water stress, the addition of allelochemicals meant an important stress only explainable in terms of *multiple stress*. Results of a long-term experiment, however, could have been very different.



*Figure 5.* Illustration of the effects of interaction of multiple stresses. In this experiment, plants were submitted to an allelochemical (hydroxamic acid BOA) and two abiotic stresses: salt and water stress. Only the combination of the three factors produced a marked decrease in  $F_v/F_m$ , a parameter usually considered as a way of measuring stress. So, this stressing condition is not attributable to any of the effects, but to the combination of all of them. An experiment to measure the effect of BOA eliminating the salt and water stress would lead to the erroneous conclusion that there is no allelopathic capacity of BOA at the tested concentration (After Sánchez-Moreiras, 2004).

## Other factors affecting the allelopathic process

The resulting phytotoxic activity of a given allelochemical in a given ecosystem will be a function of the complex interactions among the behaviour of allelochemicals and the physiological and ecological properties of both donor and target plants under interacting environmental conditions (Kobayashi, 2004).

Biological activity of allelochemicals depends on the *time* after the release into media; the greatest phytotoxic activity was found in the early stage of plant residues decomposition in most cases (Souto et al. 1994; Kitou, 1999; Ismail and Ghong, 2002). However, the phytotoxic activity of the substances released from the donor plant, can generally be modified by further metabolic reactions by microorganisms in a manner depending on the time after the release (Souto et al. 1994; Mandal, 2001).

*Seasonal changes* in allelochemicals contents are also important factors affecting the allelopathic relationships under natural conditions (Peñuelas and Llusia, 1999). Figure 6 shows the importance of diachronic changes in the relative rates of allelochemicals production (Carballeira and Reigosa, 1999). In this case, the unusual flowering dates of *Acacia dealbata* produces a peak in the phytotoxins release that makes more harm to the autochthonous underwood species because it is the local germination period for most underwood species. This difference in the production and release of allelochemicals during blooming period has been demonstrated in other species (Ahmed and Wardle, 1994). Of course, concentration of most of the allelochemicals either in the donor plant or in the interface between provider and receiver plant also depends on the genotype and the developmental stage of the plant (Reigosa et al. 1999; Wu et al. 1999).

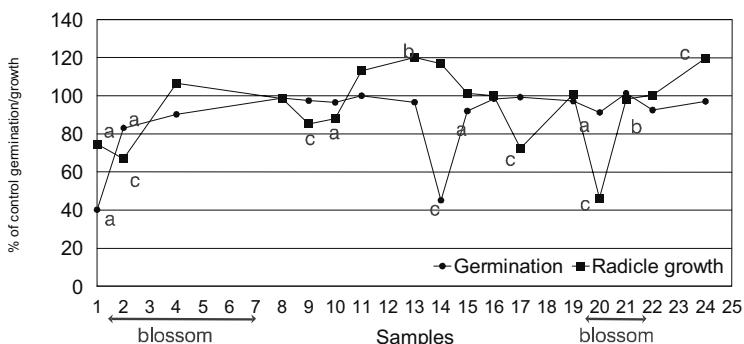


Figure 6. Importance of the moment of allelochemicals release. Effects of leachates naturally obtained from *Acacia dealbata* plants were obtained during one year in the field, and bioassayed in the lab for their phytotoxicity. During flowering periods leachates (as with throughfall and topsoil leachates) were bioactive, diminishing significantly germination and seedling growth. And the blossom period of this foreign species is really unusual in Galicia (NW Spain), just releasing allelochemicals when autochthonous plants begin germination (Carballeira and Reigosa, 1999). Letters mean significantly different to controls (a: 99%; b: 99.9%; c: 99.99%).

*Chemical constraints* are also important (Kobayashi, 2004). Although the relationships of phytotoxic activities to the concentration in the water solution was not made clear (Reigosa et al. 1999) the most important is the dilution of allelochemicals

at nanomolecular levels or lower (Legrand et al. 2003). All these factors affect the biological activity, making it a highly dynamic process that occurs on a short time-scale. So, it is suggested that allelochemical activity is induced by the concentration in natural solutions, being the amount of organic chemicals into the media dominated by environmental factors that affect the behaviour of the allelochemicals (Reigosa et al. 1999). Continuous release of allelochemicals has been shown in several studies (González et al. 1997). If degradation slows due to any environmental restriction, those allelochemicals could achieve effective concentrations in the soils, thus reaching and affecting plants.

The *transfer* of allelochemicals synthesized by plants to natural solutions (in both soil and aquatic systems) depends on diffusion and laminar advection. The amount of allelochemicals in the media will vary on the distance on the donor plant (Jose and Gillespie, 1998; Sene et al. 2000) because of physical constraints as viscosity, shear forces, and a low Reynolds number in the water (Legrand et al. 2003). To exert phytotoxic effects on other plant species, chemicals may have to move to the roots of the target plant through the soil. However, during movement, abiotic (physical and chemical) and biotic (micro-bial) soil barriers can limit the phytotoxicity of chemicals in terms of quality and quantity required to cause injury. Organic matter, reactive mineral surfaces, ion exchange capacity, inorganic ions, and abiotic and biotic factors of soil environment significantly influence allelo-chemical activity (Inderjit, 2001).

Allelopathic inhibition usually results from the *combined action* of a group of organic molecules which, in a synergic way, interfere with several physiological processes (Einhellig, 1996). Any abiotic process that interferes with accumulation or decomposition of plant material in soil will result in modifications of its chemical profile.

Soil factors as moisture, pH, organic matter content or salinity, can influence the allelochemical bioactivity or modify *soil enzyme activities* (Al-Turki and Dick, 2003). The importance of microorganisms in the mitigation and mediation of allelopathic effects has been emphasized by many investigators and the results demonstrated the importance of microbial oxidative transformations in both activating and degrading allelochemicals (Weidenhamer and Romeo, 2004). Then, hard environmental conditions, including high temperature, nutrient constraint, and periodic moisture stress that influence oxidative microorganisms or oxidative transformations on the media are involved in allelochemical evolution. Anoxia produced by flooding permits accumulation of molecules with limited effect and normally microbially removed (Premasthira and Zungsontiporn, 1996). Soil microorganisms can produce themselves allelopathic effects, and they can be affected by molecules released by the dominant plants, affecting their capacity of detoxification and their general effects, for example nitrification (Ward et al. 1997), although most times the effect of microorganisms will be detoxifying (Friebe et al. 1996; and see Chapters 14 and 15). Also, effects on ecto-

mycorrhizal fungal communities could be very important affecting higher plants (Koide et al. 1998).

## ALLELOPATHY ALONG ENVIRONMENTAL GRADIENTS

Environmental parameters that vary among locations but are, on an ecological scale of time, approximately constant or slowly changing at a specific location, may be classified as environmental gradients. Most parameters of the multidimensional abiotic and biotic environment may in some way influence the outcome of inter-specific plant competitive interactions, which may affect plant community structures in some predictable way (Tilman, 1988; Bazzaz, 1996; Grime, 2001; Damgaard, 2003).

Studied environmental gradients are specific abiotic stress factors like water availability, salinity, light, nutrient availability, heavy metal concentration, etc. In some cases biotic stresses may be assumed to be sufficiently constant on an ecological time scale to be regarded as an environmental gradient, because their effects on abiotic environment of other plants, e.g., the effect of shading trees on herbaceous plants (Damgaard, 2003). Few studies have attempted to unravel the causative factors affecting plant performance in continuous microhabitats related to allelopathy, possibly because of the complex and multiple effects that environmental factors have on local environments. The expected effects of the environment on the plant competitive interactions have been modelled using mechanistic plant models (Austin, 1990; Pacala et al. 1996; Cernusca et al. 1999), in which different plant life histories (or plant strategies) have different growth rates as a function of limiting resources. Models describe the effect of an environmental gradient on inter-specific plant competitive interactions, but allelopathic strategies are lacking.

### Phenotypic plasticity and allelopathy

The environment, besides genotype, is a source of phenotypic variation. In fact, it is the phenotype that natural selection ‘sees’ (Schlichting, 2002). The study of plant phenotypic plasticity towards abiotic stress factors grew from 1990s, but it was more recently that scientists paid attention to biotic environmental influences on the phenotype including the roles of competitors, predators and pollinators. The adaptive nature of plastic responses, this is, the links between gene regulation, physiological mechanisms and morphological plasticity are nowadays receiving some more scrutiny but still scarce (see Schlichting, 2002, for a review).

Phenotypic plasticity, or acclimation capacity, is the ability of a genotype to produce more than one alternative morphology or behaviour in response to environmental conditions. Thus, trade-offs were selected from the plastic

manifestation of traits that allow plants to maximise fitness in changing environments (Aerts, 1999; Sultan, 2000). Phenotypic plasticity may strongly influence species interactions by changing individual growth rates and vulnerability to stress factors (Agrawal, 2001). From an ecophysiological perspective, plasticity is an energetically costly trait, so that plants must maintain the genetic and cellular machinery (genes and enzymes) required to 'be plastic'.

Adaptive phenotypic plasticity in plants both to changing environments and physical gradients (Schmitt, 1997; Pedrol et al. 2000) or in different cultivars (González et al. 1997; Wu et al. 2000; Olofsdotter et al. 1999, 2002) has been examined related to secondary metabolite production. Plant populations exhibit ecotype variation in the expression of these biochemical responses (Zangerl and Berenbaum, 1990; Reigosa et al. 2002). The evolution of phenotypic plasticity is thought to result from variable natural selection in ecologically variable environments (Agrawal et al. 2002) so that the production of some allelochemicals may be constrained by genetic correlations with other traits and costs of plasticity (Agrawal, 2001; Relyea, 2002).

### **Ecophysiological costs of allelopathy**

Stress and plant productivity are interrelated such that as one stress (e.g., scarcity of nutrients) is corrected and productivity increases, other stresses (e.g., shading) increase. In general, oligotrophic (low-productivity) environments are dominated by abiotic stresses, whereas in eutrophic (high-productivity) environments, biotic stresses dominate.

To explain the evolution, distribution and diversity of allelochemicals, plant ecophysologists assume that diversion of resources to resistance or defence represents a trade-off with growth (Herms and Mattson, 1992; Cipollini, 2004). The induced response can vary greatly among plants and stress factors, but it often includes the production of numerous compounds and physical structures.

Although higher concentrations of secondary metabolites might result in a more resistant plant, the production of secondary metabolites supposes a cost for the plant (Bergelson and Purrington, 1996; Rausher, 1996; Stotz et al. 1999; Gershenson, 2002; Baldwin et al. 2002). Quantification of costs of allelopathy in plants remains a subject of controversy (Baldwin, 2002; Heil, 2002; Heil and Baldwin, 2002). We miss more experimental studies in this field since the existing results until now are dependent on the studied biological system and not susceptible to be extrapolated (Zangerl et al. 1997; Heil et al. 2000).

Of course, it is assumed that defence through allelochemicals can only be maintained over evolutionary time if the plants benefit from induction. These costs,

that reduce plant development and reproduction, might preserve genetic variation within populations by conserving the alleles that codify for high levels of defence and maximum resistance (Siemens et al. 2002). Hypotheses appear regularly in the literature as explanations for the level of plant defence, i.e. why some plants are so well defended and others are not. These hypotheses include optimal defence, carbon:nutrient balance, growth rate and growth-differentiation balance. However, there is considerable dissatisfaction with the progress with the ecological plant defence hypotheses that stems from inadequate approaches, such as failure to identify and test assumptions in experimental designs, confusing the hypotheses and their predictions, and/or choosing a subsystem (e.g., plant age or part) that may be inappropriate for the test (Stamp, 2003).

Instinctively, we should think that costs of defence would increase in stressful environments. Otherwise, extrinsic stress factors can passively limit the ability of a plant to respond most favourably to the allelopathic injure; thus, plastic responses to stress factors and allelochemical injure may interact affecting fitness (Callaway, 2002). Nonetheless, we must take into account that changing environmental factors and allelopathic relations are commonly present at the same time in any plant population (Reigosa et al. 1999). So, the induction of secondary metabolites in plants as a response to stress can be considered as a cost-saving mechanism, because they are induced as defence and used also as allelochemicals (Siemens et al. 2002; Hoballah et al. 2004). According to the stress hypothesis (Reigosa et al. 2002), inducible defences may be especially beneficial in situations where several threats act simultaneously (Gouinguené and Turlings, 2002; Hansson, 2004), and competitors affected by the stress factors increase its susceptibility. Recent works have shown empirical evidence of the selective advantages that allelopathic activity confers to many plants (Callaway and Aschehoug, 2000; Ridenour and Callaway, 2001; Hunter and Menges, 2002; Inderjit and Mallik, 2002; Oliva et al. 2002; Florentine, 2003), specially under stress conditions such as nutrient deficiency, drought, etc. (Kong et al. 2002).

To understand the costs and advantages of inducible chemical competition, one needs to study all relations that are mediated by these phytochemicals. This relates to interactions between plants, plants and herbivores, plants and other plants and plants and abiotic factors. Recent studies have shown that plant-plant allelopathy can extend to higher trophic levels (herbivores predators) (Glinwood et al. 2004; and see Chapter 10). Each of these four groups of interactions may comprise a range of interactions between individuals of different species belonging to the different groups. In fact, after the release of the phytochemicals has take place, the plant is no longer in control of who receives the chemicals (Dicke et al. 2003). Any organism in the environment can potentially be affected. Figure 7 summarizes the web of trade-offs between growth and costs of environmental relationships regarding phytochemical compounds: interference

vs. resource competition, direct or indirect active defence *vs.* passive competition, including the trade-offs with abiotic stress resistance and their multiple interactions.

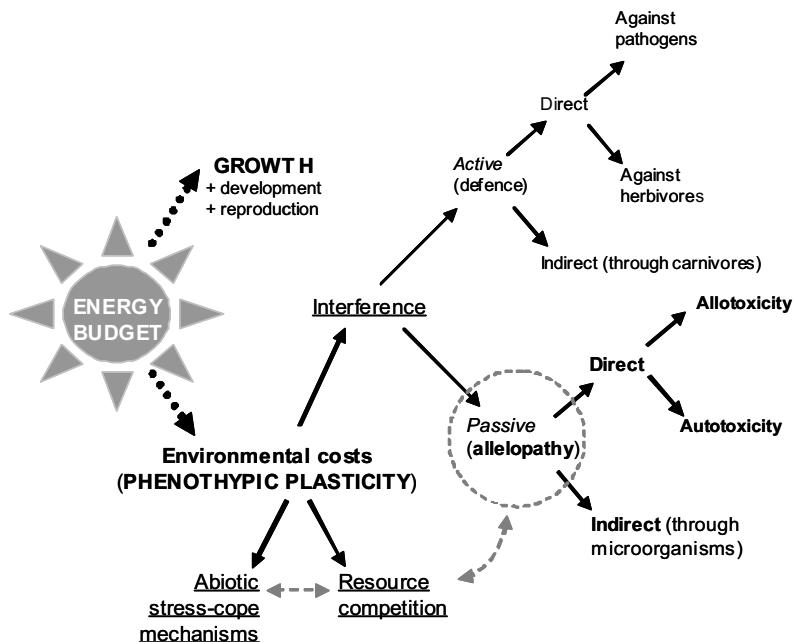


Figure 7. Trade-offs between growth and phenotypic plasticity towards environmental stresses.

Phenologic stage of the donor plant has also a direct influence on induced allelochemical production and then in the cost for the plant. Hoballah et al. (2004) showed that the cost of secondary metabolites induction in maize was only detectable in young plants or in the youngest leaves immediately after stress exposure. During maturation the treated plants compensated for the metabolic investment in early plant stages. In this sense, the metabolic cost of induced secondary metabolites involved in allelopathic relationships is evident, but this cost is acceptable in terms of fitness in the whole plant life span. A recently developed mechanistic model (An et al. 2003) proposes that there are two kinds of allelochemical productions in a plant, which are dictated by age and plant stress, and they are reflected by the corresponding dynamics in the environment. The decline of allelochemical contents in living plants with increasing age of plants may be a general case, while periodic production may be a special case.

## ALLELOPATHY × ABIOTIC STRESS: ENGINES OF EVOLUTION, SOURCES OF BIODIVERSITY

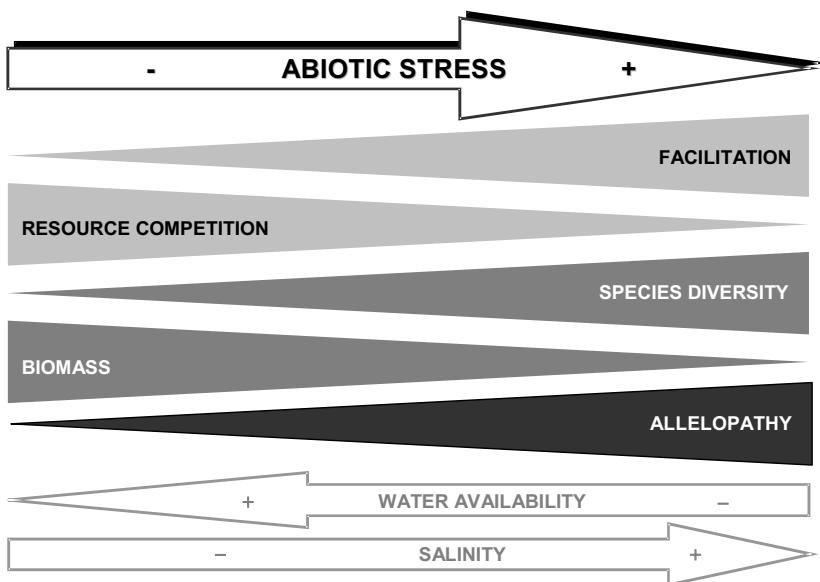
Allelopathic interactions have been proposed to have profound effects on the evolution of plant communities through the loss of susceptible species via chemical interference, and by imposing selective pressure favouring individuals resistant to inhibition from a given allelochemicals (e.g., Schulz and Wieland, 1999; Bais et al. 2004). Despite plant secondary metabolites being major determinants of species interactions and ecosystem processes, their role in the maintenance of biodiversity has still received little attention.

As both competition intensity and facilitation change along gradients of environmental stress, plant interactions are viewed as dynamic relationships, the outcome of which depends on abiotic conditions (Callaway and Walker, 1997; Brooker and Callaghan, 1998; Parker et al. 1999; Pugnaire and Luque, 2001). Evidences of facilitation phenomena among species under abiotic stress conditions have been observed concomitantly with qualitative and quantitative increases of potentially allelopathic phenolic compounds (Pedrol, 2000). As water stress increased (this caused by water depletion, salinity, or both) productivity besides resource competition intensity decreased, whereas species richness was enhanced (Figure 8).

Iason et al. (2005) investigated the relationship between chemical and biological diversity in a natural ecosystem. They considered the impact of chemical diversity in individual Scots pine trees on species richness of associated ground vegetation. Scots pine trees show substantial genetically determined constitutive variation between individuals in concentrations of a group of secondary metabolites, the monoterpenes. When the monoterpenes of particular trees were assessed individually, there was no relationship with species richness of associated ground flora. However, the chemical diversity of monoterpenes of individual trees was significantly positively associated with the species richness of the ground vegetation beneath each tree. This correlation suggests that the chemical diversity of the ecosystem dominant species has an important role in shaping the biodiversity of the associated plant community.

As said before, secondary metabolites are relevant for the plant's survival and reproductive fitness. Therefore, they represent adaptive characters that have been subjected to natural selection during evolution. There are critical qualitative differences among taxonomic groups regarding secondary metabolites. Certain specific secondary metabolite reveals an unusual metabolic pathway, and a special gene combination to control its synthesis. In fact, a common secondary metabolite pattern would provide more evidence of evolutionary proximity among taxa than morphological or physiological similarities, which can be due to evolutionary convergence. Particularly, phenolic compounds have been successfully used as taxonomic tools (Bell, 1980; Van Sumere, 1989) very valuable to clarify evolutionary

processes determining variability into specific complexes (Ardouin et al. 1988; Jay and Lumaret, 1995; de Ramón, 1995).



*Figure 8.* Summary of the changes observed in a natural community of grasses along environmental gradients of water availability and salinity (Pedrol, 2000). As water stress increases (this caused by water depletion, salinity, or both), productivity besides resource competition intensity decrease, whereas species richness is enhanced. Evidences of facilitation phenomena among species under stress conditions where observed concomitantly with qualitative and quantitative increases of secondary metabolites in *Holcus lanatus* and *Dactylis glomerata*, including phenolic compounds (Reigosa et al. 2002). Allelopathy is argued to become more important in stressful environments, thus playing a crucial role in plant community structure and species diversity.

Wink (2003) reconstructed molecular phylogenies of the Fabaceae, Solanaceae and Lamiaceae and employed them as a framework to map and to interpret the distribution of some major defence compounds that are typical for the respective plant families; quinolizidine alkaloids and non-protein amino acids for legumes; tropane and steroidal alkaloids for Solanaceae, and iridoids and essential oils for labiates. The distribution of the respective compounds appeared to be almost mutually exclusive in the families studied, implying a strong phylogenetic and ecological component. However, on a closer look, remarkable exceptions could be observed, in that certain metabolites were absent (or present) in a given taxon, although all the neighbouring and ancestral taxa express (or do not express, respectively) the particular trait. It is

argued that these patterns might reflect differential expression of the corresponding genes that have evolved earlier in plant evolution. The inconsistent secondary metabolite profiles mean that the systematic value of chemical characters becomes a matter of interpretation in the same way as traditional morphological markers. Thus, the distribution of secondary metabolites has some value for taxonomy but their occurrence apparently reflects *adaptations and particular life strategies* embedded in a given phylogenetic framework (Wink, 2003).

For the fixation of allelopathy, it is possible a non-functional accidental release of allelochemicals as by-products (Dicke et al. 2003). It seems to be clear that although allelochemicals release was incidental, once they were ecologically effective, natural selection must have operated on the donor plant to optimise the benefits of its release to the environment (Jansen et al. 2002). Recently, Winterer and Weis (2004) suggested a novel mechanism for the persistence of resistance polymorphisms that allow evolution to operate. Regarding these authors, variation in environmental stress between generations facilitates the evolution of stress resistance through assortative mating. Stress induces delayed maturation of susceptible phenotypes, segregating their fertile period from resistant phenotypes. Assortment of mates enhances the responsiveness of populations to natural selection by inflating genetic variance. Thus, positive selection and inflated genetic variance in stressful environments can cause a strong evolutionary increase in resistance. By contrast, benign environments do not segregate phenotypes, and the random mating among phenotypes deflates genetic variance, leading to a weaker response to selection against resistance, assuming that resistance is costly. When environments vary randomly from benign to stressful, populations respond asymmetrically to negative and positive selection. This asymmetry (1) accelerates fixation of a resistance allele if resistance is generally favoured (stressful generations more frequent) but delays the loss of the allele if it is generally disfavoured (benign generations more frequent), and (2) it can push a resistance allele to fixation even when long-term costs modestly exceed benefits. When resistance alleles pleiotropically delay mating, stress induced random mating has complementary effects. Serial autocorrelation in the stressor amplifies these effects. This mechanism may partly explain the preferably occurrence of allelopathy in stressing environments, and the maintenance of plasticity for allelopathic induction in changing environment (i.e., the ‘stress hypothesis’, Reigosa et al. 2002).

Emodin illustrates the wide and often overlooked potential for chemical multifunctionality in plant secondary metabolites (Izhaki, 2002). The anthraquinone emodin, identified in 17 plant families distributed worldwide, has numerous biological activities, some of which exhibit a wide spectrum of ecological impacts by mediating biotic or abiotic interactions of plants with their environment, thus having direct and indirect effects on plant survival and reproduction. Emodin in vegetative organs may help protect plants against herbivores, pathogens, competitors and extrinsic abiotic factors (e.g., high light intensities). In unripe fruit pulp, emodin may facilitate seed

dispersal by protecting the immature fruit against predispersal seed predation whereas in ripe pulp it may deter frugivores and thus reduce the chances that seeds will be defecated beneath the parent plant. It also accelerates the passage of seeds through the digestive tract, potentially reducing dispersal distance and increasing seed viability upon dispersal. Natural selection should favour secondary metabolites with multiple functions because they protect the plants against a variety of unpredictable biotic and abiotic environments. Such metabolites also enhance plant defenses by using different molecular targets of specific enemies through a variety of mechanisms of action (Izhaki, 2002).

The fact that plants synthesize more variety and quantity of secondary metabolites than animals has been related to their physical immobility, by replacing escape mechanisms against stress by chemical defence. From the assumption that some, if not all, known secondary metabolites confer some kind of selective advantage to species which synthesize them, by increasing their competitive ability in a given habitat, we can argue that synthesis of new compounds provide part of the variability on which natural selection operates, thus making possible divergent evolution of species. In that sense, whereas interactions among competition and environmental gradients are discussed as the main source of *niche diversity*, allelopathic relationships among plants by means of the synthesis and release of specific secondary metabolites can be observed as a source of *specific diversity*. In one case, interacting biotic and abiotic selective forces promote the fixation of different abilities to cope environmental stress, this is, diversity of functional types. In the case of allelopathy, ‘to wear’ an specific chemical weapon (even passive) can be a guarantee of success (particularly in invasive and exotic plant species, see Chapter 17), this is, an advantageous character to be selected and fixed. Novel biochemical weapons can also function as mediators of new plant–soil microbial interactions (Callaway and Ridenour, 2004). Moreover, the proper vegetation changes will promote potentially long-term changes in the soil (e.g., changes in soil pH) and are therefore likely to promote the re-invasion of the same and other exotics (Kourtev, 2003).

Both management of exotic plant invasions and the restoration of native communities must take into account exotic species effects on the soil. Mallik (2003) observed in boreal and temperate forest that conifer regeneration failure in the presence of dense ericaceous cover (resulting from the removal of canopy trees by forest harvesting). This fact was attributed to multiple effects of allelopathy, competition, and soil nutrient stress: The new keystone ericaceous species bring about a significant long-term habitat change by rapid accumulation of polyphenol-rich humus. Ericaceous phenolic compounds inhibit seed germination and seedling growth of conifers and, by forming protein-phenol complexes, cause a further reduction of available nitrogen of the already nutrient-stressed habitat. A low pH condition in the presence of phenolic compounds causes the leaching of metallic ions and forms hard iron pans that impair soil water movement. The phenolic allelochemicals of ericaceous

humus are also inhibitory to many conifer ectomycorrhizae. On the other hand, ericaceous plants perpetuate in the community by their stress-tolerating strategies as well as their ability to acquire nutrients through ericoid mycorrhizae (Mallik, 2003).

From the exposed evidence, allelopathy besides its interaction with other types of environmental stress can be considered as powerful engines of evolution and no ending sources of biodiversity. Nonetheless, under the perspective of conservation, destruction and changes in the use of soils (especially in tropical agroecosystems) have decreased biodiversity, bringing about the loss of valuable natural products. New methods must be generated for allelopathy as a part of the biotic resources management strategies, thus taking in mind that many different types of useful products such as natural pesticides and drugs can arise from allelopathy studies (Anaya, 1999).

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## CHAPTER 10

# ALLELOPATHY AND BIOTIC STRESSES

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## INTRODUCTION

Environmental stresses both abiotic and biotic are often implicated as very important factors changing allelopathy manifestation in nature (Einhelling, 1984, 1987, 1996, 1999, 2004; Anaya, 1999; Reigosa et al. 1999, 2002; Inderjit and Keating 1999; Inderjit and Nayyar, 2002). Moreover, Einhelling (1987, 1999), Chou (1999), Inderjit and Nayyar (2002), Reigosa et al. (2002) stated that allelopathy is more pronounced and could be important when acceptors are affected by environmental stresses, especially when they are severe. In that sense allelopathy is involved in plant adaptation processes through evolution and has a positive impact on the increase of plant tolerance/resistance to unfavourable conditions. On the other hand, however, there are claims that plants under allelopathy stress, being less vigorous, are more likely to be also less tolerant to other stressful conditions including allelopathy stress (Patrick et al. 1964; Reigosa et al. 2002 and references therein).

There is common opinion that environmental stress usually, though not always, increase allelopathic effectiveness of the target plants and this is achieved by increased concentrations of allelochemicals and/or by lowering of the threshold of allelocompound concentrations, at which the effects take place (Einhelling, 1996, 2003).

Plants exposed to biotic stresses such as pathogenic microorganisms, herbivores and small animals respond with similar defensive mechanisms by allocation of carbon skeletons from plant productivity to producing in higher concentrations and/or synthesis *de novo* molecules of defensive mode of action, in majority secondary

metabolites. These metabolites often are implicated also as allelopathically active due to the known dual- or multifunction of the secondary metabolites (Swain, 1977; Rhodes, 1994; Klepzig et al. 1996; Anaya et al. 1999; Bais et al. 2004). Plants after exposure to biotic stresses would potentially be of higher allelopathic activity not only due to higher concentration of allelocompounds but also due to a wider spectrum and/or “the right” composition/concentrations of those compounds.

Interactions between stresses and allelopathy may be considered from at least three points of view:

1. influence of stress (es) on the allelopathic potential of affected by stress plants,
2. allelopathy effects on the outcome of biotic stress(es) influence on the host plant, and
3. effects of interactions between the above on the chemistry and biodiversity of agro- and /or ecosystems.

Allelopathy, in the part of phytotoxic activity, is a biotic stress for the acceptor (receiver, target or afflicted) organisms and allelopathy as a phenomenon is widely covered in other chapters of this book. For the purpose of this chapter the term biotic stress is used in the sense of all others, besides allelopathy, biotic factors that are directly or indirectly stressful to host and/or to organisms involved in the signal transduction pathways leading to subsequent outcome. From this rule however, there is an exception i.e. autoallelopathy (autotoxicity), as generating another biotic stress included in this chapter.

This chapter will focus on presenting interactions between allelopathy and the following biotic stresses: pathogenesis, allelopathic plant residues with soil-born microbial stress, autotoxicity, herbivore infestation, competition, exotic weeds invasion stress, parasitic weeds, and stress signalling – a novel performance of allelopathy.

## PATHOGENESIS

In plants exposed to pathogenic organisms defensive mechanisms are triggered that lead to a increased production, degradation of conjugates, and/or to synthesis *de novo* of the secondary metabolites of defensive mode of action that often also show allelopathic activity. Ultimately, such plants, their litter, mulch, and residues having a higher content of allelochemicals would be of a higher allelopathic activity when allelochemicals will be released to environment (Einhelling, 1999). However, this is almost solely based on assumption that a higher content of allelochemicals would enhance allelopathic potential of donor plant. To our knowledge, except autotoxicity,

there are only two papers, in which, in purposely-designed experiments, the effect of pathogens on allelopathic activity of host plant was rigorously checked. Sutherland et al. (1999) when studied the possible allelopathic effects of perennial ryegrass (*Lolium perenne*) infected with endophytic fungus (*Neothypodium lolii* = *Acremonium lolii*) on white clover (*Trifolium repens*) showed a significant suppression of white clover by aqueous extracts of *L. perenne* associated with endophyte relative to endophyte-free extracts. The suppression varied (11 - 47%) depending on *N. lolii* strains host ryegrass cultivar, and showed concentration-dependent character. The author did not answer whether this allelopathic activity is of ryegrass, endophyte origin or both. Though, they found that used in these experiment strains in ryegrass/endophyte association differed in produced alkaloids: peramine, ergovaline and lolitrem B and that none of them could solely be responsible for the suppressed growth of white clover.

Contrary to the above and common opinion that stresses would increase allelopathic activity of the affected plant (Einhelling, 1987, 1999; Reigosa et al. 2002) Kong et al. (2002) reported the opposite. In elegantly designed studies on allelopathic activity of *Ageratum conyzoides* stressed by several factors including the infection with *Erysiphe cichoracearum* the authors demonstrated a reduced phytoinhibitory effect against all tested species: peanut (*Arachis hypogaea*), redroot amaranth (*Amaranthus retroflexus*), cucumber (*Cucumis sativus*), and ryegrass (*Lolium multiflorum*) when compared with non-infected *A. conyzoides*. Interesting is the fact that, 18 out of 24 analyzed chemical constituents of the volatiles were released to the environment in a significantly higher amounts though, without inhibitory effects on tested plant species. These allelochemicals however, had inhibitory effect on growth of 3 fungi species: sheath blight (*Rhizoctonia solani*), Chinese cabbage sclerotinia (*Sclerotinia sclerotiorum*), and cucumber gray mold (*Botrytis cinerea*).

## CROP AND WEEDS RESIDUES

Plant residues both of crops, weeds and of natural vegetation, in concerted action with soil microorganisms during decomposition can, in several ways, generate a biotic stress for new growing plants.

During decomposition of the residues microorganisms use energy, some mineral elements, water and oxygen which, if not sufficient in the soil environment, would result in competition for limited resources that as reported later, is a biotic stress.

Besides, although some products of decomposed residues are of nutritional value for the new vegetation others are phytotoxic and allelopathically interfere both with soil microbes and with newly growing plants. The problem can be more severe if, residues are from plants that during vegetation experienced environmental stresses as they are most likely to produce allelochemicals in higher concentrations (Einhelling,

1999) and subsequently when released to environment would potentially be of a higher allelopathic activity to a target plant. Moreover, Inderjit and Duke (2003) pointed out that allelochemically-enriched soils might generate chemical stress, which in turn would lead to a higher content of allelocompounds in the acceptor plants either due to the uptake or *via de novo* synthesis in response to the exposure of allelopathy stress. This was presented by Inderjit and Dakshimi (1992) who recorded a higher content of phenolics in asparagus bean (*Vigna unguiculata* var. *sesquipedalis*) grown in soil emended with *Pluchea lanceolata* as compared to *P. lanceolata*-free soil.

Allelopathic compounds released during decay of residues in the soil also enhance pathogenesis that has been recognized long ago (Patrick et al. 1964). Katz et al. (1987) studying allelopathic potential of biotypes of *Coridothymus capitatus* L. aggressive and non-aggressive to neighbouring annuals, showed an increase in the populations of actinomycetes in soil amended with shoots of *C. capitatus*. Although the increase was recorded in soils amended with both biotypes of *C. capitatus* but the increase in the case of the aggressive biotype (surrounded by annuals free-vegetation belts) was four times higher. The authors suggest possible synergistic inhibitory effects of allelochemicals emanated by *C. capitatus* and the phytotoxic activity of actinomycetes.

Allelochemicals in the soil also provoke, though indirectly, another biotic stress to receiver plants *via* the effect on vesicular arbuscular micorrhizae (VAM) - a diverse group of fungi known from benefit to plant health and productivity as well as to soil structure. Studies on the effects of aqueous shoot extracts and of root exudates of aggressive perennial grass *Imperata cylindrica* on VAM associated with 7 leguminous species (*Lathyrus aphaca*, *Medicago denticulata*, *Melilotus parviflora*, *Phaseolus vulgaris*, *Trifolium resupinatum*, *Vicia sativa*, and *Vigna radiata*) clearly demonstrated that allelopathic compounds of *I. cylindrica* reduced the VA micorrhizal colonization in all tested species (Bajwa et al. 1996; Afzal et al. 2000). Microscopic observation showed that mycelial and arbuscular infections in roots of 5 species (*L. aphaca*, *M. denticulata*, *M. parviflora*, *T. resupinatum*, and *V. sativa*) were reduced. The vesicular infections were not affected in *L. aphaca* and in *V. sativa* while in the other three species the number of vesicles increased (Bajwa et al. 1996). Studies on *P. vulgaris* and *V. radiata* showed, in addition to the above, that allelochemicals contained in aqueous shoot extracts of *I. cylindrica* also significantly reduced plant growth, yield and nodulation (Afzal et al. 2000). Allelopathic compounds are also implicated to be responsible for changes in the VA mycorrhiza infections in roots of 11 annual and 3 perennial weed species associated with *Dicanthium annulatum* (Forssk) Stapt. (Javaid et al. 1996) and though the response was variable but generally annual species were more susceptible. In most cases, similarly to the above studies, reduction was recorded although in some no response or even increased colonisations was recorded.

Aside from the direct toxic effect of allelochemicals, the suppression of the VA mycorrhiza, resulting in diminished water and nutrients uptake, may also contribute to the earlier described growth depression and yield losses in *P. vulgaris* and *V. radiata* plants. This is supported by the results of Catska (1994) who showed that inoculation of apple tree seedlings with two species of VAM fungi *Glomus fasciculatum* and *G. macrocarpum* markedly increased seedling biomass due to changes in the rhizosphere microbial composition by VAM. Also Bajwa et al. (1999) and Javaid and Bajwa (1999) demonstrated that inoculation with VAM significantly reduced the negative impact of allelopathy stress caused by *Melia azedarach* leaf extract on maize and by *Syzygium cumini* on chickpea (*Cicer arietinum L.*). Similarly, under alleopathic stress generated by *Imperata cylindrica* inoculation with two VAM species *G. mosseae* and *G. fasciculatum* improved growth and yield of winter wheat. This effect, however, was not consistent for both VAM species and all growth parameters (Bajwa et al. 2000).

Amelioration of the negative impact of allelopathy and benefit to the host by rhizosphere microbes were also attributed to ectomycorrhizal fungi (ECM). Hanson and Dixon (1987) reported enhanced survival and growth of red oak (*Quercus rubra L.*) seedlings exposed to alleopathic compounds contained in the interrupted fern frond leachates when seedlings were inoculated with – *Suillus luteus* L.: Fr.northern. Studies on Suillloid ECM fungi (Colpaert et al. 2004) proved their evolutionary adaptation to zinc toxicity – a chemical stress – and as showed by Adriaensen et al. (2003) *Suillus bovinus* (Fr.) Zn-adapted fungus protects pines from zinc stress. Since allelopathy stress to acceptor plant is of chemical nature it is possible to assume that enhanced survival and growth of red oak inoculated with *S. luteus* operates on the same or similar protective mechanism(s).

## AUTOTOXICITY

Autoallelopathy (autotoxicity, autointoxication, intraspecies allelopathic interferences), a biotic stress of allelochemical deleterious interference between plants of the same species, occurring both in natural and agroecosystems, creates a serious problem in replanting fruit trees in orchards (peach, apple, apricot, cherries, citrus), shrubs and perennial plantations (grape, asparagus, coffee, tea, alfalfa) and in some annual crops when grown successively year-by-year (reviewed by Singh et al. 1999; Yu, 1999).

Aside of autotoxicity as a biotic stress, there is evidence that problems with replanting arise not only as a direct effect of allelochemicals released to environment by plant residues but, to some extend, also due to the appearance or enhancement of biotic stress – phytopatogenesis. This was clearly demonstrated in studies on the autotoxicity in *Asparagus officinalis L.* Depressed asparagus emergence and reduced

seedling growth by allelopathic substances derived from asparagus tissues, their isolation and characterization including physiological effects on asparagus plants are well known (Hartung and Stephens, 1983; Young, 1986; Pierce and Colby, 1987; Hartung et al. 1989; Hartung et al. 1990). In addition to the above Hartung and Stephens (1983) reported a simultaneous dramatic decrease in asparagus seedling dry weights along with also dramatic increase in root rot caused by fungi *Fusarium moniliforme* (Sheld.) emend. Snyd. & Hans. and *Fusarium oxysporum* (Schlecht.) emend. Snyd. & Hans. f. sp. *asparagii* Coheand when exposed to asparagus tissues. Also Pierce and Colby (1987) observed a depressed asparagus emergence and increased infection of *F. oxysporum* f. sp. *asparagii* in the presence of asparagus root filtrate. These results clearly demonstrate that synergistic interactions between autotoxins of asparagus origin and *Fusarium* infections contribute to the decline in asparagus plantations. Similarly, in sugar cane, a population of *Fusarium oxysporum* in the rhizosphere of poorly continuously growing plants was much greater than in that of productively growing newly planted sugar cane roots or in the plant-free soil (Wu et al. 1976; after Chou, 1999). Productivity decline of continuing monocultures of mungbean is also attributed to synergistic interference of allelopathy and soil-borne plant pathogens (Waller et al. 1995; after Chou, 1999). Similarly, decline in long-term continuous corn monoculture, as reported by Turco et al. (1990), is among others, due to a higher bacterial potential for reduction of corn root vigour at an early growth stage, resulting in an increased chance for diseases. Moreover, authors on the basis of their results and of Fredrickson and Elliott (1985) suggested that the decline in corn and wheat is rather due to active microbial processes, in the presence of residues in the rhizosphere, than to building up residue origin toxins. Singh et al. (1999) in a review on autotoxicity also pointed out that in the replant syndrome, besides autoallelopathy, a key role is played by microbes, pathogens and nematodes.

## COMPETITION

Competition is another biotic stress, which triggers or changes plant allelopathic properties. Allelopathic potential of plants competing for limited resources such as nutrients, water, oxygen or growing under unfavourable light conditions will, most likely, be enhanced due to increased biosynthesis of allelochemicals and the deleterious effects of allelocompounds on target plants may be greater (Einhelling, 1999). Indeed, Kong et al. (2002) in nicely and purposely-designed experiments, showed that increased allelopathic activity of *Ageratum conyzoides* when exposed to competition with *Bidens pilosa*. The authors demonstrated, using bioassay, that volatiles released by plants growing in competition had a significant allelopathic-inhibitory effect on all tested species i.e. peanut, redroot amaranth, ryegrass and cucumber. The authors also showed that the total amount of volatiles synthesized by

*A. conyzoides* plants grown with competition, was significantly higher. It was also true for 9 among 17 detected out of 24 compounds analysed.

On the other hand, however, competition as a result of an increased plant density diminishes allelopathic properties of the leachates, volatiles and exudates of donor plant, as well as its litter, mulch and residues due to the dilution effect in density-dependent phytotoxicity. This was very well demonstrated in a target-neighbour, soybean-corn model system by Thijss et al. (1994), reviewed by Weinheimer (1996) and presented in detailed in the chapter 8 of this book.

The lowering effect of allelopathic activities, by optimising crop density, can be employed in practical application of allelopathy as a strategy for weed prevention and control in organic farming.

## EXOTIC SPECIES INVASION

A special case of competition that generate biotic stress to plants both of natural and agroecosystems is the inroad of exotic invasive species that pose a serious threat to global biological diversity as well as to yielding. *Centaurea* species are among the most destructive exotic invasive plant species to North America and there is evidence that allelopathy is one of the mechanisms through which rapid displacement of native species takes place (Callaway et al. 1999; Bais et al. 2003; Hierro and Callaway, 2003 and references therein). Bais et al. (2004) showed that roots of *Centaurea malucosa* (spotted knapweed) exude racemic catechin with (-) catechin of phytotoxic and (+) catechin of antimicrobial activity. Authors also showed that concentration of (-) catechin was more than twice as high in North America as in Europe and that European grasses were more resistant to (-) catechin than their North American counterparts (Bais et al. 2003). Hierro and Callaway (2003) pinpointed the importance of allelopathy as a mechanism of the success of invaders because native species are more likely to be “naïve” than those in origin communities to released by invaders allelochemicals as they did not co-evolve.

The biorationale of suppressing invading species with biocontrols as weapons in eliminating or reducing the invasion *via* increasing competitiveness of native against the invasive sounds attractive. However, apart from successful “green” ecological/agricultural management sometimes biocontrol herbivores are questionable. Callaway et al. (1999) reported that biocontrol herbivores might also lead to increase, *via* allelopathic relationships, competitiveness of invading species against the native ones. Authors tested the effectiveness of biocontrols *Agapeta zoegana* and *Trichoplusia ni* on *C. malucosa* grown with native species *Festuca idahoensis*. Results showed that contrary to expectations, instead of releasing the native species from the competitiveness with *C. malucosa*, reduction of the reproductive potential of the native

*F. idahoensis* took place when *C. malucosa* was infested by *A. zoegana*. Also plants of *F. idahoensis* developed significantly smaller root systems when grown together with *C. malucosa* that was infested by *Trichoplusia ni*. Both these effects subsequently increase the invasive potential of *C. malucosa*. Summing up the authors suggest that herbivore may indirectly enhance the negative impact of *C. malucosa* on native species and therefore, using biocontrols should be carefully considered before the application as it may result in an effect opposite to the expected one.

## PARASITISMS

Plants when colonized by parasitic or hemiparasitic plants are under another biotic stress because of a full or partial dependence on energy, nutrients, and water supply from the host and their influence on ecological chemistry of micro-environments. Although only a few out of over a hundred *Orobanche* ssp. and 28 *Striga* ssp. are colonising crops (cereals, legumes, crucifers, tomato, sunflowers, hemp, tobacco), they have a tremendous impact on the plants and are serious threats for agriculture in some parts of the world. Some steps of parasitism are stimulated by host plant, others exert protective response in host plants and there are essential differences in this regard between susceptible and resistant genotypes. Host-parasitic plant interactions are mediated by secondary metabolites some of which are known for their allelopathic activity (Hegazy, 1999; Hegazy and Fahmy, 1999). Thus parasitic stress would change the allelopathic properties of the host and an increased allocation of carbon skeletons into the secondary metabolites by host plants was reported for several species. Goldwasser et al. (1999), in comparative studies on resistant purple vetch cv. Popan and susceptible common vetch cv. Yovel co-cultured with *Orobanche aegyptiaca* showed an increased concentrations in the resistant genotype of both bound and free phenolics, lignin and higher activity of peroxidases by 8, 4, 2, and 2 fold respectively when compared with non-infected respective controls. Although in response to the invasion in the susceptible genotype Yovel an increase of phenolics, lignin and of activities of PAL and peroxidase also took place but it was very low and the level of lignin did not change. Authors concluded that differences in biochemistry of secondary metabolites in response to *O. aegyptiaca* were probably a part of the defence mechanism of the resistant Popany cv. forming mechanical and chemical barriers against the invading parasites.

Similarly when sunflower was infected by *Orobanche cernua* Loefl. a higher accumulation of coumarins and greater root exudation in resistant genotypes was recorded as compared to susceptible ones (Sergolini et al. 2001). According to Tsanuo et al. (2003) protection against *Striga hermonthica* in *Desmodium uncinatum* involves isoflavones from the allelopathic root exudates of *D. uncinatum*. However, as the exudates possessed compounds both stimulating germination and inhibiting radical

growth, the authors hypothesised that this combination comprises some mechanisms of protection against *S. hermonthica* what according to Khan et al. (2002) lead to suicidal germination of *S. hermonthica*.

The above described changes in the secondary metabolite chemistry, despite the fact that they are the primary steps of defensive mechanisms against parasitism, are of allelopathic nature. Changes in allelopathic properties of host plants might be also expected by an indirectly effect of changing the host secondary metabolites pool. As shown by Adler (2000) hemiparasite *Castilleja indivisa* uptakes alkaloids from the host *Lupinus texensis* and *L. albus* which potentially might result in a lower content of alkaloids.

## HERBIVORE INFESTATION

Plants when invaded by herbivores activate defence responses that consist of several mechanisms including increase in the concentration of secondary metabolites, many of which are phenolic compounds (Dalton, 1999). Phenolic compounds, on the other hand, are among those very often implicated as allelochemicals (Einhelling, 1985, 2004; Inderjit, 1996). Literature on changes in biosynthesis of phenolics as well as on other defensive secondary metabolites due to feeding by aphids, mites or caterpillar herbivore is abundant and reporting them is beyond the scope of this chapter. However, since an assumption is often made that infested plant, as having higher concentrations of phenolic compounds, would perform higher allelopathic activity when these compounds were released into the environment (Einhelling, 1999), a few examples are provided. Birch (*Betula pendula*) leaves when grazed by larvae of *Apocheima pilosaria* had higher concentrations of phenolic acids and higher activity of phenylalanine ammonia lysase (Hartley and Firn, 1989). An increase of phenolic compounds was recorded in asparagus plants (*Asparagus officinalis*) fed by asparagus aphid (*Brachycorynella asparagi* (Mordvilko) and by green peach aphid (*Myzus persicae*) (Sulzer) (Leszczynski et al. 1990) in winter wheat fed by aphids *Rhopalosiphum padi* (L.) and *Sitobion avenae* F. (Leszczynski, 1985; Niraz et al. 1988). Walters et al. (1989) reported higher concentration of anacardic acids in *Pelargonium xortorum* and concluded that it is one of the most important factors for small pest resistance in geranium. Higher resistance of tomato (*Lycopersicon esculentum* Mill.) cv. Slonka to carmine spider mite (*Tetranychus cinnabarinus*) among others is also attributed to increased concentration of free phenolics (Kielkiewicz-Szaniawska, 2003).

Recently Walker et al. (2003) in studies on metabolic profiling of root exudates of *Arabidopsis thaliana* found that 6 out of 10 compounds exuded by roots after treatment with elicitors are known from allelopathic activity.

Increased concentrations of allelochemicals due to plant exposure to pest infestation besides possible higher allelopathic activity, may also affect developmental synchrony between insect prey and predators as showed by Stamp et al. (1997) in studies on performance of *Podisus maculiventris* fed pray (*Manduca secta*) caterpillars. According to authors it seems possible to foster plant resistance against insect pests that will not be detrimental against their predators or even of some allelochemical combinations that would be strongly detrimental for insects being simultaneously favourable for the predators.

To our knowledge, only one paper reports results of study purposely designed on interactions between herbivore infestation and allelopathy (Kong et al. 2002). In this work however, the authors showed, contrary to expect and common opinion, a decrease of allelopathic potential of *Ageratum conyzoides* volatiles when *A. conyzoides* was subjected to biotic stress generated by *Aphis gossypi* feeding. Lowering of the allelopathic activity was recorded against all tested species: peanut (*Arachis hypogaea*), redroot amaranth (*Amaranthus retroflexus*), cucumber (*Cucumis sativus*), and ryegrass (*Lolium multiflorum*). This took place despite the fact that 20 out of 24 analysed chemical compounds were released in higher concentrations by stressed plants when compared to control. It is worthnoting that volatiles of *A. conyzoides* stressed plants showed inhibitory effects on three other species of insects (adult of *Tribolium confusum*, 3<sup>rd</sup> larva of *Mythimna separata*, and 4<sup>th</sup> larva of *Culex pipiens*).

## STRESS SIGNALLING: TRITROPHIC INTERACTIONS

Stress factors besides changing the allelopathic properties of a donor plant potentially may also be involved in the signal transduction pathways. Although biosynthesis, accumulation and release of secondary metabolites including allelochemicals is often orchestrated *via* signal perception by target plants, followed by transduction cascade, a novel role has been attributed to allelopathy – signalling. This takes place when released to environment substances instead of direct effects, as allelochemicals on acceptor plants (*via* physiological and biochemical mode of action) are of signalling nature, called semiochemicals or chemical signals. There is evidence that signalling in the sense of provoked changes between infested and non-infested plants and between plants and the next trophic levels such as plant-herbivores and herbivores-predators or parasitoids take place in ecosystems (Glinwood et al. 2003; Picket et al. 2003). In response to herbivore attack pathogen infection and parasitic weed invasion, quantitative and qualitative changes in semiochemicals synthesized in host plant are documented. Host plant can release semiochemicals, such as volatiles or root exudates, not only from damaged organs but also systemically from non-attacked ones being triggered by earlier step of signalling cascade (Röse et al. 1996; Picket et al. 2003). Volatiles by creating a kind of bound layer that surrounds plants take a

part in further signalling leading to regulation of pest infestation, thus participating in host specificity. These signals, when perceived by neighbouring intact plants, might elicit defence mechanism including release of secondary messengers, which in the next steps of signalling can be perceived by herbivorous insects and by their parasites (Pickett et al. 2003).

Pettersson et al. (1996) in studies on 3 species of cereals (barley, wheat and oat), and 3 species of aphids (*Rhopalosiphum padi*, *Metopolopodium dirhodum* and *Sitobium avenae*) and mildew (*Erysiphe graminum*) demonstrated that infested plants triggered, *via* volatilisation, defensive mechanism(s) against aphids and mildew in intact neighbouring plants. However, there are differences in this respect depending on cereal and aphid species. In another work Pettersson et al. (1999), studying the effects of cereals volatiles on aphid (*Rhopalosiphum padi*) antixenosis, showed that aphid-attacked plants of some barley *cvs.* emitted volatiles that induced in neighbouring intact plants of other barley *cvs.* defence mechanism(s) resulting in lowering aphid acceptance. Similarly Pare and Tuomlinson (1997) studying herbivore-induced volatiles provided evidence for active plant mediating interactions between the 2<sup>nd</sup> and 3<sup>rd</sup> trophic levels and as the authors pointed out: "this specific plant response to herbivore damage is a reliable signal to natural enemies of the herbivores as the location of their host".

Similar signalling may also operate *via* rhizosphere (Bertin et al. 2003; Pickett et al. 2003; Bais et al. 2004). Glinwood et al. (2003) showed significantly lower bird cherry-oat aphids (*Rhopalosiphum padi*) settling on barley plants *cvs.* Kara when exposed to root exudates from quack grass (*Elitrygia repens* = *Elymus repens* = *Agropyron repens*).

Parasitic weed establishment and residency on host plants besides dependence on energy, water and nutrients, is also based in part on allelopathic signalling between the hosts and invading plants (Bouwmeester et al. 2003).

These results of the chemistry of tritrophic interactions between plants, herbivores and parasites open new emerging area of exploiting allelopathy i.e. not only *via* direct physiological and biochemical mode of action but, hopefully, also through signalling. Semiochemicals released by host plants after herbivore attack may signal to predators and parasites of their potential hosts presence. Pickett et al. (2003) in an article focused on referring the future conclude that there is a chance for developing new strategies of biocontrol in agroecosystems either by: (i) crop genetic modification, (ii) external chemical imitation of plant attack that will elicit defence signalling cascade against pest, or by (iii) cultivation of genetically mixed (species, cultivars) provoking and recipient plants populations (Pickett et al. 2003).

## CONCLUDING REMARKS, RESEARCH NEEDED AND FUTURE PROSPECT

Interactions between biotic stresses and allelopathy received relatively little attention comparable to abiotic stresses and for sure to many other aspects of allelopathy thus, we may say that research on the alteration of allelopathy performance by biotic stress is in the beginning stage. This is mainly because of very complex interactions between stressed host plant and the invasive organisms; all being influenced by other variable, dynamic and also of high complexity factors of the environment. There is need for depth-in, purposely designed and rigorously conducted studies on these interactions. Simultaneously, thanks to enormous advancement in molecular biology as genomics, proteomics, and metabolomics and biotechnology together with powerful instrumentations like micro arrays the possibility of studying such complex interactions looks more realistic now. Moreover, there is chance by genomic approach to relate genes involved in biosynthesis of secondary metabolites and by genetic engineering to increase their production and/or to change their composition (Stamp et al. 1997; Wu et al. 1999; Gressel, 2000; Dixon, 2001; Inderjit and Duke, 2003; Sturz and Christie, 2003).

Therefore although, as being so complex it would not be easy to accomplish, it is a new, challenging and fascinating area of investigation for scientist involved in allelopathy.

Results of studies on allelopathy and biotic stresses interactions that trigger cascade of allelopathic interferences affecting all levels of trophic dependence, besides of their academic value, have a chance to be useful in practical application like elaborating of biocontrol agents in low chemical input agriculture or organic farming as well as in the regulation, restoration and managing of ecosystems.

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## CHAPTER 11

# PLANT SECONDARY METABOLITES. TARGETS AND MECHANISMS OF ALLELOPATHY

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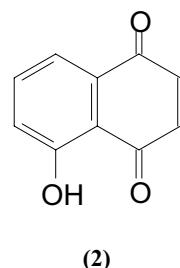
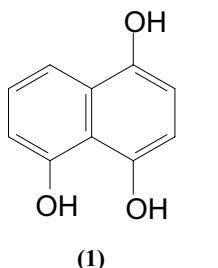
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## INTRODUCTION

Allelopathy is commonly defined as any effect: direct or indirect, stimulatory or inhibitory, mediated by a chemical compound released into the environment by a given plant or microorganism (Rice, 1984). These chemicals, termed secondary metabolites, allelochemicals, natural products or phytogrowth-inhibitors, are a major factor in regulating the structure of plant communities (Smith and Martin, 1994). Allelochemicals can be released into the environment through a variety of mechanisms: volatilization from leaves, exudation from roots, and leaching from fallen leaves and plant litter (Putnam, 1983).

The chemical interaction between plants was known for thousands of years, before the term allelopathy was coined by Molish (1937). Thus, Pliny may have been the first to record the allelopathic effects of the walnut tree (*Juglans nigra* and *J. regia*). The walnut tree is perhaps the best known allelopathic plant, causing many crops and other plants in its vicinity to wither and die. The leaves, roots, and fruits of the plant produce a hydroquinone (1), which is oxidized in the environment to juglone (2), the compound responsible for the toxic effects on other plants (Kocacaliskan and Terzi, 2001).



## Secondary metabolites

According to Whittaker (1970) and Whittaker and Finney (1971), most allelochemicals are secondary metabolites, not present in all living organisms but appearing sporadically. The production of allelochemicals is promoted by biotic and abiotic stress. Allelochemicals are referred to as secondary metabolites due to the fact that they are obtained through branching of the main metabolic pathways of carbohydrates, fats and aminoacids. Whittaker and Finney (1971) listed the chemical character of allelopathic molecules as belonging to several major groups: these include phenolic acids, flavonoids, and other aromatic compounds, terpenoid substances, steroids, alkaloids, and organic cyanides. Rice (1984) devised a classification system containing 14 chemical categories plus a miscellaneous group. Most allelochemicals derive either from acetate, or from aminoacids participating in the shikimic acid pathway. The categories proposed by Rice (1984) are (1) cinnamic acid derivatives, (2) coumarins, (3) simple phenol or benzoic acid derivatives, gallic acid and protocatechic acid, (4) flavonoids, (5) condensed and hydrolyzable tannins, (6) terpenoids and steroids, (7) water-soluble organic acids, straight chain fatty acids, (10) naphthoquinones, anthraquinones and complex quinones, (11) aminoacids and polypeptides, (12) alkaloids and cyanohydrins, (13) sulfide mustard oil glycosides and (14) purines and nucleosids. From the structural diversity of the allelochemicals, it is obvious that allelopathy must involve more than one action mechanism, different synergism patterns and diverse targets of interaction. Thus a large diversity of molecules may be allelochemicals, but not all the compounds belonging to each of the categories in the Rice classification must be allelochemicals. Furthermore, in addition or instead of acting as allelopathic agents, many of these compounds may protect plants against herbivores or against microbial infection, or function as attractants for pollinators and seed-dispersing animals.

It is a common practice to determine the allelochemical threshold concentration in susceptible plants. Allelopathy is a concentration dependent phenomenon, and allelochemicals are introduced into the environment together with a vast number of

other secondary metabolites. Thus, it is likely that synergistic effects enhance the observed activities (Putnam et al. 1986). The kinetics activity of allelochemicals is also studied.

Current allelopathy research is interdisciplinary and to make significant progress, it requires the contribution of agronomists, biologists, biochemists, ecologists, organic chemists, physiologists, soil scientists, and theoretical chemists.

Even when a given allelochemical may be tentatively identified, it is exceedingly difficult to probe its role in plant-plant interactions. Even more difficult to analyze is the primary site and the mechanism of action and to distinguish these from secondary site(s) or action mechanism(s). Measurement of the amount of an allelochemical released and absorbed is likewise complicated by interactions of the molecule with soils of different types, and by degradation or modification of the compound by microorganisms. Often, only minute quantities of the active compound can be isolated from the natural source. The participation of volatile natural products in plant-plant interactions may be easier to be demonstrated and so is the analysis of the structure.

## **Biological assays**

The most widely used biological assays for allelochemicals are seed germination and seedling growth studies. In the simplest form of these assays, seeds of the selected plant species are placed on filter papers or on agar in a Petri dish or in a small tissue culture and treated with a solution of the suspected allelochemical at various concentrations. Germination rate and seedling growth (root and shoot lengths) is then monitored versus control samples. The most important consideration in choosing or developing a bioassay for an allelopathic study is selection of the target species from both mono and dicotyledons in order to determine the potential selectivity of the allelochemicals. The most used model for weeds is lettuce (*Lactuca sativa* L.). It has been used extensively as a test organism because of its fast germination and high sensitivity (Rasmussen and Einhelling, 1979). *Lemna* sp. (duckweed) is often used to examine plant-plant interactions in aquatic environments (Elakovich, 1999), while barnyardgrass, gooseweed, and ducksalad are more relevant for study of allelopathy in rice (Dilday et al. 2000). The initial screening can be done using dose-response curves for concentration of the allelochemicals ranging from  $10^{-4}$  to  $10^{-9}$  M.

## **Target(s) and mechanisms of action of allelochemicals**

It is naive to expect that the physiological action of the numerous allelochemicals will be the same. Nonetheless, most investigations have used the common denominator

that deleterious effects in allelopathy will be expressed through a reduction or delaying of germination and stunting of growth of a susceptible plant. Apparently, allelochemicals may alter a variety of physiological processes and it is difficult to separate primary from secondary relationships when interpreting the effect of these phytotoxins on plant growth or seed germination. A further complication is that the various allelochemicals may inhibit through different mechanisms. Separating primary from secondary effects can be challenging when nothing is known about the mode of action of the compound.

The morphology of grown seedlings in the presence of allelochemicals may also yield important information. In particular, abnormal root formation such as clubbing may indicate a mode of action of compounds that are inhibitors of microtubule assembly. Stimulation of lateral root growth to the detriment of the primary root also suggests disruption of hormonal balance. Investigation of the putative effect on root cell division should be conducted by measurement effects of the compound on the mitotix index with plant roots. Subjective biological observations include root and shoot morphology and visual rating of the phytotoxic effect. Objective quantitative measurements include measurement of the length and weight of the plant parts. Leaf area measurements can provide information related to the inhibition of plant development.

Therefore, the study of physiological and biochemical roles of secondary metabolite potential targets, include: *modification of membrane structure and all type of transport receptor vs. signalling, altered features of cell morphology, interference with the cell cycle (replication, protein synthesis, mitosis), modification of phytohormone activity, perturbation of energy metabolism (respiration and photosynthesis), modification of water balance and stomata function, inhibition of pigment synthesis and/or degradation, and blockage of function for a number of enzymes, mainly regulatory enzymes.*

## MEMBRANE STRUCTURE AND TRANSPORT AS TARGET OF ALLELOCHEMICALS

Cell membranes consist of a bilayer of polar lipid molecules and associated proteins. In most cell membranes, lipids and proteins (glycoproteins) make roughly equal contributions to the membrane's mass. The lipids belong to several classes, including phospholipids, galactosylglycerides, glucocerebrosides, and sterols. These molecules share an important physicochemical property: they are amphipathic, containing both hydrophilic (water-loving) and hydrophobic (apolar-loving or water-hating) domains. When brought into contact with water, these molecules spontaneously self-assemble into higher-order structures. The hydrophilic head groups maximize their interactions with water molecules, whereas the hydrophobic tails

interact with each other, minimizing their exposure to the aqueous phase. For most membrane lipids, the bilayer configuration is the minimum-energy self-assembly structure. In this configuration, the polar groups form the interface to the bulk water, and the hydrophobic groups become sequestered in the interior. Studies of the movement of phospholipids in bilayers demonstrate that each phospholipid can diffuse laterally, rotate, flex its hydrophobic tail, bob up and down and flip-flop. Membrane lipids exist in two different physical states: as a gel and as a fluid. The change in state is known as phase transition. Gelling brings most membrane activities to a standstill and increases permeability.

Many plants survive temperature fluctuations of up to 30 °C on a daily basis. How do organisms adapt the fluidity of their membranes to suit their mutable growth environments? Membrane sterols serve as membrane fluidity “buffers”, increasing the fluidity at lower temperatures by disrupting the gelling of phospholipids, and decreasing fluidity at high temperatures by interfering with the flexing motions of the fatty acid tails. Like all cellular molecules, membrane lipids have a finite life span and have to be turned over on a regular basis. This turnover also enables plant cells to adjust the lipid composition of their membranes in response to seasonal changes in ambient temperature. Allelochemicals with detergents properties may solubilize phospholipid(s) and perturb the membrane function mimicking sterol-behavior and thus causing growth inhibition. Allelochemicals can also alter the fluidity of the membrane and thus modify the transport processes that need fluidity. There can be a direct inhibition of the interaction between carriers or transporters and the secondary metabolites resulting in transport inhibition.

Membrane proteins associate with lipid bilayers. The fluid-mosaic membrane model included two basic types of membrane proteins: peripheral and integral protein. Most recent research has led to the discovery of four additional classes of membrane proteins: fatty acid-linked, prenyl group-linked, phosphatidylinositol – anchored, and cholesterol-linked, all of which are attached to a bilayer by lipid tails. Peripheral proteins are water-soluble and can be removed by washing membranes in water or in salt or acid solutions that do not disrupt the lipid bilayer. Peripheral proteins bind either to integral proteins or to lipids through salt bridges, electrostatic interactions, hydrogen bonds or some combination of these. Some peripheral proteins also provide links between membranes and cytoskeletal systems. In contrast, integral proteins are non soluble in water. Because at least one domain lies embedded in the hydrophobic interior of the bilayers, an integral protein can be removed and solubilized only with the help of detergents or organic solvents, which degrade the bilayer.

Both the fatty acid-linked and the prenyl group-linked proteins bind reversibly to the cytoplasmic surfaces of membranes to help regulate membrane activities. Cycling between the membrane-bound and free states is mediated in most cases by phosphorylation/dephosphorylation or by GTP/GDP binding cycles.

## Membrane fluidity

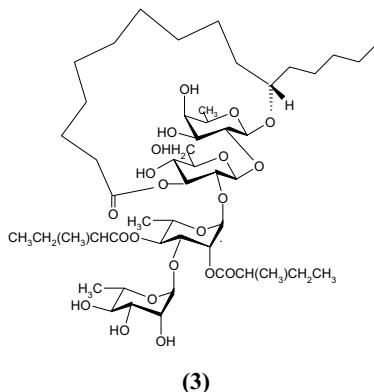
Involves the movement of not only lipid molecules but also integral proteins that span the bilayer and of different types of surface-associated membrane proteins. Collisional interactions are essential for the transfer of substrates between many membrane-bound enzymes and for electrons passage from between the electron transfer chain components of chloroplasts and mitochondria. Such movements are also critical for the assembly of multiprotein membrane complexes. In addition, many signalling pathways depend on transient interactions among defined sets of integral membrane proteins and peripheral or lipid-anchored proteins. These multiprotein assembly can be altered with allelochemicals and inhibits the normal membrane processes.

Tethering structures regulate and restrict the movement of membrane proteins, often limiting their distribution to defined membrane domains. This tethering can involve connections to the cytoskeleton and the cell wall, bridges between related integral proteins, or junction-type interactions between proteins in adjacent membranes.

A particularly striking example of the junction type of interaction occurs in the grana stacks of chloroplast membranes. Grana stack formation has been shown to affect the lateral distribution of all major protein complexes in thylakoid membranes and to regulate the function of the photosynthetic reaction centers and other components of the photosynthetic electron transport chain of PSII and PSI. Modification of the lateral distribution in the PSII complexes can affect the rate of electron transport in photosynthesis.

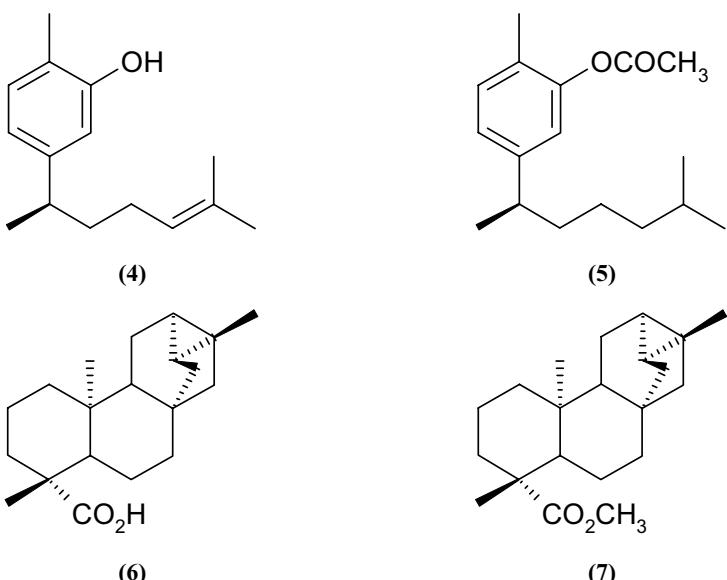
In order to eliminate weeds prior to planting the next sugar cane crop, *Ipomoea tricolor* is used as cover crop (another way to find allelopathic plants) in the State of Morelos, Mexico, because it possess allelopathic properties. Bioassay-guided analysis of *I. tricolor* extracts yielded tricolorin A (3) with a potent phytogrowth inhibitory activity. In addition, this macrolactone oligosaccharide has antibiotic and antitumor activities (Pereda-Miranda et al. 1993). Some minor components of the mixture, tricolorins B-E were identified by FAB-Mass spectrometry and high resolution of detailed NMR analysis (Bah and Pereda-Miranda, 1996). Furthermore, studies on the inhibition of plasma membrane H<sup>+</sup>-ATPase by the glycoside mixture indicated that 3 was the inhibitor compound (Calera et al. 1995). Petri dish bioassays combined with greenhouse experiments suggested that the weed suppressive activity of *I. tricolor* may involve both leaching of allelochemicals from the living plant by rain and release of allelochemicals from decaying plant matter (Anaya et al. 1995). Further studies indicate that Tricolorin A (3) behaves as uncoupler ( $U_{50} = 330 \text{ nM}$ ) in spinach chloroplasts, because it inhibited H<sup>+</sup>-uptake and ATP synthesis, and stimulates basal and phosphorylating electron flows. At higher concentrations of tricolorin A,

inhibition of photosystem II electron transport at the level of  $Q_B$  ( $I_{50} = 5 \mu\text{M}$ ) occurred. Chlorophyll *a* fluorescence analysis corroborated this finding and that the macrolactone is a structural requirement for this activity (Achnine et al. 1999). Therefore, the mechanism of action of tricolorin A (3) as allelopathic compound is by uncoupling photophosphorylation and interacting at plasma membrane  $\text{H}^+$ -ATPase. The novel macrolactone structure of the tricolorins has attracted the attention of the synthetic chemists (Larson and Heathcock, 1997; Lu et al. 1997).



*Iostephane heterophylla* (Cav) Hemsl. (Asteraceae) is widely distributed throughout Mexico, from the states of Chihuahua and Sinaloa in the north to Oaxaca in the south. Xanthorrhizol (4), a sesquiterpene of the bisabolene type, has been isolated from the roots of this plant (Aguilar et al. 1993; Mata et al. 2001) in addition to other sesquiterpenes, diterpenes, 8-hydroxy-6-acetyl-2, 2-dimethylchromene and scopoletin. Xanthorrhizol (4) possess insecticidal activity against neonate larvae of *Spodoptera littoralis* (Pandji et al. 1993). González-Bernardo et al. 2003, found the phytotoxic effect of xanthorrhizol on photosynthesis by inhibiting ATP synthesis and electron flow ( $I_{50} = 31 \mu\text{M}$ ), the site of inhibition is located at the span of  $P_{680}$  to  $Q_A$  and  $\text{PQ}$  as was corroborated by fluorescence decay data. Acetylation of xanthorrhizol indicates that the OH group is essential for interaction with the electron transport chain in the thylakoid, since 1-O-acetyl-12, 13-dihydro-xanthorrhizol (5) is almost inactive. Furthermore, the reduction of the  $C_{12-13}$  double bond may facilitate the interaction with the target redox electron transport chain or the penetration to the site of interaction, making the molecule more soluble in the lipid phase explaining why this is the most active derivative. The reduced xanthorrhizol also inhibits the  $\text{H}^+$ -ATPase. A bioguided fractionation of *Iostephane heterophylla* also led to the isolation of trachyloban-19-oic acid (6) as the compound that inhibits PSII at the level of  $Q_B$  ( $I_{50} = 25 \mu\text{M}$ ) and the

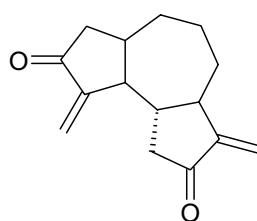
perturbation of LHC II. Chlorophyll fluorescence studies confirm the behavior of this diterpene (Hernandez-Terrones et al. 2003a). In order to understand which part of the structure is important for inhibition, methyl trachyloban-19-oate ester (7) was prepared from trachyloban-19-oic acid with ethereal diazomethane (Hernandez-Terrones et al. 2003b), this derivative presented a new site of interaction, acting as energy transfer inhibitor, blocking  $CF_0$  channel of the chloroplast ( $I_{50} = 10 \mu M$ ), because increasing concentrations of methyl trachyloban-19-oate ester restore the light dependent pH rise to a suspension of EDTA-washed chloroplasts. Methyl trachyloban-19-oate ester (7) is a noncompetitive inhibitor to well known energy transfer inhibitors DCCD and triphenyltin, therefore it has a unique site of interaction at  $CF_0$  channel.



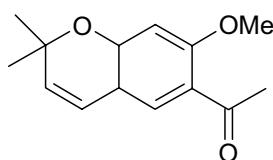
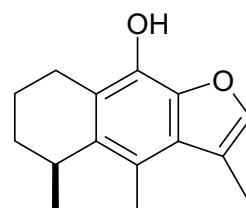
*Parthenium hysterophorus* is an aggressive, neotropical composite weed that has spread very fast in the world. It exerts negative effects on agriculture, animal husbandry, ecology and environment (Evans, 1997). Its allelopathic properties have been demonstrated (de la Fuente et al. 2000). These are attributed to the presence of parthenin (8), a sesquiterpene lactone of pseudoguayanolide nature found in various parts of the weed (de la Fuente et al. 2000). The compound is sequestered in trichomes that cover the whole plant (Picman and Picman, 1984). Parthenin is known to be phytotoxic against many plants (Batish et al. 2002). Germination of *Avena fatua* and

*Bidens pilosa* was reduced with parthenin ( $I_{50} = 625$  and  $700 \mu\text{M}$ , respectively). Further, parthenin also inhibited the growth of both weeds in terms of root and shoot length and seedling dry weight. Inhibition of root growth was greater than that of shoot growth. In addition, parthenin caused reduction of chlorophyll content and water loss in the weedy species. The site of interaction and mechanism of action is unknown.

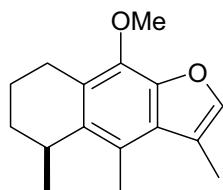
The following secondary metabolites isolated from Mexican plants: encecalin (9), and demethylenececalin (10) (Castañeda et al. 1996), cacalol (11), cacalol methyl ether (12), cacalol acetate (13), 2-acetylcacalol acetate (14) (Lotina-Hennsen et al. 1991), ivalin (19), zaluzanin C (20) isoalloalantolactone (21) (Lotina-Hennsen et al. 1992), odoratol (22),  $\alpha$ -photogedunin acetate (23),  $\beta$ -photogedunin acetate (24) (Céspedes et al. 1998), and phenylpropanoid 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene (25), methyl-ester of 3,4-dihydroxy-trans-cinnamic acid (26) has been found to act as Hill reaction inhibitors in chloroplasts because they inhibit electron flow (basal, phosphorylation and uncoupled), proton uptake and ATP synthesis. Polarographic analysis of the photosynthetic partial redox reactions indicate that, uncoupled PS I electron flow and uncoupled PS II electron transport from DPC to DCPIP of Tris washed thylakoids were insensitive to cacalol (11), ivalin (19), zaluzanin (20), doratol (22) (Achnine et al. 1998) and methyl-ester of 3,4-dihydroxy-trans-cinnamic acid (26) but they inhibited uncoupled PS II electron flow as measured from water to SiMo or DAD, and from  $\text{H}_2\text{O}$  to DCPIP at the same extent. These results indicate that the target site for these compounds is located at the water splitting enzyme.



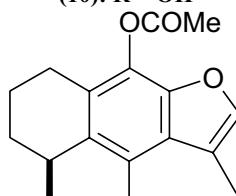
(8)

(9): R = Me  
(10): R = OH

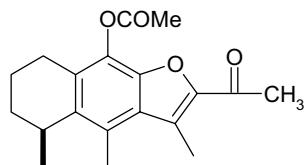
(11)



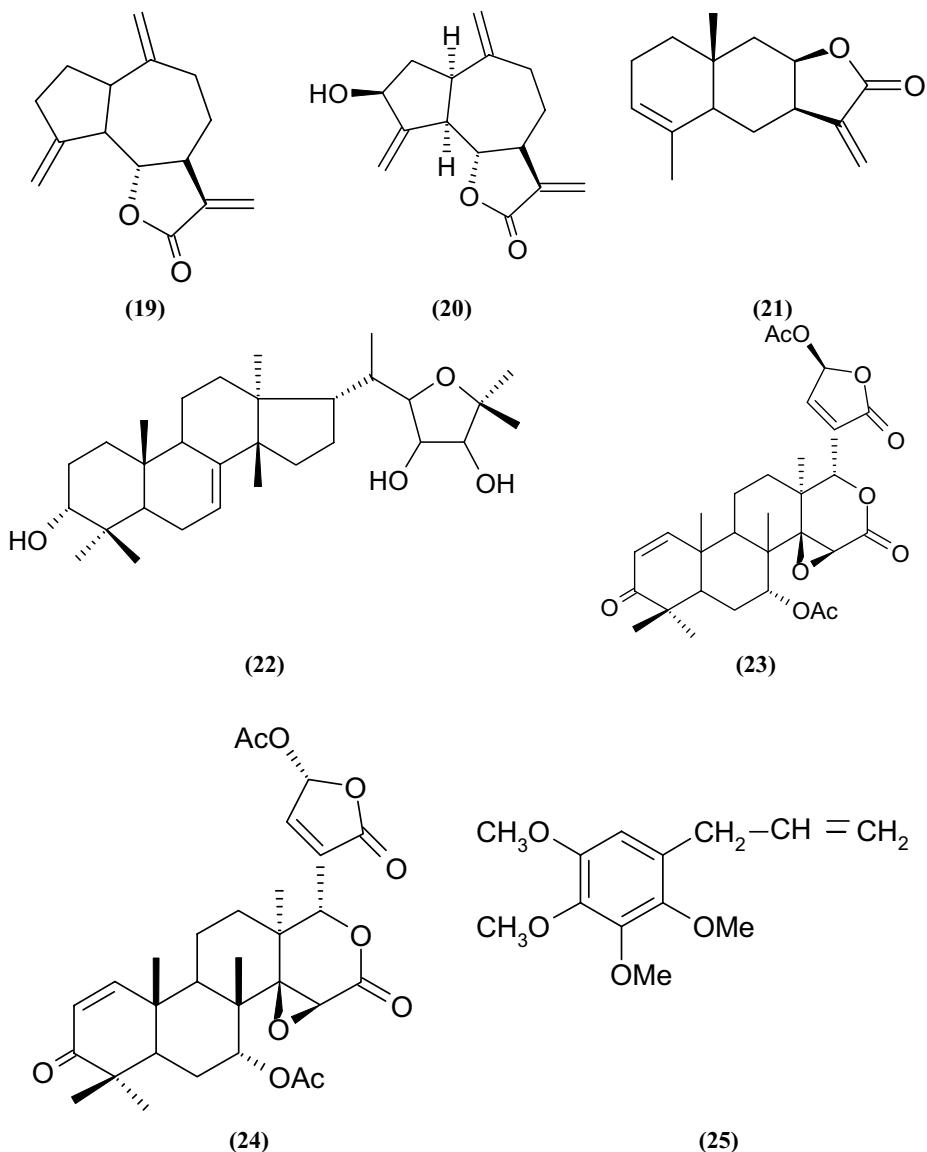
(12)

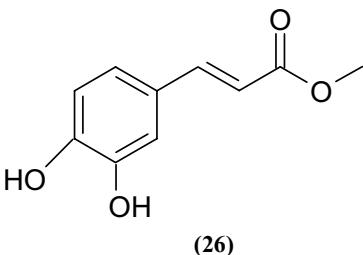


(13)



(14)



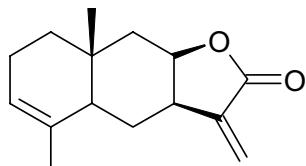


The most important findings for odoratol (22) are: (1) the natural product was found to be an inhibitor ( $150 \mu\text{M}$ ) of oxygen evolution. (2) The diol moiety at positions 23 and 24 is an important structural requirement for the inhibitory activity displayed by (22). (3) Chlorophyll a fluorescence measurements revealed a pattern similar to that resulting from hydroxylamine and Tris treatments, both well known inhibitors of the water-splitting enzyme, the presence of (22) induces the formation of a faster transient “K” between 0.8 and 1.0 ms and the formation of a quencher after 0.8 ms. These data corroborate the results obtained by polarography measurements.

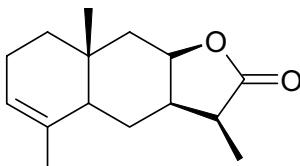
To get further evidence about the cacalol inhibition site, the redox behaviour of cacalol and its semisynthetic derivatives were studied using cyclic voltammetry. The voltammograms in a protic solvent were indicative of an electrochemically irreversible oxidation of the phenolic system. The electrooxidation of compounds 11-14 in an aprotic solvent was irreversible with similar oxidation patterns and more complex than in the protic solvent but presented similar oxidation patterns. Compounds containing an unsubstituted phenolic system presented a redox potential of  $E_{\text{pa}} = + 0.824 \text{ V}$  (V vs NHE), which resembles the potential for the water-splitting enzyme of thylakoid membrane. This result explains why cacalol inhibited the water splitting enzyme complex.

On the other hand, the site of interaction of compounds which act as Hill Reaction inhibitors, such as encecalin 9, and demethylencecalin 10 (Castañeda et al. 1998), cacalol 11, cacalol methyl ether 12, cacalol acetate 13, 2-acetylcacalol acetate 14, isoalloalantolactone 21, and 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene 25, is located in the span from  $P_{680}$  to  $Q_A$  (Jiménez et al. 1998), of the non-cyclic electron transport chain of thylakoids. Furthermore, the uncoupled electron transport from water to DCPIP (or DAD), from water to SiMo and, from DPC to DCPIP is inhibited by these compounds to the same extent. However, uncoupled PSI electron transport from  $\text{DCPIP}_{\text{red}}$  to MV is unaffected by these metabolites. The level of activity displayed by compound 25 ( $\text{IC}_{50}$  is  $2.7 \mu\text{M}$ ) is remarkable as compared to other Hill reaction inhibitors, including commercial herbicides. Therefore, this simple phenylpropanoid represents an important lead for development of new “green” herbicides agents.

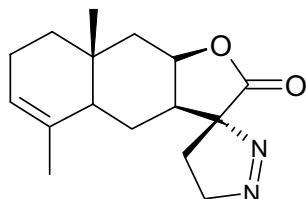
The comparison of the inhibitory effect on ATP synthesis induced by compounds 27, 28, 29, and 30 clearly indicates that the  $\Delta^{3,4}$  double bond of isoaloolalantolactone is an essential structural requirement for its inhibitory effect on photophosphorylation. However, the  $\alpha$ -methyl- $\gamma$ -lactone moiety was important but not essential for ATP synthesis inhibition.



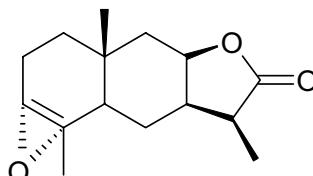
(27)



(28)



(29)



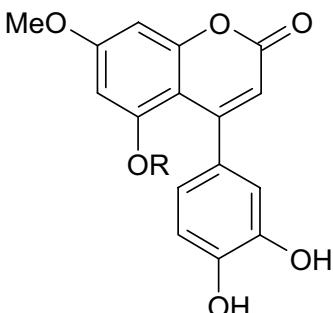
(30)

$\alpha$ -Photogedunin acetate 23, inhibits PSII electron transport from water to DCBQ without affecting PSI electron transport from DCPIP<sub>red</sub> to MV and PSII electron flow from water to SiMo. Thus, the target of  $\alpha$ -photogedunin acetate (23) is localized at Q<sub>B</sub> level. However,  $\beta$ -photogedunin acetate (24) inhibits partially, and with a similar potency of inhibition the PSI electron flow from DCPIP<sub>red</sub> to MV and from PMS<sub>red</sub> to MV. The electron flow inhibition from TMQH<sub>2</sub> to MV is similar to the inhibition of electron flow from DCPIP to MV; thus, it indicates that 24 interacts at b<sub>6</sub>f complex and in the span of P<sub>700</sub> to F<sub>x</sub>. The overall results indicate that the stereochemistry at C-23 determines the target site for interaction at the thylakoid electron transport chain. Furthermore, it is important to point out that photogedunin 23 and 24 only inhibited the redox enzymes on the thylakoid membranes when the assay media were preilluminated with actinic light during 3 minutes.

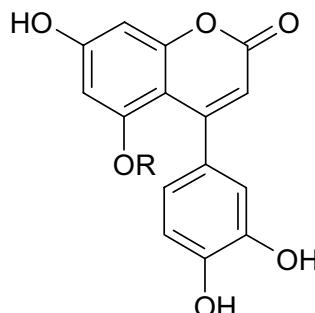
## Uncouplers

The natural 4-phenylcoumarins 31-38 (Calera et al. 1996) and some semisynthetic derivatives as well as the sesquiterpene lactones dehidrocostuslactone 39 and 240

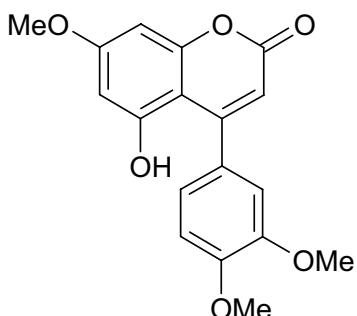
costunolide 40 acted as uncouplers in spinach chloroplasts. The glycoside 5-O- $\beta$ -D-glucopyranosyl-7-methoxy-3',4'-dihydroxy-4-phenyl-coumarin 35, 7-methoxy-5,3',4'-trihydroxy-4-phenylcoumarin 33 inhibited ATP synthesis and proton uptake. On the other hand, these compounds enhanced basal and phosphorylating electron transport. The light-activated Mg<sup>2+</sup>-ATPase was slightly stimulated by coumarins 32 and 33. In addition, at alkaline pH compound 32 stimulated the basal electron flow from water to methylviologen, but at the pH range from 6.0 to 7.5 the coumarin did not have effect. Compound 32, which possesses four free phenolic hydroxyl groups, was the most active uncoupler agent. Methylation of 36, and 37 at the phenolic groups at C-3', C-4', and C-5 resulted in a reduction or loss of the uncoupling activity. Therefore, the phenolate anions may be the active form responsible for the uncoupling behaviour of 4-phenylcoumarins.



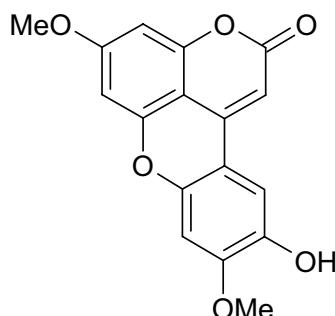
(31): R = - $\beta$ -D-galactopyranosyl  
 (33): R = H  
 (35): R = -  $\beta$ -D-glucopyranosyl



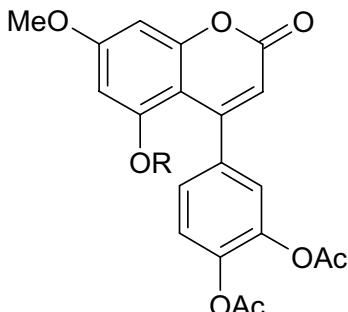
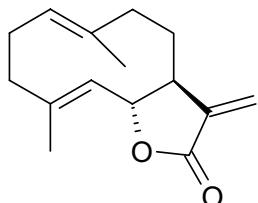
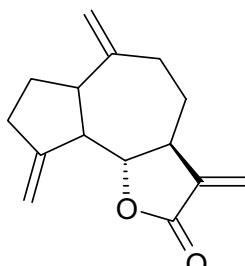
(32): R = H  
 (34): R = -  $\beta$ -D-glucopyranosyl



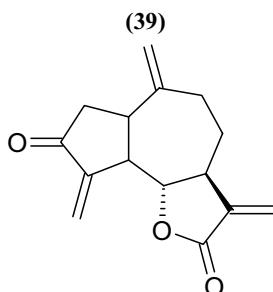
(36)



(37)

(38): R = tetra-acetyl- $\beta$ -D-galactopyranosyl

(40)



(41)

## Plasma membrane

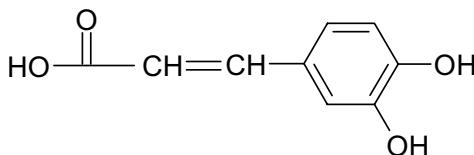
It forms the outermost boundary of the living cell and functions as active interface between the cell and its environment. In this capacity, it controls the transport of molecules into and out of the cell, transmits signals from the environment to the cell interior, participates in the synthesis and assembly of the cell wall molecules, and provides physical links between elements of the cytoskeleton and the extracellular matrix. In conjunction with specialized domains of the ER, the plasma membrane produces plasmodesmata; membrane tubes that cross cell walls and provide direct channels of communication between adjacent cells. As result of these plasmodesmal connections, almost all the living plant cells of an individual plant share a physical continuous plasma membrane.

In plant plasma membranes, the ratio of lipid classes varies remarkably among the different organs in a given plant and among identical organs in different plants. Barley root cell plasma membranes contain more than twice as many free sterol molecules as phospholipids. In barley leaf plasma membranes, the phospholipids-to-free sterol ratio is 1:3:1, whereas in spinach it is 9:1.

Most plasma membrane proteins involved in transmembrane activities such as transport and signalling, the anchoring of cytoskeletal elements to cell wall molecules, and the assembly of cellulose fibrils from cytosolic substrates are of the integral type. However, those proteins often form larger complexes with peripheral proteins. The extracellular domains of many integral proteins are glycosylated, bearing N-and O-linked oligosaccharides.

The plasma membrane H<sup>+</sup>-ATPase (P-type H<sup>+</sup>-ATPase) is the principal primary active transport system of plant cells. For example, two plant cell systems regulate the uptake of K<sup>+</sup>, which is required for growth and osmoregulation. One system corresponds to a low-affinity K<sup>+</sup>-uptake channel and a second to a high-affinity, H<sup>+</sup>-gradient-dependent K<sup>+</sup>-uptake carrier. Dehydrozaluzanin C (41) is a sesquiterpenol, with a guaiane skeleton that has been isolated from the roots of many different compositae families. It inhibits root growth and germination of plants. The I<sub>50</sub> value for lettuce root growth is 500 µM (Galindo et al. 1999). (41) Causes also rapid plasma membrane leakage in cucumber cotyledon discs. It is more active at 50 µM. Notice that the concentration needed for this activity is 10X less (41) symptoms include plasmolysis and disruption of membrane integrity, is not light dependent. Its effects on roots were reversible through the addition of various aminoacids, with histidine and glycine providing ca 40% reversion. However, a strong reversal effect was obtained with reduced glutathione, as a result of gross-reactivity with (41), it has no-effect on photosynthesis, respiration, mitotic processes and NADH oxidase activity. Thus, (41) exerts its effects on plants through at least two different mechanisms, only one which is related to the disruption of plasma membrane function. On which plasma membrane function acts dehydrozaluzanin C, still needs to be defined. Maybe a competition experiment in plasma membrane leakage with aminoacids vs (41) will give some light. Plant cell plasma membranes also contain proteins known as aquaporins, which form water channels. The leafy spurge (*Euphorbia esula*) displaces many native plant species, it has been demonstrated that small everlasting (*Antennaria microphylla*), a native perennial, is allelopathic to leafy spurge. Isolation of allelochemicals from small everlasting yields hydroquinone, a simple phenolic compound, arbutin, the monoglucoside of hydroquinone and caffeic acid (42), a phenolic acid. Both hydroquinone and (42) are inhibitory to leafy spurge seed germination, root elongation and callus culture growth. Results indicate that inhibition of growth in leafy spurge after exposure with (42) for 12 days had significantly higher leaf diffusive resistances than control plants. Transpiration was similarly affected with treated plants showing higher transpiration rates compared to controls. Chlorophyll fluorescence was significantly lower in treated plants than controls. The partial closure of the stomates of leafy spurge treated with 250 µM (42), the stable carbon isotope ratio (<sup>13</sup>C/<sup>12</sup>C) was higher than controls. These data show that a disruption of plant water relations is the primary mechanism of plant growth inhibition. The water channel aquaporin, is a suggestive target for caffeic acid.

The identification and physiological characterization of several plasma membrane receptors that bind hormones, oligosaccharides, peptides, and toxins are present at low concentrations, and thus are difficult to study. Therefore, many researchers have adapted a genetic approach to identifying and isolating receptor-protein genes.



(42)

Transport is made possible by membrane-spanning proteins within the lipid bilayer. The transport systems can be regarded as conventional enzymes in almost all respect, with the important exception that transport events are vectorial (i.e. defined by a magnitude and a direction in space). Like enzymes, all transport systems exhibit some degree of substrate-specificity and work by lowering the activation energy required for transport. In plants cells, membrane transport underpins a wide range of essential processes like:

Turgor generation. The presence of a cell wall in the vast majority of plant cells enable them to generate turgor (positive pressure). The cell wall provides structural rigidity. Turgor generation is accomplished by accumulating salts. In the mature cells of most plants,  $K^+$  accumulates in the cytoplasm and in the large central vacuole. The cations must be balanced by a corresponding concentration of anions to achieve electroneutrality; in the vacuole, the principal anion is  $Cl^-$  or malate.

Plants synthesize organic biomolecules from inorganic nutrients. These must be absorbed from the soil by roots for assimilation into amino acids and other metabolites, i.e.  $NH_4^+$ ,  $NO_3^-$ ,  $H_2PO_4^-$ ,  $SO_4^{2-}$ , trace elements such as: boron, zinc, copper, and iron, and each probably requires a specific transport system. The metabolism generates waste products that must be removed from the cytosol, i.e.  $H^+$ ,  $OH^-$  produced during assimilation of  $HCO_3^-$  and  $NO_3^-$  into organic compounds. To expel  $H^+$  from the cytosol, plants have evolved proton pumps at both the vacuolar and plasma membranes. Compartmentalization of metabolites in the cell enhances metabolic efficiency. In the mitochondrial matrix the ADP/ATP and NADH/NAD $^+$  ratio are greater than in the cytosol, this is possible thanks to specific carrier or transporters and membrane compartmentalization, which provides a substrate/product ratio that favors respiratory activity, the electron flow coupled to

ADP phosphorylation, and O<sub>2</sub> reduction to H<sub>2</sub>O. Allelochemicals may interact with one of these processes.

## Energy transduction

Membrane transport lies at the heart of the conversion of free energy into biologically useful forms. Light energy stimulates the photosynthetic electron transport chain to pump H<sup>+</sup> into the thylakoid lumen. Similarly oxidation of NADH by mitochondria provides the energy for pumping H<sup>+</sup> from the matrix into the intermembrane space. In each case, the spontaneous exergonic flow of H<sup>+</sup> back across the membrane is used to generate ATP. Understanding the mechanisms by which light and high-energy electrons are harnessed to phosphorylate ADP therefore demands knowledge of membrane transport. The energy production is a primary target for allelochemicals.

Natural occurring Annonaceous acetogenins (*ACG*) are present in the seeds of commercially-exploited tropical fruits such as ‘guanabana’ (*Annona muricata*) and ‘cherimoya’ (*Annona cherimolia*). *ACG* have also potent activity as insecticides, acaricides, fungicides, antiparasitics, herbicides and inhibitor to tumor cell growth. These compounds belong to a wide group of natural products isolated from several species of the Annonaceae family, which include more than 250 molecules with diverse chemical structures (Zafra-Polo et al. 1996a, 1996b). The mode of action of the *ACG*, the main targets are in the mitochondrial NADH: ubiquinone oxidoreductase, also known as respiratory complex I of mitochondria (Zafra-Polo et al. 1996a, 1996b; Esposti et al. 1994).

Complex I transfers electrons from NADH to ubiquinone and links this process to the translocation of protons across membrane generating an electrochemical gradient that drives the synthesis of ATP. Inhibition of complex I opens an interesting perspective for the development of a new generation of antitumor drugs with this particular mode of action.

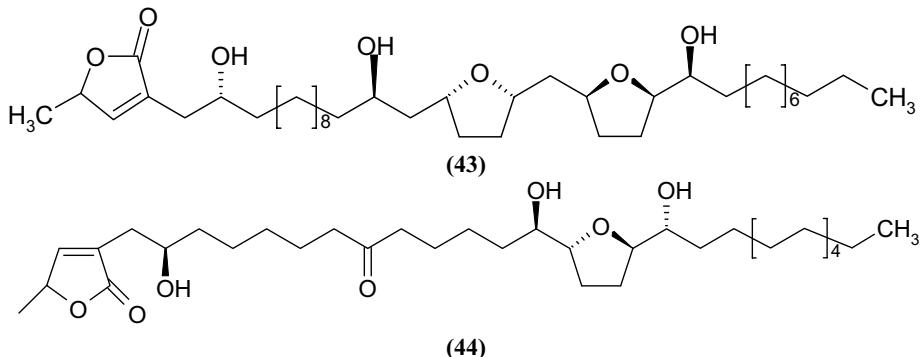
Most studies with *ACG* are done by determination of their cytotoxicity for several human-tumor cell lines (Oberlies et al. 1995, 1997). Nevertheless, growth inhibition of tumor cell cultures depends on many additional factors other than the mode of action of the molecule, such as the intracellular distribution and the diffusion across biological membranes. Studies on the target enzyme are required to better understand the mode of action of these molecules.

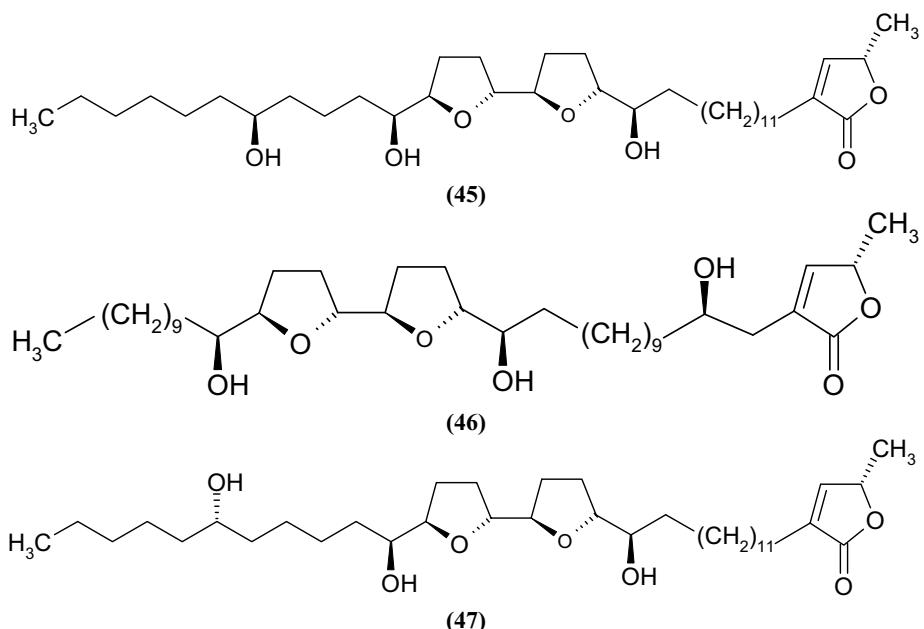
Tormo et al. (1999) established the structure-activity relationship (SAR) of Annonaceous acetogenins of the mono-tetrahydrofuranic (mono-THF) type, to perform the inhibitory studies on mitochondrial NADH: ubiquinone oxidoreductase, and NADH oxidase activities using complex I of submitochondrial particles (SMP).

The six mono-THF acetogenins present a common skeleton of 32 C with one  $\alpha$ - $\alpha'$ -dihydroxylated THF system and one unsaturated methyl- $\gamma$ -lactone moiety. They only differ by the number of hydroxyl and carbonyl groups at the aliphatic chain that links the lactone and the THF.

The results show that electron transfer inhibition, when complex I operates in physiological conditions (NADH oxidase activity) is in the same range than the most potent bis-THF ACG. However, they were less effective to inhibit the NADH: ubiquinone oxidoreductase activity measured with decylubiquinone as artificial electron acceptor. All mono-THF acetogenins showed values between those of rolliniastatins-I (43) (0.6 nM) and rotenone (5.1nM) against the aerobic oxidation of the NADH. Moreover, annonacinone (44) (Morré et al. 1995) gave an inhibitory potency very close to that of rolliniastatin-I, an ACG that belongs to the subclass of bis-THF with *threo/cis/threo/cis/erythro* relative configuration.

All six mono-THF acetogenins act as noncompetitive inhibitors confirming this displacement. Most ACG belong to this functional type of inhibitors (Zafra-Polo et al. 1996a, 1996b; Miyoshi et al.1998), but only a few acetogenins show an uncompetitive pattern (Esposti et al. 1994; Estornell et al. 1997). However, all Annonaceous acetogenins have not been studied their role on plant mitochondria, as well as, in chloroplasts, with the exception of Chávez et al. (2001), found that the bis-tetrahydrofuran Annonaceous acetogenins (squamocin (45), bullatacin (46) and motrilin (47)) behave as uncoupler-Hill reaction inhibitors and at higher concentrations these compounds disrupt the interactions between the antenna complexes and interact with PSII.





## Signal transduction

Many biotic and abiotic signals for plant growth and development trigger their respective responses by transiently increasing the concentration of cytosolic free  $\text{Ca}^{2+}$ , carried out by  $\text{Ca}^{2+}$  translocating ATPases (remove  $\text{Ca}^{2+}$  from the cytosol by pumping it across the plasma membrane and intracellular membranes) and  $\text{Ca}^{2+}$ -permeable channels (open in response to particular stimuli and allow the passive entry of  $\text{Ca}^{2+}$  into the cytosol), thereby propagating the signal.

## Organization of transport at plant membranes

Protons ( $\text{H}^+$ ) constitute one of the major energy currencies of the plant cell, on a par with NAD(P)H and ATP. At the inner mitochondrial membrane and at the thylakoid membranes, a transmembrane  $\text{H}^+$  potential is generated and used to energize the synthesis of ATP.

At all other membranes in the cell,  $\text{H}^+$ -pumps hydrolyze ATP to power the transport of protons out of the cytosol, establishing electrochemical potentials across

these membranes as well. The resulting transmembrane H<sup>+</sup> potentials are then used to power the transport of other ions and solutes across the membranes. Typically the cytosol pH is 7.5, the apoplastic pH is near 5.5, and the membrane potential difference is on the order of -150 mV. Under these particular conditions, the resulting pmf would be -268 mV. Values ranging from -200 to -300 mV are common in plant cells.

Active transport, results in the accumulation of a solute on one side of a membrane. It is thermodynamically unfavourable (endergonic), and occurs only when coupled (directly or indirectly) to an exergonic process such as the absorption of sunlight, an oxidation reaction, the breakdown of ATP or the concomitant flow of some other chemical species down its concentration gradient.

## Carriers

Specific symporters and antiporters. These are transport systems that couple the down hill (exergonic) flow of ions such as H<sup>+</sup> or Na<sup>+</sup> to the uphill (endergonic) flow of inorganic ions and solutes are called carriers.

Transporters catalyzing solute flux in the same direction as H<sup>+</sup> or Na<sup>+</sup> flux are known as symporters. Carriers also undergo conformational changes during transport. There are no long-range interactions with soluble substrates and turnover rates are greater than those of pumps, typically being about 10<sup>3</sup> s<sup>-1</sup>.

Excretion from the cytosol can be accomplished by antiporters, which exchange solutes for protons. Both symporters and antiporters tend to dissipate the pmf, and this energy is conserved in the form of an electrochemical potential for particular solutes.

## Pumps

They undergo long-range and complex conformational transition(s) that extend across large polypeptides or assemblies of polypeptides. These movements couple the metabolic reactions to those involving transport. The turnover rate of pumps is 10<sup>2</sup> molecules transported per second. Pumps have slow turnover number and generate the pmf for an array of symporter and antiporters. A square micrometer of membrane may include several hundred to several thousand pump proteins, while it typically contains only 1 to 10 channel proteins. The rapid turnover rates and diffuse distribution of channels facilitate electrophysiological analysis of single channel molecules. The *proton pumps* at the vacuolar and plasma membranes are electrogenic. They create electrical current because the ions they remove from the cytosol carry charge. Therefore, this H<sup>+</sup>-pumping ATPase not only contributes directly to the chemical

component of the pmf, the  $\Delta\text{pH}$ , but also tends to make the electrical component,  $V_m$  more negative.

## **Ion channels for $\text{K}^+$ , $\text{Ca}^{2+}$ and anions**

The net direction of ion flux through a channel is determined solely by the electrochemical force acting on that ion. Channels, in contrast to pumps and carriers, do not undergo conformational changes during transport and can catalyze ion fluxes of  $10^6$  to  $10^8 \text{ s}^{-1}$ . The rate of  $\text{Na}^+$  movement through the acetylcholine receptor ion-channel is linear with respect to the extracellular  $\text{Na}^+$  concentration; the process is not saturable in the way that transporter-catalyze.

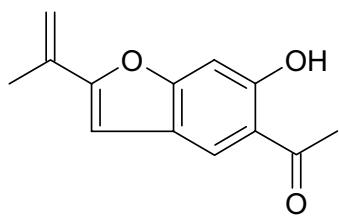
## **F-type $\text{H}^+$ -pumping ATPases are found in plants at the inner mitochondria and thylakoid membranes that synthesize ATP**

These two kinds of membranes contain proton-pumping electron transport chains driven by the redox potential and by light energy respectively. The pmf established by the electron transport chain serves to drive  $\text{H}^+$  flow back through the F-type ATPase, thereby resulting in ATP synthesis. One sector of a F-type ATPase called  $F_0$  in plant mitochondria and  $CF_0$  in plant chloroplasts, transverses the membrane and forms an  $\text{H}^+$ -conduit. The other sector of the enzyme called  $F_1$  in mitochondria and  $CF_1$  in chloroplasts, readily dissociates from the transmembrane sector, contains adenine nucleotide-binding sites and can hydrolyze ATP *in vitro*. The flow of  $\text{H}^+$  through  $F_0$  drives long-range conformational transitions in  $F_1$  that result in the synthesis of ATP. Crystallographic studies of the  $F_1$  sector  $\alpha_3\beta_3\gamma$  complex (located primarily on the  $\beta$  subunits), show three distinct conformations. At any given time, one binding site is in an open conformation, another is binding a nucleotide loosely, and the third is binding a nucleotide tightly, the conformational model for proton-driven ATP synthesis, which postulates that ATP is synthesized by F-type ATPase through a process of rotational catalysis (Lores Arnaiz, 1998). According to the conformational model, ADP and inorganic phosphate first bind to the open nucleotide-binding sites. Proton flow through the  $F_0$  sector of the enzymes causes the central  $F_1$  subunit ( $\gamma$ ) to rotate, altering the conformation of all three nucleotide-binding sites. The tight binding site opens and releases ATP into the aqueous medium, the open site is converted to a loose site, and the loose binding site forms a tight pocket in which ATP is formed spontaneously. In net terms, a total of three or perhaps four protons are admitted through the  $F_0$  sector for each ATP synthesized.

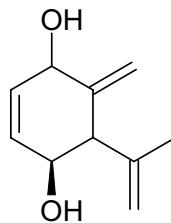
## Energy transfer inhibitors

Here it was found that the F-Type H<sup>+</sup>-pumping ATPase from chloroplast is a good target of interaction for allelochemicals, thus the following secondary metabolites Euparin 48, piquerol A 49 (Méndoza et al. 1994), dehidrocostus lactone 50, costunolide 51, 7-oxo-7-deacetoxygedunin 52, gedunin 53, and 5-O-β-D-galactopyranosyl-7-methoxy-3', 4'-dihydroxy-4-phenylcoumarin 31 behave as energy transfer inhibitors, that is, F-Type H<sup>+</sup>-pumping ATPase inhibitors. All these compounds inhibited the Mg<sup>2+</sup>-ATPase activity of the bound membrane thylakoids, as do energy transfer inhibitors. The target was located at CF<sub>0</sub> in the case of 52, 53 and 35, because the proton uptake was re-established as the concentration of the inhibitor was increased in the assay medium, when the assay was done in CF<sub>1</sub> washed thylakoids. In the case of the tetrnortriterpenoid 52 it was found that the ketone group at C-7 was an important structural requirement for the inhibitory activity exerted on the enzyme H<sup>+</sup>-ATPase, since 53 possessing a 7α-acetoxy group was a less potent inhibitor to photophosphorylation.

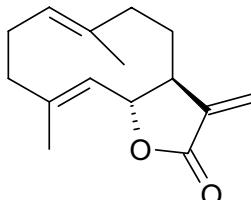
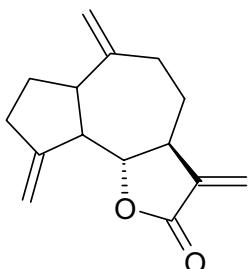
Comparison of the type of activity displayed by the limonoids 22, 23 and 24, revealed that the nature of the heterocyclic ring at C-17 determines the mechanism and the target of this type of compounds. In the case of gedunin type, which possesses a furan ring at C-17, was observed to behave as energy transfer inhibitor. However 23 and 24 possessing a ketal ring at the same position behave as Hill reaction inhibitors.

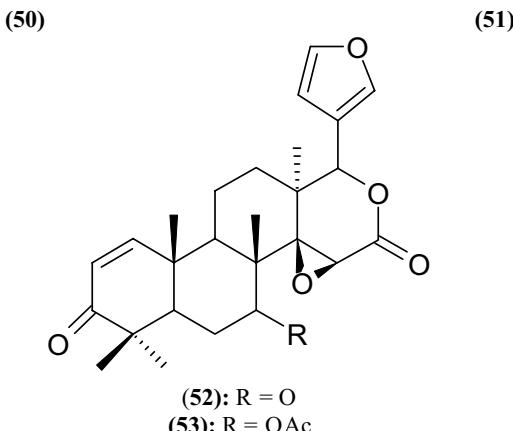


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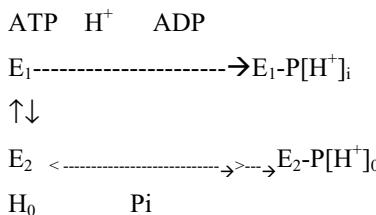




### P-type ATPase, the plasma membrane H<sup>+</sup>-pumping ATPase

It is a single polypeptide of about 100 KD that both binds ATP and catalyses H<sup>+</sup> transport, it is encoded by 10 genes in arabidopsis and tobacco. Isoforms of P-type H<sup>+</sup> ATPase appear to serve distinct function and may be expressed differentially in specific tissues and during particular phases of development.

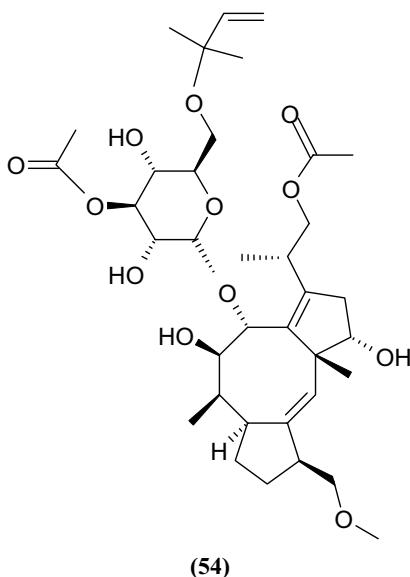
The pmf generated by the plasma membrane P-type ATPase powers transport through a variety of carriers; it also influences ion channel activity through its impact on V<sub>m</sub>. The resulting acidification of the cell wall in turn activates expansins, proteins that loosen H<sup>+</sup>-bonds within the wall and allow turgor-generated growth. It also produces an electrochemical gradient across the plasma membrane. Electrochemical gradient consists of an electrical potential (*inside negative*) and a concentration gradient (*a pH gradient outside acidic*). Many metabolic pathways result in the net production of H<sup>+</sup>, some of which must be removed to prevent acidification of the cytosol. This is done by the plasma membrane H<sup>+</sup>-ATPase. The plasma membrane ATPase pumps a single H<sup>+</sup> out of the cell for each MgATP hydrolyzed. During hydrolysis, the  $\gamma$ -phosphate of ATP becomes transiently but covalently bound to an aspartyl residue on the enzyme, forming an acyl-phosphate. Hydrolysis of this acyl-phosphate bond provides the driving force for the reaction cycle, in which different conformations of the enzyme, known as the E<sub>1</sub> and E<sub>2</sub> states, expose the H<sup>+</sup>-binding site to alternate sides of the membrane.



The conformational change that exposes the bound H<sup>+</sup> to the apoplast is linked to additional conformational transitions that lower binding-site affinity, permitting H<sup>+</sup> dissociation.

This family of P-type enzymes includes the fungal plasma membrane H<sup>+</sup>-ATPase, the Na<sup>+</sup>/K<sup>+</sup>-exchanging ATPase ubiquitous in animal cell plasma membranes, Ca<sup>2+</sup>-ATPases of the plasma membranes of animals and plants, and the H<sup>+</sup>/K<sup>+</sup>-exchanging ATPase of mammalian gastric mucosa.

All P-type ATPases are inhibited by orthovanadate (H<sub>2</sub>VO<sub>4</sub>), which forms an analog of the E-P transition state and blocks the reaction cycle. In tomato and tobacco there are tissue specific H<sup>+</sup>-ATPases, for example AHA3 is expressed selectively in phloem companion cells, the micropyles and the developing seed funiculus. In contrast, AHA10 is expressed mainly in developing seeds, especially in the tegument surrounding the embryo. From expression studies in yeast mutants that lack native plasma membrane H<sup>+</sup>-ATPase activity, it is known that AHA1 and AHA2 have Km values for ATP of 0.15 mM, whereas the Km of AHA3 is 10-fold higher. Sensitivities to orthovanadate also differ. Regulation of plasma membrane H<sup>+</sup>-ATPase is mainly an activation in response to lowered cytosolic pH. More profound perhaps, is the impact of the C-terminus cytosolic region of the enzyme, which forms an auto inhibitory domain and whose function has been identified by either tryptic cleavage or genetic modifications that activate the H<sup>+</sup>-ATPase considerably. A striking twist to C-terminal H<sup>+</sup>-ATPase inhibition has been observed with fusicoccin (54) (an inhibitor produced by *Fusicoccum amygdali*). Fusicoccin stimulates plasma membrane H<sup>+</sup>-ATPase, in a whole range of plant cell types. Fusicoccin and trypsin-induced activation of the plasma membrane H<sup>+</sup>-ATPase exhibit similar kinetic characteristics and are non additive, indicating that fusicoccin may activate the enzyme by relieving C-terminal auto inhibition. A separate mechanism of activation of H<sup>+</sup>-ATPase is associated with auxin-induced stimulation of proton pumping. In this case, auxin up-regulates expression of the pump. Induction of H<sup>+</sup>-ATPase expression correlates with the auxin-responsiveness of proton pumping in the tissue.



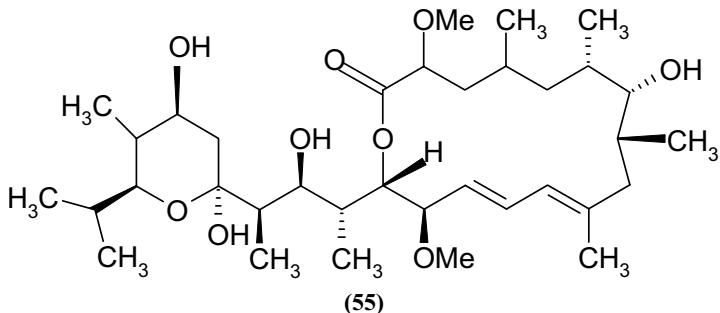
## Calcium-pumping ATPases

They are distributed in the plasma membrane, the ER, the chloroplast envelope, and vacuolar membranes. These enzymes pump  $\text{Ca}^{2+}$  out of the cytosol, thereby maintaining the cytosolic concentration of free  $\text{Ca}^{2+}$  at about 0.2  $\mu\text{M}$ . This low concentration of free  $\text{Ca}^{2+}$  has to be maintained in cells in order to prevent precipitation of phosphates. In eukaryotes it has become a base on which to build stimulus-response coupling pathways. All  $\text{Ca}^{2+}$ -ATPases are P-type ion-motive ATPases. *Arabidopsis*, has an ER-type  $\text{Ca}^{2+}$ -ATPase. In plasma membranes,  $\text{Ca}^{2+}$ -ATPase activity is enhanced by calmodulin. One of the  $\text{Ca}^{2+}$ -ATPases has been associated with the chloroplast inner envelope membrane; another resides in the vacuolar membrane. The vacuolar  $\text{Ca}^{2+}$ -ATPase has a calmodulin-binding domain, present as an extension at the N terminus, as for example the plant endomembrane "PM-type"  $\text{Ca}^{2+}$ -ATPases. The cytosol negative membrane potential opposes the export of divalent cations with double the effective force imposed on monovalent cations. The free energy of the  $\text{Ca}^{2+}$  electrochemical potential difference across the plasma membrane, roughly  $-60 \text{ KJ mol}^{-1}$ , may actually exceed the free energy input available from ATP hydrolysis ( $-50 \text{ KJ mol}^{-1}$ ). The enzyme also catalyzes  $\text{Ca}^{2+}/\text{H}^+$  exchange.

## Vacuolar and other membranes are energized through vacuolar H<sup>+</sup>-ATPases, a V-type H<sup>+</sup>-ATPase

Plant vacuoles contain a highly acidic solution, with a pH near 5.5, about 2 to 3 units lower than that of the cytosol. Proton pumping into the vacuolar lumen not only energizes the membrane carrier-mediated transport but also generates the low pH of the vacuole, whereas protease, glucosidases, phosphatases and nucleotidases with acidic pH optima reside. Proton pumping is catalyzed by vacuolar type (V-type) H<sup>+</sup>-ATPases. The sequence analysis has demonstrated that these enzymes are distant relatives of F-type H<sup>+</sup>-ATPases. V-type ATPase operates solely in the direction of ATP hydrolysis. The ratio of H<sup>+</sup> translocated per ATP hydrolyzed has been measured as 2 or below when luminal pH is low. The V-type enzyme can be separated into a soluble V<sub>1</sub> sector, analogues to F<sub>1</sub> and including the adenine nucleotide-binding sites, and a membrane-bound V<sub>0</sub> sector, analogous to F<sub>0</sub> and composing the H<sup>+</sup> pathway through the membrane. V-type H<sup>+</sup>-ATPases are specifically inhibited by the macrolite antibiotic baflomycin A<sub>1</sub> (55). It's a hydrophobic compound produced by Streptomyces and interacts with the V<sub>0</sub> sector of the enzyme. An acidic luminal pH of all cellular organelles except mitochondria and chloroplast, contribute to vesicle sorting and hence to membrane trafficking and protein targeting. Therefore the V-Type H<sup>+</sup>-ATPase could be a good candidate for allelochemicals interactions.

H<sup>+</sup>-pumping inorganic pyrophosphatase (H<sup>+</sup>-PPase) ubiquitous at the vacuolar membrane of plants. Could be also a good candidate for allelochemical interactions.



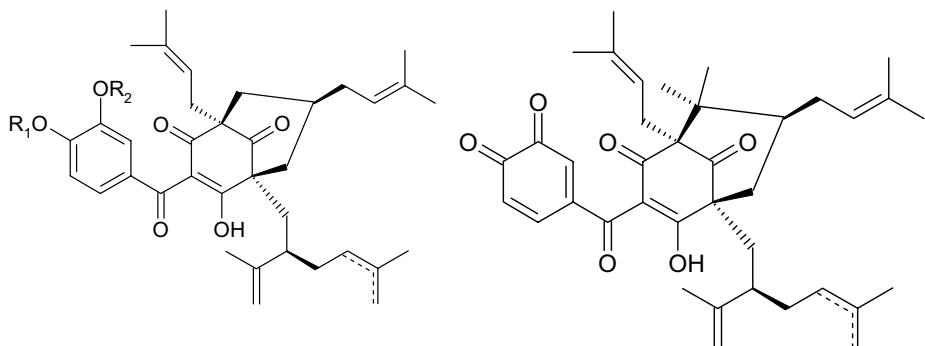
## Tubulin/microtubule system

It is involved in many biological phenomena, such as formation of the mitotic spindle, constitution of the cell cytoskeleton, and axonal transport. Therefore, any substance able to interact with this system represents a potential inhibitor of cell replication. This is a promising target to search for anticancer agents from the plant kingdom.

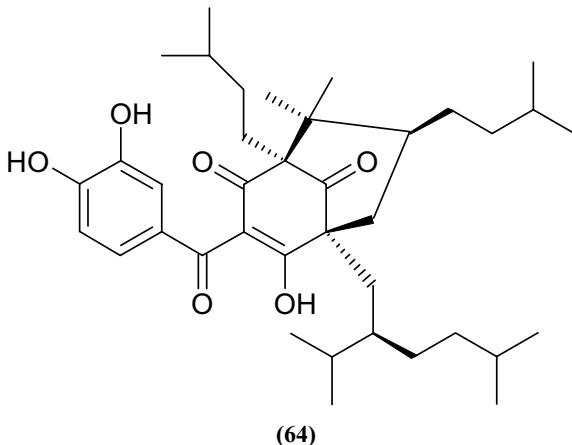
Roux et al. (2000), have found the microtubule disassembly inhibitory properties of the frown polyisoprenylated benzophenones xanthochymol (56) and guttiferone E (57), isolated from the fruits of *Garcinia pyrifera*. Furthermore, observation by electron microscopy of the microtubules assembled in the presence of mixture 1 (56+57) and cooled to 0°C exhibited a classical pattern for microtubules; this last observation is contrary to that shown by paclitaxel, that in the same condition did not promote assembly of tubulin at 0°C. The IC<sub>50</sub> value was 2 µM for mixture 1. A struture-activity relationship study, found that etherification of the enol by methylation or cyclization led to a complete loss of activity on tubulin. The same is true if both hydroxyls at C-13 and C-14 are methylated or oxidized (58 and 59). However, some activity is preserved if only one of the hydroxyl groups at C-13 or C-14 is methylated (compound 60 and 61 cited in Roux et al. (2000), ethylated (62) or glycosylated (63). Hydrogenation of the double bonds (compound 64) also led to a total loss of activity. Therefore, the catechol and enol portions of the molecule are not the entire pharmacophore responsible for the biological activity, the lipophilic domain having the unsaturated prenyl chains is also essential because the octahydro derivative 64 is not active, although the catechol and enol parts are not modified.

However, the IC<sub>50</sub> values for the cell growth inhibitor (for the cytotoxicity on KB cells) were similar for all the compounds studied. Therefore, it appears that cytotoxicity is probably not related to the interaction of the products with tubulin inside the cell. The tubulin/microtubule system assembly or disassembly is the preferred target for anticancer allelochemicals.

Xanthine oxidase (XO) catalyzes the formation of uric acid from the purines hypoxanthine and xanthine, and is responsible for the medical condition known as gout. Gout is caused by the deposition of uric acid in the joints leading to painful inflammation. Inhibititon of XO leads to a remission in gout (Chiang et al. 1994). XO also serves as an important biological source of oxygen-derived free radicals that contribute to oxidative damage to living tissues that are involved in many pathological processes such as inflammation, atherosclerosis, cancer and aging (Chiang et al. 1994; Cos et al. 1998). Therefore, in-vitro bioassays are used to examine test materials for XO inhibition, as inhibitors of XO may be potentially useful for the treatment of gout or other XO-induced diseases (Goodman and Gilman, 1990).

(56) : R<sub>1</sub> = R<sub>2</sub> = H, Δ<sup>36</sup>(57) : R<sub>1</sub> = R<sub>2</sub> = H, Δ<sup>35</sup>+(58) : R<sub>1</sub> = R<sub>2</sub> = Me(60) : R<sub>1</sub> = Me, R<sub>2</sub> = H(61) : R<sub>1</sub> = H, R<sub>2</sub> = Me(62) : R<sub>1</sub> = Et, R<sub>2</sub> = H(63) : R<sub>1</sub> = β-D-Glc, R<sub>2</sub> = H

(59)



(64)

Sweeney et al. (2001), studied extracts from Australians plants and assayed them for inhibition of the enzyme xanthine oxidase. The tree species of *Clerodendrum floribundum* R. Br., *Eremophila maculata* (Ker Gawler) F. Muell and *Stemodia grossa*

Benth all exhibited IC<sub>50</sub> values less than 50 µg/mL. The most active plant species examined was *Clerodendrum floribundum* R. Br, extract E3 with an IC<sub>50</sub> of 6.00 µg/mL. This active plant may contain bioactive natural products useful in the treatment of gout or other xanthine oxidase induced diseases. The target of these unknown allelochemicals seems to be XO.

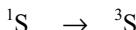
## SINGLET OXYGEN AND FREE RADICALS PROCESSES IN PLANTS

Singlet oxygen (<sup>1</sup>O<sub>2</sub>), a potential product of photochemical reactions of many allelochemicals and chloroplasts, is a damaging agent to all living organisms. How is the formation and control of <sup>1</sup>O<sub>2</sub> within the plants? Does it possibly have a role in plant defense?

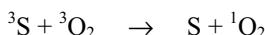
The ground state of molecular oxygen has two unpaired electrons with parallel spin. The triplet state is rare in ground state molecules and because electrons occupying the same orbital must have opposed spin (the Pauli exclusion principle), the reaction of ground state oxygen with most substances is restricted. The activation of oxygen involves overcoming this spin restriction to reactions. Reduction of <sup>1</sup>O<sub>2</sub> leads to the potentially toxic superoxide anion, hydrogen peroxide and the hydroxyl radical. Electronic excitation of oxygen molecules, involving spin inversion, results in excited states with an unopposed spin, designated as singlet states <sup>1</sup>O<sub>2</sub>. Two excited singlet states of oxygen occur. However, the first singlet state (<sup>1</sup>Δg) excitation energy of 0,98 eV, its lifetime is long enough to allow chemical reaction. Therefore, the first excited singlet state of molecular oxygen is the one involved in certain photooxidative, photodynamic and biological processes. The major mechanism of <sup>1</sup>O<sub>2</sub> formation in biological systems is by energy transfer from photoexcited compounds. The absorption of a photon by such sensitizer results in an excited singlet state, with a very short lifetime (10<sup>-6</sup> – 10<sup>-8</sup> sec):



which by intersystem crossing, involving spin inversion, may be relaxed to a longer lived triplet state (Ca 10<sup>-3</sup> sec):



Molecular oxygen in a non spin restricted reaction, can quench such a triplet state by energy transfer resulting in <sup>1</sup>O<sub>2</sub> and the regeneration of the ground state sensitizer



The relatively low excitation energy of its first excited singlet state allows oxygen to quench the triplet states of a variety of compounds. Photosensitizing compounds, capable of the efficient population of triplet states, one of diverse origin and structure

and are active in regions of the electromagnetic spectrum ranging from the near UV, through visible to the near infra-red (Knox and Dodge, 1985). Singlet oxygen is responsible for type II photodynamic reactions of exogenous and endogenous sensitizers in biological systems, although direct reaction of the excited sensitizer with a substrate (a type I reaction) can occur (Knox and Dodge, 1985).

Singlet oxygen is a metastable entity of  ${}^1\text{O}_2$  that varies from 2 to 4  $\mu\text{sec}$  in  $\text{H}_2\text{O}$  to 25-100  $\mu\text{sec}$  in non-polar solvents (cited in Knox and Dodge, 1985). Therefore,  ${}^1\text{O}_2$  potentially is a damaging agent in membrane where it is generated by allelochemicals. Evidence has been presented indicating the increased lifetime of  ${}^1\text{O}_2$  in the hydrophobic interior of the membrane relative to the aqueous environment of the cell. Although  ${}^1\text{O}_2$  has no spin restriction to reaction, it is not indiscriminantly reactive. Its electrophilic nature results in chemical reaction with compounds with heavily substituted double bonds or an electron-rich functionality. In addition,  ${}^1\text{O}_2$  may be physically quenched in its ground state by a variety of substances. As a consequence of this selectivity these are certain biomolecules that are cellular targets for action of  ${}^1\text{O}_2$ . Specific aminoacids, most notably histidine, methionine and tryptophan, are susceptible to oxidation by  ${}^1\text{O}_2$  with the possible consequence of enzyme inactivation. Membrane destruction, a common feature of photodynamic reactions, is due to lipid peroxidation initiated by the formation of hydroperoxides from the reaction of  ${}^1\text{O}_2$  with unsaturated fatty acids. Of the nucleic acid, guanine is particularly sensitive to attack by  ${}^1\text{O}_2$ . others classes of compounds may quench as well as react with  ${}^1\text{O}_2$  for examples phenol,  $\alpha$ -tocopherol (vit E), carotenoids and certain amines. Ascorbate acts as an effective quencher of superoxide (Knox and Dodge, 1985), hydroxyl radical and  ${}^1\text{O}_2$ . These compounds protect the cell for the  ${}^1\text{O}_2$  damaging.

Singlet oxygen and secondary plant substances. Certain defensive allelochemicals are capable of photosensitizing reactions that involve the transfer of light energy to oxygen, such as  ${}^1\text{O}_2$ , which is used for their own defense. Other secondary plant products (Knox and Dodge, 1985) may have a physiological role in that they protect the plant against damaging photodynamic reactions by quenching the excited singlet state of oxygen. Here, some of the secondary metabolites involved in  ${}^1\text{O}_2$  generations will be described.

## QUINONES

The hypericins, occurring in the genus *Hypericum* (St John's Wort) are responsible for the photosensitization occurring when grazing animals ingest these plants. The condition of intense skin irritation is known as hypericism. *Hypericum* is contained in specialized trichome glands, presumably as a protection against auto toxicity, located on flowers, stems and leaves. Evidence has been presented that hypericin, isolated from the calyx of *Hypericum hirsutum*, is capable of the generation

of  ${}^1\text{O}_2$  and hence lipid peroxidation. The photodynamic reactions of hypericin are promoted by visible light, predominantly in the region of 500-600 nm. Hypericin (65) has been shown to be phototoxic to the larvae of *Aedes atropalpus* mosquito. Fagopyrin (66), a hypericin derivative, occurs in the flowers of buck wheat (*Fagopyrin esculentum*), and gives rise to a photogenic condition in animal herbivores that is comparable to hypericin. Cercosporin (67) a fungal toxin, is produced by a member of the genus *Cercospora*, which includes fungi that are responsible for leaf spot diseases of a wide range of plants of economic importance. The photodynamic action of this toxin results in lipid peroxidation and membrane damage that resembles that of the pathogen. Evidence indicates that (67) is capable of photochemical generation of  ${}^1\text{O}_2$  by this secondary metabolite.

Furanocoumarins (psoralen) (68), characteristic of the Rutaceae and Apiaceae, possess photosensitizing activity in mammalian skin. This photosensitizing activity arises from their ability to photobind to pyrimidine bases of DNA, resulting in crosslinks, when irradiated by long wavelength UV light [320-400 nm]; an activity that does not involve molecular oxygen. However, it is apparent that furanocoumarins are also capable of photodynamic reaction involving oxygen. They are found in all parts of the plants and are generally located in oil gland and ducts, seed coats and leaf surface wax.

Interestingly, angular furanocoumarins although capable of binding to DNA are unable to induce crosslink but do appear to be more efficient generators of  ${}^1\text{O}_2$  than linear furanocoumarins.

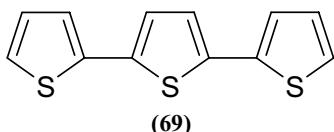
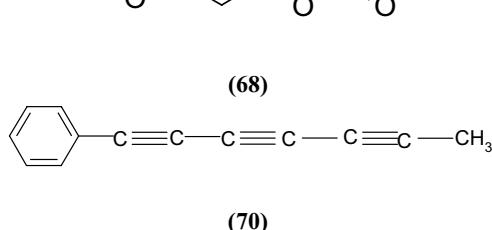
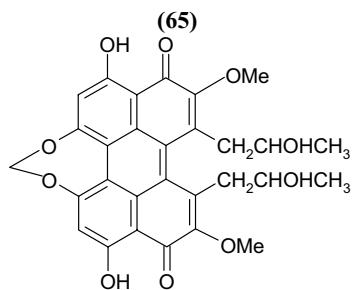
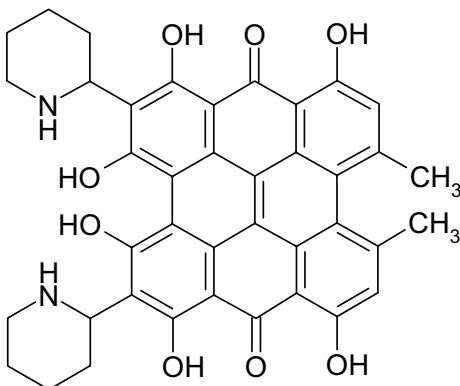
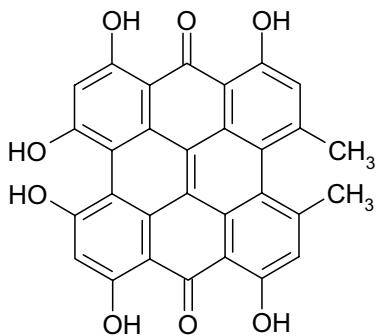
The polyacetylenes and their thiophene derivatives are a diverse class of compounds occurring predominantly in the Compositae. These compounds are also activated by near UV light (320-400 nm), but differ to furanocoumarins in that they are not capable of interacting with DNA. Polycetylenes are found in all parts of the plants including the cuticle but are frequently restricted to specific organs such as the roots.

Isolation of  $\alpha$ -Terthienyl (69) is isolated from the roots of marginol (*Tagetes*), its nematocidal activity was demonstrated to be greatly enhanced by light and  $\alpha$ -terthienyl is efficient generator of  ${}^1\text{O}_2$  and the primary mechanism of action is the photodynamic disruption of membranes. Its phytotoxicity to microorganisms, insects and plants has been demonstrated. Therefore, maybe it is an allelopathic agent with an herbicidal potential.

Phenylheptatriyne (70) found in the leaves of *Bidens pilosa*, is the most studied polyacetylene, and also displays phototoxic action and antifeedant activity.

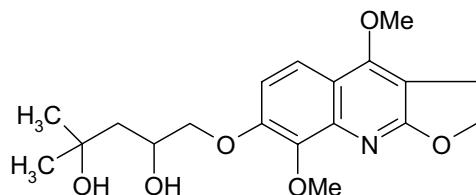
In contrast, photosensitization involving straight chain and ring-stabilized polyacetylenes such as (70) appears to be predominantly non-photodynamic under anaerobic conditions. The biological activity of these secondary metabolites against a

wide range of organisms suggests that they may have a protective role within the plant, especially against insect predators.

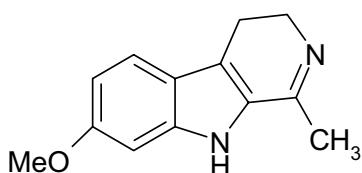


Furanoquinoline alkaloids such as dictamine (71) (Rutaceae) are phototoxic against microorganisms and mosquito larvae. Several  $\beta$ -carboline alkaloids such as harmaline (72) and isoquinoline (73) and alkaloids such as bereberine (74) are also phototoxic. Benzofurans and chromenes, from species of the genus *Encelia* are capable of yielding phototoxic reactions. Photoactivation by UV light of a range of isoflavanoid phytoalexins has been reported and appears to involve free radicals with

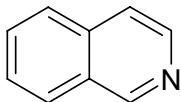
$^1\text{O}_2$ . The production of  $^1\text{O}_2$  during the autooxidation of tannins has been proposed to have a protective role by their fungistatic and deterrent effects.



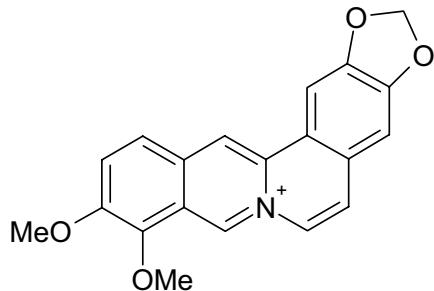
(71)



(72)



(73)



(74)

Secondary metabolites from plants of diverse biogenetic origin are capable of the photogeneration of  $^1\text{O}_2$  suggesting the widespread use of this potent toxic agent as protective and defensive, and may be a potential herbicide. It is also possible that  $^1\text{O}_2$  damage involves many plant stress conditions such as high light, limited water, presence of herbicides. The specificity of the allelochemicals that generate  $^1\text{O}_2$  is required in order to avoid an unspecific phytotoxicity.

## ACKNOWLEDGMENT

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## CHAPTER 12

# MITOCHONDRIA AS A SITE OF ALLELOCHEMICAL ACTION

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## INTRODUCTION

Seed germination is a critical step in the propagation and cultivation of most crop species. Several environmental factors, including light, temperature, oxygen and chemicals, are able to regulate both the capacity and the germination rate. The successful completion of germination depends, on the other hand, on fundamental cellular activities such as the transport of water, ions and other solutes, protein and RNA synthesis and respiration. The latter phenomenon produces ATP, which is a reactant in many cellular processes. An interference with ATP production is, thus, extensive to the ATP-requiring activities and it will impair seed germination and plant growth. It is generally accepted that mitochondrial oxidative phosphorylation provides ATP during the first hours of imbibition and for the subsequent steps of germination (Morohashi and Suguimoto, 1988). Mitochondria can be considered, thus, as a potential site of allelochemical action. The question if mitochondria are the primary or the secondary site of action of allelochemicals can only be answered if the effects of these compounds are fully characterized and quantified. In this chapter we describe our experience in the evaluation of the effects of some allelochemicals on the respiratory activity of isolated mitochondria and intact tissues from seedlings.

## Experimental measurements of respiration

The study of the mitochondrial functions has gained great impulse after it was possible to measure, in a continuous way, variations in oxygen concentration of a medium containing mitochondria or submitochondrial particles. This became possible thanks to a polarographic device which is generally called “oxygen electrode” (Chance and Williams, 1955). The most frequently used oxygen electrode is the Clark type electrode, which consists in a platinum cathode separated from the silver anode by an electrolyte solution (generally KCl). The platinum electrode is polarized to approximately 0.7 volt. Oxygen is reduced on the platinum surface, generating a small electric current in the direction of the silver cathode. Within certain limits this electric current is proportional to the oxygen concentration at the surface of the platinum electrode. In practical terms the combined platinum-silver electrode is inserted into a device made of glass or plexiglass consisting in two concentric chambers. In the internal chamber the mitochondrial suspension or other materials are placed in an appropriate reaction medium. The external chamber is used to circulate thermostated water in order to control the temperature. The electrode is positioned in such a way that its terminal, coated with a Teflon membrane, is in contact with the incubation system. The internal chamber is closed and the homogeneous distribution of oxygen in the medium is assured by magnetic stirring (Bracht et al. 2003). This equipment allows measuring oxygen consumption by mitochondria, submitochondrial particles, isolated cells, tissue pieces and microorganisms.

Although the oxygen electrode measures only the rate of a single reaction, i.e., the transfer of electrons to oxygen, informations about several mitochondrial processes can be obtained by modifying the incubation conditions so that a particular process becomes the limiting step of respiration. By changing the kind and the concentrations of substrates, co-factors and by adding specific inhibitors, different processes can be evaluated. These include: activities of components and segments of the respiratory chain, ATP-synthase activity, proton permeability of the membrane, adenine-nucleotide transport across the membrane, several dehydrogenase activities and transport of substrates across the membrane (Nicholls and Ferguson, 1992).

The measurement of respiration with plant tissues is a relatively easy task. When tissue slices are added to the incubation system they respire at the expense of endogenous substrates, mainly carbohydrates. In such a system, evidently, several factors can control respiration. These factors are the size of the sugar reserves, the rate of sugar import from the reserves and the activities of several metabolic pathways, such as glycolysis, the pentose monophosphate pathway and the glyoxylate cycle, which provide NADH and succinate to the respiratory chain. Moreover, although the mitochondrial respiration represents almost the total tissue respiration, other oxygen

consumption reactions can be contributing. This contribution varies from tissue to tissue and includes mainly photorespiration, peroxidases and lipoxygenases. The obvious advantage of measuring the mitochondrial oxidative activity in intact tissues is that it is strictly adjusted to the *in vivo* conditions and needs. However, the complexity of plant intact tissues is likely to present a serious obstacle for a correct interpretation. For this reason, in the efforts of finding out if the mitochondrial respiratory chain is or not a physiologically relevant site of allelochemical action, it is desirable to perform experiments with both isolated mitochondria and intact tissues.

## **Isolation of mitochondria**

Mitochondria can be isolated from a diversity of plant materials, including leaves, potato tubers, bean hypocotyls, endosperm and embryo. The procedures generally employed are based on the classical method of Bonner (1967). Fresh materials are cut into a chilled medium containing basically an osmoticum for the maintenance of their structure such as mannitol, sucrose or sorbitol, a buffer system (pH 7.0 –7.5) and substances such as ethylene diamine tetraacetate (EDTA), cysteine and defatted serum albumin. The preparation of a mitochondrial fraction displaying biochemical and physiological integrity is not easy. The crucial point in the whole procedure is the grinding of the material. The forces and the times used in the grinding procedure must be optimized for each material in order to avoid extensive rupture of the mitochondrial membranes. This in turn leads to a low yield of mitochondria, which can be as low as 1 to 3% of the total tissue mitochondria. This is a serious difficulty because, in order to produce an adequate amount of mitochondrial suspension, a large volume of fresh material is required. Nearly 500 maize seedlings (3 day-old) are required, for example, to obtain a considerable amount of good quality mitochondria from primary roots. Another difficulty is the contamination of preparations by extra-mitochondrial components released during the grinding procedure. A wide variety of harmful substances is released, particularly vacuolar contents and the products of specific enzymes. Free fatty acids such as linolenic and linoleic acids released by the action of acyl hydrolases are the most problematic contaminants in mitochondrial preparations from plants (Douce, 1985; Loomis, 1974). The strategies used to minimize the effects of these substances can be the following: a) maintenance of a low ratio of tissue/extraction medium; b) quick adjustment of pH after grinding; c) rapid separation of the organelles from soluble components of the cellular brei; d) addition of anti-oxidant substances such as cysteine and ascorbate; e) addition of defatted serum albumin (0.1-1% w/v) which binds effectively anions and lipids; f) addition of EDTA, which inhibits the activity of  $\text{Ca}^{2+}$ -dependent lipases. After tissue disruption, the pH of the homogenate is rapidly adjusted to 7.2 and after an initial low-speed centrifugation (nearly 1000g), the mitochondria are sedimented at higher centrifugal forces (10000–20000g). The sediment is then washed by repeated suspension and sedimentation. The

mitochondrial preparation resulting from these procedures is termed washed mitochondria.

A washed mitochondria preparation consists of mitochondria with a high degree of intactness and functionality. However, depending on the kind of tissue, considerable amounts of other subcellular organelles, such as microbodies, amyloplasts, thylakoids and peroxisomes as well as soluble enzymes (vacuolar proteinases, lipolytic enzymes, acyl hydrolases, catalases and lipoxygenases) contaminate the preparations (Bligny and Douce 1977). Besides the rapid deterioration of the mitochondrial membranes due to the actions of these enzymes, the presence of enzymes catalyzing oxygen-consuming reactions such as lipoxygenases and catalases is particularly critical for measurements of the respiratory activity. Further purification of the mitochondrial suspensions is, thus, an indispensable procedure.

The purification by centrifugation on a sucrose gradient has been applied to various sources of mitochondria including potato tubers, bean hypocotyls and pea leaves (Douce et al. 1972). However, the introduction of nontoxic silica-sol (Percoll) has provided a faster procedure with several additional advantages over the sucrose density gradient method. Isosmotic and low-viscosity conditions can be used and the passage of washed mitochondria through a Percoll density gradient removes extramitochondrial membranes as well as contaminating enzymes (Neuburger et al. 1982). A continuous self-generated gradient or a discontinuous gradient (Jackson et al. 1979) can be employed. The advantage of the discontinuous gradient is that a lower centrifugal force is required, only 7000g against 40000g for the continuous gradient. A high-speed centrifuge is, thus, not required. We have been using successfully the Percoll discontinuous gradient to purify mitochondria from embryo and seedling tissues (Sert et al. 1998; Takahashi et al. 1998; Abraham et al. 2000, 2003a,b).

## Interference of allelochemicals with mitochondrial respiration

Allelochemicals from phenolic acids, quinones, coumarins, terpenoids and flavonoids have been shown to affect the respiratory activity of intact plant tissues and isolated mitochondria. The potency of the action of allelochemicals on isolated mitochondria has been shown to be amply variable. However, the comparison is not precise due to the different experimental conditions used by the various investigators. Besides the different tissues and species from which the mitochondria were isolated, different media and substrates were used. In general terms, however, it can be concluded that the quinones sorgoleone and juglone are the most active compounds, being effective in isolated mitochondria at concentrations near to 1.0  $\mu\text{M}$  (Koeppe, 1972; Hejl et al. 1993; Rasmussen et al. 1992). Flavonoids are active at the concentration range of 20 to 1000  $\mu\text{M}$  (Stenlid, 1970; Ravanel, 1986; Ravanel et al. 1981; 1986; 1990; Takahashi et al. 1998) and the phenolic acids affect mitochondrial metabolism at relatively high 270

concentrations (millimolar range) (Van Sumere et al. 1972; Demos et al. 1975; Sert et al. 1998; Abraham et al. 2003b). The potency of the monoterpenes, on the other hand, has been shown to vary within two orders of magnitude (50-5000 µM) (Abraham et al. 2000, 2003a). The mechanisms of action of allelochemicals have demonstrated to be also amply variable and most of them have multiple sites of action. Figure 1 illustrates the sites of action of some allelochemicals within mitochondria. Sorgoleone has been shown to inhibit mitochondrial electron flow at the b-c<sub>1</sub> complex (Rasmussen et al. 1992) and juglone induces alternate pathways to oxygen reduction (Hejl et al. 1993). Studies performed with several flavonoids have demonstrated an uncoupling activity (Ravanel, 1986; Ravanel et al. 1986; Takahashi et al. 1998), inhibition of oxidative phosphorylation (Koeppe and Miller, 1974), inhibition of electron flow at the level of complexes I (Ravanel et al. 1981) and III (Takahashi et al. 1998), inhibition of phosphate uptake (Takahashi et al. 1998) and inhibition of the activity of exogenous NADH-dehydrogenase (Ravanel et al. 1986; 1990). Phenolic acids have also been shown to interfere with the mitochondrial metabolism at different levels, including inhibition of electron flow, inhibition of phosphate and Ca<sup>2+</sup> transport (Demos et al. 1975) and inhibition of L-malate oxidation (Sert et al. 1998). Salicylic acid stimulates the ADP-limited electron transport by acting as uncoupler at low concentrations (< 1.0 mM). At higher concentrations, however, it inhibits the electron flow from the dehydrogenases and the ubiquinone pools (Norman et al. 2004). Abraham et al. (2000, 2003a) demonstrated that monoterpenes stimulate respiration and impair the respiratory control suggesting that they act as uncouplers.

Table 1 allows comparing potency and mode of action of some allelochemicals from a variety of classes on mitochondrial respiratory parameters. The data were obtained from experiments performed in our laboratory with purified mitochondria isolated from maize (*Zea mays* L.) root and/or soybean (*Glycine max* L. Merril) hypocotyl axes (Sert et al. 1998; Takahashi et al. 1998; Abraham et al. 2000, 2003a,b). In these experiments a mixture of substrates (L-malate, succinate, L-glutamate and NADH) was used. It is believed that in the presence of these substrates, the total oxidative capacity of the mitochondria is attained (Day et al. 1988).

### Actions of allelochemicals on intact tissue respiration

Whether the phytotoxic effects on the whole plant are due to a direct action on mitochondria still remains unclear for most allelochemicals. The adverse effects on mitochondria depend on the access of the allelochemical to the mitochondria, which, in turn, depends on the permeability of the plant cell membrane to the compound as well as on its possible chemical transformations within the cells. For most allelochemicals data on these issues are lacking. They are important, however, because it is evidently possible that the compound never attains those concentrations

within the cell at which the compound is active on isolated mitochondria. This occurs possibly with those allelochemicals which cause adverse effects on isolated mitochondria only at relatively high concentrations (millimolar range), such as ferulic, vanillic and coumaric acids, caffeine, rutin and the monoterpenes camphor, eucalyptol and limonene. It should be considered, however, that the tissue concentrations may be higher than those ones in the medium as it was recently demonstrated for salicylic acid (Norman et al. 2004). Salicylic acid accumulates into the cultured tobacco cells up to 6- or 10-fold the externally applied concentration.

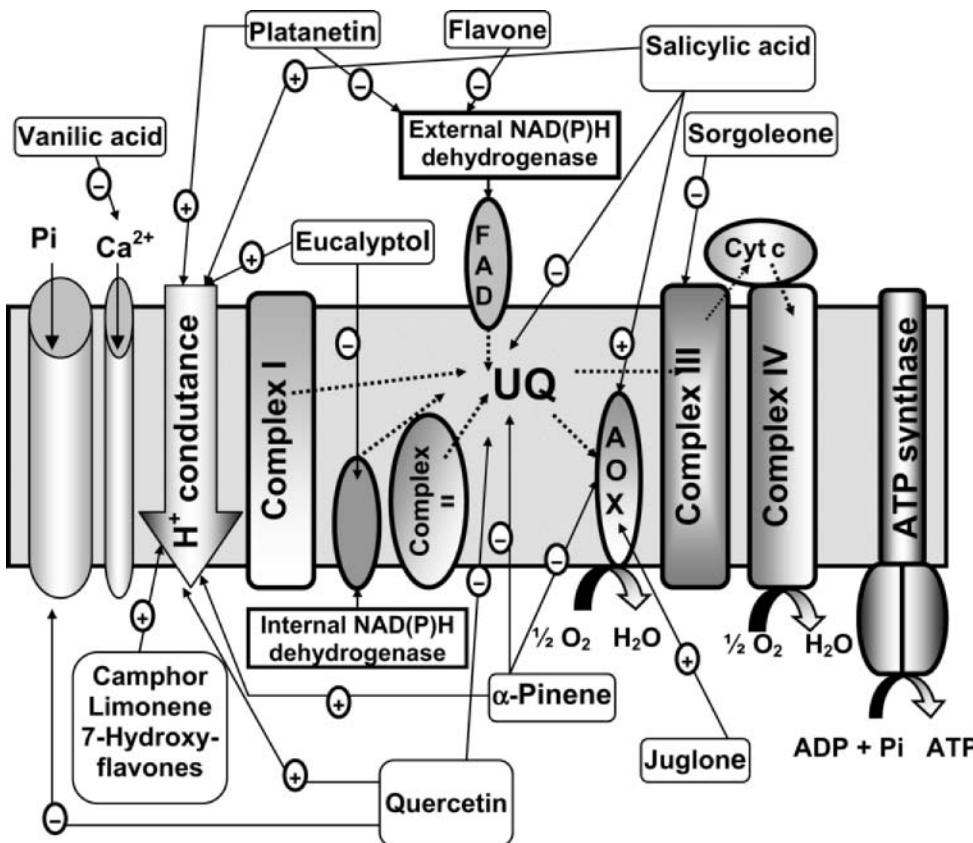


Figure 1. Main sites of action of some allelochemicals within the plant mitochondria. Activation is indicated as + and inhibition as -.

Analysis of the respiration of tissues grown in the presence of allelochemicals may help to provide the information if the adverse effects on mitochondria are or not manifesting in intact tissues. Changes in the respiration of intact tissues are, however, more difficult to interpret. Besides the influence of extramitochondrial processes the respiration of intact plants is often cyanide-resistant, an indication that the mitochondrial alternative oxidase (AOX) is present (Bendall and Bonner, 1971). The extent to which changes in the respiratory rates alter the efficiency of ATP synthesis depends,

*Table 1.* Effects of some allelochemicals on respiratory parameters of isolated mitochondria. Respiratory activity was measured in the presence of a mixture of substrates (L-malate, succinate, L-glutamate and NADH). Unless indicated, the effects were observed in mitochondria isolated from both maize root and soybean hypocotyl axes. The maximal concentration assayed was 10.0 mM for all compounds.

Compound	Altered parameter	Active concentration ( $\mu\text{M}$ )
Coumaric acid	Reduction of respiratory control <sup>a</sup>	$\geq 5000$
Ferulic acid	—	NS
Vanilic acid	—	NS
Camphor	Stimulation of state IV respiration	> 5000
Eucalyptol	Stimulation of state IV respiration	$\geq 5000$
Limonene	Stimulation of state IV respiration and Inhibition of state III respiration	$\geq 1000$
$\alpha$ -Pinene	Stimulation of state IV respiration and Inhibition of state III respiration	$\geq 50$
Caffeine	—	NS <sup>a</sup>
Coumarin	Reduction of ADP/O ratio <sup>a</sup>	$\geq 5000$
Quercetin	Stimulation of state IV respiration Inhibition of FCCP-uncoupled respiration and of NADH- and succinate-oxidase	> 500
Rutin	—	NS
Flavone	Inhibition of state III and IV respiration	> 250

<sup>a</sup> = maize root mitochondria; NS = non significant.

thus, on the partition of the electron flux between the cytochrome oxidase pathway (COX) and the alternative pathway of electron transport. Although the role of AOX in thermogenic plants is well-characterized (Meeuse, 1975), in non-thermogenic plants it has not yet been well defined. AOX is thought to allow carbon flux through the citric acid cycle when ADP is limiting, thereby providing carbon skeletons for other cellular processes or minimizing the production of reactive oxygen species (ROS) by the respiratory chain (Wagner and Moore, 1977). The partition between COX and AOX in

whole plant tissues can be estimated by using the cytochrome pathway inhibitor potassium cyanide (KCN) and an alternative oxidase inhibitor salicylhydroxamic acid (SHAM). Recently, an  $^{18}\text{O}$ -discrimination method has been used to measure the alternative oxidase in vivo under steady-state conditions (Robinson et al. 1995). This method requires, however, a highly complex apparatus.

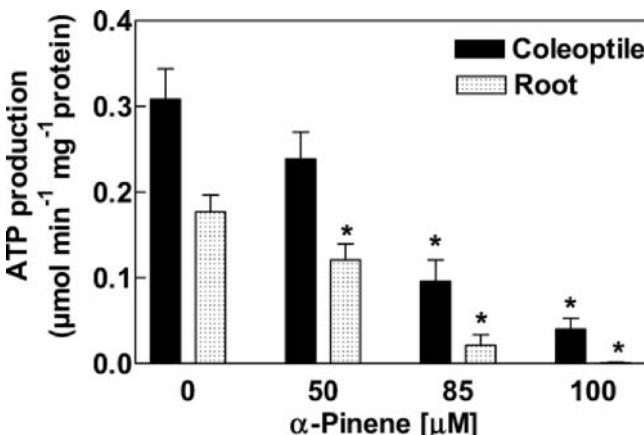
Due to the influence of these different processes on respiration in intact tissues, inconsistencies are frequently found when comparing the effects in isolated mitochondria and intact tissues. For juglone, for example, stimulation of respiration has been observed in isolated mitochondria, whereas inhibition of respiration of leaves and excised roots from several plant species has been reported (Perry, 1967; Koeppe, 1972; Neave and Dawson, 1989; Hejl et al. 1993; Jose and Gillespie, 1998). Salicylic acid causes stimulation or inhibition of the tobacco whole cell respiration depending on the concentration at which it is applied (Norman et al. 2004). The uncoupling action and the inhibition of electron flux observed in isolated mitochondria could be an explanation for these effects, but salicylic acid has also been shown to stimulate the alternative pathway respiration (Rhoads and McIntosh, 1993), an action which is probably influencing the rate of respiration in the intact tissue.

### **The action of the monoterpene $\alpha$ -pinene on the respiration of isolated mitochondria and intact tissues**

In an attempt to examine how the action on mitochondria can affect the respiratory activity in intact tissues and its possible significance for seedling growth, we have performed an integrated study with the monoterpene  $\alpha$ -pinene, the most active compound among all allelochemicals previously assayed in our laboratory (Table 1). Alfa-pinene is one of the major components of essential oils of several aromatic species, including the major forest tree species of the United States (Geron et al. 2000) and of Mediterranean plants (Llusia and Peñuelas, 2000). It may account for approximately 50% of the total monoterpene emission from many areas (Geron et al. 2000). The interaction of  $\alpha$ -pinene with plants of several species is, thus, a highly probable event in nature. Evidence that monoterpenes perturb plant respiration had already been presented by Muller et al. (1968, 1969). These authors have demonstrated that the exposure of *Cucumis sativus* to emanations from *Salvia leucophylla* leaves diminishes oxygen uptake of seedlings and excised roots.

We have found that  $\alpha$ -pinene causes severe impairment of the ATP production capacity of mitochondria isolated from maize seedlings (Figure 2). The ID<sub>50</sub> is  $80.2 \pm 5.5$  and  $62.9 \pm 5.4 \mu\text{M}$  in mitochondria from coleoptile and roots, respectively.

The changes caused by  $\alpha$ -pinene in the respiratory parameters support the conclusion that uncoupling of oxidative phosphorylation and inhibition of electron



*Figure 2.* Effects of  $\alpha$ -pinene on ATP synthesis by mitochondria isolated from coleoptiles and primary roots of maize. Mitochondria ( $0.2 \text{ mg protein ml}^{-1}$ ) were incubated at  $25^\circ\text{C}$  in reaction medium containing  $10 \text{ mM L-malate}$ ,  $10 \text{ mM L-glutamate}$ ,  $300 \mu\text{M}$  thiamine pyrophosphate ( $\text{TPP}^+$ ) and  $200 \mu\text{M NAD}^+$ . The reaction was initiated by the addition of  $5 \text{ mM ADP}$  and stopped after  $10 \text{ min}$  by the addition of  $0.3 \text{ M perchloric acid}$ . The content of ATP in the neutralized extract was determined using the hexokinase and glucose-6 phosphate dehydrogenase method (Lamprecht and Trautschold, 1974). Each data point is the mean  $\pm$  S.E.M. obtained with 5-6 mitochondrial preparations. \* $p \leq 0.05$ , ANOVA with Duncan's multiple range test.

transfer are the primary causes for the ATP production impairment. Alfa-pinene in the concentration range of  $50$  to  $500 \mu\text{M}$  exerts similar effects on oxygen consumption irrespective of the source of mitochondria (maize coleoptile or primary root) or of the substrate (L-malate, succinate or NADH). As illustrated by a typical experimental result in Figure 3, at least two kinetic phases in the dose-response curves can be distinguished (Abrahim et al. 2003a). At concentrations lower than  $250 \mu\text{M}$ ,  $\alpha$ -pinene stimulates respiration in state IV and inhibits respiration in state III. At concentrations higher than  $250 \mu\text{M}$ , the effects of  $\alpha$ -pinene on both state IV and state III respiration are inhibitory. At concentrations higher than  $100 \mu\text{M}$  a complete suppression of the respiratory control ratio is evident and in the presence of  $50 \mu\text{M}$   $\alpha$ -pinene a tendency toward lower values of the ADP/O ratios is apparent (inserted graph in Figure 3). The uncoupling effect is also supported by the decrease caused by  $\alpha$ -pinene on the transmembrane potential as revealed by changes in safranine binding to energized mitochondria (Abrahim et al. 2003a).

Inhibition of electron transfer is suggested by the observation that in the presence of the uncoupler carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone (FCCP),  $\alpha$ -pinene causes only inhibition of respiration (Figure 3). Since it was demonstrated that

$\alpha$ -pinene does not affect the activities of L-malate dehydrogenase and succinate dehydrogenase (Abrahim et al. 2003a) and that it inhibits oxygen consumption irrespective of the substrate, it is highly probable that it acts on electron transfer at a specific point between the quinone pool and oxygen.

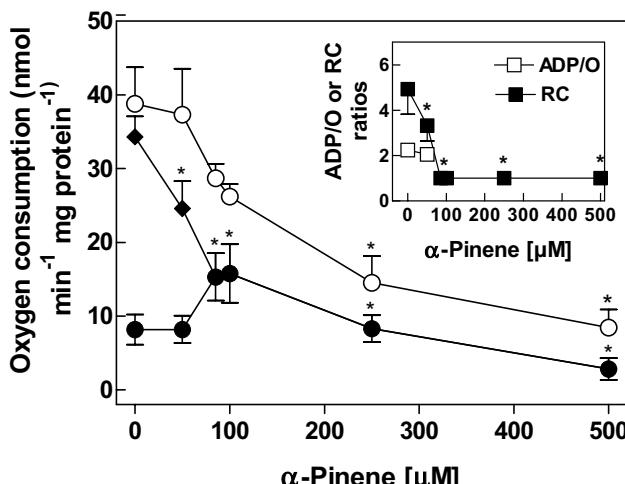


Figure 3. Effects of  $\alpha$ -pinene on the respiratory activity of mitochondria isolated from primary roots<sup>S</sup> of maize. Mitochondria from primary roots (0.24–0.64 mg protein) were incubated at 25°C in reaction medium. The reaction was initiated by the addition of 10 mM L-malate and the oxygen consumption was followed polarographically over approximately 5 min. After this time 150 nmol of ADP were added. ADP addition was repeated twice. Each data point is the mean  $\pm$  S.E.M. obtained with 4–6 mitochondrial preparations. (◆—◆), rates of oxygen consumption in the presence of 100  $\mu$ M ADP (state III respiration, second ADP addition); (●—●), rates of oxygen consumption in the absence of ADP (state IV respiration, after the second ADP addition); (○—○) rates of oxygen consumption in the presence of 20  $\mu$ M FCCP. \*p < 0.05, ANOVA with Duncan's multiple range test.

If the actions observed in isolated mitochondria also occur in intact tissues, one can expect changes in tissue respiration as well as impairment of growth and development of the plant.  $\alpha$ -pinene indeed affects maize seedlings growth as it has been revealed by experiments in which 3-days-old seedlings were grown in liquid nutrition medium in the absence and in the presence of 250  $\mu$ M  $\alpha$ -pinene for 6, 12, 24 or 48 h. As shown in Figure 4, the root length is reduced as soon as 6 h after treatment and a reduction in the fresh weight of the primary roots and coleoptiles appears at 24 h of treatment. The root length is reduced by approximately 60% at 6 h of treatment, 87% inhibition occurring at 48 h. The maximal inhibition, about 30%, has been found in the fresh weight of primary roots and coleoptiles at 48 h of treatment.

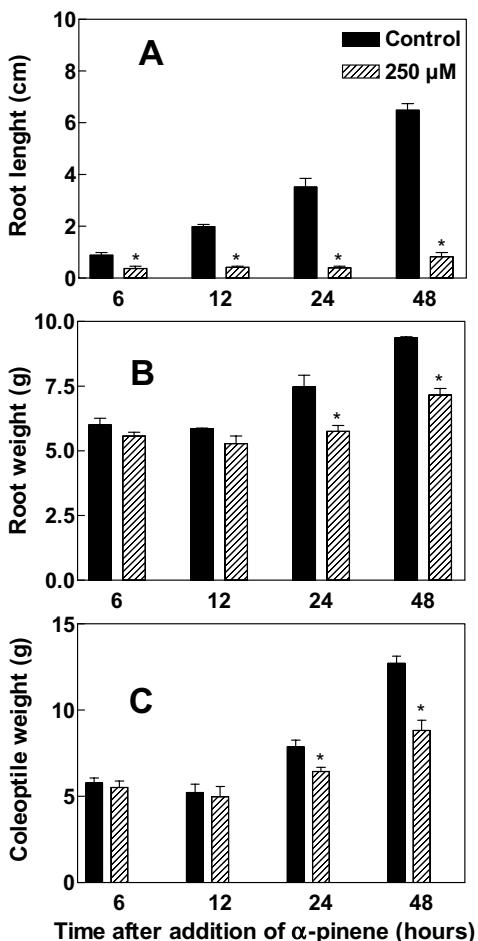
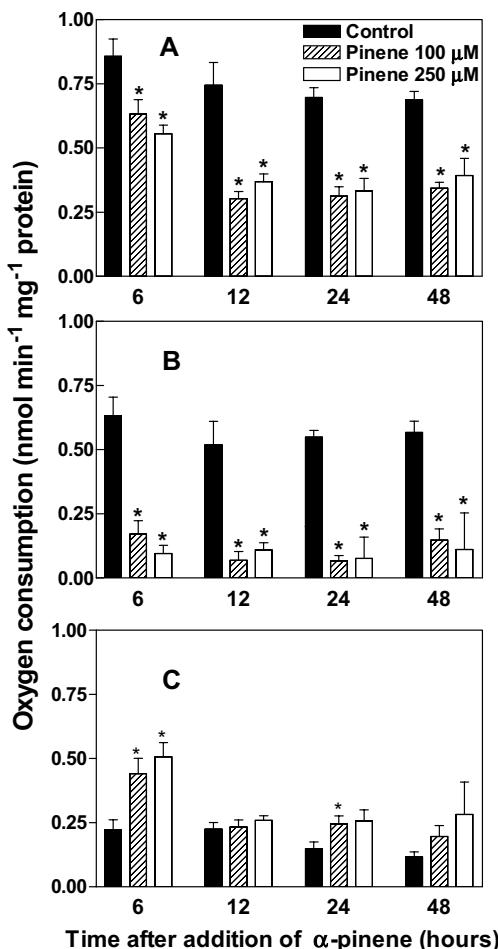


Figure 4. The effects of  $\alpha$ -pinene on the length of primary roots (A), fresh weight of primary roots (B) and fresh weight of coleoptiles (C). The roots of three-day-old seedlings were kept immersed in a continuously aerated nutrition solution and grown at a temperature of 25°C on a 16 h/light-8 h/dark regime at 230  $\mu\text{mol m}^{-2} \text{s}^{-1}$  foton flux. Alfa-pinene (250  $\mu\text{M}$ ) was added to the nutrient solution and at each time interval (6, 12, 24 or 48 h) primary roots and coleoptiles were excised and the length and the fresh weight were measured. (\* $p < 0.05$ ) Significant differences between  $\alpha$ -pinene-treated and untreated seedlings were determined by ANOVA with Duncan's multiple range test

The respiration rates of maize seedlings that were grown in the presence of  $\alpha$ -pinene are also changed significantly as revealed by experiments performed with excised root apices and illustrated by Figure 5. In the presence of 100  $\mu\text{M}$   $\alpha$ -pinene there is a significant decrease in the overall oxygen consumption rate of nearly 25% at 6 h and 60% at 12 h when compared with untreated plants (Figure 5A). In the presence of 250  $\mu\text{M}$   $\alpha$ -pinene oxygen consumption is decreased by 35% and 50% at 6 h and 12 h, respectively. No further reduction is noted in roots that had been exposed to  $\alpha$ -pinene 100 or 250  $\mu\text{M}$  for 24 or 48 h. The oxygen consumption measured in the presence of KCN provides an estimate of the cytochrome oxidase pathway (COX) capacity (KCN-sensitive respiration, Figure 5B) and of the extramitochondrial oxidases plus mitochondrial alternative oxidase (KCN-insensitive respiration, Figure 5C). The degrees of inhibition of the cytochrome oxidase pathway are higher than those ones of the overall respiration. At 6 h of treatment with 100  $\mu\text{M}$   $\alpha$ -pinene, for example, 73% inhibition is observed in the KCN-sensitive respiration, whereas the overall oxygen consumption is inhibited by 26%. This discrepancy results from the fact that  $\alpha$ -pinene exerts a stimulatory effect on the KCN-insensitive respiration (Figure 5C). This stimulatory effect is more pronounced at 6 h, but it is evident for all periods of treatment. It is improbable that this effect is due to an increase of the mitochondrial alternative oxidase activity because in isolated mitochondria  $\alpha$ -pinene does not increase the KCN-insensitive respiration. Actually it exerts an inhibitory effect. In the presence of 100  $\mu\text{M}$   $\alpha$ -pinene, for example, the KCN-insensitive respiration is reduced by 86%. On the other hand, an increased activity of the lipoxygenase was detected in root apex extracts indicating that this enzyme may account, partly at least, for the stimulation of the KCN-insensitive respiration. An increase of nearly 54% was found, for example in the lipoxygenase activity in root extracts from seedlings grown for 6 h in the presence of 85  $\mu\text{M}$   $\alpha$ -pinene.

It seems likely, thus, that the inhibitory effect of  $\alpha$ -pinene on the respiration of intact tissues is consequence of its effects on mitochondria. The root apex is composed by tissues with high metabolic activity. The mitochondrial respiration is limited by the adenylate levels, a condition which is equivalent to state III respiration of isolated mitochondria. State III respiration is indeed strongly reduced by  $\alpha$ -pinene in a concentration range (50-250  $\mu\text{M}$ ) similar to that one which inhibits tissue respiration and impairs plant growth. Mitochondria can be considered, thus, a likely site of  $\alpha$ -pinene allelochemical activity.



*Figure 5.* The effects of  $\alpha$ -pinene on respiration of excised maize root apices. Primary root apices (6 mm in length) were excised from seedlings grown in the absence or in the presence of  $\alpha$ -pinene as described in the legend of Figure 4. They were added without delay to the nutrition solution in the oxygen electrode vessel and oxygen consumption was recorded polarographically, at 25°C, using a Clark-type electrode. (A) Rate of oxygen consumption in the absence of KCN; (B) difference between the rates of oxygen consumption measured in the absence and in the presence of 1.0 mM KCN; (C) rate of oxygen consumption in the presence of KCN. The data are the mean $\pm$ SEM of 4-8 experiments. (\* $p < 0.05$ ) Significant differences between  $\alpha$ -pinene-treated and untreated seedlings were determined by ANOVA with Duncan's multiple range test.

## Future directions for examination of the role of mitochondria in the mechanisms of action of allelochemicals

Apart from the essential function of mitochondria in energy-generation processes within the cells, mitochondria are involved in such complex processes as oxidative stress and programmed cell death. The reactive oxygen species (ROS) have been implicated in biotic and abiotic stresses in plants (Lamb and Dixon, 1997) and mitochondria are the major source of ROS formation. The main mechanism responsible for mitochondrial ROS production is the respiratory chain in complexes I and III, forming superoxides, which subsequently dismutate to hydrogen peroxide (Braidot et al. 1999; Kowaltowski and Vercesi, 1999). Plant mitochondria also produce ROS by autoxidation of the flavin-semiquinone of the external NADH dehydrogenase (Rich and Bonner, 1978). Normally, ROS are decomposed or their peroxidation products are neutralized by natural defense systems consisting of specialized enzymes such as catalase, peroxidase, superoxide dismutase and glutathione peroxidase. However, under conditions of increased ROS generation, they can accumulate, exerting damaging actions on the cells, including peroxidation of membrane lipids, oxidative damage to proteins and DNA (Halliwell and Gutteridge, 1984) and opening of the mitochondrial permeability pore, an event linked to apoptotic cell death (Ellis et al. 1991; Green and Reed, 1998). The precise mechanisms of the mitochondrial pathway of apoptosis in plants have not yet been totally elucidated (Pennel and Lamb, 1997). However, it has been demonstrated that several factors facilitate this process, including ROS induced membrane permeability transition (MPT), decrease of membrane potential (Jabs, 1999), depletion of ATP coupled to  $\text{Ca}^{2+}$  influx (Jones, 2000; Tiwari et al. 2002) and elevation of intramitochondrial  $\text{Ca}^{2+}$  levels (Levine et al. 1996). Similar signaling mechanisms have been implicated in necrotic and apoptotic cell death. Because some allelochemicals have been demonstrated to interact with mitochondria, the possibility that they could act directly or indirectly by promoting oxidative stress and inducing subsequent necrosis or apoptosis should be considered. There is some evidence indicating that  $\alpha$ -pinene causes oxidative stress in maize roots. It activates KCN-insensitive respiration in intact tissues and in isolated mitochondria it uncouples electron transport from ATP synthesis, leads to a decrease in the membrane potential and inhibits the alternative oxidase pathways. All these actions have been demonstrated to induce ROS generation (Popov et al. 1997) and are also common properties of several apoptosis-inducing agents (Jabs, 1999). The increased activity of lipoxygenase observed in intact roots is also in agreement with this interpretation. The peroxidative attack may damage membranes, resulting in the release of substrates for lipoxygenases, including polyunsaturated fatty acids (Hildebrand, 1989). Connections between lipoxygenase activity and cellular proliferation, apoptosis and necrosis have been indeed demonstrated (Spiteller, 2003).

In summary we hypothesize that some allelochemicals are toxic to plants or microorganisms by inducing oxidative stress, necrosis or apoptotic cell death through their actions on mitochondria. Clearly, specific assays are needed in order to establish these phenomena as the exact mechanisms of action in allelopathy. These assays should include morphological studies (Mittler et al. 1997) as well as measurements of mitochondrial ROS production (Cathcart et al. 1983), levels of peroxidation products (Buege and Aust, 1978; Jocelyn, 1987), permeability transition pore opening (Forte et al. 2001) and cytochrome c release (Sun et al. 1999).

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## CHAPTER 13

# WEED GERMINATION, SEEDLING GROWTH AND THEIR LESSON FOR ALLELOPATHY IN AGRICULTURE

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## INTRODUCTION

The question of paradigm in the science of allelopathy is under discussion in the latest years (Reigosa et al. 1999; Inderjit, 2002; Mallik, 2002). Although it is well known that the plant physiologist Hans Molisch in 1937 coined the term allelopathy, the actual subject of his work has been neglected. Indeed, he was caught by an horticultural problem: the induction of ripening by early-ripening apples and pears on fruits from late-ripening varieties when stored together. Molisch demonstrated that the substance responsible for ripening induction was ethylene. He also demonstrated that root growth of vetch (*Vicia sativa* L.) and pea (*Pisum sativum* L.) seedlings is inhibited when seeds were germinated under a jar together with some apples. Molisch in his book reported: *The described phenomenon that one plant can influence another, play an important role in physiology, so it deserves an appropriate term. For this I coin the word allelopathy from the Greek words “allelon”, meaning mutual and pathos, meaning harm or “affection”. The shorter word allopathie is appropriate too but it is already present in literature as opposite of homeopathy* (Molisch, 1937).

Successively, Molisch's definition was adopted in a broader view by the botanist Elroy Rice who was encouraged by some studies that demonstrated the role of allelopathy in the field. Rice formal definition of the term was: *any direct or indirect harmful or beneficial effect by one plant (including microorganisms) on a another through production of chemical compounds that escape into the environment* (Rice, 1984). This shift of paradigm implies a growing complexity to ascertain an allelopathic

phenomenon in the ecosystems where a dynamic web of surprising interrelationships among organisms exists at all scales. Indeed, the term allelopathy is perceived differently by researchers of different specializations and his differences in worldview has created much confusion. For example, an ecologist's perspective of allelopathy is quite different from that of natural product chemist, biochemist, plant physiologist, agronomist, weed scientist, microbiologist, and experimental botanist (Mallik, 2002). Unfortunately only rarely have these varied professional talents been assembled in experimental programmes. By way conclusive proofs of allelopathy in the field remain few, and use of field relevant bioassay has been regularly called for (Inderjit, 2002).

Seed germination is the most widely used bioassay in allelopathy and the literature pertaining to the use of this bioassay and its general suitability for the determination of allelopathic activity among species has been reviewed by Leather and Einhellig (1986), Inderjit (1995) and Romeo and Weidenhamer (1988). These authors pointed out that there is little standardization governing seed germination bioassays. Most research is centered on a few plants, especially agricultural seeds to discover biochemical and physiological aspects of seed germination. This raises the question as to what extent do the results obtained in the lab with these seeds which have been subjected to man's selection can be extrapolated to the wild plants in the field.

This chapter reviews the biological characteristics and germination responses of three major weeds: Purslane (*Portulaca oleracea* L.), Lambsquarter (*Chenopodium album* L.) and Redroot pigweed (*Amaranthus retroflexus* L.), with a focus on how their reproductive strategies indicate a vulnerability to allelopathic control. We have also included some unpublished data obtained in our lab.

## WEEDS FROM A BOTANICAL PERSPECTIVE

Weed species may be defined as those plants "out of place" from an anthropocentric point of view. A fundamental basis for sound weed management in agriculture is to know:

- a) the species present and the level of infestation;
- b) biology and ecology of the prevalent species;
- c) interference of the prevalent weed species;
- d) technically effective, economically viable and environmentally safe methods of control (Labrada and Parker, 1994).

Table 1 lists 14 species considered as the most serious weeds from a worldwide perspective on the basis of their distribution and prevalence in crops. It is difficult to

classify weeds on a narrow set of botanical (e.g., morphological, phenological or taxonomic) criteria. However, a look at the table with taxonomic and ethnographic evidence shows that important weeds and crops are closely related and took origin from a common ancestor (i.e., *Avena fatua* and *A. sativa*; *Sorghum halepense* and *S. vulgare*). Moreover, some weed seeds such as Barnyard grass, Goosegrass, Lambsquarter, and Redroot pigweed were important sources of food in the past (Harlan, 1992). Most of monocot crops and weeds are Gramineae therophytes whose seeds are the only overwintering living structures. The two worst weeds Purple nutsedge and Bermuda grass are geophytes. Finally, dicot weeds such as Purslane, Lambsquarter, and Redroot pigweed are taxonomically related belonging to the same order of Centrospermae.

## **REPRODUCTIVE STRATEGIES OF THREE MAJOR WEEDS: Purslane (*Portulaca oleracea* L.), Lambsquarter (*Chenopodium album* L.) and Redroot pigweed (*Amaranthus retroflexus* L.)**

Seed dispersal and seed germination are critical phases in the life-cycle of the plant, during these phases the forces of natural selection have a maximum opportunity to exert their influence. These clues will be evaluated into three annual weeds: purslane, lambsquarter and redroot pigweed.

### ***Portulaca oleracea* L**

Purslane is a succulent herb with stems that may grow erect or prostrate, depending on light conditions. It is one of the weeds which have been most successful in colonizing arable lands worldwide (Allard, 1965). The plant is a major obnoxious weed of 45 crops in 81 countries and the ploughable layer of the soil cropped with maize contains about 220.000 purslane seed per m<sup>2</sup>. Purslane fruit is a capsule apparently simple structured with a pyxidium and a caliptra. Indeed, its inner structure reveals the contrivance of the plant in order to reach an effective seed dispersal, which contribute to its weediness in arable lands. Figure 1 shows the morpho-functional aspects of the purslane fruit. The ovaric cavity is divided in two chambers, the lower one is wide, dehiscent and contains numerous seeds (50-70). The upper chamber is tiny, globous, and contains few seeds. During ripening this tiny chamber is included in the nipple-shaped operculum of the capsule, by means of a tight narrowing across the ovaric apex. The capsule is circumscissed in hot days when the operculum falls, retaining inside 2-4 seeds, along with the calyx, dried petals, stamen and styles (together called "the caliptra"). Moreover, the withering petals, stamens and styles produce a glue-like substance turning the operculum into a fruit absolutely indehiscent.

Table 1. The Worst Weeds of the World (from Holm et al. 1977, modified)

Rank	Species	Taxa and Biological Form
1	<i>Cyperus rotundus</i> L. (Purple nutsedge)	Monocot., Cyperales, Cyperaceae, Geophyte
2	<i>Cynodon dactylon</i> (L.) Pers. (Bermuda grass)	Monocot., Graminales, Gramineae, Geophyte
3	<i>Echinochloa crus-galli</i> (L.) Beauv. (Barnyard grass)	Monocot., Graminales, Gramineae- Therophyte
4	<i>Echinochloa colonum</i> (L.) Link. (Jungle rice)	Monocot., Graminales, Gramineae, Therophyte
5	<i>Eleusine indica</i> (L.) Gaertner (Goosegrass)	Monocot., Graminales, Gramineae, Therophyte
6	<i>Eichhornia crassipes</i> (Mart.) Solms. (Water hyacinth)	Monocot., Liliiflorae, Pontederiaceae, Hydrophyte
7	<i>Portulaca oleracea</i> L. (Purslane)	Dicot., Centrospermae, Portulacaceae, Therophyte
8	<i>Chenopodium album</i> L. (Lambsquarter)	Dicot., Centrospermae, Chenopodiaceae, Therophyte
9	<i>Digitaria sanguinalis</i> (L.) Scop. (Crabgrass)	Monocot., Graminales, Gramineae, Therophyte
10	<i>Convolvulus arvensis</i> L. (Field bindweed)	Dicot., Tubiflorae, Convolvulaceae, Hemicryptophyte
11	<i>Sorghum halepense</i> (L.) Pers. (Johnson grass)	Monocot., Graminales, Gramineae, Geophyte
12	<i>Imperata cylindrica</i> (L.) Beauv. (Cogon grass)	Monocot., Graminales, Gramineae, Geophyte
13	<i>Avena fatua</i> L. (Wild oat)	Monocot., Graminales, Gramineae, Therophyte
14	<i>Amaranthus retroflexus</i> L. (Redroot pigweed)	Dicot., Centrospermae, Amaranthaceae, Therophyte

Legend: Geophyte: a perennial plant that is deeply embedded in the soil substrate.

Hemicryptophyte: a plant having buds at the soil surface and protected by scales, snow, or litter.

Hydrophyte: aquatic plant, floating or rooting in the mud.

Therophyte: an annual plant whose seed is the only overwintering structure.

This phenomenon was discovered by the Italian botanist Federico Delpino, who

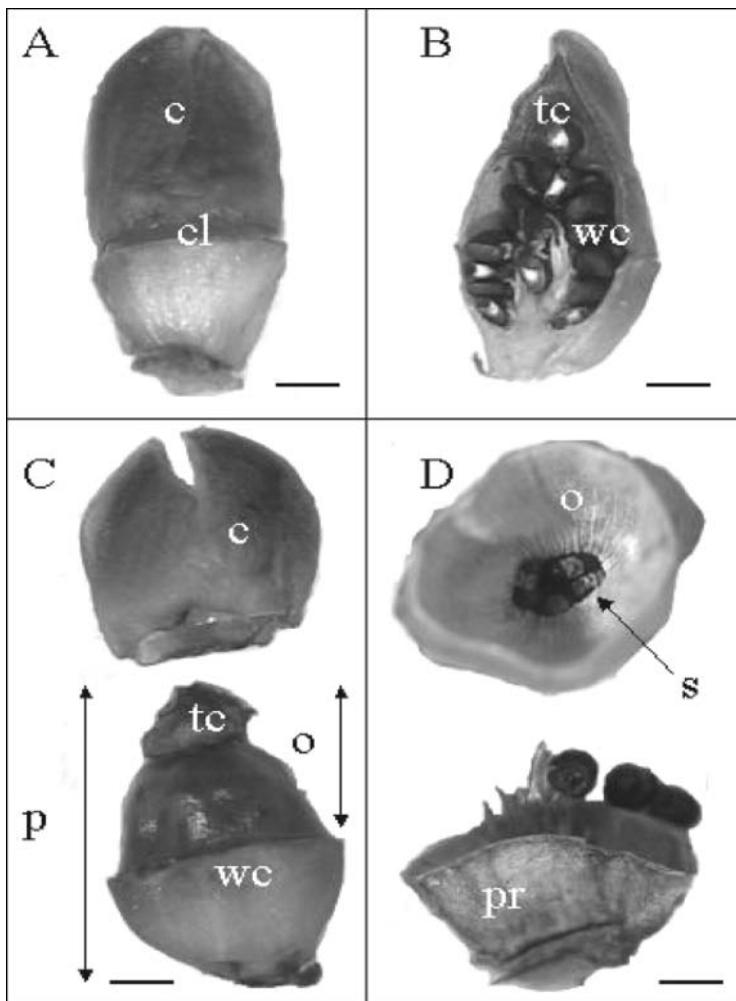


Figure 1. Stereomicrographs showing the morpho-functional aspects of purslane fruit. A) Capsula with the calytra (c) and the circumscission line (cl); B) Longitudinal section of the capsule with the upper tiny chamber (tc), containing few seeds and the lower wide chamber (wc), containing numerous seeds; C) Unripened capsule with the calytra (c), the pyxidium (p), with a tiny chamber (tc) narrowing at the apex and a lower wide chamber (wc); the operculum (o) covers the pyxidium. D) The operculum (o) retaining few seeds (s); pyxidium remnant (pr); Bars = 1mm.

referred it by the term Heteromericarpia, providing the first evidence that one of the ten most widespread weeds has fruits with effective seed dispersal (Delpino, 1903). In fact, the seeds in both chambers are the same but their fate is different. Those from the wide chamber of the pyxidium gradually spread around the plant while those of the opercule will fly away from the plant. Paradoxically, most of the worst weeds of the cultivated fields have seeds with no obvious dispersal aids (Zimmerman, 1976).

We have also studied the anatomical and ultrastructural aspects of *in vitro* germination of the purslane seeds using scanning electron microscopy (Figure 2). A dry quiescent purslane seed and the successive changes when it is moistened have been reported here. The outer integument (testa) of the seed coat is formed by dead cells sculptured on the surface with stellulae (Figure 2A). The peripheral face of the testa presents an opening: the micropyle and a residue of the funiculus of the placenta which functions as elaiosome, an edible appendage of seeds dispersed by ants. Surprisingly, the first structure that protrudes from the micropyle of the moistened seed, is not the radicle, as happens in seeds of other species, but the endosperm (Figure 2B). When germination proceeds further, the radicle breaks the endosperm and protrudes, completing rapidly the early stages of primary structure development viz., root hairs and hypocotyl elongation (Figure 2C). Usually these three phases require 25, 30 and 40 hours respectively. The radial section of purslane seed shows the embryo surrounded by a thin endosperm layer and curved around the starch hard perisperm (Figure 2D).

Weed scientists were not aware of the endosperm protrusion. Perhaps the phenomenon was overlooked because they recorded only the end of germination, when the radicle emerges, which is visible with the naked eye. Our step by step morphological investigations of purslane germination process, has revealed the endosperm protrusion (Aliotta et al. 1996).

### ***Chenopodium album L***

As purslane lambsquarter is reported to be one of the most successful colonizing species. The plant is an erect, rigid, pale-green herb growing to 2 meter in rich, moist soil, strongly tap-rooted. During the long photoperiods of 16 to 18 hours in the temperate zone, the plant grow vigorously for a long time and attains great size before it is induced to flower by oncoming short days. Because the plant has no special seed dispersal system, most of its seeds are deposited near the mother plant: such deposition causes it to grow in patches in crops. The seeds are commonly distributed as impurities in crop seeds. Lambsquarter thrives on all soil types. It attains its greatest size on fertile heavy soils rich in nitrate. Fruit is an utricule (a seed covered by the thin papery pericarp which often persists). Propagation of *Chenopodium album L.* is always from seeds. There is considerable heteromorphy in the seeds. Their color in one plant 290

may be black and shiny, brown, and brownish green. It is believed that there is a correlation between the amount of dormancy in a seed and its color (Holm et al. 1977). A needle puncture on the seed coat in the micropylar region of the seeds overcome their dormancy (Aliotta et al. 2002). Figure 3 shows the morphology of lambsquarter seed and its germination after scarification. Germination begins 24 hours of moistening and the first structure that protrudes from the micropyle of the seed is the endosperm layer which covers the radicle. When the germination proceeds further, the radicle breaks the endosperm and protrudes. After 96 hours the seedling is well developed.

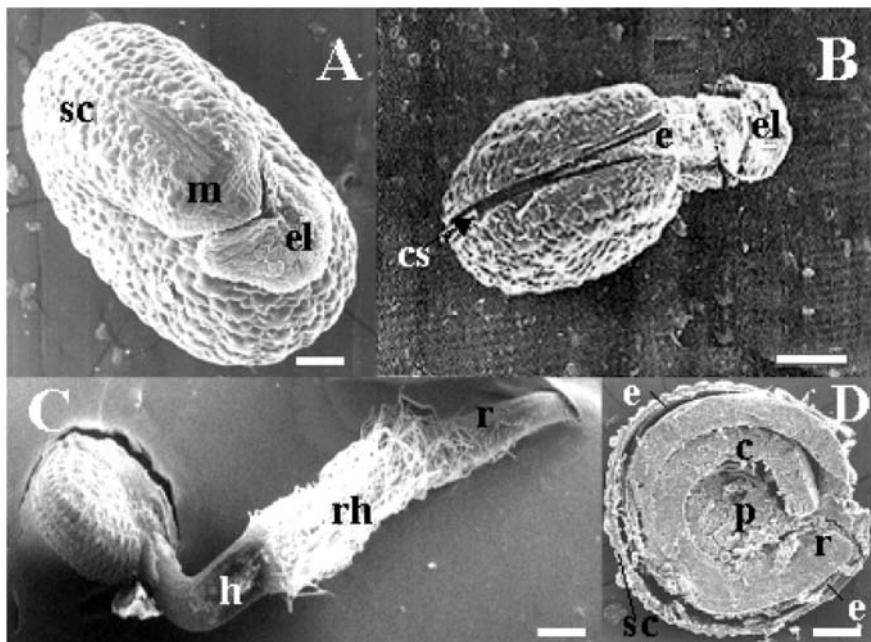


Figure 2. SEM micrographs of the whole purslane seed (A), Bar = 200 $\mu$ m. Endosperm protrusion (B), Bar = 250  $\mu$ m. Seedling growth (C), Bar = 250 $\mu$ m. Radial section (D), Bar = 150  $\mu$ m. sc, seed coat; m, micropyle; el, elaiosome; e, endosperm; cs, circumscission line; h, hypocotyl; r, radicle; rc, root cap; c, cotyledons; p, perisperm; rh, root hairs.

### *Amaranthus retroflexus L*

Some species of the genus *Amaranthus* are serious weeds worldwide viz., *Amaranthus retroflexus*, *A. spinosus*, *A. viridis*, *A. hybridus*, *A. lividus* and *A. blitoides*.

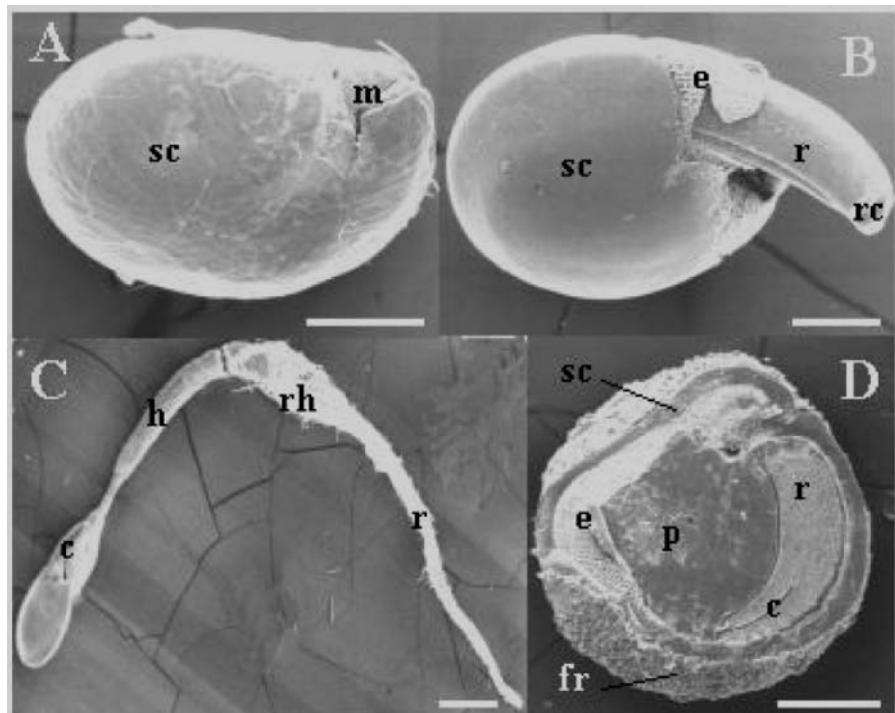


Figure 3. SEM micrographs of the whole lambsquarter seed (A), Bar = 300  $\mu$ m. Endosperm and radicle protrusion (B), Bar = 300  $\mu$ m. Seedling growth (C), Bar = 1mm. Radial section (D), Bar = 350  $\mu$ m.m, micropyle; e, endosperm; fr, fruit residue; h, hypocotyl; r, radicle; rc, root cap; c, cotyledons; p, perisperm; rh, root hairs; s, seed coat.

The *A. spinosus*, *A. retroflexus* and *A. viridis* are among the worst weeds. *A. retroflexus* and *A. spinosus* compete with other weeds and grasses in pastures, crop and roadsides.

*A. retroflexus* is the species most widespread. It has exhibited resistance to triazines herbicides and has developed resistant biotypes, therefore since 1990's, its biology and allelopathic properties are intensely studied (Suma et al. 2002). *A. retroflexus* is a monoecious, erect, finely hairy, freely-branching, herbaceous annual growing to 2 m tall; taproot pink or red, depth varies with soil profile; leaves alternate, egg-shaped up to 10 cm long; flowers numerous, small, borne in dense blunt spikes 1 to 5 cm long, densely crowded onto terminal panicle 5 to 20 cm long; tepals 5, much 292

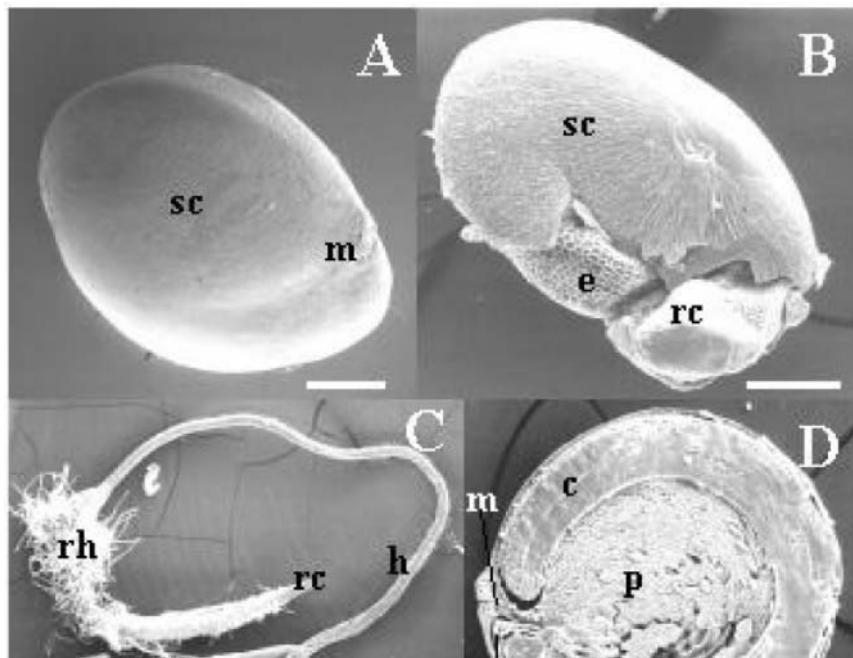


Figure 4. SEM micrographs of the whole redroot pigweed seed (A), Bar = 250 µm. Endosperm and radicle protrusion (B), Bar = 250 µm. Seedling growth (C), Bar = 300 µm. Radial section (D), Bar = 100 µm. m, micropyle; e, endosperm; h, hypocotyl; r, radicle; rc, root cap; c, cotyledons, p, perisperm; rh, root hairs; s, seed coat.

longer than fruit, 1 pistil and 5 stamens; style branches erect or a bit recurved; fruit a utricle, membranous, flattened, 1.5 to 2 mm long, dehiscing by a transverse line at the middle, wrinkled upper part falling away; seed oval to egg-shaped, somewhat flattened, notched at the narrow end, 1 to 1.2 mm long, shiny black or dark red-brown. Figure 4 shows the morphology of redroot pigweed seed and its germination after scarification.

It should be noted that the three weeds have the same seed embryology. In fact, the seeds are lenticulars, seed coat usually black and its peripheral face presents an opening: the micropyle. The embryo is curved around the starchy hard perisperm and covered by a thin endosperm. It is interesting the role of endosperm, which during germination protrudes covering the radicle. Recently, we have demonstrated that phenolic compounds of olive oil mill wastewater and the aqueous extract of Rue (*Ruta*

*graveolens* L.), induce an inhibitory growth delaying, modifying, and locking cellular activity of endosperm of purslane, lambsquarter and redroot pigweed. Our studies suggest that the outermost living structures of the seed (i.e., seed coat or endosperm) are the primary subject of the allelochemicals (Aliotta et al. 1999, 2000, 2002).

## ALLELOPATHY AND WEED CONTROL

Seed and growth parameters of the three weeds studied are reported in table 2. They are discussed considering eventual vulnerability points. Seed size and weight of purslane, lambsquarter and redroot pigweed are very small and the numerous seedlings emerge best from a depth of 0.5 to 1.0 cm. By contrast, most agronomic crops have much larger seeds and they are usually planted at 3 to 5 cm of depth. These difference in emergence depth and seed size between weeds and crops makes possible the implementation of allelopathy for natural weed management (Mohler, 2001). Crop residues can provide selective weed control through their physical presence on the soil surface and through the release of allelochemicals. A more fruitful approach has been to use waste products from plants that are processed for food or oil (Aliotta et al. 2001; Duke et al. 2002; Bhowmik and Inderjit, 2003).

The suppression of smaller-seeded weeds by allelochemicals may be the result of two processes. First, at least from germination until emergence, the surface-to-volume ratio of a small seeded species is usually greater, and therefore its exposure per unit mass to allelochemicals in the soil is also greater. Second, when residue is used as a mulch, the allelopathic toxins are released onto the soil surface and may not diffuse very deeply into the soil profile (Mohler, 2001), Barnes and Putnam (1986) showed that percent germination and root elongation of several species decreased as the layer of soil separating seeds from rye residue decreased from 15 to 0 mm. To have any potential for emergence, a small-seeded weed or crop must germinate near the soil surface, but under an allelopathic mulch, this is where the toxins are most concentrated. In contrast, larger-seeded crops are planted more deeply, and thus germination and initial root growth may occur in a less toxic environment. Both hypotheses require testing by careful experimentation (Mohler, 2001).

Once small-seeded weeds have germinated and established in the field starts the crop-weed interference. As can be seen in table 2 Purslane, lambsquarter and redroot pigweed have higher values of growth parameters such as: biomass added per unit time (RGR), and leaf weight ratio (LAR), than sunflower. The values of net assimilation rate (NAR), are almost similar. That is, differences in growth rate due to seed size were attributable to morphology rather than physiology. Because small-seeded weeds have a higher RGR than larger seeded crops, they tend to catch up in size eventually. As an extreme example, the initial 500-fold difference in the seed size of maize and redroot pigweed, may be reduced to a two-fold difference in the size of the mature

plants if each species is allowed to grow without competition (Mohler, 1996). At the emergence, the crop has a greater leaf area and a larger root system than the weed. Therefore the crop's absolute growth rate is initial greater, and usually remains greater for at least several weeks (Zimdahl, 1980).

*Table 2.* Seed and growth parameters of three major weeds in arable lands and sunflower.

Parameters	<i>Portulaca oleracea L.</i>	<i>Chenopodium album L.</i>	<i>Amaranthus retroflexus L.</i>	<i>Heilanthus annuus L.</i>
Seed weight (mg)	0,12±0.01	0.38±0.02	0.37±0.03	61±2.3
Number seeds m <sup>-2</sup>	120000	4000	68000	-
Seedlings m <sup>-2</sup>	420±15	90±5	300±10	-
Seedling type	Epigeal	Epigeal	Epigeal	Epigeal
RGR*	0.461	0.298	0.349	0.197
LAR*	180	224	198	140
NAR*	0.220	0.254	0.298	0.241
LWR*	0.430	0.674	0.597	0.495

Notes: RGR: relative growth rate = g increase in plant weight g<sup>-1</sup> plant weight day<sup>-1</sup>.

LAR: leaf area ratio = cm<sup>2</sup> leaf area g<sup>-1</sup> plant weight.

NAR: net assimilation rate = g increase in plant weight dm<sup>-2</sup> leaf area day<sup>-1</sup>.

LWR: leaf weight ratio = g leaf weight g<sup>-1</sup> plant weight.

\*Correlation with ln (seed weight), significant at  $p < 0.05$  level.

Sources: From Aliotta et al. 2001; Seibert and Pearce, 1993; and Zimmerman, 1976.

Use of the initial advantage conferred to the crop by relatively large size and high absolute growth rate is a key concept in ecological weed management. A major strategy in most annual crops is to design the cropping system so that the initial size advantage still holds at the time the crop and weeds grow into physical contact. With few exceptions, both crops and weeds are adapted to open habitats, and both are intolerant of shade ( Bello et al. 1995). Consequently if the crop is in the superior position, it will suppress the growth of the weeds, whereas if the weeds grow above the crop canopy, then yield reduction is likely to be severe.

Recently, it has been examined the role of allelopathic crop residues, natural compounds and weed-suppressing cultivars, as well as rhizosphere interactions involving higher plants (Birkett et al. 2001; Duke et al. 2002; Bhownik and Inderjit, 2003). Results suggest that allelopathy offers a real promise for practical weed management, especially if we join the different expertise involved.

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## CHAPTER 14

# ALLELOPATHY: A SOIL SYSTEM PERSPECTIVE

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## INTRODUCTION

Soils are very diverse in composition (e.g., minerals, clays, organic matter, moisture, nutrition) and behavior. Some are acidic, others alkaline, some reduced, some strongly oxidized. In addition soils contain numerous living organisms (e.g., microbes, fauna) and plant organs (e.g., roots, rhizomes, tubers, bulbs). Thus, if we wish to understand a given process within a given soil, for example an allelopathic interaction, then that process must be viewed and understood within the specific environmental constraints of that soil. This makes each soil essentially unique from any other soil. To generalize on a larger scale than just a single soil, researchers studying the mechanisms of allelopathic interactions have focused on how the actions of allelopathic agents are influenced by similar or corresponding soil characteristics for a range of soils. Thus, for example, there have been a number of publications on how allelopathic agents interact with soil fractions or specific soil components such as clays (Greenland, 1965; Whitehead et al. 1983; Dao, 1987; Lehman et al. 1987; Dalton, 1999), soil microbes (Blum and Shafer, 1988; Smith and Ley, 1999; Blum, 2003), and roots (Glass and Böhm, 1971; Harper and Balke, 1981; Blum and Rebbeck, 1989). This reductionist approach is useful, but has its limits because this approach excludes numerous interactions of the various soil components. However, there is now sufficient data for the behavior of the individual soil components, particularly in regard to their interactions with simple phenolic acids, to determine the value of such a reductionist approach to our understanding of the role of organic acids in the more complex environment of soils. Doing this is particularly pertinent at this time since there has been some uncertainty expressed as to the importance of organic acids, such

as phenolic acids, as allelopathic agents in field soils (Smith and Ley, 1999; Blum, 2003).

In this chapter I describe soil system characteristics of interest (i.e., nature of mineral soils with emphasis on soil organic matter, soil organisms, soil processes, and root anatomy, morphology, growth and development), and discuss how water soluble allelopathic agents, particularly organic acids, are influenced by these soil system characteristics. The scope of this presentation, due to the complexity of the topic, is limited to simple organic acids and mineral soils of temperate regions of the world. In addition, I lean heavily on the phenolic acid literature, because it is among the most comprehensive and the behavior of phenolic acids in soils is a good indication of the behavior of other potential phytotoxic organic acids, such as acetic acid, butyric acids, citric acid, formic acid, fumaric acid, lactic acid, malonic acid, propionic acid, tannic acids, and tartaric acid (Patrick, 1971; also see Rice, 1984).

## MINERAL SOILS

### Nature of mineral soils

Soils have structural and biological properties that distinguish them from rocks (i.e., parent material) and the sediments from which they originate. They are dynamic systems providing plants with support, water, nutrients, and air and housing extensive populations of fauna and microbes that are involved in recycling organic materials (i.e., decomposition) produced by living organisms. At all spatial and temporal scales the major influences defining the environment that exists within soils are dependent on the physical, chemical and surface properties of their components (e.g., minerals, organic matter, water, gases, living organisms) and their spatial distribution (Lavelle and Spain, 2001).

Ultimately the origin of soils can be traced back to the weathering of igneous (e.g., granite, diorite), sedimentary (e.g., limestone, sandstone), and/or metamorphic (e.g., slate, marble) rocks. Weathering of rocks leads to the formation of coarse to fine grained particles (i.e., gravel, sand, silt, and clays) composed mainly of minerals.

The minerals of rocks are nearly always distinct crystalline substances which are mostly compounds of oxygen, silicon, and aluminum, sometimes with appreciable amounts of iron, calcium, sodium, potassium, or magnesium. For example, on average these eight elements compose 98% of igneous rocks while minor elements (e.g., titanium, phosphorous, manganese) generally are less than 1% (Greenland and Hayes, 1978). Silicon oxide is the most abundant mineral in all igneous rocks while the other six elements vary with the mineral composition of igneous rocks. The dominant

minerals in limestone, sandstone, slate and marbles are calcite ( $\text{CaCO}_3$ ), quartz ( $\text{SiO}_2$ ), clays, and calcite, respectively (Brady, 1984).

The size distributions of the coarse to fine particles determines the soil texture (e.g., clay, clay loam, sandy clay loam), particle density (weight of the solid particles divided by the volume of the particles [does not include pore space]) and bulk density (weight of the soil divided by the total volume [includes pore spaces]). The bulk density for mineral soils ranges from 1 to  $1.8 \text{ g/cm}^3$  (Brady, 1984). Because organic matter is highly porous and has a particle density of 1.2 to  $1.5 \text{ g/cm}^3$ , the incorporation of organic matter into mineral soil will generally decrease both particle and bulk density. A typical mineral soil will contain 45% minerals, 5% organic matter, and 50% pore space (Hartel, 1998).

It takes a long time (thousands to millions of years) to form the coarse to fine grained minerals that make up a soil. Much of this material is eventually transported by water, wind, and/or gravity down slopes and deposited to form soils with sufficient depth for the evolution of horizons. Horizons form due to the accumulation and incorporation of organic matter in the top soil layer, the decomposition of this organic matter, the transformation of soil minerals by physical and chemical weathering, and the capillary and/or gravitational movement of water soluble and water suspended substances from the top soil layer to the layers below (Foth, 1990). There are five major recognized horizons, each with potential subhorizons. Any given soil may or may not have all of these horizons. The major horizons are the following:

- a) the O horizon - undecomposed and partially decomposed organic layer that forms just on top of the mineral soil,
- b) the A horizon - top most mineral layer containing humified organic matter,
- c) the E horizon - zone just underneath the A horizon where maximum eluviation of clay, iron, and aluminum oxides and the concentration of resistant minerals such as quartz occurs,
- d) the B horizon - region of maximum accumulation of materials such as iron and aluminum oxides and silicate clays, and
- e) the C horizon - zone of unconsolidated materials (Brady, 1984). Most biological activity (e.g., location and activity of roots, microbes and fauna), including allelopathic interactions, occurs within and at the interfaces of the O and A horizons.

## Soil pH

An important property of any soil is its pH (Bardy, 1984). Acid soils have a pH < 7 and alkaline soils have a pH > 7. The pH of mineral soils typically ranges from about 3.5 to about 8.5. Organic soils may have a lower pH. As pH drops below 6, aluminum can occupy a significant portion of the cation exchanger phase of soils, (Buol et al. 1997), while exchangeable bases (e.g.,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$ ) are more dominant at higher soil pH (i.e., base saturation is greater). Soils with pH between 8 and 8.5 typically contain calcite. Higher pH levels (>9) can occur in arid-zone soils with high levels of soluble salts, particularly sodium. Thus, there is a general trend of decreasing base saturation and increasing saturation with acidic ions ( $\text{Al}^{3+}$  and  $\text{H}^+$ ) as pH decreases. The sources of protons that contribute to declining soil pH and increasing soil acidity (protons and exchangeable Al present) include atmospheric deposition of acids such as  $\text{H}_2\text{SO}_4$  and  $\text{HNO}_3$  generated from atmospheric reactions between water and gaseous  $\text{NO}_x$  and  $\text{SO}_x$  from fossil fuel emissions,  $\text{H}_2\text{CO}_3$  produced from aqueous dissolution of atmospheric  $\text{CO}_2$  or biologically-produced soil  $\text{CO}_2$ , and biological activity (e.g., respiration, production of organic acids, nitrification of mineralized N or ammonium fertilizers, and imbalances in cation and anion uptake by plants). The rate of soil acidification is related to the rates of acid inputs versus the soil buffering capacity. Soil pH is mainly buffered by dissolution of calcite and other carbonates (pH > 7), cation exchange of bases by  $\text{H}^+$  and  $\text{Al}^{3+}$  (or its hydrolysis species) (pH 7 to 5), dissolution of Al-bearing minerals (pH < 5) and dissolution of Fe-bearing minerals (pH < 4) (van Bremen et al. 1983; Hesterberg, 1993). Phytotoxicity of simple organic acids is most evident under acidic conditions when organic acids are protonated, i.e., neutral in charge, and is lost when organic acids are partially dissociated under neutral and basic conditions, i.e., negatively charged (Harper and Balke, 1981; Blum et al. 1985; Shann and Blum, 1987a; Lehman and Blum, 1999; Blum et al. 1999).

## Cation and anion exchange capacity, surface charges, and adsorption

Soil colloids (organic matter and clay minerals [phylllosilicates such as kaolinite, vermiculite, smectites, micas, chlorites]) and oxides (Fe-, Al-, and Mn-oxides) have a mixture of positively and negatively charged sites on their surfaces to which are attracted a range of ions, both organic and inorganic (Lavelle and Spain, 2001). In most soils the negative charges predominate to give a net negative charge to the soil. A net positive charge may occur in certain soils, particularly at low pH. These charges are of considerable importance to plant nutrition since they determine cation and anion exchange capacity of the soil. Organic matter in mineral soils provides between 20 to 80% of the cation exchange capacity (Wagner and Wolf, 1998). Pure organic matter

has a cation exchange capacity (CEC) of 240 centimole (+)/kg and an anion exchange capacity (AEC) of 1 centimole (-)/kg (Hartel, 1998). Pure smectite and kaolinite, two clays, have a CEC of 118 and 7 and an AEC of 1 and 4, respectively. Since soils are composed of various mixtures of clays, sand, silt, (sand and silt per se contribute little to CEC and AEC) and organic matter, the CEC and AEC values vary considerably from soil to soil.

For the soil organic matter, 75% of the negative charges are pH-dependent. These originate from the partial dissociation of phenolic ( $C_6-OH$ ), enolic (-OH), and organic acid moieties (-COOH) of humus (Brady, 1984). Clay minerals possess several types of negative charges. At the edges of clay particles, hydroxide (-OH) radicals become dissociated (-O<sup>-</sup>) at neutral to high pH. In addition, negative charges arise as a result of isomorphic substitution of aluminum ( $Al^{+3}$ ) by other divalent cations, such as magnesium ( $Mg^{+2}$ ) and iron ( $Fe^{+2}$ ) or substitution of silicon ( $Si^{+4}$ ) by aluminum ( $Al^{+3}$ ), resulting in permanent negative charges which are neutralized by surface adsorption of cations (Brady, 1984). For smectite and kaolinite 5% and 57% of the negative charges are pH dependent, respectively.

For soil organic matter protonated groups such as (-OH<sub>2</sub><sup>+</sup>) and (-NH<sub>3</sub><sup>+</sup>) may yield minor amounts of positive charges, but the overall charge for soil organic matter is negative (Bohn et al. 1985). Clay minerals posses several types of positive charges. The positive charges arise from the protonation of hydroxyls on the edges of silicate clays and on the surface of iron and aluminum oxides (e.g., goethite[FeOOH], gibbsite [Al(OH)<sub>3</sub>], hematite [Fe<sub>2</sub>O<sub>3</sub>], ferrihydrites [poorly crystalline Fe-oxide], non-crystalline Al-hydroxide, and allophanes). The resulting AEC is inversely related to soil pH (Foth, 1990). Thus, the same site can be negative, neutral, or positive depending on pH. Comparative levels of charges for pure gibbsite and goethite are 6 (+) and 6 (-) centimole/kg, and 4 (+) and 4 (-) centimole/kg, respectively (Brady, 1984). However, since the charged sites on clays and organic matter are counter balanced by appropriate cations or anions, soils are electroneutral.

It is also important to understand that many of the “adsorption processes”, particularly those involving chemisorbed ions such as trace metal cations (e.g., Cu<sup>+2</sup>, Zn<sup>+2</sup>, Pb<sup>+2</sup>) and anions (e.g., H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, MoO<sub>4</sub><sup>-2</sup>) do not require CEC or AEC per se, but are a result of chemical reactions at clay surfaces (Hesterberg, 2002; Brown and Parks, 2001). The amount adsorbed depends on the concentration of the metal ion, the affinity of that ion to the clay surface relative to the soil solution, pH, and presence of competing ions.

Finally, sorption/desorption of simple organic acids in soils and thus their potential roles as allelopathic agents are also influenced in major ways by cation and anion exchange, surface charges, and chemisorption. Please see subsequent section entitled: “Sorption/Desorption of Water Soluble Organic Acids” for details.

## Soil aeration

The soil atmosphere contains various amounts of nitrogen, carbon dioxide, oxygen, water vapor and a number of other gases. Oxygen is of particular importance to most living soil organisms and roots. Oxygen is required for oxidation-reduction reactions of aerobic fauna, microbes and roots, and for the determination of the reduction and oxidation states of chemical elements in the soil. The concentration of oxygen in the soil is determined by the amount of oxygen in the air-filled pore spaces, biotic and abiotic oxygen demand, and oxygen diffusion rates. Oxygen concentrations decline with depth of soil. Low oxygen content of soil is frequently associated with:

- a) high oxygen demand by decomposers and roots,
- b) poorly-drained soils or well-drained soils after a heavy rain,
- c) soils with limited pore spaces (compacted soils, poor soil structure), and
- c) low oxygen diffusion rates.

Perhaps the best measure of aeration status of a soil is the oxygen diffusion rate (Brady, 1984). This rate ( $\text{g O}_2 \times 10^{-8}/\text{cm}^2/\text{minute}$ ) indicates how fast oxygen can be replenished within soil. The rate is largely determined by concentration gradients of oxygen between the atmosphere (21%  $\text{O}_2$ ) and soil atmosphere or oxygen gradients from one region to another within the soil, and volume and types (micro- vs. macropores) of pore spaces filled with air or water. In general when oxygen diffusion rates decrease to  $20 \text{ g O}_2 \times 10^{-8}/\text{cm}^2/\text{minute}$  root growth ceases (Brady, 1984). Normal growth of shoots requires oxygen diffusion rates of around 30 to 40  $\text{g O}_2 \times 10^{-8}/\text{cm}^2/\text{minute}$ .

There is an inverse relationship between oxygen and carbon dioxide in the soil atmosphere. Carbon dioxide concentrations are usually greater in the soil (up to several hundred times) than the air (0.03%  $\text{CO}_2$ ) and they are a product of faunal, microbial and root respiration (Brady, 1984). Since carbon dioxide dissolves in water and forms carbonic acid, carbon dioxide levels can influence soil solution pH (source of  $\text{H}^+$ ), the solubility of soil minerals and organic substances, and the phytotoxicity of organic acids.

Finally the presence or absence of oxygen will dramatically modify the end products of decomposition. Greenwood (1961) found that whenever oxygen concentrations were less than  $3 \times 10^{-6} \text{ M}$  aerobic microbial activity was inhibited. Aerobic decomposition of soil organic matter will produce oxidized elements (e.g.,  $\text{CO}_2$ ,  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$ , ferric oxides, manganic oxides) while anaerobic decomposition, which is much slower, will produce reduced elements (e.g.,  $\text{CH}_4$ ,  $\text{N}_2$ ,  $\text{NH}_4^+$ ,  $\text{H}_2\text{S}$ ,  $\text{S}_2^-$ , ferrous oxides and manganous oxides). In addition, under anaerobic conditions phytotoxic levels of ethylene, lactic acid, butyric acid, formic acid, benzoic acid

cinnamic acid derivatives (e.g., p-hydroxybenzoic, ferulic, respectively), amines, and many other intermediate breakdown products of soil organic matter will also be produced and accumulated in the soil (Patrick, 1971; Brady, 1984).

## Nature of soil organic matter

Living organisms (i.e., plants, microorganisms, fauna) add a variety of organic matter and recycle organic matter in soils. For example the addition of organic matter by higher plants results from:

- a) leaching of leaves, twigs, stems, flowers, and/or fruits by precipitation events,
- b) deposition, leaching, and incorporation of leaves, twigs, stems, flowers, and/or fruits on top of or into the soil,
- c) root exudates, secretions and lysates,
- d) production of mucilaginous materials by roots and associated rhizosphere microbes,
- e) sloughing of root cells and root cell mortality, and
- f) decomposition and leaching of roots, rhizomes, bulbs, and corms by gravitational and capillary water.

Estimated inputs range from 2000 to 7000 kg/ha/year for conifer and mixed forests, 5000 to 7000 kg/ha/year for deciduous and broad-leaved forests, and 500 to 7000 kg/ha/year for grasslands (Rodin and Bazilevich, 1968a). Estimates for cultivated land range from 1000 to 3500 kg/ha/year if we use net primary productivity as an estimate of inputs (Whittaker and Likens, 1975).

A portion of the soluble organic matter added to soil by higher plants will be taken up by functional roots, but most of this organic matter, both soluble and insoluble, will be utilized by soil fauna and microorganisms as an energy and nutrient resource for their growth, development and reproduction. While utilizing these resources organic matter is altered and a variety of new and unique organic substances (molecules, compounds, polymers) are produced. Soils, thus, contain complex mixtures of organic substances composed for example of: lipids, fatty acids, waxes, water soluble carbohydrates (mono- and disaccharides), hemicellulose, cellulose, polyuronides, organic acids, melanins, phenolic acids, lignin, proteins, amino acids, DNA, RNA, nucleotides, vitamins, tannins, hydrocarbons, keratin, chitin, humic acids, fulvic acids and humin, to name a few (Flaig, 1971; Paul and Clark, 1989; Lavelle and Spain, 2001). Since these organic substances have different stability/turnover rates in the soil (Wagner and Wolf, 1998), a somewhat quasi-steady state in soil organic matter is reached, i.e., the more stable substances begin to dominate the organic matter

composition (e.g., waxes, cellulose, lignin, humic acids, fulvic acids, humin, melanins, chitin, keratin). Well-drained mineral soils will contain organic matter ranging from 1 to 10% by weight (Brady, 1984).

## Functions of soil organic matter

Soil structure arises when individual clay particles cohere to each other more than to adjacent clay particles surrounding them and form soil aggregates. These soil aggregates can cluster to form compound aggregates. The resulting hierarchical structures of aggregates are classified either as microaggregates ( $<250\text{ }\mu\text{m}$  equivalent cylindrical diameter (ECD)) or macroaggregates ( $>250\text{ }\mu\text{m}$  ECD). Colloidal forms of organic matter derived from plants, microbes and fauna, oxides of iron and aluminum, and highly-disordered silicate clay minerals help to cement and hold these aggregates together (Lavelle and Spain, 2001). Larger macroaggregates ( $>2\text{ mm}$ ), on the other hand, are frequently held together by a three-dimensional network of small roots, root hairs, mycorrhizal hyphae, and hyphae of saprophytic fungi. Aggregates are surrounded by inter-connected pore spaces that permit the infiltration and movement of water, solutes, and gases through the soil matrix. Lower resistance to root growth associated with soil porosity also leads to faster root growth (Barely, 1962; Taylor, 1971; Greenland and Hayes, 1981). Soil aggregation is not only important for maximizing root growth, increasing infiltration rates of precipitation, reducing runoff and erosion, and increasing soil aeration, but also turns out to be one of the most important factors controlling faunal and microbial activity and soil organic matter turnover. Most soil organisms involved in organic matter turnover exist on the outside surfaces of aggregates and in the pore spaces between them; relatively few reside within microaggregates (Paul and Clark, 1989). The size of the entrance determines the accessibility to pores. Occupancy is also determined by water content of the pores.

The importance of cation and anion exchange capacity in nutrient retention was described in a previous section. Organic matter also conserves nutrients in organic forms by reducing rapid nutrient depletion from the root zone, primarily the O and A horizons. In fact, soil organic matter supplies nearly 50 to 80% of the phosphate, 80% of the sulfur, a large part of boron and molydenum, and nearly all of the soil nitrogen absorbed by plants from unfertilized, temperate soils (Brady, 1984; Bohn et al. 1985). Soil organic matter also forms stable complexes with  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ , and other polyvalent cations increasing availability of trace elements to higher plants (Stevenson, 1982).

Organic matter gives soil a darker color. This increases absorption of solar radiation and average soil temperature. Assuming nothing else is limiting, an increase in average soil temperature should mean greater metabolism, growth and/or reproduction for soil organisms and roots during the growing season. Such increases

in temperature can also lead to earlier root growth in the spring and continued root growth into late fall/early winter since soil temperature is one of the primary regulating factors for root growth (Lyr and Hoffman, 1967).

Organic matter buffers soil pH in the slightly acid, neutral and alkaline range which helps to maintaining a more uniform pH environment (Stevenson, 1982). This is important since many soil processes, including the phytotoxicity of organic acids, are pH dependent.

Finally, depending on concentration and soil solution chemistry, soluble organic ions, molecules and/or compounds in soil solutions can act as plant, microbe, and faunal growth inhibitors and/or promoters. This, of course, has been the primary focus of researchers studying allelopathic interactions.

## **Distribution of organic matter and plant roots**

The location of organic matter is primarily near the soil surface (litter and leachates) and in the root zone (primarily the O and A horizons). Input of litter in the temperate areas of the world tends to be seasonal, mostly in the autumn. Turnover of this litter is regulated by the environment and is species and tissue specific and thus quite variable, but most turnover occurs in the spring and early summer. Horizontal distribution of aboveground litter is largely determined by plant species, density and distribution, and litter input, movement, fragmentation, soil incorporation, and decomposition.

Since for higher plants 40 to 85% of the primary productivity occurs below ground (Fogel, 1985) and that is also where most of the interconversions and synthesis of new organic substances occur, root distribution becomes an important determinant of organic matter distribution in the soil. Root mortality and turnover is highly variable, but is frequently associated with drought and precipitation events, respectively. Most roots (numbers and biomass) are located in the upper 30 cm of the soil and root numbers and biomass decline rapidly with depth thereafter (Kutschera, 1960; Böhm, 1979). However, that is not to say that roots cannot reach considerable depths. For example, roots of common crops such as corn, soybean, sorghum, and wheat growing in good arable soil can easily reach 2 m or more in depth (Kutschera, 1960; Böhm, 1979; Rendig and Taylor, 1989). Native grassland species growing in the western United States also easily reach 2 to 3 m (Weaver, 1920, 1926) and fir trees ranging from 4 to 181 years of age have attained root depths of 1.8 to 3 m (Schultz, 1978). Horizontal distribution of roots can essentially be continuous as for a turf grass system, fairly regular but clumped (rows vs. interrows) as for crop monocultures such as corn or soybean, or continuous to clumped depending on the total plant density and the type of natural community.

The release and production of allelopathic agents in the soil should closely correspond to the distribution of organic matter in the soil (i.e., primarily in the O and A horizons). In addition, since leaf litter can be more or less phytotoxic than root litter (Lehman and Blum, 1997; Staman et al. 2001; Blum et al. 2002), their functional toxicity (i.e., toxicity under field conditions), and horizontal and vertical distribution should provide considerable insight about the sources and locations of potential allelopathic agents.

## Soil solutions

Soil water content in the root zone is dynamic and is largely a product of precipitation events minus surface runoff, deep drainage, and evapotranspiration (Kramer and Boyer, 1995). In some instances ground water can also be important. There are three liquid forms of soil water:

- a) hygroscopic or bound water that is unavailable to soil organisms or roots,
- b) capillary water that is available to soil organisms and roots, and
- c) gravitational water, water above field capacity, that is of limited value to soil organisms and roots due to its mobility. In unsaturated soils, gravitational water will move vertically by gravity and horizontally by capillary action to become hygroscopic or capillary water. The ratio of these three forms of water, for any given soil, is determined by soil texture (e.g., proportions of sand, silt, clay), soil structure (e.g., pore space) and water content. Movement of water in soils occurs through a complex continuum of pores which vary in size and shape. Since micropores hold water at a higher tension than meso- and macropores, preferential flow of water occurs through meso- and macro pores (Jardine et al. 1990). Soil profiles may also be important in this regard particularly when these are interrupted by rocks or hard-pan layers which can modify or limit the movement of water. Too much water (i.e., water above field capacity) for extended periods can lead to a water saturated, anaerobic, and highly reduced soil environment. Too little capillary water for extended periods can lead to a highly oxidized and droughty soil environment.

Soil solution is defined as the water containing various concentrations of inorganic and organic substances and gases in a dissolved or suspended state located in soil pores (Adams, 1974; Greenland and Hayes, 1981). Because of different sizes and locations of soil pores, soil solutions can be continuous to discontinuous. Due to the variation in soil composition and soil pore structure, soil solutions at slightly different locations can contain very different amounts of dissolved substances. Concentrations of substances dissolved in the soil solution are determined by sources (e.g., clays, organic matter, roots), inputs (e.g., mineral weathering, organic substances released by

roots, microbes and/or fauna, organic matter decomposition, cation and anion exchange reactions, dissolution of precipitates), physicochemical nature of the soil solution (e.g., pH, temperature, types of aqueous complexes [ion pairs] between cations and anions), and losses (e.g., leaching, root uptake, faunal and microbial utilization and conversions, cation and anion exchange reactions, precipitation). At any point in time soil solutions will contain only a tiny fraction (less than 2%) of the total inorganic and organic substances/matter in the soils (Bardy, 1984). Soils, however, are remarkable systems in that as dissolved cations, anions, or neutral substances are removed from the soil solution (e.g., root uptake, sorption) they are replaced by other cations, anions, or neutral substances (e.g., root exudates, secretions, and proton pumps, desorption) as long as soil solution chemistry does not change dramatically and adequate sources are present. From a standpoint of plants this replacement is particularly important since nutrient elements in most soil solutions are far less than needed for plant growth. This replacement may also be very important for the expression of allelopathic effects. However, concentrations of dissolved substances that are too high can lead to drought stress, salinity stress, and/or toxicity.

Another important property of any soil solution is its acidity, neutrality, or alkalinity. These states are normally related to pH. Solution pH can determine the solubility and the ionic state of both inorganic and organic substances. The following four examples should demonstrate the importance of this solution property:

- a) At pH values below 5, aluminum, iron, and manganese can be soluble in sufficient concentrations to be toxic to plant growth (Moore, 1974; Foth, 1990).
- b) Soil phosphorus is never readily available to plant roots, but its availability is generally highest in a range centering around pH 6.5 (Brady, 1984).
- c) Simple phytotoxic phenolic acids, such as ferulic acid, have a  $pK_a$  of approximately 4.5 (see Blum et al. 1999). This means that at pH 3.5 ferulic acid is 90% protonated (neutral) and 10% negatively charged, at pH 4.5 the ratio is 50/50, at pH 5.5 the ratio is 10/90, and at pH 6.5 the ratio is 1/99. All indications are that root contact and uptake of phenolic acids, such as ferulic acid, occur when molecules are in their protonated state and are repelled or not taken up in their negative state (Harper and Balke, 1981, Blum et al. 1985; Shann and Blum, 1987a; Lehman and Blum, 1999). The influence of solution pH on root/microbe - ferulic acid (or for that matter other organic acids) toxicity can thus be substantial. and
- d) Most soil organisms have a preferred pH range (Paul and Clark, 1989). For example, bacteria generally prefer a near neutral pH range, while fungi prefer a more acidic range. Actinomycetes, on the other hand, do not tolerate acidic conditions very well. Earthworms prefer a neutral to slightly basic pH range and litter feeding arthropods prefer a more acidic range (Lavelle and Spain,

2001). Thus solution pH can have an enormous influence on organic matter turnover rates in soil. Plant species can also be categorized by the pH range they can tolerate or grow best in. For example, alfalfa and clover grow best in moderately acidic to slightly alkaline soils and blueberries and azaleas grow best in strongly acidic soils (Brady, 1984). I am not aware of any research data on how plant preferred pH ranges may relate to susceptibility of allelopathic agents, such as organic acids, under acidic conditions.

## Sorption/desorption of water soluble organic substances

The mineral and organic components of soil are capable of reversibly and/or non-reversibly (i.e., fixed into the recalcitrant organic matter) sorbing mineral ions, and water-soluble organic substances (ions, molecules, and compounds), including enzymes. Sorption of inorganic ions was described in a previous section. Soluble organic substances and enzymes reach these surfaces by diffusion (over very short distances) or by means of mass flow of gravitational and capillary water driven by gravity or “transpirational pull” of plants, respectively. Organic substances and enzymes sorbed to clay or humic constituents, for example, are protected from microbial utilization and hydrolysis by soil enzymes in the soil solution (Lavelle and Spain, 2001). This reversible and non-reversible sorption of organic molecules and enzymes can thus dramatically influence soil solution chemistry, and thus allelopathic interactions. The type of sorption process and its strength will depend on the molecular size, polarity, and ionization of an organic substance. Nonpolar, polar non-ionic, and organic electrolytes (ions) are generally weakly, weakly, and strongly sorbed, respectively, to clay and humus surfaces (Singer and Munns, 1999).

The state (i.e., cationic, anionic or protonated [nonionic] forms) of the soluble organic electrolytes in soil solution and thus their potential for sorption depends on their chemical composition, their  $pK$  values, and soil solution chemistry (e.g., solution pH, other cations and anions present). Cationic and anionic forms can bind to negatively charged (e.g., clays, organic particles) or positively charged sites (e.g., Al and Fe oxides) on soil surfaces, respectively (Greenland, 1965, 1971; Watson et al. 1973; Lehman et al. 1987; McBride, 1987). Multivalent cation bridges can immobilize anionic forms to negatively charged sites (Greenland, 1965, 1971).

Protonated forms can be sorbed (e.g., chemical partitioning, ligand exchange, oxidative reactions) by soil organic matter (Chiou, 1989; Hassett and Banwart, 1989) and/or polymerized into humic substances in the soil (Martin et al. 1972; Martin and Haider, 1976; Haider et al. 1977; Shindo and Huang, 1984; Wang et al. 1986). Sorption may also occur through hydrogen bonding and van der Waal's forces (Greenland, 1965, 1971).

The binding strengths of the resulting complexes vary considerably. Some complexes (e.g., those resulting from hydrogen bonding, van der Waal's forces, anion exchange or cation bridging) are easily disrupted (i.e., desorbed); others (e.g., those resulting from ligand exchange, oxidative reactions with mineral surfaces, polymerization) are fixed into recalcitrant organic matter or bound onto clays and thus may be unavailable for re-entering the soil solution. Clearly, sorption/desorption is a major factor in determining soil solution concentrations of allelopathic agents.

Finally, sorption/desorption is not only limited to inorganic and organic ions. At typical soil pH values (pH 5 to 8) surfaces of soil microorganisms are predominantly negatively charged and can go through similar sorption/desorption processes in soil (Hartel, 1998). This is also true for primary root surfaces since substances in cell walls such as pectins and many xylans and arabinogalactans contain uronic acid residues making cell walls predominantly acidic and negatively charged (Fry, 1988). How important this sorption/desorption by microbe and root surfaces may be to the expression of allelopathic effects is presently not known, however, I suspect that it could be of substantial importance.

## **SOIL ORGANISMS**

### **Bulk-soil fauna**

Soil contains a variety of animals ranging from microfauna (e.g., protozoa, amoeba), mesofauna (e.g., nematodes, small worms, insect larvae, mites, springtails) to macrofauna (e.g., beetles, ants, termites, spiders, centipedes, earthworms, small mammals) (Brady, 1984; Foth, 1990; Singer and Munns, 1999; Lavelle and Spain, 2001). They have a variety of functions, aside from their own growth, development and reproduction, including fragmentation of litter and organic particles which increases the surface area for microbial action, regulation of soil microbial populations and each other (i.e., predation), utilization and conversion of organic matter (i.e., decomposition), aeration of the soil, mixing of organic matter throughout the soil, soil aggregate formation, nutrient cycling, modifying the carbon/nitrogen ratio of the soil, and feeding on roots (i.e., herbivory), to name a few. Thus soil fauna not only expedite decomposition and release/loss of potential allelopathic agents, but also compete for resources with soil microorganisms and with plant roots.

The estimated number of soil fauna, excluding nematodes and protozoa, may total well over 1.5 million/m<sup>2</sup> for a typical mineral soil (Kevan, 1965). Nematodes ranged from 1.8 to 120 million/m<sup>2</sup> in a European grassland soil (Kevan, 1965) and from 1.7 to 6.3 million/m<sup>2</sup> in a variety of forest soils (Sohlenius, 1980). Macfadyen (1968)

determined, based on a variety of studies, that soils on average contain just below 1 million protozoa per m<sup>2</sup>.

## Bulk-soil microorganisms

Species of microorganisms (i.e., algae, bacteria, actinomycetes, and fungi,) in soil are very diverse, have a broad range of metabolic capacities, catalyze reactions that can change soil physical and chemical properties, decompose organic matter and synthesis/release/loss of potential allelopathic agents, and/or cause various root diseases (Brady, 1984; Foth, 1990; Albrecht, 1998; Singer and Munns, 1999; Smith and Ley, 1999; Lavelle and Spain, 2001; Blum, 2003). Populations of microbial species range from few per gram of soil to millions per gram of soil. Bacteria are the most abundant attaining populations in excess of 10<sup>8</sup>/g soil, representing 10<sup>4</sup> to 10<sup>6</sup> different species (Wollum, 1998). Algae and cyanobacteria located primarily near the surface or just below the surface range from 10<sup>3</sup> to 10<sup>8</sup>/g soil, although numbers of 10<sup>3</sup> to 10<sup>4</sup> are much more common. The actinomycetes and fungi range between 10<sup>6</sup> to 10<sup>7</sup> and 10<sup>4</sup> to 10<sup>6</sup>/g soil, respectively. The relative population density of a given microbial species in the soil is determined by predation and by its ability to reproduce and compete with other organisms and organs (e.g., fauna, microbes, and roots) for resources whenever physical and chemical requirements are above the required minimum level for that species. Most microbial species will form inactive resting states (i.e., quiescent) and/or survival structures (e.g., spores, endospores, sclerotia; i.e., dormant stages) whenever conditions fall below their minimum requirements. These quiescent and dormant stages can exist in soil for very long times and become reactivated as soon as their minimum resource requirements are exceeded. Actually, microorganisms spend most of their lives just surviving. For example, the average bulk-soil bacteria may be active 3 years out of 30 years (Lavelle and Spain, 2001). Finally the physical, chemical, and biological properties of soils are constantly changing. As soil properties change, so do the activities of microbial species within the soil.

Another biological entity in the soil are the viruses that in fertile agricultural soil may reach 10<sup>11</sup> viruses/g soil (Angel and Gagliardi, 1998). Such numbers and the fact that they require a living host, such as bacteria, fungi, insects, plants or animals suggests that they play a significant role in soils but few functions besides disease and gene transfer have been identified.

## DECOMPOSITION

Decomposition, a series of oxidation-reduction reactions, involves two simultaneous, but complementary, processes: mineralization and humification (Lavelle and Spain, 2001). Both processes are a result of faunal and microbial activities within the soil. Mineralization is a catabolic process which ends when elements contained in organic matter are finally released in inorganic forms, such as nitrate, ammonium, phosphate and sulfate. Humification, on the other hand, is an anabolic process by which organic substances are condensed into recalcitrant organic polymers (polymerization) such as humus (e.g., humic acids, fulvic acids, humin). Both processes are important to soil productivity in that the former influences root, microbial and faunal behavior, soil solution chemistry, formation and losses of inhibitory and/or stimulatory concentrations of allelopathic agents, and regulates the fluxes of available ions to soil organisms and roots while the latter regulates the accumulation of stabilizing (i.e., recalcitrant) organic matter in the soil.

Most natural communities eventually reach a steady state between input and loss of litter. Once a steady state has been reached the turnover rate of the litter can be calculated as follows: average standing crop of litter divided by annual input of litter. Based on this ratio, estimates of turnover rates of 1.6 to 5 years have been determined for deciduous forest litter, 16 to 20 years for pine forest litter, and 0.8 to 1.5 years for grassland litter (Rodin and Bazilevich, 1968 a, b).

Decomposition of plant residues under aerobic and anaerobic conditions proceeds via the formation of simple organic substances which are then broken down further. Under aerobic conditions these compounds disappear very rapidly from the soil. However when oxygen is deficient these organic molecules tend to accumulate in the soil to toxic levels. Among the compounds formed under these conditions are: methane, hydrogen sulphide, ethylene, lactic acid, butyric acid, formic acid, benzoic and cinnamic acid derivatives (e.g., p-hydroxybenzoic, ferulic, respectively), amino acids, and many other intermediate breakdown products (Patrick, 1971; Brady, 1984). Anaerobic soil environments would occur in heavy, poorly aerated or waterlogged soils and in soils after extended periods of heavy precipitation. Pockets of anaerobic conditions would also be found in aerated soils, particularly associated with clay and organic particles or clay-organic aggregates in the soil. Greenwood (1961) found that water-saturated soil aggregates greater than 3 mm in diameter had no oxygen at their center and that whenever oxygen concentrations were less than  $3 \times 10^{-6}$  M that aerobic microbial activity was inhibited. Oxygen diffusion is about 10,000 times faster in air than through water (Greenwood, 1961) and thus variations in the oxygen concentrations in moist aerobic soils is very likely to be highly variable. Concentrations of phytotoxins should thus also be highly variable in such soils since these would be inversely related to oxygen concentration.

## ROOTS

### Roots and root systems

Defining what constitutes a root is not as easy as it would first appear, since there are a variety of specialized root types (e.g., fleshy roots, aerial roots, pneumatophores). This discussion, however, will focus on the common root types, woody and non-woody tap, fibrous, and adventitious roots. Such roots are normally below ground (with the exception of some portions of some adventitious roots), have no leaf organs or buds, no nodes or internodes, and usually no stomates, but may have lenticels (Esau, 1965; Fahn, 1982). Such roots also have a root cap that covers the apical meristem, lateral roots that arise from the pericycle, adventitious roots that arise from tissues other than the pericycle, and may or may not have secondary growth. Finally, root diameter and surface characteristics of individual roots varies widely within species and between species.

The initial development (i.e., primary growth) of roots, no matter their origin, can be categorized by the following root zones:

- a) embryonic zone - location of apical meristem (i.e., zone of cell division) covered by a root cap,
- b) elongation zone - where cells elongate and begin to differentiate,
- c) root hair zone - the most active zone for root uptake in which the primary tissues (i.e., xylem, phloem, pericycle, endodermis, cortex, epidermis) have fully matured and root hairs have developed from epidermal cells,
- d) zone of suberization - zone in which suberin, a waxy material, covers the outer surface (epidermis) of the root reducing root uptake, but not eliminating it; this is also the area of the root where lateral roots are formed from the pericycle, and finally
- e) the zone of decay of the cortex (loss of epidermis, cortex, and endodermis) and development of secondary tissue (i.e., secondary xylem and phloem, periderm).

Associated with the embryonic zone are four transitory meristems involved in root growth:

- a) root initials which produce the three other transitory meristems,
- b) the protoderm which produces the epidermis,
- c) the procambium which produces the vascular tissue (e.g., primary xylem and phloem), and

- d) the ground meristem which produces the ground tissue (e.g., cortex in dicotyledonous roots and cortex and/or pith in monocotyledonous roots).

Secondary growth, when present, for roots depends on the vascular cambium which produces secondary xylem and phloem, and the pericycle (just underneath the endodermis) which is the origin not only of lateral roots but also the periderm (composed of three tissues - cork cambium [phellogen] which produces cork cells to the outside [phellem] and cork parenchyma cells to the inside [phelloderm]). The vascular cambium, phloem and periderm together form what is commonly called bark. Since most root meristems (embryonic zones) can continue to generate young undifferentiated tissue for long periods of time and maturation along the root axis is gradual, there is a gradient of different developmental maturities along any individual root.

Root systems of monocotyledonous plants (e.g., cereals and grasses) consist of seminal roots, which are composed of the primary root and roots arising within the nodes of the embryo, and crown roots, which arise from the crown and subsequent stem nodes (Kutschera, 1960; Klepper, 1987; Rendig and Taylor, 1989; Moore et al. 1998). Some or all of the seminal roots may die whenever an adequate crown root (adventitious) system has been produced. The frequency of branching of seminal and adventitious roots ranges from none to several orders of branching. The resulting root system is referred to as a fibrous root system.

Herbaceous dicotyledonous root systems originate from the primary root which forms a tap root with many branching lateral roots. Root systems range from systems with a dominate tap root to systems where the tap root atrophies and branching lateral roots take over. Eventually, however, with time the dominance of the tap root is generally lost for most root systems (storage roots are one exception) and lateral roots (i.e., secondary, tertiary, etc.) take over to form what is referred to as a diffuse root system (Klepper, 1987; Kutschera, 1960; Rendig and Taylor, 1989; Moore et al. 1998). As a general rule roots that develop early in ontogeny (e.g., tap roots) tend to be oriented in the vertical direction but secondary, tertiary, etc. lateral roots tend to be oriented in the horizontal direction, at least initially. The depth of the root system is determined, in part, by the amount of root branching, the greater the branching the shallower the root system.

Perennial dicotyledonous species develop root systems just like herbaceous dicotyledonous species, but then secondary growth leads to the development of a framework of “long term” roots (i.e., woody roots or perennial roots) which continue to grow and initiate new lateral and adventitious roots throughout the soil whenever the environment is appropriate (Lyr and Hoffman, 1967; Moore et al. 1998; Charlton, 1996). Formation of these lateral and adventitious roots, referred to as feeder roots or fine roots, is common in the upper horizons (i.e., O and A horizons) of the soil. The

feeder roots (mycorrhizal or non-mycorrhizal) generally have a considerably shorter life span (a couple of months to years depending on species) than woody roots. Feeder roots either die or in the case of some non-mycorrhizal roots convert to woody roots (Bloomfeld et al. 1996). The resulting root systems, therefore, consist of a mixture of different types of roots and ages of roots. The balance of the types of roots and ages of roots for a given perennial species varies with plant age, season and soil environment. However, no matter what the balance of root types and ages an adequate feeder roots system is essential for survival.

The central point, different species have very different root types (herbaceous, woody, fine, coarse), root systems (fibrous, tap, diffuse), and horizontal and vertical distributions. Knowing what these differences are when studying plant-plant interactions is important since such differences will provide insight into the exploitative-competitive nature of species for soil resources, the proximity of their roots to each other, and thus their potential for chemical interactions.

## **Root morphology, rhizoplane, and rhizosphere**

Normally the root tip is covered with the root cap which is the site of detection of gravity and provides protection to the root meristem as the root grows through the soil (Sievers and Braun, 1996). The outer surface of roots is the epidermis which produces root hairs in the root hair zone that eventually becomes suberized or is lost with periderm formation (Hofer, 1996). The surface of the root is referred to as the rhizoplane while the area just around the root is referred to as the rhizosphere (Campbell and Greaves, 1990; Kennedy, 1998; Pinton et al. 2001). Associated with both the rhizoplane and rhizosphere are bacteria, actinomycetes, fungi, and fauna (Kennedy et al. 1998; Lavelle and Spain, 2001). These organisms are supported by the inorganic and organic secretions, exudates, lysates and mucilage products produced by the root (Rovira et al. 1979; Neumann and Romheld, 2001).

More specifically the rhizosphere consists of the soil matrix immediately surrounding the root (Rovira and Davey, 1974; Rovira et al. 1979; Kennedy, 1998; Lavelle and Spain, 2001). It is frequently characterized by gradients in soil moisture, pH, organic matter, nutrients, microorganisms, fauna, and soil gases. In each instance there are distinct differences between the rhizosphere and bulk soil brought about by the action of roots. Soil moisture, oxygen and nutrient levels are generally lower in the rhizosphere than in the bulk soil. Organic matter, microorganisms, fauna (particularly microfauna) and carbon dioxide levels are generally higher in the rhizosphere than the bulk soil. Changes of plus or minus one pH unit are not uncommon between the rhizosphere and the bulk soil (Jaillard et al. 1996). Roots maintain neutral electrical conditions in the rhizosphere by balancing uptake and losses of anions and cations.

The extent of the rhizosphere ranges from a few millimeters in the absence of mycorrhizae to several centimeters in the presence of mycorrhizae.

Bacteria, fungi, and protozoa populations are greatest on the rhizoplane and decline rapidly with distance from the root (Papavizas and Davey, 1961; Curl and Harper, 1990). Rouatt and Katzenelson (1961) found roughly  $10^9$  colony forming units of bacteria/g root dry mass on the rhizoplane, and a range of  $10^8$  to  $10^9$  in the rhizosphere. The ratio of rhizosphere to bulk soil colony forming units were 24 for red clover, 6 for oats and wheat, and 3 for maize and barley. Rouatt et al. (1960) found wheat rhizosphere to bulk-soil ratios of 24 for bacteria, 112 for fungi, 2 for protozoa, 125 for ammonifiers, and 1260 for denitrifiers. Populations of bacteria, fungi, and protozoa in the wheat rhizosphere were  $10^9$ ,  $10^6$ , and  $10^3$  colony forming units/g soil, respectively.

These organisms serve numerous functions in the rhizosphere, among them:

- a) decomposition (mineralization and humification) of organic residues and production and utilization of potential allelopathic agents,
- b) enhancing plant growth through production of plant hormones,
- c) increasing nutrient availability,
- d) enhanced nutrient use efficiency, and
- e) protection against root pathogens and possibly phytotoxins.

While some of these associated species of microorganisms cause disease others form symbiotic relationships with roots (e.g., mycorrhizae and nodules). With few exceptions primary roots (roots without secondary growth) of most plant species form ectomycorrhizae and/or endomycorrhizae, a symbiotic relationship between fungi and roots (Wilcox, 1996). Ectomycorrhizae benefit roots by providing a mechanical and chemical barrier, the mantle, that protects against root pathogens. Both ecto- and endomycorrhizae have associated with them a hyphal system that increases the soil volume exploited for nutrients and water and thus increase nutrient uptake and drought resistance. Another example of a symbiotic relationship is the formation of nitrogen fixing nodules by *Rhizobium* species on roots of some species of the *Leguminosae* or by actinomycetes on roots of some species of the *Ulmaceae*, *Betulaceae*, and *Casuarinaceae* (Vance, 1996). Without question the primary roots are the most biologically active in terms of growth and mortality, uptake and loss of water, nutrients, and soluble organic substances, and microbial and faunal activity.

In general, roots with secondary growth (growth in diameter and the formation of a periderm) live much longer and form what is referred to as a woody or perennial root system which provides plant support and the place for continued formation of new lateral and adventitious roots such as feeder roots that may or may not become

mycorrhizal. Feeder roots are primary roots and thus are biologically very active. For woody roots the suberized cork cells of the periderm substantially reduce biological activity for these roots by partially isolating the living cells of these roots from the soil environment. However, their role to water, nutrient, and organic substance uptake or loss should not be entirely ignored since the periderm may contain cracks, crevices, and lenticles (Kramer and Bullock, 1966; Chung and Kramer, 1975 ).

The nature of the root surface morphology (e.g., unsuberized, suberized, with or without a periderm, types and location of root hairs, mycorrhizal or non-mycorrhizal) will clearly influence the outcome of allelopathic interactions since these surface characteristics will determine the nature of rhizoplane and rhizosphere, and thus the likelihood of contact between allelopathic agents and root surfaces. The range of differences between the bulk soil and the rhizosphere/rhizoplane suggest that bulk-soil physicochemical properties (e.g., nutrition, pH, presence or absence of allelopathic agents) may provide a poor basis for determining the presence or absence of allelopathic interactions.

## **Root growth**

Root growth of herbaceous species occurs primarily during the vegetative phase of shoot development and declines rapidly during the reproductive phase of plant development. In the case of soybeans, for example the growth rate during pod fill is about half the rate during the period of vegetative growth (Rendig and Taylor, 1989). Root systems are extremely plastic so that development of all root systems is very irregular and largely regulated by the supply of energy, carbon and growth regulators from the shoot and the surrounding soil environment. Soil conditions that regulate root growth are texture (portion of sand, silt, and clay), structure (size and distribution of pores and channels), temperature, pH, water content, oxygen supply, available nutrient supply, and presence or absence of concentrations of toxic and/or growth promoting substances, pathogens, and herbivores. These soil factors operate simultaneously and interactively with each other and with the shoots in regulating root growth and thus root distribution (Klepper, 1987). Roots generally proliferate rapidly where soil conditions are good and slowly, or in extreme cases not at all, where conditions are poor or inhibitory. This behavior leads to what is referred to as compensatory root growth, growth associated with exploitation of available resources by one portion of a root system that compensates for a lack of available resources by another portion of a root system (Brouwer, 1981, 1983). In addition, such differential growth also allows for the rapid exploitation of patches of high nutrient supply in the soil (Fitter, 2002). This root system plasticity, the ability of individual root meristems to respond quickly to changing soil conditions, is a fundamental adaptive characteristic of roots which ensures maximum acquisition of resources and adequate anchorage for plants.

Root initiation and growth for perennial species in many instances starts earlier in the spring than shoot growth and ends after shoot growth in the fall (Lyr and Hoffman, 1967). However, for some species, conifers in particular, root initiation and growth occurs throughout the year whenever the soil environment is appropriate. Thus root initiation and growth are very irregular over time and vary substantially from year to year because they are largely regulated by the soil environment as long as energy and carbon reserves are adequate. Here also compensatory root growth and the ability for roots to exploit patches of high resource supplies by feeder roots are common.

Root growth is essential for plant survival since:

- a) new root zones for active uptake of water and nutrients (i.e., root hair zone), must be continuously produced due to maturation of tissues along the root axis (i.e., root hair zones eventually lose their root hairs and become suberized or covered with a periderm),
- b) resources such as phosphorus, which are highly immobile in soil, are rapidly depleted around roots, and root growth is thus required for a continuous supply of these type of resources,
- c) growth is needed to compensate for root mortality and herbivory. Thus anything that impacts root growth, including allelopathic agents, will influence the success or failure of a plant.

## **Root life span**

Like all plant organs, roots will pass from birth to death. Thus the size and population structure of a root system is determined by the birth and death rates of individual roots. Based upon optimization theory, total plant growth should be greatest when a root system maximizes water and nutrient acquisition per unit of resource supplied by the shoot (Eissenstat and Yanai, 2002). Thus if roots were initiated in the most favorable soil patches and became inactive or were shed when they were no longer efficient in nutrient and water acquisition, then maximum plant growth could be maintained. The ability of a root system to efficiently capture and utilize resources thus depends on root growth, root turnover (birth minus death of individual roots), root distribution, spatial and temporal heterogeneity of water, oxygen and nutrients in the soil, and physiological activity of individual roots comprising the system which changes with root age, energy and carbon supply, temperature and resource availability. Such cost-benefit analysis of root life span suggests that plants control the life span of their roots and ignores other external factors such as disease

and herbivory aboveground and belowground. In fact, these factors also have a role in determining root life spans. The bottom line is that there is enormous variation in root life span (days to years) and the source of this variation is not well understood for the “normal range” of environmental conditions experienced by plants. The average functional life of higher order roots for soybean plants, for example, is roughly 10 to 20 days (Rendig and Taylor, 1989). For trees the median life span of the finest roots can range from <20 days in fast-growing trees to >1 year for slow-growing trees (Eissenstat and Yanai, 1997). Root diameter is also important, for example, the median life span of 0.1 to 0.2 mm diameter apple roots was approximately 40 days, while it was >240 days for 0.5 to 1.1 mm diameter roots (Wells and Eissenstat, 2001).

How the life span of individual roots and thus root distribution may influence plant responses to allelopathic agents is not known. This is an area that deserves attention, particularly for seasonal or longer term studies.

### Absorbing zone of roots

Roots have a variety of functions. Among them are anchorage of the plant, storage of energy and carbon reserves, site of growth regulator synthesis (e.g., abscisic acid, cytokinins, auxins) and interconversions (e.g., gibberillic acids), synthetic activities (e.g., nitrogen fixation, conversion of inorganic nitrogen to amino acids, synthesis of organic acids) and absorbing water and nutrients. Here we are chiefly concerned with roots as absorbing organs including uptake of organic substances.

For primary roots without mycorrhizae, little water, nutrients, and organic substances enter near the tip of the roots because of the presence of the root cap, the high resistance of the dense protoplasm there, and the lack of xylem tissue for transporting these substances away from the site. Beyond the root hair zone the xylem tissue is functional but suberization and/or lignification of the endodermis, hypodermis, when present, and epidermis can seriously reduce the entrance of water, minerals, and organic substances. Thus maximum rates of uptake take place in the root hair zone (Kramer and Boyer, 1995). This is not to say that suberized roots do not take up water, nutrients or organic substances. They do. Chung and Kramer (1975) observed for 1-year-old pine seedlings that removal of unshuberized roots reduced the root surface area by 42%, the rate of water uptake by 23%, and  $^{32}\text{P}$  uptake by 47%. Thus considerable amounts of uptake occurred by way of the suberized roots.

Hyphae (essentially functioning like root hairs) of endomycorrhizae are found along the entire root surface of herbaceous monocotyledonous and dicotyledonous roots and spread directly from the root surface to the surrounding soil. For perennial species hyphae of endo- and/or ectomycorrhizae are located only on the fine or feeder roots (i.e., primary roots, roots without a periderm) of woody species. For

ectomycorrhizae the root surface is surrounded by a layer of hyphae called the mantle and hyphae spread out from it into the surrounding soil. (Smith and Read, 1997).

For roots with secondary growth the presence of the periderm will seriously reduce the entrance of water, nutrients and organic substances. However, Kramer and Bullock (1966) suggested that, in fact, some water and nutrients may actually be taken up by woody roots since the periderm of woody roots may contain cracks, crevices, and lenticles.

There are three pathways by which water, nutrients, and organic substances can enter primary roots. The first is by way of the intercellular spaces. This is referred to as apoplastic movement. However, a portion of the intercellular spaces are filled with gas since the complete infiltration of these spaces by water leads to abnormal growth (Burström, 1959; 1965). The movement of water, nutrients, and organic substances terminate at the endodermis because the Caspary strip surrounding the cells of the endodermis seals the intercellular spaces of the endodermis. The second pathway can be initiated at the epidermis with or without root hairs, anywhere within the cortex, and/or at the endodermis. Water, nutrients, and organic substances cross a cell membrane and then are transported or moved from cell to cell by way of plasmodesmata. This is referred to as symplastic movement. The third is by way of fungal hyphae. This would be a combination of symplastic and apoplastic movement. Since hyphae do not penetrate cell membranes and terminate in the root cortex, water, nutrients, and organic substances would have to leave the hyphae and enter the intercellular spaces of the cortex and then pass through cell membranes in the cortex or the endodermis before moving on towards the vascular tissue of the root. Hyphae of ectomycorrhizae are only located in the root intercellular spaces of the cortex. Hyphae of endomycorrhizae are also located in the intercellular spaces of the cortex but hyphae also penetrate cell walls at various places and form arbuscles between the cell wall and the cell membrane (Smith and Read, 1997).

The influence of mycorrhizae on plant nutrition, water relations, and other aspects of plant physiology (e.g., Pfleger and Lindemann, 1994; Smith and Read, 1997; Blum et al. 1999) suggest that mycorrhizae will have a substantial impact on plant responses to allelochemical agents. However, I am not aware of any direct comparisons of water and nutrient uptake by roots with and without mycorrhizae in the presence and absence of allelopathic agents.

## **Cell membranes**

Cell membranes are highly impermeable to charged inorganic and organic substances (i.e., ions), but nonpolar molecules such as hydrocarbons and oxygen, and small uncharged polar molecules such as water and carbon dioxide readily pass

(diffuse) through the membrane core (Moore et al. 1998; Taiz and Zeiger, 2002). Diffusion, however, could not account for the rates of water movement across membranes. The discovery of water channels, aquaporins, in membranes appears to have resolved this issue. The movement of water in this instance is regulated by phosphorylation (Assmann and Haubruck, 1996). Larger molecules and ions require channels, composed of membrane proteins surrounding an aqueous core (diffusion - passive transport) or protein transporters (facilitated diffusion - passive transport; symporters and antiporters - active transport) to move through membranes (Maathius and Sanders, 1992). Channels appear to be primarily involved in the movement of specific ions or water although there is some evidence to suggest that they may also be involved in transport of organic acids (Walker et al. 2003). Transporters (in the past called carriers) also move specific ions but tend to specialize in moving specific organic molecules across membranes (Taiz and Zeiger, 2002). For example sucrose, amino acids, and a variety of other metabolites are taken up by symport with protons. Larger metabolites such as flavonoids, anthocyanins, and secondary products of metabolism are transported by ATP-binding cassette (ABC) transporters (Martinoia et al. 2002; Taiz and Zeiger, 2002; Walker et al. 2003). ABC transporters have been found in the plasma membranes of plants, animals and microbes, and the tonoplast of plants (Nikaido and Hall, 1998; Theodoulou, 2000). Transport by ABC transporters consumes ATP directly.

There has been very limited research on how allelopathic agents modify membrane behavior directly (e.g., Glass and Dunlop, 1974), a subject that clearly deserves much more attention. Indirect evidence such as water and ion uptake studies (Glass, 1973, 1974; McClure et al. 1978; Harper and Balke, 1981; Einhellig et al. 1985; Lyu and Blum, 1990; Lyu et al. 1990; Bergmark et al. 1992; Booker et al. 1992; Lehman and Blum, 1999), however, suggests that membrane perturbations are an important aspect of plant response to allelopathic agents, such as phenolic acids (see section of “Plant Responses” for additional details).

## Root-root connections

There is a general tendency to think that the root system of each plant is an independent entity. This, however, turns out not to be the case. For example, herbaceous monocotyledonous and dicotyledonous roots and primary roots associated with woody dicotyledonous roots of a variety of species can be interconnected by hyphae associated with mycorrhizae. These types of connections are frequently not species specific (Francis and Read, 1984; Simard et al. 1997; Smith and Read, 1997). Such connections have been shown to transfer water, nutrients, and carbon compounds (Brownlee et al. 1983; Francis and Read, 1984; Read, 1997; Simard et al. 1997). For woody species there is an additional transfer mechanism which in many instances is

also not species specific, the root graft (Graham and Bormann, 1966). These occur primarily when secondary growth of woody roots press woody roots growing side by side against each other and continued secondary growth leads eventually to connections of the secondary vascular tissue. Once vascular tissues are connected, anything moving in the xylem and phloem can move from plant to plant, including toxic or stimulatory concentrations of organic substances. The direction of water and carbon movement in hyphae and graft connections are determined by source-sink relationships (i.e., the strength of the sink).

I am not aware of any research regarding root connections and the transfer of organic phytotoxins (potential allelopathic agents). By definition, however, this type of root to root transfer between two individuals would not constitute an allelopathic interaction.

## **Root-shoot relationships**

The functional equilibrium model hypothesizes that growth of roots and shoot systems are in strict harmony with one another and thus the ratio between them is predictable under a great variety of external conditions (Brouwer, 1983). More specifically when resources (water, nutrients) are limiting to roots, root growth is favored and when resources ( $\text{CO}_2$ , solar radiation) are limiting to shoots, shoot growth is favored. These changes in allocation patterns turn out to be relatively strong when nutrient supplies are varied by fertilization but changes in morphology and physiology tend to be more common for a wide range of natural environmental variations, including solar radiation, nutrients, water and  $\text{CO}_2$  (Reich, 2002). Rather than making large adjustments in allocation to roots or shoots, plants tend to change tissue morphology, anatomy, metabolism and root turnover to alter their ability to capture and/or retain resources. For example, root diameter of P-deficient barley plants was about a third compared to those supplied with phosphorus and exhibited prolific development of elongated root hairs which were essentially absent in P-supplied plants (Lefebvre and Glass, 1982). Such changes in root surface area could have important implications to allelopathic interactions.

## **ORGANIC ACIDS IN SOIL SYSTEMS: SOME ADDITIONAL OBSERVATIONS**

### **Allelopathy**

Molisch (1937) coined the term allelopathy to describe both positive and negative chemical interactions of plants and microorganisms mediated through the environment

(Willis, 1985; Molisch [translation], 2001). The terminology describing these interactions and the definition of allelopathy have, however, varied somewhat over time (see Grodzinsky, 1971; Whittaker and Feeny, 1971; Rice, 1974, 1979, 1983; 1984; Willis, 1985). Unfortunately even Molisch's definition is open to interpretation. The inclusion of "chemical interactions mediated through the environment" in the definition suggests that the effects of physicochemical modifications of soil resulting from the addition of organic matter/substances to soil are excluded. In addition, substances used only as a source of nutrients, carbon, or energy (Whittaker and Feeny, 1971) and substances that are directly transferred between plants (i.e., mycorrhizal hyphae and root grafts) are also excluded.

## Plant responses

There is considerable literature on proposed mechanisms of action of individual organic acids, particularly phenolic acids (Rice, 1984; Einhellig, 1986, 1995, 2002; Prasad and Devi, 2002). In each case, the primary site of action appears to be at the root cell membrane level (i.e., function like contact herbicides), but ultimately, through a cascade of effects, these root cell membrane perturbations lead to a disruption of plant nutrition, water relations, and/or energy fixation (Glass and Dunlop, 1974; Harper and Balke, 1981; Bergmark et al. 1992; Booker et al. 1992; Einhellig, 1986, 1995, 2002; Blum et al. 1999; Prasad and Devi, 2002). There is, therefore, nothing unique about plant responses to inhibitory concentrations of these type of allelopathic agents when compared to a variety of other environmental stresses. The literature on mechanisms for stimulatory concentrations of allelopathic agents is generally lacking, but one would expect similar, but positive, types of influences on plant nutrition, water relations, and/or energy fixation (Rice, 1986). Data on how inhibitory, neutral, and/or stimulatory concentrations of various organic substances in soil solutions interact to modify plant behavior is also essentially lacking.

It may be argued that inhibitory concentrations of phenolic acids should actually function like systemic herbicides since allelopathic agents such as phenolic acids are taken up by roots and distributed to other parts of the plant (Glass and Böhm, 1971; Harper and Balke, 1981; Shann and Blum, 1987a; Lehman and Blum, 1999). By definition systemic agents are translocated throughout the plant from point of uptake (in this case roots) to the site of action (e.g., chloroplasts of leaves). Thus, as long as concentration and toxicity of the agents are sufficient, contact by any part of a root system should lead to a reduction in growth of the entire plant. However in terms of actual plant response, it appears that inhibitory concentrations of allelopathic agents, like simple phenolic acids (i.e., cinnamic and benzoic acid derivates) act more like contact agents than systemic agents. This is based on the following observations:

- a) There is a direct linear relationship between the level of root contact of inhibitory concentrations of phenolic acids and plant inhibition (i.e., leaf expansion, nutrient uptake, water uptake) and very poor to no relationship between phenolic acid uptake and plant inhibition (Klein and Blum, 1990; Lyu and Blum, 1990; Lehman et al. 1994; Lehman and Blum, 1999).
- b) Phenolic acid concentrations are highest at the root level, partly due to high levels in the apoplast, and are generally much lower (roughly 90 to 95% lower) by comparison in leaf tissue (Shann and Blum, 1987b; Blum unpublished data) and
- c) Plants readily utilize, inactivate, sequester, and dilute allelopathic agents, such as phenolic acids, once they enter roots (Bates-Smith, 1956; Harborne, 1982; Goodwin and Mercer, 1983; Shann and Blum, 1987b; Balke et al. 1987). This suggests that the amounts of phenolic acid in soil solutions taken up by a root and subsequently distributed to the rest of the plant are far too low for a systemic action. I suspect that this is also true for other potential phytotoxic organic acids. However, this topic has not been adequately explored and deserves much more attention.

It has been recognized that factors such as root predation, root disease, and competition need to be eliminated before allelopathic interactions can be identified as the causative agent (Willis, 1985; Weidenhamer et al. 1989; Blum et al. 1999) although clearly this is not an all or nothing proposition. What has not always been recognized or characterized is the role of direct root connection by way of hyphae or grafts. There is, however, considerable evidence for intra and interspecific transfer of water, nutrients, and carbon in the literature (Brownlee et al. 1983; Francis and Read, 1984; Read, 1997; Simard et al. 1997). Clearly this is an area that deserves attention. If nothing else, it represents another avenue by which substances can be transferred from root to root. Given our present definition of allelopathy, intra- and interspecific root to root transfers through root grafts or hyphae associated with mycorrhizae would not qualify as allelopathic interactions since the transfer bypasses the soil environment. That being the case, these transfers would have to be excluded just like competition.

## **Soil organic matter**

The nature of the organic matter, both soluble and particulate, is very complex containing a broad range of organic ions, molecules, compounds, and polymers. The addition of this organic matter to soil can change soil structure (e.g., formation of soil aggregates), aeration, water holding capacity, microbial and faunal activity, mineral nutrition, soil solution chemistry, soil horizon development, and soil temperature. All of these changes brought about by soil organic matter will influence the size and distribution of root systems and their ability to capture resources for plant growth,

development, and reproduction. Thus organic substances associated with allelopathic interactions constitute only one of a large number of organic-matter-influenced soil processes that affect root activity and growth. This then is one problem researchers in allelopathic interactions face in establishing the presence of and characterizing allelopathic interactions in the field, or, for that matter, in laboratory bioassays (Lehman and Blum, 1997; Blum, 1999).

## Water soluble allelopathic agents

To start with, all soluble organic and inorganic substances in and released from plants and modified by soil fauna and microbes can theoretically be stimulatory, neutral, or inhibitory depending on their concentrations and the sensitivity of the receiving roots (see Rice, 1979). Clearly some substances are much more effective in eliciting a response at lower concentrations than others but even that can vary dramatically depending on the soil environment. For example, the growth of cucumber seedlings was inhibited more by multiple treatments of 0.5 µmol/g soil ferulic acid than *p*-coumaric acid in Portsmouth B-horizon soil (Gerig and Blum, 1991) but just the opposite occurred in Cecil A-horizon soil where ferulic acid at that concentration had no effect on cucumber seedling growth (unpublished data). When we add the fact that:

- a) plants and plant debris release/lose (e.g., excretions, lysates, leaching, decomposition) very complex mixtures of soluble inorganic and organic substances,
- b) *p*-coumaric acid is more inhibitory to morning-glory seedling growth in the presence of non-inhibitory concentrations of glucose than in the absence of glucose (Pue et al. 1995),
- c) increasing soil nitrate levels decreases the inhibitory effects of *p*-coumaric acid but increases the inhibitory effects of methionine on morning-glory seedlings (Blum et al. 1993),
- d) soil solution concentrations of individual phenolic acids released/lost from plants or plant debris are invariably well below their level of toxicity, but that effects of individual phenolic acids in mixtures can be additive at soil solution concentrations (Blum et al. 1989, 1993; Lyu et al. 1990; Pue et al. 1995; Blum, 1996), and
- e) the level of inhibition of cucumber seedling leaf expansion and net nutrient and water uptake is directly related to the proportion of the root system in contact with individual or mixtures of phenolic acids (Klein and Blum, 1990; Lyu and Blum, 1990; Lehman et al. 1994), it must be concluded that trying to assign

allelopathic interactions under field conditions to a single compound or even groups of similar compounds, such as organic acids, is a questionable activity. After all, the resulting observed plant effects (inhibitory or stimulatory) will be determined by the functional ratio of promoters (substances at concentrations that stimulate) and inhibitors (substances at concentrations that inhibit) in the soil solution. Thus more research emphasis needs to be given to understanding how soil solution dynamics, the influences of promoters and inhibitors in soil solutions, and the influences of neutral substances on promoter/inhibitor activity impact root processes.

## **Distribution of allelopathic compounds**

The distribution of organic matter and the associated distribution of mixtures of allelopathic agents is important to plant response because:

- a) effects of water soluble allelopathic agents, such as organic acids, appear to be local in nature (i.e., act like contact agents), and thus, a sufficient part of the root system must be impacted for an inhibitory effect to be observed aboveground (Klein and Blum, 1990; Lyu and Blum, 1990; Lehman et al. 1994),
- b) roots, litter and organic matter, the major sources of allelopathic agents, are frequently unevenly distributed both vertically and horizontally in soil, and
- c) after initial establishment, plant root growth and distribution is largely regulated by the soil environment.

Roots grow poorly or not at all in soil zones that are inhibitory to them but generally grow where soil conditions are adequate or good for growth. The ability for roots to minimize direct contact with or essentially avoid inhibitory soil zones or pockets altogether is clearly very beneficial to herbaceous species whenever allelopathic agents are unevenly distributed in the soil. Stimulation of root growth in fertilized soil zones and substantial inhibition or avoidance of roots in inhibitory soil zones has been well established by the implant soil mass technique (sometimes also referred to as the mesh bag technique) using fertilizer, herbicides, and aluminum enrichment (Lund et al. 1970; Rechcigl et al. 1987; Lin and Myhre, 1990). Species with woody roots have even a greater ability to minimize interactions with allelopathic agents because of the protection of their periderm and their ability to produce new primary roots in fertile or non-inhibitory soil zones or pockets. This may also help to explain why seed germination and seedling establishment are much more susceptible to water soluble allelopathic agents in soils than are mature plants even when sources of allelopathic agents are unevenly distributed. Seeds and very young seedlings with their small root systems have a very limited ability to avoid sources of allelopathic agents in close proximity. However no matter the type or size of plant, a reduction in

aboveground growth or in extreme cases mortality will occur whenever root growth or function of a sufficiently large portion of a root system is suppressed.

## Stability, turnover rates, and sinks

The focus of allelopathic research has primarily been on soluble, more biochemically active substances. It should not be surprising that such substances have very rapid turnover rates (short residence time) in aerobic and nutrient rich soil environments (conditions found in most laboratory bioassays) and can, at times, be readily leached below the root zone. Rates of leaching would be largely determine by the amount of gravitational water generated by precipitation events, soil porosity, soil solution pH, and solubility and pK of substances of interest. However, under anaerobic conditions or in anaerobic soil pockets that are frequently found in field soils, organic substances would tend to accumulate because under these conditions input would be greater than losses (Patrick, 1971). Since most roots would not actively grow in such anaerobic soil areas, allelopathic agents would have to be transferred to these roots by mass flow of soil solutions.

Turnover rates or losses of biochemically active substances in the soil solutions would be determined by rates of sorption, polymerization, oxidation and reduction rates, uptake and/or utilization by soil fauna, microflora, and roots. Such losses would be greatest in aerobic soils where a number of soil processes (e.g., sorption, uptake, polymerization) and sinks (e.g., roots, microbes, fauna, clay, organic matter) actively compete for and remove soluble organic substances from the bulk- and rhizosphere-soil solutions. Even individual roots are competing for these substances (i.e., the greater the number of roots per unit area the fewer organic substances there will be per root; Weidenhamer et al. 1989; Thijs et al. 1994; Sinkkonen, 2001). This competition between soil processes and sinks and the afflicted root is important to allelopathic interactions because effects of mixtures of allelopathic agents are concentration (i.e., input-losses), soil environment, and receiving root dependent. Given this competitive environment, how frequently would roots growing in aerobic soil environments interact with allelopathic agents, such as organic acids, compared to microorganisms, fauna, clays, and organic matter?

From the standpoint of surface areas, clays, organic matter and soil microbes in bulk soil have much greater surface areas than roots, thus greater opportunities to interact with allelopathic agents. In addition, for allelopathic agents to reach the root surface they must not only pass through the bulk soil but also the rhizosphere and the rhizoplane, which also have large microbial and faunal populations that can potentially utilize allelopathic agents as a carbon and energy source. However, one should not be entirely misled by the number of organisms. For example, many of the bulk-soil microbes are inactive at any given point in time and only a small percentage of the root

surface is actually covered by bacteria. The percent cover for bacteria on roots of eight plant species ranged from 4% to 10%. (Rovira et al. 1974). The percent cover for fungi was about 3% for *Lolium* and *Plantago*. For *Pinus radiata* roots, bacterial cover ranged from 1% to 16% for four day old seedlings to 37% for a 90 day old root segment (Bowen and Theodorou, 1973). In spite of this, I would suggest that certain conditions/mechanisms would probably have to exist before potential allelopathic agents, such as simple organic acids, could reach a given root surface at toxic concentrations. A partial list of such potential conditions/mechanisms follows:

- a) The sorption of soil surfaces needs to be at a steady state with the soil solution or soil surfaces must be saturated, i.e., the supply is greater than the demand by soil sinks. This steady state is concentration dependent (Blum et al. 1994). A sorption steady state or saturation of soil sinks is likely to occur for the immediate areas surrounding decaying organic fragments within the soil or just underneath rapidly decaying litter. Such conditions may also occur in moist to wet soils with large inputs of aboveground and belowground litter. These soils are likely to have many anaerobic sites where organic molecules can accumulate since faunal, microbial, and root activity is very limited in anaerobic sites. Mass flow resulting from “transpirational pull” could bring these substances from anaerobic sites to roots growing in more aerobic sites. Thus adequate supplies of soil water and rates of transpiration by the receiving plant will be extremely important in determining the movement of allelopathic agents to its root system.
- b) The right combination of soil aggregates can also enhance root contact with allelopathic agents. Mobility of water and solutes in soils occurs through a complex continuum of pores of various sizes and shapes. Since different size pores hold solutions at different tensions, solute concentrations tend to vary among pore sizes (Jardine et al. 1990). Micropores tend to retain solutes more than meso- and macropores. Jardine et al. (1989) observed that significant concentrations of soil reactive tracers such as  $Mg^{2+}$ ,  $NH_4^+$ , and dissolved organic carbon were not sorbed by water-saturated soil after simulated precipitation events due to preferential flow of soil solutions through larger (meso- and macropores) soil pores. In extreme cases they were able to demonstrate that these reactive tracers behaved identically to non-reactive tracers such as  $NO_3^-$  and  $Br^-$ . They concluded that solute mobility in saturated soil was largely controlled by preferential flow. Under such circumstances mass flow resulting from “transpirational pull” could rapidly bring allelopathic agents from areas of higher concentration to roots and lead to a bioconcentration of these agents in the rhizosphere and at the rhizoplane.
- c) Root systems have an extensive mycorrhizal-hyphal system in close proximity to sources of allelopathic agents. Since fungi generally prefer acidic environments the presence of organic acids may expedite the growth of mycorrhizal hyphae

into these areas. These hyphae could transport more inhibitors (substances at concentrations that inhibit) than promoters (substances at concentrations that stimulate) to roots. Recall that substances transported by hyphae to roots are released into the intercellular spaces of the cortex before they can interact with root cell membranes. There are no direct connections between hyphae and plant cell membranes. Hyphae in this case function as a transport system.

- d) It is also possible that hyphal membranes are impacted by inhibitory concentration of phenolic acid ( $pK_a = 4.5$ ) and other organic acids (e.g., acetic acid  $pK_a = 4.8$ , citric acid  $pK_a = 3.1$ , formic acid  $pK_a = 3.8$ , and tartaric acid  $pK_a = 3.0$ ; Sposito, 1989) similarly to what has been observed for root cell membranes. In that case reductions in water and nutrient uptake by hyphae could actually lead to reductions in plant growth with minimal direct root surface contact by allelopathic agents. and/or
- e) More inhibitors (substances at concentrations that inhibit) than promoters (substances at concentrations that stimulate) are transported to root surfaces, inhibitors are transported faster than promoters to the root surfaces, and/or more inhibitors than promoters are generated in the rhizosphere or on the rhizoplane. Soils do modify rates of movement of individual inorganic and organic substances by differential sorption and desorption. For example, substances with a higher affinity to clays or organic particles than organic acids could prevent, or at least reduce, the sorption of organic acids and thus maintain toxic concentrations. Microorganisms associated with the rhizoplane and rhizosphere have also been shown to convert non-toxic substances to toxic ones, but more frequently from toxic to non-toxic substances (Liebl and Worsham, 1983; Blum, 1998, 2003).

## FINAL OBSERVATIONS

Given the level of complexity of soil systems it will be very difficult to demonstrate with any certainty that concentrations of simple organic acids (e.g., acetic acid, butyric acids, citric acid, formic acid, fumaric acid, lactic acid, malonic acid, phenolic acids, propionic acid, tannic acids, and tartaric acid) found under field conditions function as allelopathic agents until transport and interactions of inhibitors (substances at concentrations that inhibit) and/or promoters (substances at concentrations that stimulate) and soil solution chemistry is understood. Presently our ability to isolate and test soil solutions from most soils on root processes is not feasible without radically changing the nature of the soil solution (e.g., adding water to obtain sufficient amounts for testing). Under the right conditions, however, traps and lysimeters could be used to characterize and collect soil solutions, respectively, for testing root responses to simulated or real time soil solutions (Soon and Warren, 1993; 330

Weidenhamer, 1996; Dalton, 1999). That may turn out to be the only reasonable way to answer the question: Are all the inorganic and organic ions, molecules and compounds in the soil solution surrounding a particular root system inhibitory, neutral or stimulatory to plant growth?

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## CHAPTER 15

# MICROORGANISMS AND ALLELOPATHY: A ONE-SIDED APPROACH

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## INTRODUCTION

Plant-microbe interactions shape the conditions that sustain life. Highly multiple and multifaceted in character, they are crucial for the function of ecosystems, be they natural or man-made. Allelopathic interactions constitute a large and very important part of them<sup>1</sup>. As Rice stated (1974), the salient point concerning allelopathy is that its effect depends upon a chemical compound (mainly a secondary metabolite) being added to the environment. This is the crucial issue that distinguishes allelopathy from competition, as the latter involves removal or reduction of some factor from the environment that is required by other species (or by individuals of the same species) sharing the habitat. An analogy that would provide insight into this fundamental difference in the case of negative effects is the following: allelopathy is armed war, competition is economic war. In the case of plant-microbe allelopathic interactions, the allelopathic agent may be either the plant or the microbe. Given the multitude of microbes, of secondary metabolites, of habitats, where the two interacting agents co-occur, and of processes in which they are involved, we had to reduce the scope of this article to a manageable extent. Therefore, we will not cover the entire spectrum, but confine ourselves to discussing interactions of this character

- in the terrestrial environment, primarily in natural/semi-natural ecosystems,
- among higher plants and microorganisms,

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<sup>1</sup> There is a number of definitions of allelopathy. In this article, we adopt the broad definition, including plants and microorganisms, positive and negative effects (Rice, 1974).

- with plants being the contributors of allelochemicals.

Our approach is, therefore, one-sided. We will deal with interactions that are mediated by secondary metabolites produced by plants, and only on special occasions by microbes. It is also ecosystem-oriented. Our primary concern in presenting and discussing evidence of such interactions is their meaningfulness in terms of ecosystem function.

We have grouped the various cases of allelopathic interactions involving microbes into (i) those related directly or indirectly with decomposition and nutrient cycling, and (ii) those related with plant defense. In the first, we have included topics regarding immediate effects of plant allelochemicals on microbial growth and structure of microbial communities, as well as on processes related with decomposition, the nitrogen cycle, and nutrient availability to plants. We also discuss some of the consequences of such effects as expressed on other components and aspects of an ecosystem, like failure of natural regeneration or afforestation due to primary effects on mycorrhiza. Regarding plant defense, we discuss about constitutive, inducible and signal chemicals, and extend our discussion to the costs involved. We present cases regarding defense strategies and chemicals associated with the different plant parts, and we also deal with plant-animal interactions that are mediated by plant-microbe interactions, as is the case for ruminant herbivores.

## Delivery system of allelochemicals<sup>2</sup>

By definition, any allelopathic interaction involves at least one allelochemical. In the particular cases that we examine, it is required that the potential allelochemical of the plant-contributor must come into contact with the sensitive microbe-target. Such chemicals are liberated from live or decaying plant biomass through volatilization, leaching, root exudation or decomposition of plant residues, further affecting positively or negatively microbes and the processes in which they are involved. In general, roots appear to be a more important source than above-ground parts. The organic compounds released by plant roots are reviewed by Inderjit and Duke (2003). In the soil environment, these chemicals can be adsorbed by soil particles, what restricts their mobility. They may also be transformed into more active, less active or entirely inactive compounds. Retention of their activity is one of the crucial issues regarding allelopathic interactions.

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<sup>2</sup> There is lack of consensus in identifying a compound as allelochemical. In this article, we will use the term as defined by Reese (1979) and we will refer to non-nutritional chemicals produced by one organism that affects growth, health, behaviour or population biology of other species.

## DECOMPOSITION AND NUTRIENT CYCLING

### Processes related with the nitrogen cycle

#### a. Nitrogen fixation

A number of studies showed direct inhibitory activity of plant secondary metabolites, either as such or in plant extracts, on the growth of nitrogen-fixing microorganisms, symbiotic or free living. Rice (1964) reported inhibition of growth of *Rhizobium* strains by numerous plant species, with most inhibitory being *Adropogon scoparius*, *Ambrosia elatior*, *Euphorbia corollata*, *Cenchrus pauciflorus*, *Erigeron strigosus*, and with roots being more inhibitory than other parts. Aqueous extracts of different plant parts of the grass *Aristida adscensionis*, dominant in NW India, inhibited growth of *Rhizobium*, isolated from *Indigofera cordifolia*, a common associate of the grass (Murthy and Nagodra, 1977). Many weed species have been demonstrated to chemically inhibit the free-living *Azotobacter* and various potential nitrogen-fixing cyanobacteria, such as *Anabaena*, *Nostoc* and *Schizothrix* (Rice, 1974). Extracts from *Andropogon virginicus* and other species commonly found in the first two stages of succession of old fields in Oklahoma inhibited the growth of *Rhizobium leguminosarum*, *Azotobacter chroococcum* and *A. vinelandii* (Rice, 1965, Rice and Pancholy, 1972, 1974). Dawson and Seymour (1983) reported inhibition of *Rhizobium japonicum* and *Frankia* by juglone in *in vitro* cultures. Phenolic compounds, such as gallotannins, chlorogenic and isochlorogenic acid, were involved in these negative interactions.

Obviously, effects on nitrogen-fixing microorganisms cannot always be inhibitory. Stimulation of nitrogen-fixers by secondary metabolites of legumes is a valid hypothesis to test. Evidence for such an activity is given by Gitte et al. (1978) and Nutman (1965). *Rhus glabra* and *Chenopodium album* were also found to strikingly stimulate the growth of *Anabaena* (Rice, 1974), whereas aqueous extracts of *C. album* and *Sertaria viridis* to enhance growth of *Bradyrhizobium japonicum* (Mallik and Tesfai, 1987).

Apart from the direct effect of secondary metabolites on the growth of nitrogen fixers, there is a lot of information concerning nitrogen fixation itself and related processes. However, it is not always clear whether the effect produced results from the interaction of plant secondary metabolites and microorganisms and is not due to the interplay of other important factors. For example, higher plants (such as *Ambrosia plisostachya*, *Aristida oligantha*, *Bromus japonicus*, *Digitaria sanguinalis*, *Euphorbia supina*, *Helianthus annuus*) were found to be very inhibitory to the nitrogen-fixing bacteria, and also to inhibit nodulation and nitrogen fixation of several legumes (Rice, 1974, and relevant references therein included). Similarly, rice, *Argopyron repens*, *Parthenium hysterophorus*, *Imperata cylindrica*, *Hemarthria altissima*, *Ipomoea*

*lacunosa*, *Pluchea lanceolata* (Mallik, 1999, and relevant references therein included) were found to reduce nodulation and/or nitrogen fixation. Effects on nodulation and nitrogen fixation could be seen as deriving from the growth inhibition of nitrogen-fixing bacteria. However, as Weston and Putman (1985) showed, regarding the allelopathic effect of *Agropyron repens* on *Glycine max* and other legumes, the decreased nodulation and concomitant nitrogen fixation observed in legumes was the result of root-hair growth inhibition and not of growth inhibition of the symbiotic microorganisms.

In nitrogen-poor soils, the rate of nitrogen-fixation regulates the amount of available nitrogen. Factors affecting the survival or metabolism of any of the nitrogen-fixing microorganisms would probably also affect competition between plants with different nitrogen requirements (Rice, 1974) and would therefore be responsible for plant-plant indirect interactions.

### **b. Nitrification**

In the mid-30's, Richardson (1938) found that in grassland soils of England the level of ammonium-nitrogen was greatly higher than the level of nitrate-nitrogen, and that the ratio of ammonium to nitrate nitrogen increased with the age of the sward. He also reported that grasses and other plants absorb the ammonium-nitrogen as readily as the nitrate. Following that, Theron (1951) studied the failure of rehabilitation by grasses of wornout soils in South Africa, despite their luxuriant growth at the initial stages. After a number of experiments, he found that the nitrate content in the soil fell as vegetation developed. He concluded that perennial grasses and other plants inhibit nitrification and that this inhibition is probably due to bacteriostatic excretions of living roots.

Neal (1969) studied the effect of root extracts of fourteen grass and forb species appearing at different stages during the succession process in the grasslands of Alberta, Canada, on *Nitrosomonas* (oxidizing ammonium nitrogen to nitrite) and *Nitrobacter* (oxidizing nitrite to nitrate). Extracts of early-stage species proved inhibitory to both bacteria (more to *Nitrobacter*), whereas only one climax species (*Stipa comata*) inhibited these bacteria appreciably. Evidence for low nitrate content in grasslands and increase of inhibition of nitrification with the progress of succession came from many different geographic areas, suggesting that it is a widespread phenomenon (Rice, 1974, and relevant references therein included). It thus appeared likely that the strong inhibition of nitrification in the later stages of old-field succession aids in the build-up of available nitrogen, in the form of ammonium-nitrogen, which finally enables the higher nitrogen-requiring climax species to invade.

Based on several studies, Rice (1974) proposed a scheme explaining the succession process in oldfields and rangelands, which is meaningful in terms of

nitrogen conservation, implies a two-stage allelopathic interference (plants → microbes → plants), and results in vegetation patterning. Among others, this scheme was based on the striking inverse correlation existing between soil ammonium- and nitrate-nitrogen: ammonium-nitrogen increased from a low content in the first successional stages to a high content in the climax, whereas nitrate-nitrogen followed exactly the opposite pattern. In parallel, counts of nitrifiers were high in the first successional stages and low in the climax. Soil tests indicated that low rates of nitrification in the climax were not due to differences in pH, texture or organic matter content. The opposite trends of the two nitrogen ions could be explained as resulting from their physico-chemical properties: as the ammonium ion is positively charged, it is adsorbed on the negatively charged colloidal micelles, thus preventing leaching below the depth of rooting; as the nitrate ions are negatively charged, they are repelled by the colloidal micelles in the soil and are readily leached below the depth of rooting or washed away in surface drainage. All these combined would mean that inhibition of nitrification would help in nitrogen conservation. As reduction of nitrate to ammonium requires energy, inhibition of nitrification would also conserve energy. Given the evidence that many species can use ammonium-nitrogen as effectively or even more than nitrate-nitrogen, conservation of both nitrogen and energy could be the driving force for succession to proceed in the direction of inhibition of nitrification (Rice, 1974).

More recently, low nitrification rates in a ponderosa pine (*Pinus ponderosa*) forest were attributed to direct inhibition by monoterpenes leached from pine litter (White, 1986). Bremner and McCarty (1993) argued that the low nitrification rates were not due to allelopathic effects but to the presence of phenolics, tannins and monoterpenes, which initiate heterotrophic immobilization of  $\text{NH}_4^+$  resulting in decreased availability of  $\text{NH}_4^+$  for nitrifying bacteria and thus lower nitrification rates. This was supported by a soil study in a Norway spruce (*Picea abies*) forest, as increased microbial respiration and decreased nitrification were observed after addition of monoterpenes (Paavolainen et al. 1998). Evidence for allelopathic suppression of nitrification was provided by Ward et al. (1997), who found inhibition of nitrification in pure cultures of nitrifying bacteria, in presence of redwood (*Sequoia sempervirens*) monoterpenes, but not of glucose (Krummel & Harms, 1982), what seems to eliminate the possibility of heterotrophic competition. However, Strauss and Lamberti (2002), who studied the microbial decomposition of dissolved organic carbon and examined how nitrification is affected by different carbon sources, including glucose and leaf leachates from 18 temperate forest tree species, found that nitrification rates decreased as carbon concentration increased. Glucose and white pine (*Pinus strobus*) leachates strongly suppressed nitrification. The fact that these two carbon sources resulted in similar nitrification rates is inconsistent with both the heterotrophic competition hypothesis and the allelopathy hypothesis. If carbon quality alone was responsible for variable nitrification responses (heterotrophic competition hypothesis), there should be

much lower nitrification rates in the treatments that received glucose than those receiving white pine leachate. If chemical inhibition alone was responsible for variable nitrification responses (allelopathy hypothesis), there should be much lower nitrification rates in the treatments that received the white pine leachate (because of high monoterpene concentration) than those receiving glucose. The authors suggest that both mechanisms function concurrently: high quality carbon induces competition between heterotrophic and nitrifying bacteria for  $\text{NH}_4^+$  and some functionality of nitrifying bacteria is hindered via direct allelopathy from certain compounds in litter leachate.

In another study, Thevathasan et al. (1998) examined the effect of juglone on soil ammonification and nitrification, given the concerns that walnut based intercropping systems may be influenced by nitrification inhibition due to the presence of juglone. No inhibition effect was observed either in laboratory experiments or under field conditions.

Studies of nutrient cycling in natural systems span more than 100 years. In the earlier years, attention was mostly given to the measurement of the pools of nutrients in plants and soil and of the return of nutrients from plant to soil in litterfall. In the later years, attention was mostly concentrated on the processes of nutrient cycling, and particularly on those that sustain the supply of nutrients. Concerning nitrogen, variability in rates of nitrification were interpreted in different ways including allelopathy, but its importance remains still uncertain (Attiwill and Adams, 1993). A lot of work is required in order to have a clear understanding of the contribution of allelopathy in regulating nitrification, and in a wider context the whole spectrum of nitrogen transformations and possibly those of other macronutrients.

## Processes related with decomposition and the soil microbial community

Microorganisms have an extraordinary capacity to convert, synthesize, eliminate, detoxify or utilize secondary metabolites as carbon and energy sources. They often use them rapidly and efficiently, responding to changes in resource quantity and quality, via enzyme induction, mutation, or alterations in the structure of their communities.

Some compounds are rapidly transformed in the soil, whereas others remain unchanged for long. The active life of allelochemicals depends on the edapho-climatic conditions; their interaction with soil microbes is crucial for that. Degradation products can have increased, decreased or no activity at all compared to the molecules, they originate from. There is a huge amount of experimental work investigating the effects of residues or decaying material from various plant sources on other plants. Such plant-plant interactions involve microbial mediation during the different stages of the decomposition process. The soil sickness problem of various crop plants, deriving

from toxins accumulated in the soil from live biomass or plant residues, may also involve microbial mediation. We are not going to present examples of such indirect allelopathic effects. We will instead put emphasis on the changes of the soil microbial community and its activity, as induced by plant secondary metabolites, focusing on essential oils and monoterpenoids that we have studied in depth.

Aromatic plants make a remarkable contribution to the flora and vegetation of the Mediterranean environment. Very common among them are representatives of Lamiaceae. In this environment, decomposition is limited by the unfavourable combinations of temperature and rainfall. If the antimicrobial activity of essential oils held for soil microorganisms, decomposition would be critically affected, as biological constraints (essential oils inhibiting microorganisms) would function on top of physical ones (climate). Therefore, a major goal of our work has been to understand how essential oils, known to possess antimicrobial activity, affect soil microorganisms and concomitantly the decomposition process.

We have found that soil respiration was activated in presence of *Coridothymus capitatus* and *Satureja thymbra* essential oils and that this increase was a primary and not a secondary effect; these essential oils did not kill some microorganisms thereby providing substrate easily decomposable to others, but directly activated soil bacteria (Vokou et al. 1984). We further examined how a number of individual constituents (oxygenated or not) of these essential oils affect soil respiration. We found that all lead to a similar response increasing CO<sub>2</sub> release from soil samples. Some were immediately active, some others after a time lag (Vokou and Margaris, 1988). Given these responses, we examined the effect of essential oils of largely different chemical composition, from aromatic plants indigenous or not on soil samples of different origin. The essential oils tested were rich in carvacrol and/or thymol, in carvone-dihydrocarvone, in 1,8 cineol-camphor, and in linalool-linalyl acetate. The soil samples were collected from the area beneath the wild growing aromatic plants, from open spaces without a shrub layer, but also from areas that had not been exposed to essential oils (e.g., from cultivated fields). In spite of their different chemical composition, all essential oils activated soil respiration to a comparable degree. Also, soils not previously exposed responded similarly to those supporting aromatic plants (Vokou and Liotiri, 1999). We further examined the rich-in-fenchone essential oil of *Lavandula stoechas* (Vokou et al. 2002). The activation of soil respiration in presence of this essential oil or of fenchone was accompanied by a remarkable increase of the soil bacterial population, by approximately three orders of magnitude. The increased soil respiration can thus be attributed to the increase of the soil bacterial population and its concomitant activity. The bacterial population changed not only in size but also in composition. One bacterial strain, tentatively identified as *Aeromonas hydrophila* had an overwhelming participation in the treated soils: more than 70% as compared to less than 15% in the control ones. This strain proved very tolerant in antimicrobial

bioassays. There was also substantial evidence that it can use monoterpenoids as carbon and energy source. These results indicate shift of the microbial population balance in soil in favour of particular bacterial strains, tolerant and/or able to catabolize monoterpenoids.

The net result of the above studies is that the antimicrobial activity of essential oils and of individual terpenoids is not expressed against soil microbial communities. In their presence, soil metabolism is enhanced since some bacteria can readily utilize them. We estimated that 64% of the monoterpenoid-carbon added is respired, whereas 36% contribute to increasing the soil organic content (Vokou et al. 1984). This is consistent with the average yield coefficient of  $0.35 \text{ mg dry weight mg}^{-1}$  substrate consumed predicted by Lynch (1979). We also estimated the degradation rate of these compounds, under favourable conditions (temperature, moisture) to be around  $1.7 \text{ g m}^{-2} \text{ day}^{-1}$  (Vokou and Margaris, 1988). Such a rate allows fast degradation and supports evidence indicating that there may be no residue problem with terpenoids when used in pest control (Lydon and Duke, 1995).

Given the responses that we get, we can argue that essential oils determine the successional stages during the decomposition process of the litter of aromatic plants. Bacteria, able to utilize essential oils, like *A. hydrophila*, must be among the initial colonizers of the rich-in-essential-oil litter of these plants. Activated in autumn, after the first rainfalls that follow the dry Mediterranean summer, they grow using monoterpenoids as their carbon and energy source. Those inhibited by such toxic to many microorganisms substrate will be later colonizers; they will establish in the litter only after the initial colonizers consume/upgrade it.

Such enhanced microbial activity, rapid utilization, and shift of the microbial population balance in soil have also been reported for other allelochemicals. If nutrition, moisture, pH, and temperature are appropriate, addition of *p*-coumaric acid into a carbon-limited environment will stimulate rapid microbial utilization of the compound (Blum et al. 1999). Use of high concentrations will induce a strong, but artificial, selection for *p*-coumaric acid utilizing microbes. Relating this with the inhibitory activity of phenolic compounds on germination and growth of plants, researchers have suggested that unreasonably high concentrations of phenolic acids are required in soil systems for inhibition of seedlings in nature. However, Blum et al. (1999) provide evidence from their work and that of other researchers that mixtures of phenolic acids, at low individual concentrations, may be more active as plant-growth inhibitory agents than high concentrations of single phenolic acids. They also argue that, as ideal soil environments with high concentrations of a single phenolic acid are extremely unlikely in nature, conclusions based on observations under such ideal conditions must be viewed with skepticism.

## Mycorrhiza

Most higher plants do not have roots. With only a few exceptions, like the representatives of the family Cruciferae, they have mycorrhiza. These are associations of roots with fungi occurring mainly in the form of sheaths or vesicular arbuscules. Fungi are primarily suppliers of minerals to the host. Studies with sheathing mycorrhiza have shown that phosphorus, nitrogen, and calcium move along the fungal hyphae into the host roots (Harley and Smith, 1983). Vesicular arbuscular mycorrhiza enhance phosphorus uptake. But Newsham et al. (1994) found that the main benefit offered by arbuscular mycorrhizal fungi to the annual grass *Vulpia ciliata* was not phosphate uptake but protection against root pathogenic fungi.

Plant-plant allelopathic interactions are often assumed to be the causal agent of failures of forest natural regeneration or of afforestation efforts. These interactions may be direct or indirect, through mediation of microorganisms. Given the advantages that mycorrhiza forming fungi offer to the host plants, there is substantial research effort regarding their manipulation as inocula for tree seedlings that are used in afforestations.

*Kalmia angustifolia* is an ericaceous shrub that frequently invades black spruce (*Picea mariana*) clear-cuts in central Newfoundland. On many sites, where *K. angustifolia* grows, black spruce seedlings become chlorotic and stunted. Allelochemicals of *K. angustifolia* were found to affect the growth and development of black spruce but also the growth of certain ectomycorrhizal fungi associated with the tree (Yamasaki et al. 1998). Mallik and Zhu (1995) studied the effect of extracts of leaves and humus of *Kalmia* as well as of eight phenolic acids isolated from these extracts on black spruce root growth. The effect was always inhibitory. In contrast, several isolates of ectomycorrhizal fungi were tolerant to *Kalmia* leaf extracts and four were stimulated. Black spruce seedlings inoculated with these fungal isolates had better growth in presence of *Kalmia* plants than the control plants (Mallik, 1999), with one isolate causing 2-3 fold increase in seedling biomass. Given these results, Inderjit and Mallik (2002) argue that there is no compelling direct evidence to support plant-plant allelopathy as a mechanism of *Kalmia* interference to black spruce and that the synergistic interaction of ecological factors may better explain the interference mechanism, as organic molecules released from *Kalmia* into the environment may influence soil mineralization, mycorrhizae, nutrient dynamics, and soil microbial ecology.

The failure of natural regeneration observed in subalpine spruce (*Picea abies*) forests has also been examined. Phenolic compounds (catechol, protocatechuic acid, *p*-hydroxybenzoic acid and *p*-hydroxyacetophenone) produced by *Vaccinium myrtillus*, *Athyrium filix-femina*, and *Picea abies* (predominant species of spruce

forests in the Alps) and present in humic solutions were tested on respiration of *Cecococcum graniforme* and *Laccaria laccata*, two spruce mycorrhizal fungi. They proved to have biological activity even at extremely low concentrations, what suggests a possible contribution of these plant species to allelopathic inhibition of mycorrhizal fungi (Pellissier, 1993; Boufalis and Pellissier 1994). Phenolics in the humic solution were also tested on *Hymenoscyphus ericae* (symbiont of *V. myrtillus*) and *Hebeloma crustuliniforme* (symbiont of *P. abies*). As *H. ericae* responded better than *H. crustuliniforme*, it was concluded that this can explain the dominance of *V. myrtillus* among understory species (Souto et al. 2000). Horton et al. (1999) provided evidence that the establishment of *Pseudotsuga menziesii* in the *Arctostaphylos*-dominated but not in the *Adenostoma*-dominated chaparral of California, is due to the ectomycorrhizal fungi that are associated with *Arctostaphylos*.

Ericaceous plants are well known to suppress conifer regeneration. These plants produce large quantities of polyphenolic materials that can bind soil organic N as calcitrant protein-phenol complexes. They are also known to be in symbiotic associations with a variety of mycorrhiza, some of which are specifically adapted to nutrient-poor habitats, can utilize protein-N that is complexed with tannic acid or use tannin as a carbon source (that most other mycorrhiza cannot). It seems, therefore, that ericaceous plants are better equipped than conifers in using the organic and inorganic resources of their environment (Mallik, 1999, and references therein). Among other mechanisms that operate, the interaction of Ericaceae and mycorrhizal fungi plays a major role in determining nutrient-cycling, and further the dynamics of vegetation.

Representatives of other than Ericaceae families also suppress conifer growth. Fennoscandian plant communities dominated by the evergreen dwarf shrub *Empetrum hermaphroditum* appear to have negative effects on forest regeneration. Water extracts of leaves of *E. hermaphroditum* were tested (Nilsson et al. 1993) regarding development and nitrogen uptake by roots and mycorrhizae (*Paxillus involutus* Batsch) of Scots pines (*Pinus sylvestris*). Among seedlings that did not receive *E. hermaphroditum* extract, mycorrhizal plants grew better than nonmycorrhizal plants, had a higher shoot to root ratio, and three times faster nitrogen uptake. A low concentration of *E. hermaphroditum* extract impaired nitrogen uptake; it was reduced to one-third and one-tenth in mycorrhizal and non-mycorrhizal plants, respectively. It also strongly inhibited the growth of *P. involutus*. The spread of mycorrhizal infection and uptake of nutrients by roots and mycorrhizae were more sensitive to the extract than seed germination and radicle growth.

Another example of impeded growth that may be mediated by plant-mycorrhiza interactions is the case of red oak (*Quercus rubra*). In many acid soils, where red oak is planted, seedlings cannot establish because of the aggressivity of the grass *Molinia caerulea*. In their experiments, Timbal et al. (1990) found that *Molinia* was responsible for a reduced growth of red oak but also for modifications in the mycorrhizal status of

the roots, suggesting an indirect allelopathic effect involving the ectomycorrhizal symbionts.

Given the widespread occurrence of mycorrhiza, their close association with their plant-symbionts and the benefits they offer to them, factors that affect their growth and metabolism, such as plant allelochemicals, are likely to have serious repercussions on the vegetation structure and soil dynamics. We believe that this is a very promising aspect of allelopathic research with a high potential of giving fruitful results.

## PLANT DEFENSE

Though lacking an immune system, plants are surprisingly resistant to diseases. They do so by mobilizing an array of mechanisms, from production and accumulation of antimicrobial agents to programmed cell death (hypersensitive response) or induction of systematic immunity (Figure 1).

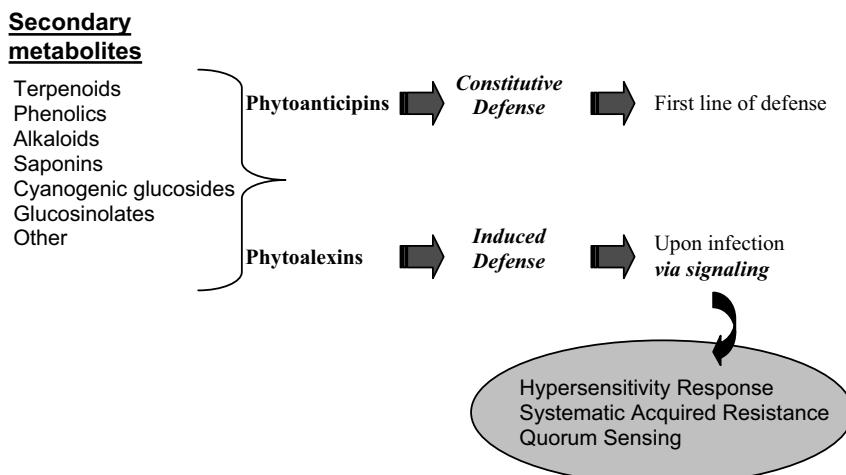


Figure 1. Chemical defenses of plants against microbes

The huge variation in the chemical composition of plant species is often explained in terms of response to biotic and abiotic factors affecting them. Plants have evolved in presence not only of a large number of other plant species, but also of grazing animals, insects, microbes, nematodes, and other organisms. Unable to move in less hostile environments, they often respond to environmental pressures by producing chemicals that influence interactions in favour of them. These phytochemicals most often offer multiple resistance as they affect biological processes that are not unique to a species

or a group of species. Because of this general activity, they may be also toxic to the producing plant. Therefore, they must be sequestered or continuously exuded to avoid self poisoning. Glands, resin ducts, idioblasts, and specialized cell layers seem to be the most lucrative sources of secondary compounds having autotoxic properties.

The successful invasion of a pathogen depends on its ability to circumvent or avoid plant defense chemicals. The plant-pathogenic fungus *Stagonospora avenae* is able to detoxify the steroidial toxic saponins of *Avena sativa* and infect it (Wittstock and Gershenson, 2002). The strategy to avoid defenses is exemplified by some fungi that can germinate and remain quiescent on plant tissues till the antifungal compounds are decreased to nontoxic levels on the tissues and then begin the invasive growth (Osbourn, 1999).

A vast array of secondary metabolites have been examined regarding their antimicrobial activity, mainly in *in vitro* studies. Essential oils and various classes of phenolic compounds make the bulk of these studies. Only between 1987-2001, there were more than 500 reports about the antimicrobial properties of essential oils and of their constituents against bacteria and fungi (Kalemba and Kunicka, 2003). This is explained by the fact that their antimicrobial properties have been known for centuries; this is why essential oils find a wide application in folk medicine, food flavouring and preservation. Research regarding the contribution of other families of secondary metabolites to plant defense against microbes are not as numerous. Regarding alkaloids, among the few accounts that address the issue to a certain extent are the following: Biavatti et al. (2002) found that furanoquinoline alkaloids from the South Brazilian endemic plant *Raulinoa echinata* have antifungal activity against *Leucoagaricus gongylophorus*, the symbiotic fungus of leaf-cutting ants. Park and Choi (1999) studied the bactericidal and fungicidal properties of the cork tree (*Phellodendron amurense*), rich in berberine. Nagaoka et al. (1995) reported that fungitoxic compounds related to the resistance of tomato against soil-borne disease are alkaloids. Oliva et al. (2003) reported high to moderate activity of quinoline and quinolone alkaloids isolated from *Ruta graveolens* on a number of fungi species.

Large screening projects studying the effect of extracts from a great number of plants species on various microorganisms characterized the early years of allelopathic research. However, as stated by Rice (1974), most of these efforts concern inhibition of possible human pathogens and, with few exceptions, they are not meaningful in terms of ecology. Literature survey shows that such unfocused research is still done and published in our days. But, in parallel, many elaborate, hypothesis-testing studies have increased our understanding of the ways that plants defend themselves from pathogen attacks.

## **Phytoanticipins vs. Phytoalexins**

Secondary metabolites with a phytoprotective role are formed prior or subsequent to pathogen infection. In other words, they are part of constitutive or inducible defense mechanisms, respectively. Constitutive defense chemicals are usually described as phytoanticipins (or prohibitins or pre-infection metabolites). The term includes a large number of compounds produced through different metabolic pathways; they constitute the first line of plant chemical defense against pathogens. Phytoalexins, on the other hand, are inducible compounds. They are synthesized in response to pathogen attacks and accumulate into injured plant tissues soon after infection; their production involves *de novo* enzyme synthesis. Usually, constitutive antimicrobial compounds and induced phytoalexins can be detected in the same plant.

Both phytoanticipins and phytoalexins include different classes of structurally unrelated compounds that belong to all three major groups of secondary metabolites - phenolics, terpenoids and nitrogenous compounds. Phytoanticipins usually serve as multi-purpose protective agents, against not only microbes but also other pests and abiotic stresses. Synthesis of phytoalexins is a rather widespread defense strategy of plants, primarily against fungal pathogens, and is encountered both in gymnosperms and angiosperms. Nevertheless, there are plant families, like the legumes, which are more efficient than others, like Rosaceae and Curcubitaceae, in producing phytoalexins (Ingham, 1981; Grayer and Harborne, 1994). More than 300 chemical structures have been characterized as phytoalexins. Within a plant family, phytoalexins are usually chemically related to already existing constitutive defense agents (Harborne 1999).

The synthesis of constitutive or induced defense chemicals seems to be related with the frequency and severity of pathogen attacks. Plants that are likely to suffer frequent or serious damage may be better off investing mainly in constitutive defense, whereas plants that are attacked rarely may rely predominantly on induced defenses (Wittstock and Gershenson, 2002). The same compound can serve as phytoanticipin in one species and as phytoalexin in another. Not only species but also plant parts may differ regarding the content of defense chemicals; one compound may accumulate in plant organs that frequently become targets and be absent in other, less susceptible organs.

There are also mixed strategies, whereby compounds that form part of the constitutive plant defense and naturally occur within healthy plant tissues raise dramatically their concentration after a microbial attack.

Genetically transformed plants can provide insight on the protective role of phytoalexins and phytoanticipins. For instance, several phytoalexin-deficient mutants

of *Arabidopsis thaliana* with reduced levels of the indole camalexin were tested regarding their disease resistance. One mutant (*pad-3*) was more susceptible to the fungal pathogens *Cochliobolus carbonum* and *Alternaria brassicola*, but not to other fungi or to pathogenic strains of *Pseudomonas syringae*. Other mutants had differing susceptibilities to those pathogens (Glazebrook and Ausubel, 1994). Introduction of the novel phytoalexin resviratrol (originating from grapevine) into alfalfa (*Medicago sativa*) resulted in reduced symptoms of the leaf spot disease caused by the pathogen *Phoma medicaginis* (Hipskind and Paiva, 2000), while over-expression of isoflavone O-methyltransferase in the same plant resulted in more rapid and increased production of the phytoalexin medicarpin, after infection of *P. medicaginis* (He and Dixon, 2000). Such results show that secondary metabolites serve as defense compounds of a plant against its pathogens, but also that the relationship between phytoalexin production and disease resistance is complex and highly pathogen-dependent (Dixon, 2001).

## Signaling

Phytoalexin synthesis is conjugated with a series of events that happen upon pathogen attack. In this series, the early defense response is the rapid cell death in the site of invasion, the activation of gene encoding pathogenesis-related (PR) proteins, the emergence of phytoalexins localized in or near the brown, necrotic cells, and the reinforcement of structural barriers (Kombrink and Schmelzer, 2001; Pierce et al. 1996); the major compound that straightens cell walls is lignin, a phenolic polymer.

In addition to localized resistance response, plants can synthesize a signal at the site of infection, which spreads along in both infected and non-infected plant parts and leads to an increased and long lasting resistance. Salicylic acid plays a key role as a signal molecule; it sharply increases its concentration after attack. Expression of PR proteins and other reactions follow, and a systematic immunity throughout the whole plant is established known as systematic acquired resistance (SAR) (Feys and Parker, 2000; Kunkel and Brooks, 2002).

Apart from their contribution to constitutive and induced defenses, phenolic compounds have been accredited with another highly specific and extremely important role, that of xenognosins. The critical stage in the establishment of a host-pathogen interface is host recognition or xenognosis. Several xenognostic agents controlling both pathogenesis and symbiosis across a diverse range of organisms have been discovered. The aromatic ring and the phenolic functional group are common features (Tok et al. 1997). Some of the molecules recognized as xenognosins are not perceived directly but after they are converted to oxidation products. This is the case of xenognosin A and B, which signal plants to switch from vegetative to parasitic growth, but after they are converted to quinones [e.g. to 2,6 dimethyl-1,4-benzoquinone

(DMBQ)]. Other xenognostic agents, like acetosyringone that induces the transition to the pathogenic mode in the oncogenic *Agrobacterium tumefaciens*, appear to be recognized directly by receptor proteins. The signals that initiate the host-specific legume-*Rhizobium* interaction are flavanioids, such as luteolin (Siqueira et al. 1991, Tok et al. 1997, and relevant references included in both reviews).

Another type of secondary-metabolite mediated plant-microbe interactions, recently revealed, is linked with quorum sensing (Fray, 2002). Many bacteria regulate various processes in concert with their population size. A cell-to-cell communication system operates with the aid of diffusible signal molecules, which allows bacteria to express important traits, such as pathogenicity, swarming, antibiotic production, only when the signaling molecules reach a critical threshold, which coincides with establishment of a large population (a point at which the population is described as 'quorate'). These signals that are produced and perceived by bacteria make the population to behave as a single unit. The quorum sensing signals most commonly used by Gram-negative bacteria are N-acylhomoserine lactones (AHLs). There is ever increasing evidence that some plants can synthesize furanones and some other compounds not yet identified that either stimulate or inhibit AHLs. Also, transgenic plants producing high levels of AHLs had dramatically altered susceptibilities to infection by pathogenic *Erwinia* species (Fray, 2002). Interference of plant secondary metabolites with bacterial signaling constitutes another very intriguing aspect of plant defense mechanisms.

## **Plant parts and defense strategies**

The phyllosphere is subject to deposition of every single microorganism that happens to circulate in the atmosphere through dry or wet precipitates or through another organism acting as vehicle. For bacteria, it is estimated that the rate of deposition would yield a cumulative immigrant population of about  $10^4$  cells per month on an average sized leaf (Lindow, 1996).

Plants have to face this load of bacteria, pathogenic or not, and also all other organisms that turn up on their aerial parts. Several *in vitro* bioassays have shown that various constitutive metabolites occurring in leaves exhibit antimicrobial activity against phytopathogens. Such bioassays cannot prove any actual protection of plants from microbes but provide information on the potential contribution of these metabolites in plant defense.

Trying to relate secondary chemistry and microbial infection, we have examined the influence of secondary metabolites on the bacterial colonization of the phyllosphere of four aromatic plants occurring in the Mediterranean area (Karamanolis et al. 2000). To this aim, we estimated the total bacterial population and that of ice

nucleation active bacteria (INA) of their phyllosphere, the leaf content in essential oils and their qualitative and quantitative composition, as well as the amount of surface phenolics. We further studied the effect of the leaf secondary metabolites of these plants on two INA bacteria, *Pseudomonas syringae* and *Erwinia herbicola*. We found that the differences in the bacterial colonization of their phyllosphere were related to the plants' content of secondary metabolites and their antimicrobial activity, as recorded in the *in vitro* bioassays. Microbes could grow abundantly on the phyllosphere of plants with weak antimicrobial activity but not on others, close-by that have strong activity. A similar pattern seems to exist for other, non-aromatic plants of the Mediterranean area (Karamanolis et al. in press).

Recently, the relation between secondary metabolites and phyllosphere fungi was examined by Talley et al. (2002) in sagebrush (*Artemisia tridentata*), a widely distributed species. The authors examined the following three hypotheses a) that populations in habitats favourable to fungi have a greater antifungal activity and a greater number of antifungal secondary metabolites than populations in drier habitats, b) that the same antifungal metabolites have alternative functions, and c) that the extent of fungal disease found in the field is a function of the abundance of fungal pathogens in the sagebrush habitat and the level of antifungal defenses in the sagebrush population. Their results confirmed the first hypothesis. Regarding the second, although it was impossible to rule out all alternative functions, they found no evidence of protection from ultraviolet light or oxidation. The third hypothesis was not confirmed; the incidence and severity of fungal disease were similar in moist and dry habitats, possibly reflecting an equilibrium between plant defense and fungal attack, as sites with greater fungal abundance compensated with more effective secondary metabolites. The authors conclude that fungi seem to serve as important selective agents shaping the sagebrush defensive chemistry.

Monoterpenes seem to play an important role in resistance to infection of conifers. In several tree species, their concentration was found to increase following failed attack by Scolytidae beetles and their associated fungi (Cook and Hain 1986, Paine et al. 1987). Supportive evidence that these compounds act as defense chemicals against both insects and pathogens is offered by Klepzig et al. (1996), who demonstrated inhibition of fungal propagule germination, mycelial growth and beetle tunneling by monoterpenes. Among the latter,  $\alpha$ -pinene seems to play a major role. In bioassays with red pine (*Pinus resinosa*) extracts, lower  $\alpha$ -pinene concentrations were highly correlated with increased beetle and fungal success (Klepzig et al. 1995).

Both phytoanticipins and phytoalexins are produced in leaves. For example, rice plants produce a wide variety of chemicals against fungal infections. Some, like silicate, alkylresorcinol and a number of fatty acids behave as phytoanticipins. Others belonging to flavonoids and diterpenoids behave as phytoalexins (Grayer and Kokubun, 2001; Susuki et al. 1996). Coumarin, scopoletin and ayapin, potent

antifungal compounds, have been reported as phytoalexins in tobacco plants. Scopoletin and ayapin levels increase under pathogen attacks by *Helminthosporium carbonum* in sunflower (*Helianthus*). Also, diterpenic acids, widespread among the different species of *Helianthus*, have fungicidal properties. A mixture of angleloyl grandifloric acid and kaur-16-en-19-oic acid showed the highest inhibitory activity ever reported against *Verticillium dahliae* and *Sclerotium sclerotinum* (MIC=10 ppm), two pathogenic fungi of the sunflower (Macias et al. 1999).

As for the phyllosphere so also for the rhizosphere, both phytoanticipins and phytoalexins of various chemical structures have been detected. The avenasins, saponins of oat, were found to deter the fungal pathogen *Gaeumannomyces graminis*, causing severe yield losses in wheat and barley (Osbourne, 1996), from inducing pathogenicity to the plant. They are constitutive defense chemicals accumulated in oat, mainly in the root epidermal cells.

Chlorogenic acid has antifungal properties inhibiting the growth of *Phytophthora parasitica*. When the plant is affected by several fungi but also under extremely cold conditions, production of chlorogenic acid is stimulated leading to increase of its concentration in tobacco roots (Macias et al. 1999, and relevant references therein included).

A number of studies aiming at eliciting phytoalexins in the root systems resulted in production of various chemicals. Infected bulbs of *Allium cepa* produced two aliphatic diones, which are not sulfur based like the majority of *Allium* secondary metabolites (Tverskoy et al. 1991). *Sanguisorba minor* is the only representative of Rosaceae that gave positive response in phytoalexin elicitation; a simple phenolic compound was produced in roots (Kokubun et al. 1994). Among the Compositae, tubers of *Polymnia sonchifolia* produce three different acetophenones, when inoculated with *Pseudomonas cichorii* (Harborne, 1999). In another study, banana rhizomes infected with *Fusarium oxysporum* were able to synthesize four different phytoalexins, the musanolones with a very specific phenalonone-type structure (Luis et al. 1996). One of the early findings in phytoalexin history (Kuc, 1982) was that potato tubers synthesize at least twenty different phytoalexins of sesquiterpenoid structure.

Seeds can be divided into two major groups: those that readily germinate and those that remain dormant. The latter would decompose, were they not equipped with mechanisms preventing them from decay. A hard, water impermeable seed coat, as exhibited by representatives of legumes, Geraniaceae, Rosaceae, Cistaceae, may be the solution. This mechanism missing, there should be alternatives to keep seeds intact in the soil environment.

Secondary metabolites with antimicrobial activity that are present in seeds could function as preservatives preventing them from decay. Rice (1979) believed that this is

probably one of the most important and universal ecological roles of allelopathy in perennial plants growing in natural ecosystems, though limited amounts of research done in this field do not reflect its importance. As phenolic compounds with antimicrobial activity are widespread in fruits and seeds (particularly in the seed coat and the external layer of fruits), he concluded that although this evidence does not relate directly to the prevention of seed decay, it certainly has an important indirect relation.

Flavonoids, terpenoids, and nitrogenous metabolites are major components of plant seeds. Conjugated forms of compounds are soluble in water and can be easily released following imbibition. As Ndakidemi and Dakora (2003) argue, once in the soil, these metabolites are first in line to serve as eco-sensing signals for suitable rhizobia and arbuscular mycorrhizal fungal partners required for the establishment of symbiotic mutualisms and they may serve as defense molecules against pathogens. An improvement in our understanding of seed chemistry would permit manipulation of these molecules for effective control of pathogens and enhanced acquisition of N and P via symbiosis with soil rhizobia and arbuscular mycorrhizal fungi.

Saponins are an important source of antifungal triterpenoids and steroids. Some exhibit fungicidal activity at very low concentrations, even below 5 ppm (Oleszek, 1999). The distribution of particular saponins in the seeds of *Medicago* species, shows considerable variation. Relatively high frequency of hemolytic seeds were found in the sections *Spirocarios*, *Lupularia*, and *Geocarpa*. The author provides nice and ecologically meaningful explanations regarding this variability, suggesting that the high frequency is a response to an increased need for protection from both microbes and insects. Yet, more direct proof is missing. Similar is the case of *Cenchrus setigerus*. This is a common fodder grass of arid and semiarid regions of India that produces seeds during September-October. The seeds remain buried in sand for a long period, especially during unfavorable environmental conditions (when the monsoon fails in subsequent years). As its spikelets are extremely rich in cyanidin glycosides, the authors imply that these compounds constitute the defense system that keeps seeds viable for the next irregular monsoon (Parihar and Mal, 1999).

Overall, despite the seemingly very interesting case of allelopathic interactions between seeds and microbes, little research has been conducted till now.

## **Ecological cost**

Defense mechanisms are essential for plant survival, but are costly in terms of nutrients and energy. For instance, nitrogen is required for the synthesis of alkaloids but also of aminoacids. When nitrogen is not sufficient, plants have to find the golden mean in covering the requirements for growth-reproduction and defense.

High energy expenses are also required. The metabolic pathways associated with production of defense chemicals are usually complex, involving several enzymes. In terms of energy, terpenoids are more expensive than any primary (and secondary) metabolite (Gershenson, 1994). The energy cost becomes even higher as the plant has to find means by which to avoid self-poisoning. One strategy is to develop differentiated cell structures where to store toxic defense compounds that are released upon tissue damage. Another strategy is to store such compounds as inactive precursors, for example as glucosides, separately from activating enzymes. The glucosinolates found in the representatives of the order Capparales are compartmentalized separately from their activating enzyme, the thioglucosidase myrosinase (Wittstock and Gershenson, 2002). Recent studies in *Arabidopsis thaliana*, showed that glucosinolates are stored in high amounts in sulfur-rich cells that are found between the phloem and the endodermis of the flower stalk, whereas myrosinase is localized in adjacent phloem parenchyma cells (Koroleva et al. 2000, Andréasson et al. 2001). In response to tissue breakdown, the glucosinolates contact myrosinase and are hydrolyzed into an unstable aglycon, which in turn re-arranges in a variety of biologically active compounds including substituted isothiocyanates (mustard oils), nitrile, thiocyanates and oxazolidinethiones. These products act as fungicidals and bactericidals (Mithen and Magrath, 1992; Osbourn, 1996), and may also function as precursors to a group of indolyl-phytoalexins that are induced in *Brassica* (Rouxel et al. 1989).

Given these requirements, plants have to “wisely” allocate the resources available, be they nutrients or energy, if they are to maximize their fitness.

## **Microbial mediation of plant-herbivore interactions**

Microbial mediation of plant-herbivore interactions is widespread. Microbes can modify the suitability of a plant to a herbivore by altering the plant's nutritional and defensive chemistry. For example, mycorrhizal and nitrogen-fixing mutualists of plants can adversely affect plant resistance to herbivores; grasses are traditionally considered as possessing few chemical defenses, but fungal endophyte-infested grasses contain fungal-derived alkaloids that defend the plant against herbivores; microorganisms on the phyllosphere or rhizosphere of a plant can alter insect behavior and growth since insects can depend on microbial symbionts for detoxification of plant chemicals (Krischik and Jones, 1991).

Many herbivores utilize microbial fermentation to digest food substrates. The chemical make-up of plants has striking effects on digestibility and nutritional value to ruminants (Riddle et al. 1996) because of the effects of certain phytotoxins on microorganisms in the rumen of the animals. Murray et al. (1996) investigated whether simple non-tannin phenolic compounds found in heather (*Calluna vulgaris*) have an

adverse effect on rumen microbial activity. They found that arbutin had positive effects, whereas orcinol and quinol had negative effects on *in vitro* digestion by rumen microorganisms and are likely to influence feeding ecology. Cattle and sheep, in contrast to goats that readily graze leafy spurge (*Euphorbia esula*), either avoid the plant entirely or graze it reluctantly. This is attributed to aversive chemicals, possibly of diterpenoid character, in leafy spurge. Kronber and Walker (1993) argued that the diet selection differences among these ruminant species are due to differential activity of microorganisms in their rumen.

Patterns of plant-animal associations cannot be understood by considering the plant and the herbivore as an isolated pair of interacting organisms. In many plant-animal interactions, same as in many plant-plant interactions, the direct target of allelochemicals are microorganisms that act as mediators of both plant resource suitability to the herbivore and the capacity of the herbivore to use the plant resource.

## PROMOTION OF MICROBIAL INFECTION

Promotion instead of inhibition of microbial infection by plant secondary metabolites may be beneficial for the producing plant. For example, addition of cassava (*Manihot esculenta*) exudates stimulated growth of diazotrophic bacteria *in vitro* (Balota et al. 1995). These bacteria in turn stimulated the mycorrhizal *Glomus clarum*. The beneficial effects could be related to growth promoting compounds able to stimulate plant susceptibility to mycorrhizal infection, spore germination or growth of the mycelium being able to increase the contact between fungal hyphae and plant roots and, consequently, to increase mycorrhizal colonization. Cases of beneficial effects involving symbiotic microorganisms are also presented in other parts of this article. But there are others, where no such benefits are obvious.

Evidence for plant allelochemicals conditioning roots for pathogen invasion has since long accumulated. The release of allelochemicals from plant residues in plots of continuous crop cultivation or from allelopathic living plants may induce the development of specific allelopathic bacteria that utilize these plant excretions (Barazani and Friedman, 2001). An example is that of *Asparagus* (Yu, 1999, and relevant references therein included). Old plantings of continuously cropped *Asparagus* give lower yields, of inferior quality. As fungicide and fumigation treatments did not prevent stunting and wilting of asparagus, autotoxicity has been suggested as one of the possible causes. Experiments by various researchers provided evidence of allelochemicals (both hydrophobic and hydrophilic) produced by various plants organs (roots included) that limit its growth. An interaction with root phytotoxins and two common *Fusarium* species affecting roots and crown has been reported. *Fusarium* virulence increased in the presence of the phytotoxins. It has, therefore, been suggested that autotoxic substances not only exert detrimental

influences on *Asparagus* itself but seem to predispose the plant to infection by *Fusarium*.

## CONCLUDING REMARKS

There is a vast array of plant-microbe interactions mediated by plant secondary metabolites. Some of them can be defined allelopathic, but only if we adopt the broad definition of allelopathy, including both plants and microorganisms, both positive and negative effects. Such interactions related with plant defense, involving phytoanticipins, phytoalexins or signal chemicals, can be ascribed as allelopathic in the broad sense. In other cases, even if we adopt Reese's definition of allelochemicals, i.e. non-nutritional chemicals produced by one organism that affect growth, health, behaviour or population biology of other species, we cannot describe a plant metabolite as positively allelopathic to a microbe, when the latter can utilize it, since we violate the 'non-nutritional' criterion of the definition. If we adopt the stricter definition of allelopathy, as plant-plant chemical interference, we will realize that the most important effects of compounds released into the environment by plants on other plants are not direct. Most often, secondary compounds of a plant-contributor first affect the soil processes, biotic and/or abiotic. Changes in these are then expressed (or not) on a plant-target. The link between donors and targets are primarily microorganisms, and the effect indirect.

Regarding the methodology and results of allelopathic research, we would like to make the following comments. We have a lot of circumstantial evidence concerning secondary-metabolite-mediated interactions. When we study isolated organisms and factors, we can have precision, predictability and eventual control of the interaction to our benefit. This is manifested in an exemplary way in the case of antibiotics. But if we are interested in processes occurring in complex environments, we are still far from understanding, never mind predict and control, the net result. Secondary metabolites do not affect only one target organism or only one target-process. Competitive relationships in a certain environment may also change as a side-effect of allelopathic interactions, and in addition, production of active metabolites heavily depends on various stress conditions, of either biotic or abiotic character (Anaya, 1999; Reigosa et al. 1999). The multiplicity of simultaneously running processes and interacting organisms makes the outcome quite uncertain.

Let us take one case to exemplify what we mean. It is that of carvacrol, an oxygenated monoterpenoid that we have intensely investigated regarding its allelopathic and other effects (Table 1). This secondary metabolite is produced in large quantities by aromatic plants in arid/semi-arid environments like the Mediterranean. Under the commercial name oregano, a large number of plant taxa belonging not only to the genus *Origanum* but also to other genera and other than Lamiaceae families are

used, with only prerequisite being the high content of carvacrol in their essential oil (Kokkini et al. 1989).

Table 1. Studies that we have conducted with carvacrol and/or carvacrol-rich essential oils of Mediterranean aromatic plants against various target species and processes.

Compound/ aromatic plant	Target process	Target organism /target plant part	Response	Source
<i>Thymus capitatus</i> , <i>Satureja thymbra</i>	Essential oil seasonal yield	Leaves, branches, flowering buds, flowers, litter	Large variability among plant parts and seasons	Vokou and Margaris, 1986a
<i>T. capitatus</i> , <i>S. thymbra</i>	Seed germination	<i>Cucumis sativus</i> , <i>Citrullus lanatus</i>	Low inhibition	Vokou and Margaris, 1982
	Seedling elongation		Strong inhibition	
<i>T. capitatus</i> , <i>S. thymbra</i>	Seed germination	<i>Hymenocarpus circinatus</i> ,	Low inhibition	Vokou and Margaris, 1982
	Seedling elongation	<i>Astragalus hamosus</i> , <i>Medicago minima</i>	Moderate inhibition	
<i>T. capitatus</i> , Carvacrol	Seed germination	<i>T. capitatus</i>	Strong inhibition	Vokou and Margaris, 1986b
	Seedling elongation		Strong inhibition	
<i>T. capitatus</i>	Spatial distribution	<i>T. capitatus</i>	Random or normal	Vokou and Margaris, 1986b
Carvacrol	Seed germination	<i>Lactuca sativa</i>	Moderate inhibition	Vokou et al. 2003
	Seedling elongation		Strong inhibition	
Carvacrol+ <i>p</i> -cymene, Carvacrol+ $\gamma$ -terpinene	Seed germination	<i>Lactuca sativa</i>	Synergistic effect	Vokou et al. 2003
	Seedling elongation		No synergism	
<i>Origanum onites</i> , <i>O. vulgare</i> subsp. <i>hirtum</i>	Sprout emergence	Potato tubers	No effect	Vokou et al. 1993
	Sprout elongation		No effect	
<i>T. capitatus</i> <i>S. thymbra</i>	Soil respiration	Soil microbes	Manifold increase	Vokou et al. 1984, Vokou and Liotiri, 1999
Carvacrol	Soil respiration	Soil microbes	Manifold increase	Vokou and Margaris, 1988
<i>S. thymbra</i>	Growth	Soil bacteria	Manifold increase	Vokou et al. 1984

Compound/ aromatic plant	Target process	Target organism /target plant part	Response	Source
<i>S. thymbra</i>	Spore germination	<i>Penicillium citrinum</i>	Strong inhibition	Vokou et al. 1984
<i>S. thymbra</i>	Mycelial growth	<i>Mucor hiemalis</i>	Strong inhibition	Vokou et al. 1984
<i>O. vulgare</i> subsp. <i>hirtum</i>	Growth	Bacterial strains isolated from the surface of potato tubers	Strong inhibition	Vokou et al. 1993
<i>T. capitatus</i> , <i>S. thymbra</i> , <i>O. onites</i> , <i>O. vulgare</i> subsp. <i>hirtum</i> , <i>O. dictamnus</i>	Growth	<i>Erwinia carotovora</i>	Moderate to strong inhibition	Vokou et al. 1993
<i>O. vulgare</i> subsp. <i>hirtum</i>	Colonization	Phyllosphere bacteria	Low	Karamanolis et al. 2000
<i>O. vulgare</i> subsp. <i>hirtum</i>	Colonization	Ice nucleation active bacteria	Absence	Karamanolis et al. 2000
<i>O. vulgare</i> subsp. <i>hirtum</i> , Carvacrol	Growth	<i>Erwinia herbicola</i> , <i>Pseudomonas syringae</i>	Strong inhibition	Karamanolis et al. 2000
<i>O. vulgare</i> subsp. <i>hirtum</i>	Foraging behaviour Consumption	Snails ( <i>Helix aspersa</i> , <i>H. lucorum</i> , <i>Eobania</i> <i>vermiculata</i> )	Repellence Very low	Vokou et al. 1998
<i>S. thymbra</i> , <i>T. capitatus</i>	Pollination	Bees and other pollinating insects	No apparent effect	Petanidou and Vokou, 1993

Carvacrol is low to moderately active in inhibiting germination and very active in inhibiting seedling elongation of various test plants like *Lactuca sativa*; in combination with *p*-cymene or  $\gamma$ -terpinene it has synergistic effects on seed germination but not on seedling elongation of this species (Vokou et al. 2003). In contrast to its moderate effect on seed germination of other species, it is highly inhibitory to seed germination (and growth of seedlings) of aromatic plants like *Thymus capitatus* that produce it in high quantities. Yet, in a study in a Mediterranean-type ecosystem, although seedling emergence of *T. capitatus* was remarkably lower than that of the other woody species (despite the high number of seeds that it produced), 60% of the seedlings emerged under the parent plant (Argyris, 1977). Tested on potato tubers, carvacrol did not exert any noticeable effect on sprout emergence and elongation, in contrast to other oxygenated monoterpenoids that were very inhibitory. Regarding microbes, carvacrol suppressed the growth of bacterial strains isolated from the surface of potato tubers, of

disease inducing bacteria like *Erwinia carotovora*, of ice-nucleation-active bacteria that cause extensive damages in agriculture like *Pseudomonas syringae* and *Erwinia herbicola*, but strongly activated soil microorganisms inducing a two - to sixfold increase of soil respiration. Finally, it had a repellent effect against grazers, such as snails.

With so many intervening factors and so many interferences, how can then one explain the abundance of carvacrol-rich aromatic plants in the Mediterranean environment? Each of the piecemeal questions that directed our research found an answer, but they are all partial answers, and the big question still remains unanswered. We believe that this is the case with many others researches. The issue becomes even more complicated as allelopathic activity under field conditions is often due to the joint action of mixtures of allelochemicals rather than to one allelochemical, with synergistic or antagonistic interactions possibly playing a major role (Einhellig, 1995; and relevant references therein included). But we study individual allelochemicals and more rarely mixtures of very few, when in natural settings, root exudates and foliar leachates may contain many compounds belonging to different chemical families, e.g., terpenoids, phenolics, alkaloids, polyacetylenes, etc. How these compounds interact and which is the net result of these interactions? Examining various factors and processes independently, we provide valuable pieces of information, but we tend to either overestimate or underestimate the importance of each one or ignore how they may enhance each other or interfere in another way.

Reigosa et al. (1999) argue that allelopathy can be important only under a limited set of specific conditions. Inderjit et al. (2002) argue that the understanding of the joint action of phytotoxins in allelopathy research is mainly hindered due to the lack of a well-defined reference model and methodological problems. If these are so, we have not only to find better tools for our work but primarily re-examine our conceptual basis. A plant secondary metabolite is an allelochemical, if it affects a target-plant species either directly or indirectly, and the effect is allelopathic. It is also an allelochemical with respect to microbes, not at the level of individual bacterial strains but at the level of the microbial community, the structure of which changes, often dramatically, in its presence. We should not visualize allelopathy as merely adhering to a plant-plant direct interference and concomitantly try to prove its importance under natural settings or separate it from competition. We agree with Inderjit and Weiner (2001) that most of the phenomena broadly referred to as allelopathic interference will be better conceptualized and investigated in terms of soil chemical ecology. The ever increasing body of knowledge regarding chemical communication (another aspect of the non-nutritional use of chemicals) within and among species, with microorganisms being major partners, should also be attached in the allelopathy corpus. Allelopathy exists in nature in various ways. We should expand its scope to include all pertaining aspects of chemical ecology.

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## CHAPTER 16

# ECOLOGICAL RELATIONSHIPS AND ALLELOPATHY

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## INTRODUCTION

### The release of allelochemicals is diverse

Plant ecology studies the relationships between plants and their environment, including other plants. Those relationships consist of abiotic and biotic factors that affect plants, and the effects of plants on those factors. Light availability, temperature, moisture conditions, different soil characteristics such as nutrient availability, and other organisms are the most important of those factors (Crawley, 1997). Effects of a plant include alterations in those factors in the vicinity of the plant. A plant may change light availability beneath its foliage, it may affect the quality and quantity of plant-available nutrients, and it may alter numerous other processes in its vicinity. In particular, a plant may fight (or attract) other plants, pathogens, herbivores and predators that may damage or benefit it or neighbouring vegetation. The processes may occur as the plant lives or after its death as decomposition proceeds. Chemical compounds may be produced and released during any of these processes, and they may show allelopathic activity in plant-plant interactions (Rice, 1984).

### Plants and micro-organisms have coevolved

Invertebrate and microbial communities usually colonize newly exposed land surfaces before autotrophs, and thus become the first established community assemblage on bare areas (Hodkinson et al. 2002). Those heterotrophic communities

conserve nutrients that facilitate the establishment of autotrophic plants. Thus, it is likely that for hundreds of millions of years, higher land plants have constantly lived in close contact with soil micro-organisms. Therefore, every process that releases phytochemicals from plant tissue to soil medium has evolved while the released phytochemicals have been prone to microbial activity. Microbial degradation may have neutralized, reduced or amplified any phytotoxic effect of a plant on another plant (Williamson and Weidenhamer, 1990). However, soil bacteria are superior competitors for neither allelochemicals nor nutrients: plants can but bacteria cannot store nutrients for later use; and soil bacterial populations are regulated effectively by predation. For these reasons, ecologically significant amounts of allelochemicals may recurrently have been able to affect the root cells of target plants even in naturally coevolved plant communities (Williamson and Weidenhamer, 1990; Wardle and Lavelle, 1997; Mallik, 2002). As a result of this coexistence between plants and soil micro-organisms, any species releasing allelochemicals must have evolved in presence of the bacterial degradation of the released allelochemicals in its original plant community. Furthermore, the plant may have evolved to minimize the probability that target species and soil micro-organisms are quickly able to detoxify the compounds released. The abundant microbes in a plant community, on the other hand, should have evolved to abide and utilize the phytochemicals that are released from those plant species with which they have evolved (see Schmidt et al. 2000). Although it has been argued that microbial activity in soil inevitably leads to inactivation of all allelopathic agents before these affect target species, there nowadays exists evidence suggesting that several unrelated plant species have evolved to detoxify plant-born allelochemicals in soil-root interface (see below).

## TWO DIFFERENT PERSPECTIVES

### Evidence for allelopathy

The unequivocal proof of the existence of allelopathy has traditionally been considered to require (see Fuerst and Putnam, 1983; Blum et al. 1999):

1. that the active compound or its precursor is released to environment from living or dead tissues of a donor plant (= the plant that is the origin of the allelochemicals involved).
2. that the quantities released from the donor plant are sufficient to cause a growth alteration in the target plant.
3. that the allelochemical(s) is/are received by the target plant in sufficient quantities to cause the growth alteration.

4. that the drift of allelochemicals from the donor plant to the target plant can be explicitly traced in nature.

In an ecological and evolutionary context, it is hardly necessary to assume that all these steps always remain distinguishable once evolved. If allelopathy has been evolutionary significant for both counterparts of the interaction, and if the goal of the donor species is to inhibit the growth of the target species, an evolutionary arms race should be going on between these two. The outcome of the race may drastically depend on environmental conditions, and it may change as new adaptations evolve. Depending on the stage of the race, the target species may be harmed in a way that is ecologically significant, or it may not be harmed at all. Therefore, two separate processes and two different objectives are distinguishable from the four steps presented above. The problem under interest may be approached using either the perspective of studying a donor species, or the perspective of studying a target species (Koricheva and Shevtsova, 2002; Reigosa et al. 2002).

## **Objectives of different perspectives**

When using the target species perspective, it is relevant to test whether a potential target species reacts to the existence of potential allelochemicals in the vicinity of it, on its surface, or in it, and whether the response of the target species is ecologically significant (Box 1). The target species may respond by growth alteration, or by detoxifying the compounds via a chemical process that is not used for other purposes (Einhellig, 1996; Weidenhamer, 1996; Schulz and Wieland, 1999). The growth response provides direct evidence that allelopathy is currently ecologically significant, while the latter response strongly suggests that allelopathy has affected the evolution of the target species. In addition, the latter response may require resources that cannot be allocated elsewhere, which may affect plant fitness (see Crawley, 1997). If either of these responses is observed, it may not always be necessary to show that there nowadays exists a donor species that is capable of releasing allelopathic agents in effective quantities within the present-day distribution of the target species, because the tolerance of the target species, growth conditions, or species distribution may have changed after the response evolved. Obviously, this does not justify unrealistic speculations about what might have occurred in the past (see Romeo, 2000).

Another and totally separate process is to use the perspective of the donor species (Box 1). In here, it is relevant to prove that a potential donor species releases phytochemicals owing to the presence of a probable target species, or that the release of chemical compounds is induced by the presence of a potential target species. Donor plants may distinguish the presence of target plants by recognizing specific phytochemicals produced by these (Sunderland, 1960; Logan and Stewart, 1992), or they could simply react on environmental fluctuation in abiotic conditions (for

example a change in soil pH) that is usually caused by a target plant; many plant species tend to change abiotic conditions around their roots (Waisel et al. 1996). In an alternative strategy, a potential donor species releases continuously, or recurrently, compounds that have (or had) a significant effect on a target species that interferes with the donor species in natural circumstances. In such a case, abiotic fluctuation in environmental conditions may regulate the toxicity of the release (Muller and del Moral, 1966; Nilsson et al. 1998). The evolution of the target species may or may not have been affected by the compounds released, depending on how important the selective pressure caused by the allelopathic compounds has been during the evolution of the target species. Obviously, allelopathy may have been crucial for the success of a donor species, and completely irrelevant for the success of its target species.

The perspective of studying a donor species:	The perspective of studying a target species:
<ul style="list-style-type: none"> <li>-is/was it capable of releasing allelopathic agents?</li> <li>-does/did the release benefit it?</li> <li>-are other explanations for the release secondary?</li> <li>-are there any signs of specific adaptations (target identification, spatial change in release, etc.)?</li> <li>-are other explanations for the adaptations secondary?</li> </ul>	<ul style="list-style-type: none"> <li>-how often a target species has to cope with allelopathic agents?</li> <li>-does/did the target species suffer/benefit from the release?</li> <li>-are other explanations for the effect secondary?</li> <li>-are there any signs of specific adaptations (detoxification, spatial change in growth, etc.)?</li> <li>-are other explanations for the responses secondary?</li> </ul>

⇒ Proof beyond a reasonable doubt

Table 1. The major questions approved when studied potential allelopathic interactions from the perspective of either the donor or the target species.

### The perspective of the target plant

It is important to distinguish the difference between the ecological relationships and experimental designs that are related to the target plants from those that are related to the donor plants. The former relationships have been explored in ecological

literature in recent years. Plant ecologists have successfully used root chambers, activated carbon, and different densities of target plants to state that several species must cope with allelopathy in natural situations, and to estimate the implications of allelopathy on target plants (Mahall and Callaway, 1992; Nilsson, 1994; Thijss et al. 1994; Sinkkonen, 2001). Callaway and Aschehoug (2000) found that in the vicinity of a Eurasian knapweed [diffuse knapweed (*Centaurea diffusa* Lamarck)] the growth of grass species from North America is decreased more than the growth of closely related grass species from native communities of *C. diffusa*. Further, root growth of one of the North American target species [Idaho fescue (*Festuca idahoensis* Elmer)] was slowest in contact with the roots of *C. diffusa*, but the effect was decreased when soil had been treated with activated carbon (Ridenour and Callaway, 2001). The most likely reason for the phenomenon observed is inhibitory substances, the concentration of which is gradually diluted as the distance from the roots contacted increases (note that activated carbon *per se* does not exclude the possibility that exuded phytochemicals immobilize significant amounts of nutrients in the vicinity of donor roots, and that the proportion of nutrients immobilized gradually decreases as distance from donor roots increases in carbon-free treatments). Another elegant way to elucidate the existence of allelopathy is to plan a density-dependent experimental design in which target plants grow at different densities in soil that contains a homogenous concentration of naturally found, potential allelochemicals.

Ecological relationships connected to a target species can be approached with an alternative strategy: to prove if initial target species are able to detoxify potential allelochemicals, and to state that the detoxification ability has not been evolved due to other ecological factors. Detoxifications of degradation products of hydroxamic acids in many dicotyledonous plant species seem to prove evidence for the usefulness of this strategy. Hydroxamic acids are widespread defence chemicals in the Poaceae (Niemeyer, 1988). They are well-known for their multiple roles in pest and disease resistance and for their allelopathic activity against other plants (Niemeyer, 1988; Chiapusio et al. 1997; Frey et al. 1997). Benzoxazolinone is a phytotoxic and abundant metabolite of hydroxamic acids. The detoxification ability of benzoxazolinone varies between related dicotyledonous species considerably, depending on the probable frequency of contact with hydroxamic acid producing grasses in the evolutionary history of the species (Schulz and Wieland, 1999). Several plant species have probably coevolved to detoxify allelopathic agents that originate in hydroxamic acid releasing members of Poaceae, or they may have evolved to utilize rhizosphere microbes that detoxify the allelopathic agents. Since benzoxazolinone does not occur in soil independently from hydroxamic acid producing plants (or their residue), this special case provides reliable evidence of the significance of allelopathic interactions in natural circumstances.

## The perspective of the donor plant

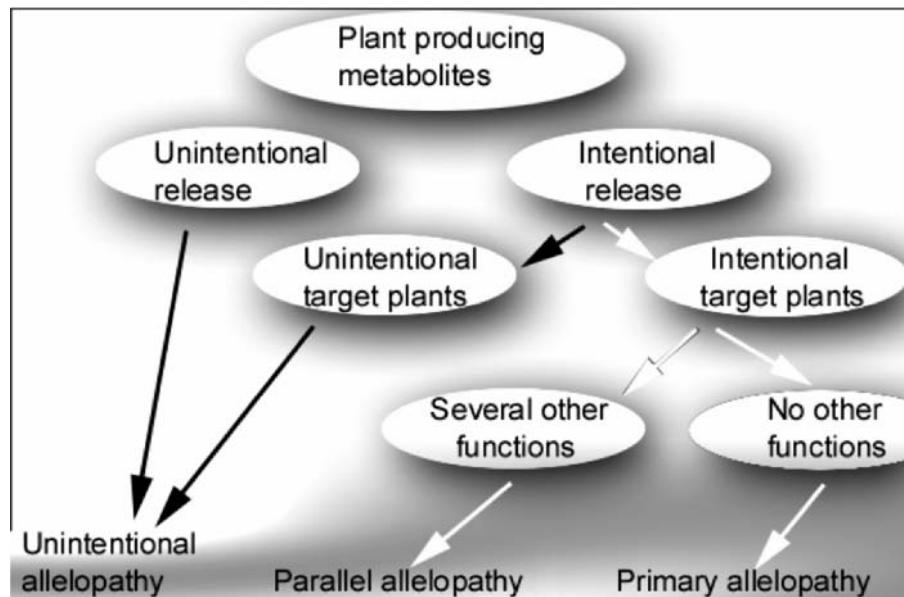
On the contrary to target species' relationships, ecological relationships of the donor species have been studied seldom. To prove that allelopathy is evolutionary significant one should be able to show that a plant releases, synthesizes or allocates phytochemicals in order to affect the growth of other plants (see Siemens et al. 2002), not just that the released compounds have toxic effects on other plants in suitable environmental conditions (see Seigler, 1996; Romeo, 2000). The latter phenomenon may always turn out to be an ecologically insignificant side effect (or even a trade-off) of a primary function that has been significant in the evolution of the donor species. If causalities are not clear, it is often easy to deny the evolutionary significant role of allelopathic function if potential allelochemicals have concurrent functions in the ecology of a donor species (see Pellissier, 1995; Brierley et al. 2001). Due to these reasons, it might be valuable to study whether the release of allelopathic agents is an evolutionary stable strategy (ESS) for the potential donor species (see Maynard-Smith, 1972, 1989). An ESS is a strategy that is adopted by most of the population and that cannot be displaced by a rare mutant (a hypothetical example could be increased exudation of specific allelochemicals through root tips to nutrient patches *versus* unaltered exudation of specific allelochemicals through root tips to nutrient patches). This evolutionary approach has been used successfully to explain ecological relationships in different environments. For example, allelopathic interactions between different species of algae may explain how multiple species can coexist in a seemingly uniform environment (Czárán et al. 2002), and allelopathy by two interfering land plant species may allow the coexistence of these (Dubey and Hussain, 2000; Amarasekare, 2002).

The difference between the perspective of studying a donor plant and the perspective of studying a target plant is as large as the difference between the perspectives of studying an herbivore and studying a plant that the herbivore consumes (Koricheva and Shevtsova, 2002). In recent ecological literature, many studies dealing with plant secondary metabolites and plant-herbivore interactions examine the problem under interest using either of the perspectives, not both of them (see Hartley and Jones, 1997). If approached simultaneously, the two perspectives are separated consciously (see Alliotta and Branca, 1996). For some reason, however, a mixed use of two different perspectives was common when plant secondary metabolites and plant-plant interactions were approached in the 20<sup>th</sup> century. This may partially have caused the bias of allelopathy research towards non-ecologically oriented questions (see Bever et al. 1998; Pellissier, 1998; Watkinson, 1998; Carral Vilaríño, 2002).

## EVOLUTIONARY HISTORY OF ALLELOPATHIC EFFECTS

### Three different possibilities

Ecologically significant, allelopathic interactions of plant-born secondary metabolites can be divided to three categories in natural circumstances, based on the



*Figure 1.* Three ecologically different categories of allelopathy. In primary allelopathy, phytochemicals are released primarily for allelopathic functions, while in parallel allelopathy phytochemicals are released for several ecologically significant functions. Evolutionary responses have usually occurred before the relationships indicated by white arrows become possible.

evolutionary history of the donor species (Figure 1). First, chemicals released for non-allelopathic purposes may reduce or ameliorate the fitness of unintentional target plants, or an unintentional release of phytochemicals, for example due to physical damage, may result in growth alterations in other plants. This category can be called unintentional allelopathy. Second, chemicals could be released for several ecologically relevant purposes, one of which is allelopathy against other plants. This category can be described as parallel allelopathy. Parallel allelopathy may be a result of recurrent unintentional allelopathy that has led to an adaptation in the donor species. Parallel allelopathy may be spatial or temporal: the same compound may be used for different

purposes on different parts of a plant, or at different times, respectively. Third, chemicals could be released mainly or solely owing to their allelopathic activity against other plants. This primary allelopathy may not be as common as the other categories since phytochemicals often have multiple roles in plant ecology (see Seigler and Price, 1976; Hartley and Jones, 1997; Nitao et al. 2002). Ecological relationships connected to these three categories differ from each other, and they may be approached using different experimental designs. In all cases, either target or donor plants, or both, have adapted or will possibly adapt to the selective pressure caused by the allelochemicals released.

## Unintentional allelopathy

Unintentional allelopathy occurs when phytochemicals affect a plant just by coincidence. Environmental conditions may change so that phytochemicals are released in larger quantities, they persist longer in soil, or they are degraded less than earlier (An et al. 2002). A mutation or a series of mutations may change the quality [for example the addition of a methoxy group to bibenzyl skeleton in crowberry (*Empetrum nigrum* L.); see Nilsson et al. 2000] or quantity [for example differences in the amount of isothiocyanates in rape (*Brassica napus* L.); see Choesin and Boerner, 1991] of chemicals released. As a result, phytochemicals may become effective allelochemicals against other plants. A plant may grow in a novel environment where the effects of the released phytochemicals are not the same as in its original environment. Changes in allelopathic relationships of a donor plant may occur if the plant has colonized a new plant community (Schulz and Wieland, 1999), or a new geographical area (Callaway and Aschhousg, 2000). There, soil microbes may degrade phytochemicals released from the alien species less efficiently than within its native distribution, or indigenous vegetation may lack the ability to tolerate the novel phytochemicals. Unintentional allelopathy may occur in agricultural systems as man brings together crop and weed species with separate evolutionary histories (Schulz and Wieland, 1999). If unintentional allelopathy increases the fitness of the donor plant, the phenomenon may become recurrent. Eventually, the result will be parallel or primary allelopathy. On the contrary, if an unintentional target species is sporadically affected by phytochemicals released from another species for whatever reason, the probability of adaptation will remain low (see Gardner et al. 1998).

The Petri dish germination test of lettuce is a classic example of an experimental design that tests for the potential of a compound to beget unintentional allelopathy. In this experiment, lettuce (*Lactuca sativa* L.) seeds are allowed to germinate on filter paper at different concentrations of phytochemicals from a non-agricultural species. Germination of seeds is recorded regularly, and finally the size of the seedlings is measured. The result usually indicates that the selected compounds show allelopathic

potential at certain concentrations against lettuce germination and seedling growth on Petri dishes (see Inderjit and Dakshini, 1995; Inderjit and Weston, 2000).

Rabotnov's coevolutionary hypothesis states that allelopathy is likely to be less significant in coevolved plant communities than in a case of a native and an alien species (Rabotnov, 1974; Mallik, 2002; Reigosa et al. 2002). Rabotnov's hypothesis can be regarded to state that the possibility of drastic allelopathic effects is higher in unintentional allelopathy than in a case of intentional target species, or that target species will eventually win the arms race between donor and target species, possibly with the aid of soil microbes. Currently there is evidence that contradicts Rabotnov's hypothesis in certain plant communities, or at least suggests that donor species may be successful in the race for an evolutionarily significant period of time (Mallik, 2002). Despite this, it may take time before the release pattern of allelochemicals of an alien donor species achieves an ESS in a new geographical area, and before native plant species and microorganisms adapt to the new selective pressure. Further, if allelochemicals of the alien species had multiple defensive roles within the species' original distribution area, the significance of other than allelopathic roles may be diminished in the new area if the alien species escaped its native pests when it entered the new area.

## **Parallel allelopathy**

Earlier chapters in this book have described the great diversity of phytochemicals that potentially are involved in allelopathy. The compounds described often have multiple roles, and the significance of different roles of many phytochemicals is still unexplored. For instance, it is not clear what are the main causes of variation in the levels of phenolic compounds in plants. Traditionally, phenolic levels have been thought to be mediated by plant-herbivore interactions, but phenolics also protect plants from photodamage, affect nutrient availability in certain plant communities, and are one of the major groups of potential allelopathic agents (Northup et al. 1995; Blum et al. 1999; Close and McArthur, 2002; Nitao et al. 2002). Low molecular weight organic acids (LMWO) often increase the availability of nutrients and minerals to plants, particularly the solubility of soil phosphate, iron, calcium and magnesium, and they tend to change rhizosphere pH (Jungk, 1996). Citric acid is a typical LMWO that is released through roots of many dicot plant species and that interacts with the environment of the donor plant in multiple ways. Roots of white lupin (*Lupinus albus* L.) have been shown to excrete citric acid in phosphorus deficiency (Dinkelaker et al. 1989). This compound has also been associated with the inhibition of plant growth by asparagus (*Asparagus officinalis* L.) in a mixture of several LMWO's (Hartung et al. 1990). Despite this, it is not currently known whether allelopathic effects of citric acid have been significant enough to modify the production and release of this compound

through the roots of the donor species. If they have been, allelopathy of citric acid is parallel with other significant ecological functions of the compound.

Another case where allelopathy is a likely consequence of the release of plant secondary metabolites for multiple purposes is phenolic compounds of trees and dwarf shrubs in boreal and hemiboreal forest plant communities (see Pellissier and Souto, 1999). There, polyphenols often control nitrogen release from plant litter. The phenomenon was first established with polyphenols from bishop pine (*Pinus muricata* D. Don) (Northup et al. 1995). Beneath pine trees, organic forms of nitrogen dominate in acidic environments where nitrogen is of short supply, and mineral forms reach high concentrations in nutrient-rich environments. Since mycorrhizae of pine are adapted to utilize dissolved organic nitrogen efficiently, this phenomenon may minimize nutrient losses from infertile and acidic soils and, eventually, help to explain the convergent evolution of tannin-rich plant communities throughout the world (Northup et al. 1995; Lipson and Näsholm, 2001; Miller and Bowman, 2003). As the previous chapters have shown, tannins and other phenolics are one of the most studied potential allelopathic agents found in nature.

When dealing with parallel allelopathy, it must be taken into account that the behaviour of donor plants is always a compromise between the allelopathic function and non-allelopathic functions of the phytochemicals. Therefore, optimal responses of donor plants to the presence of target plants may not be found always, and target plants may be able to cope with the compounds released to some extent (Schulz and Wieland, 1999).

## Primary allelopathy

Current evidence strongly suggests that many plant secondary metabolites are like squirrel's hands – they have evolved for multiple purposes. Despite this, primary allelopathy may be found in nature. Primary allelopathy occurs when a compound is produced and released only or mainly because of its effects on other plants (see Figure 1). If the compound is used for other than allelopathic purposes, these are secondary roles that have not affected the concentration, amount and allocation of the compound in the plant and from the plant. Primary allelopathy may be a result of a sudden mutation, a gradual decrease in the importance of other ecological roles of the compound, or a selective pressure that has led to the synthesis of chemical compounds targeted solely against the growth of other plants.

Primary allelopathy is often ecologically significant in interactions between parasitic plants and their hosts (Rice, 1984). As far as I know, there are no known examples of primary allelopathy in which phytotoxins affect through soil-root interface. However, a bibenzyl Batatasin-III in northern crowberry [*Empetrum nigrum*

ssp. *hermaphroditum* (Hagerup) Böcher] fulfils several properties of a primarily allelopathic chemical. First, it accumulates into specific glands on leaf surface; if the primary function of the compound were protection from sunlight or low temperatures, it should be spread evenly in the leaf instead of accumulating in specific glands. Second, it is leached by water from these glands; if the primary function were herbivore avoidance, natural selection should favour individuals whose batatasin-III concentration does not fall rapidly during the period of active growth (see Nilsson et al. 1998). Surplus production of batatasin-III is also an unlikely explanation since *E. nigrum* is capable of accumulating high concentrations of other toxic compounds in old stem tissues (Monni et al. 2000), and concentration of batatasin-III is highest in

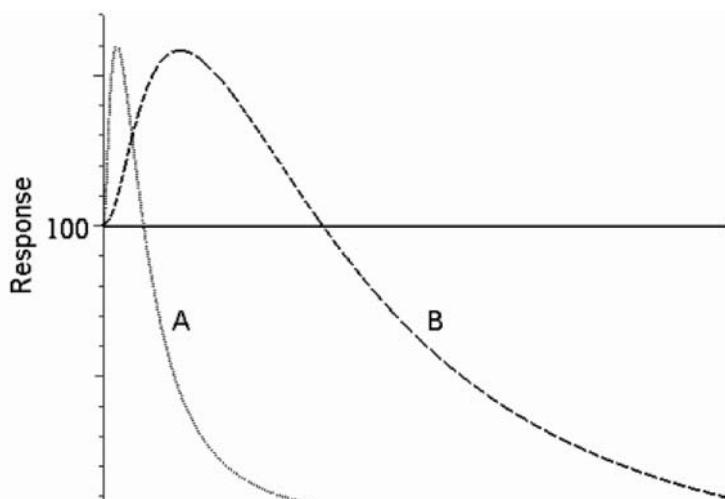


Figure 2. The response of two target species to a toxin at different concentrations as percentages of the response of conspecific plants grown in the absence of the toxin. If natural concentrations are usually low, species A is likely to benefit more than the species B. If high concentrations occur frequently, the situation may be the opposite one. Units are arbitrary.

green leaves. If nutrient immobilization were the primary reason for production of batatasin-III, natural selection should have favoured the release of a compound with a lower  $pK_a$ -value than 10 of batatasin-III since compounds with low  $pK_a$ -values have their highest activity in acidic conditions when the risk of leaching of inorganic ions is high (Northup et al. 1995; Lehman and Blum, 1999; Wallstedt et al. 2002). Further, *E. nigrum* ssp. *nigrum* produces another but less toxic bibenzyl (Nilsson et al. 2000). Unlike the northern subspecies, *E. nigrum* ssp. *nigrum* does not dominate ground vegetation anywhere in boreal forests (Tybirk et al. 2000). Due to these reasons, batatasin-III is a candidate for a primarily allelopathic compound. This, of course, does

not mean that the primary function of other phenolic compounds released from the glands on the leaves of *E. nigrum* ssp. *hermaphroditum* is allelopathy, nor does it suggest that the levels of batatasin-III in soil are sufficient to inhibit plant growth without synergistic action with the other compounds released from the glands.

## ECOLOGICAL RELATIONSHIPS AND EVOLUTION

### Dilution of allelopathic effects in nature

Typically, the response of an organism to a toxin is stimulation at low concentrations (Figure 2). As the concentration increases, stimulation gradually turns to inhibition. At very high concentrations, the organism may die. The effect of allelochemicals on plants usually follows this pattern (Carballeira et al. 1988; An et al. 1993). In certain cases, stimulation has not been observed at low concentrations, but theoretically it might occur if the concentration used were low enough (An et al. 1993; Sinkkonen, 2001).

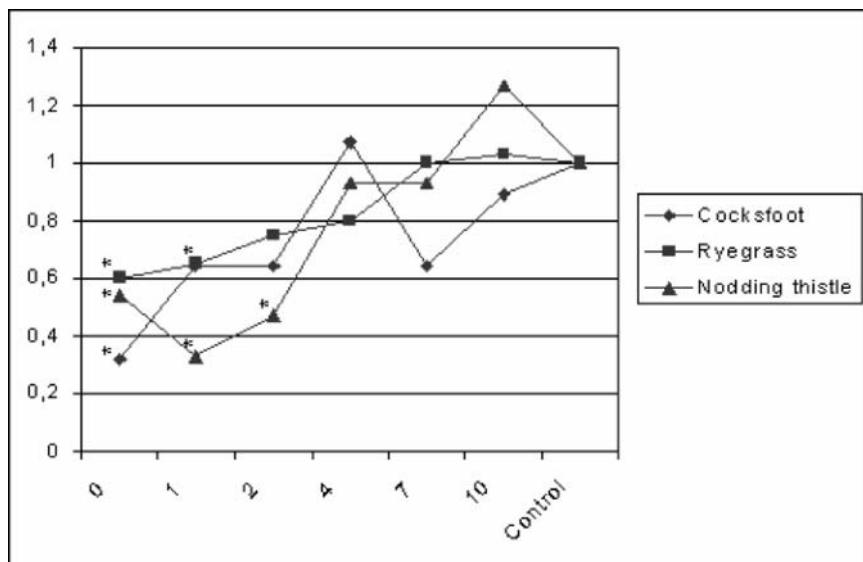


Figure 3. Relative germination of target species (germination of treated plants / germination of control plants) at varying distances (mm) from nodding thistle (*Carduus nutans* L.) seeds on soil. Controls germinated without nodding thistle seeds. \* = significantly different from control ( $p < .05$ ). Data from Wardle et al. (1991a).

Due to the dose-dependence of chemical interference, donor plants inevitably face problems when they intend to optimise the release of allelochemicals in a fluctuating environment. Declivity of microhabitat, distance from the source of allelochemicals (Figure 3), and the density of target plants may drastically change the dose received by a single target plant. Around a single donor plant, concentrations of water soluble allelochemicals are likely to be higher downhill than uphill, which may totally mediate the outcome of the allelopathic effect (Carballeira et al. 1988). In a close proximity to the source, the outcome is often growth inhibition (Wardle et al. 1991a; Mahall and Callaway, 1992; Huang et al. 2000; Callaway and Aschelhoug, 2000), but stimulation of plant growth has also been observed (Søgaard and Doll, 1992).

The methodology and principles of the density-dependence of allelopathic interactions are thoroughly discussed in Chapter 5. The ecological consequences of this phenomenon are diverse, and they differ depending on the perspective used. Since the balance between allelopathic stimulation and inhibition depends on target plant density and the phytochemical concentration in the soil, target species may tend to evolve towards stimulation at higher phytochemical concentrations (Sinkkonen, 2001). However, a possible cost of this tendency is weak stimulatory effects at low concentrations, and at high plant densities. Therefore, the tolerance of a target species may even decrease, if plant densities are permanently high or phytochemical concentrations low (Figure 2). Further, because seeds of several species usually germinate close to each other in nature, sensitive target species may benefit from their insensitive and abundant neighbours that inactivate most of the allelochemicals at high plant densities (see Thijs et al. 1994).

Allelopathic effects of decaying plant material are also density-dependent. Fresh plant residue usually contains potential allelochemicals, and donor species may even have evolved to utilize the toxic properties of their own residue in order to ensure the survival of the offspring (Wardle et al. 1998). As microorganisms begin the decomposition process, allelochemicals are often released from dead plant material, and later leached by rainfall or further metabolised to inactive compounds, depending on abiotic conditions (An et al. 1996; An et al. 2002). The process often brings on growth inhibition of intended or unintended target plants, but the residue of some plant species tend to stimulate the growth of ensuing plants (Wardle et al. 1991b; An et al. 1996). There is often a short stimulatory period in the beginning of the decomposition process, followed by a longer inhibitory period that gradually turns again to stimulation at the end of the residue decomposition process (An et al. 1996). However, depending on the density, the target plants may share the allelochemicals so that inhibition does not occur (Figure 4).

## Target selection

Since it is likely that all target species do not evolve similar strategies in order to be able to grow under the influence of allelopathic agents released from a single donor species, the donor species has to find an optimal strategy to negatively affect the growth of interfering plants as efficiently as possible in continuously and unexpectedly fluctuating environment. If an ESS is achieved only by releasing allelochemicals, it should either be the mean of the optimal strategies against every abundant target species, or a high phenotypic plasticity in the release of allelochemicals of the donor species. Since different plant species utilize different microhabitats (Crawley, 1997; Miller and Bowman, 2003), and since certain plant species more probably change growth conditions unfavourable to potential donor species than other plant species (Ohlson et al. 2001), indigenous target species may have been of different importance during the evolution of allelopathy in a donor species. As a result, allelopathic effects of donor species can be expected to be most severe against those target species that have had a high potential to diminish the fitness of the donor species in natural situations. This may explain why a single donor species may have fatal effects against certain species and weak or no allelopathic effects against other potential target species (see Wardle et al. 1998; Quested et al. 2003).

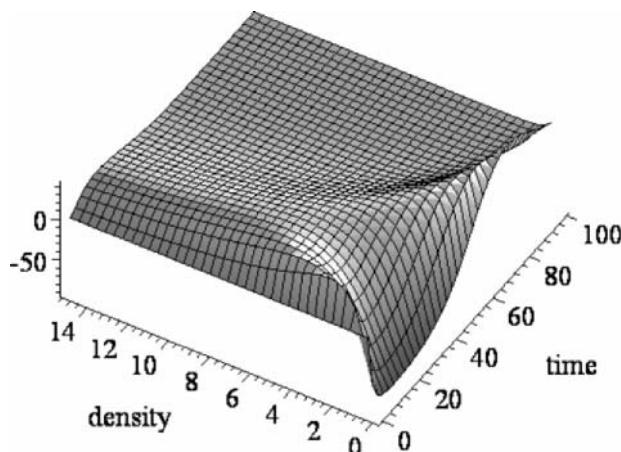


Figure 4. The relationship between the decomposition time of allelopathic residue, target plant density, and the weight of target plants. Control (no residue) = 0. Units are arbitrary. Based on Sinkkonen (2003).

## Sudden shocks versus constant stress

Plants are nasty neighbours: they are unpredictable. The size, age, and vigour of donor and target plants substantially determine how much allelochemicals can be released and how much can be tolerated. Multiple, simultaneous stresses may modify the response of plants (Hartley and Jones, 1997), and chemical interference may affect synergistically with other factors causing stress. There is substantial spatial and temporal variation in the concentration of allelochemicals and in the release pattern of allelochemicals among species and individuals, and even within individuals (Table 1). Due to this, both donor and target plants often live in a patchy environment. This natural patchiness does not disqualify the importance of various dilution mechanisms or target selection; it merely complicates the evaluation of the significance of allelopathic interactions.

*Table 2.* Examples of intrinsic and extrinsic sources of variation in allelochemistry of donor species that excrete compounds against other species (see also Hartley and Jones, 1997).

Type of variation	Reference
Spatial within individual donor plants	(Collantes et al. 1999)
Diurnal within individual donor plants	(Åhman and Johansson 1994)
Spatial from individual donor plants	(Zackrisson and Nilsson 1992)
Seasonal from individual donor plants	(Molina et al. 1991)
Rain-induced release from donor plants	(McPherson and Muller 1969)

Variation that is related to the release of allelochemicals may be selected for several reasons. First, communities of soil microorganisms seem to be able to acclimate and adapt to chronic concentrations of allelochemicals, which obviously increases degradation (Blum, 1996; Schmidt et al. 2000; Staman et al. 2001). Second, evidence from plant-pesticide studies suggests that target species rapidly evolve high resistance if toxic compounds are continuously or repeatedly present at high concentrations in the environment (Gardner et al. 1998). The evolution of resistance may be delayed or may not occur against artificial phytotoxins if the toxins are released infrequently and at varying quantities (Gardner et al. 1998). This strategy may also minimize the costs of producing a toxin. Third, several different, simultaneously affecting toxins may delay the prevalence of resistant genotypes

(Gardner, 2002). Therefore, if qualitatively and quantitatively alternating mixtures of allelochemicals are present irregularly, acclimatisation and adaptation to allelochemicals may be delayed. Hence, biotic conditions may favour the strategy of sudden shocks, instead of the strategy of constant stress in the release of allelochemicals.

### Conflict of interests

An and co-workers (2001) studied the biological activity of all allelochemicals identified from residues of *vulpia* [*Vulpia myuros* (L.) C. C. Gmelin]. They found out that the majority of compounds possessed low or medium biological activity and these compounds also contributed most of the *vulpia* phytotoxicity, while compounds with high biological activity were in the minority and only present at low concentration (An et al. 2001). They also found out that if allelochemicals were used in proportions found in the residue, the growth inhibition in the target species was higher than if the same allelochemicals were used in equal proportions. Thus, at least some donor species may be able to release “an optimal cocktail” of allelochemicals, which gives the best input-output relation, which usually leads to the highest possible inhibition. Further, natural selection may act faster for resistance against (or detoxification of) abundant soil phytochemicals than against less abundant compounds in soil.

Often there may be a trade-off between growth and plant defence, such as allocation of resources to the production of allelochemicals (Hartley and Jones, 1997). In other cases, such a trade-off may not exist, and an increase in the production of allelopathic agents also ameliorates plant's competitive ability (Siemens et al. 2002). In both cases, donor species may be unable to optimise their allelopathic effect by maximizing the exudation. Instead, to achieve an ESS, donor species must maximize the difference between benefits and costs of producing the allelopathic effect. Obviously, target plants aim the opposite: to maximise the difference between benefits and costs of resisting the allelopathic effect. In fact, to be an ESS, benefits of resistance should be higher than the costs in any target species.

### CONCLUDING REMARKS

It is easy to imagine how a plant may benefit from the ability to release compounds that harm other plants on purpose. A plant could hinder and even kill other plants so that these never had a chance to begin to limit its' growth (see Wardle et al. 1998). A plant could avert other plants' growth so that these were not able to consume limiting resources as efficiently as otherwise (see Weih and Karlsson, 1999). A plant could warn other plants, and thus “advice” them to grow elsewhere (see Mahall and

Callaway, 1992). Since the production of secondary metabolites for one purpose may reduce the allocation of resources elsewhere, a plant would benefit even more if it were capable of concentrating the release of allelochemicals temporally and spatially. The inhibition could take place at a special time when the target plants are most sensitive, or it could concentrate on nutrient patches so that competing plants were not able to utilize the nutrients as efficiently as otherwise. Currently, however, much of the research has concentrated in considering whether allelopathy exists as a natural phenomenon. Since this may not be of the highest importance anymore, the upcoming ecologically oriented research on allelopathy will hopefully concentrate on determining the ecological and evolutionary significance of allelopathic interactions compared to other evolutionary processes that affect plants. Hence, it is still worthwhile to agree with the view of Muller (1969): "Agronomists and horticulturists have long been more willing than ecologists to consider allelopathy as a significant phenomenon. This disparity is now decreasing as ecologists encounter increasingly effective evidence of allelopathy. A major influence in this development has been the recent availability of powerful analytical chemical techniques... Thus, allelopathy has emerged as a natural phenomenon which must be considered in any attempt to understand that intricate pattern called 'ecological process'."

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## CHAPTER 17

# RESISTANCE AND SUSCEPTIBILITY OF PLANT COMMUNITIES TO INVASION: REVISITING RABOTNOV'S IDEAS ABOUT COMMUNITY HOMEOSTASIS

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## INTRODUCTION

One of the strangest but most interesting phenomena in ecology is the tremendous increase in abundance that some species experience when introduced to new ecosystems by humans (see Louda et al. 2003). Although most species that establish in new ecosystems appear to be well behaved and act more or less like the native species, some become overwhelming dominants – far more abundant than they are in their native regions. Equally impressive is the collapse of many native communities when new species are introduced (see D'Antonio and Vitousek, 1992; Mack et al. 2000; for reviews on community-level effects of plant invasions). Diversity can be transformed to virtual monocultures (Braithwaite et al. 1989; Malecki et al. 1993; Meyer and Florence, 1996; Bruce et al. 1997). The extreme nature of invasive success and native collapse suggests that unusual and very powerful ecological processes and mechanisms are at work. The goal of this chapter is to scrutinize the mechanisms that drive the impressive “transmogrification” (Watterson, 1988) of some exotic invaders from minor components at home to competitive monsters away from home and how this transmogrification devastates native plant communities.

The list of plant species that transmogrify from good citizens to outlaws is remarkable. For example, *Heracleum mantegazzianum*, appropriately called giant hogweed in North America, is a relatively rare endemic restricted to a small and remote part of the Caucasus Mountains in the Republic of Georgia (Kolakovskiy, 1961; Z. Kikvidze, pers. com.). Introduced as a “garden curiosity” because of its 50 cm

inflorescences and 6 m height, it is now a major problem in continental Europe and Great Britain and a federally listed noxious weed in North America where it forms dense monocultures. One of the most invasive plants of wild lands in California, *Centaurea solstitialis* (Howald et al. 1999; DiToamso and Gerlach, 2000; Rejmánek, and Reichard 2001), only occurs in discrete, highly disturbed patches in its native Turkey (O. Eren and J. Hierro, pers. obs.). In invaded regions, *C. solstitialis* appears to have substantial competitive abilities against the locals and occurs at much higher densities than at home (Dukes, 2001; Hierro et al. unpublished data). *Cytisus scoparius* is a minor weed where it is native in western and central Europe, but when introduced to New Zealand, Australia, and the United States it became a major invader of pastures and forest and a target of biocontrol programs (Paynter et al. 1998; Memmot et al. 2000). Similarly, *Echium plantagineum* is an occasional weed in its native range in the Mediterranean region; however, after its introduction to Australia as a garden plant in the 1800s, it has spread to infest and dominate large areas of agricultural land, becoming one of the Australia's worst weedy species (Grigulis et al. 2001). A rare South American legume, *Mimosa pigra*, sharing an uncannily similar etymology with giant hogweed, has spread throughout many other tropical areas of the world after human introduction. *Mimosa pigra* has been particularly successful in tropical Australia where it forms dense monospecific stands with an understory composed only of conspecific seedlings (Miller and Lonsdale, 1987; Braithwaite et al. 1989).

The primary hypotheses for the remarkable success of many exotics as community invaders relative to their success in their native communities are: 1) they have escaped the consumers that control their population growth - the “natural enemies hypothesis” (Darwin, 1859; Williams, 1954; Elton 1958), 2) certain invaders can take advantage of ‘empty niches’ that no local natives utilize (Mack et al. 2000; Dukes, 2001; Sakai et al. 2001), 3) because of a longer history with humans, invaders can take advantage of disturbances more efficiently than natives or even engineer disturbances to their own advantage (Simberloff and Von Holle, 1999; D’Antonio et al. 1999; Mack et al. 2000); and 4) invaders have undergone rapid natural selection in response to new environments which makes them unusually aggressive (Blossey and Nötzold 1995; Mack et al. 2000; Gaskin and Schaal, 2002). However, as described below many highly successful invaders do not always behave in ways that support these hypotheses. The puzzling behavior of many successful exotic invaders provides an unparalleled opportunity to examine fundamental ecological theory including the relative importance of top-down versus bottom-up control of communities, the nature of niches, the importance of local adaptation, and the individualistic versus holistic nature of plant communities. In this chapter we focus on the latter issue and argue that the peculiar behavior of many exotic species may challenge our conceptual paradigm for plant communities.

## THE INDIVIDUALISTIC PARADIGM OF PLANT COMMUNITIES AND THE TRANSMOGRIFICATION OF INVADERS

Most community ecologists appear to hold the general view that communities are fundamentally organized by the struggle for resources among more-or-less stochastically dispersed species with physiological adaptations to a particular subset of abiotic conditions, and that are consumed from time to time by members of other trophic levels (Lortie et al. *in review*). Various modern syntheses of Henry Gleason's individualistic theory (Gleason, 1926) have been stated and restated in many ways, but perhaps it is best to first consider what was written by Gleason himself:

The sole conclusion that we can draw from all the foregoing considerations is that the vegetation of an area is merely the resultant of two factors, the fluctuating and fortuitous immigration of plants and an equally fluctuating and variable environment"..."an association is not an organism, scarcely even a vegetational unit, but merely a *coincidence*".

This quote may represent one extreme end of the range of positions on the nature of communities, but recent attempts to define generally accepted individualistic concepts are similar:

"The alternative view (to Clements) pioneered by Gleason, perceives vegetation as an assemblage of individual plants belonging to different species with each species distributed according to its own physiological requirements as constrained by competitive interactions." Moore (1990)

This strong individualistic perspective has been challenged (Callaway, 1997; Lortie et al. *in review*), and for many ecologists neither the holistic or individualist *conceptual* perspectives correspond with a modern comprehensive *empirical* understanding of current plant communities (Callaway, 1997; Inouye and Stinchcombe, 2001; Lortie et al. *in review*). The point of emphasizing the individualistic paradigm in this chapter is not to prepare for its total rejection - to the contrary, the individualistic theory has provided a conceptual platform for outstanding advances in the study of resource competition - but rather to propose that the theory has permeated our thinking

as ecologists to the extent of limiting the range of hypotheses that we explore for ecological phenomena, such as the transmogrification of some introduced species. Recognition of the possibility that we may wear individualistic blinders may open up new avenues of studying plant invasions, as well as suggesting modifications of our conceptual paradigms of communities (Lortie et al. *in review*).

If plant communities really are individualistic, then the ways in which exotic invaders transmogrify must also be individualistic, and are not likely to include co-evolved and unique interactions among coexisting *plant* species in natural communities. In other words, if communities are individualistic then the rules of dispersal, competition for resources, consumer interactions, and physiological adaptation must explain the transmogrification of invasive species and the collapse of invaded communities. On the other hand, if interactions over long periods of time among plants within a community drive natural selection in the direction of homeostasis, and the arrival of new plants disrupt this homeostasis, then communities are not fully individualistic.

The natural enemies hypothesis does not conflict with the individualistic paradigm. And although this is by far the most common explanation for the unusual success of invasive plants, there are many reasons to doubt that the natural enemies hypothesis provides an answer for all, or even most, successful exotic plant invasions. The common failure of introduced biocontrols to control their targets, by far the most frequent outcome (Louda et al. 2003; Pearson and Callaway, 2003), strong compensatory responses of some invaders to defoliation and damage (Müller-Schärer, 1991; Steinger and Müller-Schärer, 1992; Callaway et al. 2001), and the lack of evidence for consistent and strong top-down regulation in many natural ecological systems suggest that the natural enemies hypotheses does not definitively explain a large proportion of invasive transmogrifications. The consistency with individualism does not seem to correspond with strong explanatory power.

The empty niche hypothesis does not conflict with the individualistic paradigm for plant communities either. Some invaders must certainly benefit from empty niches, to the extent that this could explain how they are able to be dominants in recipient communities (e.g., Dyer and Rice, 1999; Dukes, 2001), but this does not explain how the nastiest invaders appropriate the niches of *other* species. The crucial question to be answered is how some invasive plants transmogrify after introduction to competitively exclude their new neighbors and establish monocultures that persist for decades. Again, a hypothesis that is comfortably consistent with individualistic theory does not seem to provide strong explanatory power for transmogrification.

The occupation of anthropogenically-disturbed habitats by species better adapted to the effects of humans does not conflict with the individualistic paradigm, and most exotic invaders obviously benefit a great deal from disturbance. However, many

exotic plants are able to displace natives in relatively undisturbed natural communities. Furthermore, many of these same exotics do not respond to disturbance in the same way in their native ecosystems, and many exotic invaders appear to create stable and near permanent monocultures after disturbance - completely unlike disturbance-adapted natives that are replaced by late successional species.

One of the most recent hypotheses for the disproportionate success of species in new regions is that some of these species are actually unique genetic entities; created by the exposure of old genotypes to new environments or the mixing of previously isolated genotypes. While intriguing and undoubtedly occurring (Lee, 2002; Gaskin and Schaal, 2002; Hänfling and Kollman, 2002) the rapid evolution of such dominant organisms would suggest that local adaptation is remarkably insignificant. Considering that ecologists have spent much of the last hundred years trying to understand how species have evolved to be adapted to the environments in which they occur, transmogrification by rapid evolution is hard to accept as a general phenomenon for invasive success. Furthermore, the hypothesis that newly evolved genotypes are disproportionately and consistently more disruptive to intact communities than the original inhabitants is not fully consistent with individualistic theory – if “new” genotypes have a unique ability to extirpate locally adapted native communities this suggests that there must be something special about species that have lived together over long periods of time.

## THE ‘NOVEL WEAPONS’ HYPOTHESIS

Here, we propose and discuss the possibility of another, but not mutually exclusive, hypothesis for the transmogrification of species when they invade novel ecosystems – the ‘novel weapons’ hypothesis (Callaway and Aschehoug, 2000; Bais et al. 2003). Specifically, we argue that some invasive plants may succeed because they bring novel mechanisms of interaction, primarily chemically mediated, to natural plant communities. There are several major barriers to thorough consideration of this hypothesis. First, and rather significant, there is not much empirical evidence. Second, as presented in detail above, plant communities are widely thought to be “individualistic”, that is composed of plant species that have similar adaptations to a particular physical environment. The novel weapons hypothesis conflicts with this as it suggests the possibility that natural plant communities may be somewhat tightly knit, and perhaps even coevolved, entities. The third controversial aspect of the novel weapons hypothesis is that the only truly effective and novel weapons (or at least the ones that we can think of) that could vary dramatically among taxa are allelopathic (i.e., biologically active chemicals that are produced by plants and released into their environments), a form of interaction with a tawdry history (Callaway, 2002) that is

often dismissed from serious consideration in plant community theory in favor of resource-driven interactions.

The novel weapons hypothesis has its own murky history. T.A. Rabotnov (1982), an ecologist at Moscow State University, proposed a decidedly non-individualistic “evolutionary approach to the study of allelopathy”. He argued that plants could evolve in response to the chemicals exuded from the roots or washed from the leaves of their neighbors, stating that the

“resistance of plant species to the vital secretions of other components of biocenoses [communities], including saprotrophic organisms, has been created through the acquisition of properties by plants preventing the harmful action of secretions of other organisms, or through the detoxification of toxic substances by soil saprotrophic and ecrisotropic [rhizosphere] organisms.”

Although shaped by a rather dogmatic perspective on the importance of allelopathy, and based on virtually no experimental evidence what-so-ever, Rabotnov raised the rather provocative idea that plant communities (and their associated microbes) themselves held some of the answers to invasions. With more certainty than warranted, Rabotnov stated that over evolutionary time, “allelopathically neutral” or “allelopathically homeostatic” biotic systems form, in which allelopathic interactions are so insignificant that they have no effect on the composition of the communities. In other words, plants and microbes adapt to the chemicals produced by their neighbors (much like they rapidly adapt to herbicides and other chemicals) and therefore allelopathy may not be very important in most natural communities. Rabotnov’s ‘logical’ conclusion to this rather ballsy claim was that “disturbed homeostasis” occurs when interactions take place among species without an evolutionary history. According to Rabotnov, adaptation to allelopathic exudates is the foundation of community homeostasis and the lack of adaptation to allelopathic exudates is the foundation for the loss of such homeostasis. Rabotnov did not connect his speculations with the current global explosion of exotic invasive plants, but the implications are fascinating. Is it possible that some invasive exotic plants are spreading through and replacing native plant communities because they produce and release harmful chemicals that the native inhabitants have never experienced? If so, ecology must be prepared for a major realignment of community theory and new approaches to the management of invasives.

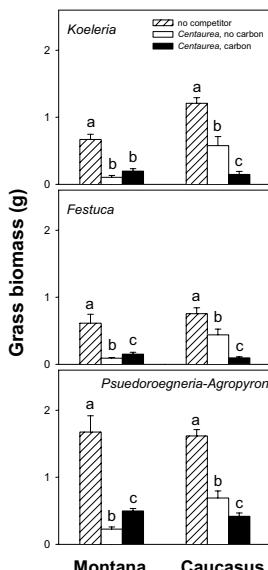
Rabotnov based his claims on a small number of non-quantitative, and in some cases hardly substantiated observations. First, he noted that *Eucalyptus* trees introduced to California and Western Europe had strong negative effects on

Californian and European native herbaceous species, whereas in their native lands of Australia and Tasmania it has “no allelopathic action on indigenous species of grasses.” How he knew the latter is somewhat mysterious. He argued a similar case for walnut trees (*Juglans*), famous for their allelopathic effects when introduced as a crop around the world, but which form a “well-developed grass cover” beneath them in their native Kyrgyzstan and Uzbekistan. Again, we must take Rabotnov at his word. Apparently unaware of the body blow delivered by Bartholomew (1970) to the early allelopathy scene, including *Salvia* (see Callaway, 2002), Rabotnov noted that *Salvia leucophylla* had important allelopathic effects on introduced species of annual grasses that did not have an evolutionary history with *Salvia*. Finally, he suggested that the effect of *Pteridium aquilinum*, bracken fern, on annual grasses without a common evolutionary history was also evidence for allelopathic damage to evolved community homeostasis.

Rabotnov’s empirical case was weak, but we now know much more about allelopathy and even biogeographical variation in the effects of invaders and the chemicals they produce – and Rabotnov’s conjectures may no longer seem so wacky. Collectively, plants produce a compositionally diverse array of over 100,000 different low-molecular-mass natural products, also known as secondary metabolites, many of which appear to be species-specific (Bais et al. 2001, 2003; Flores et al. 1999). This rich diversity is likely due to selection pressures for the acquisition of resources, herbivory, microbial interactions, and perhaps even interactions with other plants. The evidence for allelopathic effect of some of these chemical products is increasing. Root exudates may initiate and manipulate biological and physical interactions between roots of different species and between plant roots and soil organisms. Furthermore, many studies suggest that allelopathy may contribute to the ability of particular exotic species to become dominants in invaded plant communities (see Hierro and Callaway, 2003, for a review of allelopathy and plant invasion). But most importantly, recent experiments have demonstrated that some invaders may exude allelochemicals that are relatively ineffective against neighbors in natural communities, but highly inhibitory to plants in invaded communities.

## EVIDENCE FOR THE ‘NOVEL WEAPONS’ HYPOTHESIS

To our knowledge, biogeographic variation in the interactions between invasive plants and other plant species has only been explored with two species in the genus *Centaurea* and *Alliaria petiolata* (garlic mustard). While relatively rare in native communities, *Centaurea* species, such as *C. maculosa* (spotted knapweed), *C. diffusa* (diffuse knapweed), *C. solstitialis* (yellow starthistle), *C. melitensis*, and the closely related *Acropitilon repens* (Russian knapweed), are among the most widespread and destructive grassland invaders in the world (Maddox et al. 1985; Griffith and Lucey,



*Figure 1.* Natural concentrations of (-)-catechin in rhizospheres of *Centaurea maculosa* and native grasses in regions invaded by *C. maculosa* (North America) versus the origin place of this plant (Europe). Values are means + 1 SE. Reprinted from Bais et al. 2003.

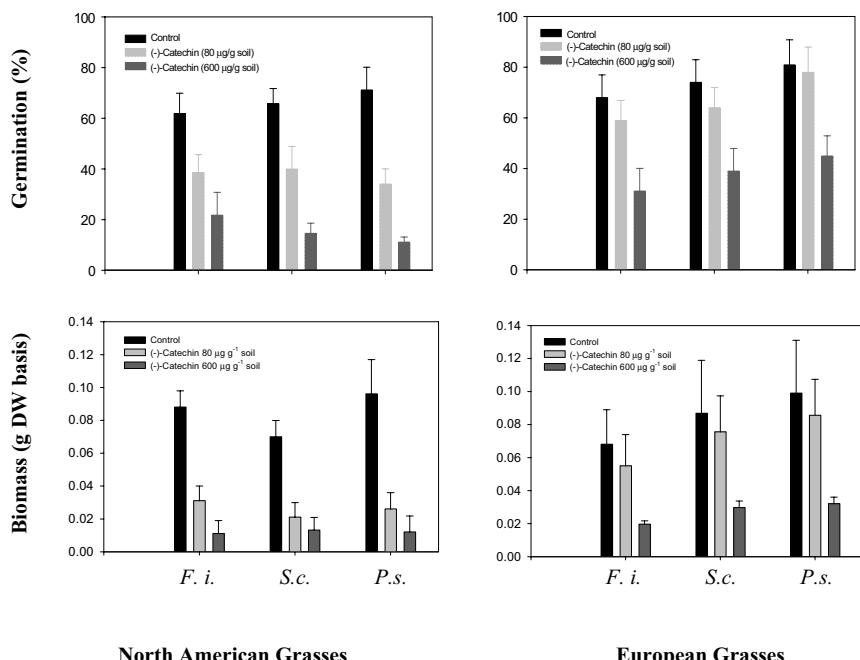
1991; Sheley and Jacobs, 1997; Whitson et al. 1992). Furthermore, much is known about chemical interactions among *Centaureas* and other species. Most interesting is that even among this closely related group of species the chemicals that appear to drive their allelopathic effects are derived from completely different pathways. For example, (-)-catechin, a rare metabolic product so far only found elsewhere in the bark of cotton plants and produced via the flavinoid pathway, appears to be the weapon of choice for *C. maculosa* (Bais et al. 2002; 2003). *Centaurea diffusa*, on the other hand, wields 8-hydroxyquinoline, a compound not previously described as a natural metabolic product (Vivanco et al. *in review*). The root exudates of *A. repens* contain 7,8-benzoflavone ( $\alpha$ -naphthoflavone), a phytotoxin unrelated to either of the others and not previously known as a natural product (Stermitz et al. *in press*). Interestingly, each of these chemicals has powerful negative effects (in laboratory conditions) on the two species that do not produce them, but no effect at all when applied to the species that produces it – an answer to one of the strongest criticisms of allelopathy (i.e., allelopathic plants should also be negatively affected by their own chemicals, Williamson, 1990). Self-resistance is understood at the cellular level for *C. maculosa* (Bais et al. 2003). Considered as a whole, the production of these very different

metabolic by-products and their very different effects suggest wide-open possibilities for the novel weapons hypothesis.

Of the *Centaurea* species, allelopathic mechanisms are best understood for *C. maculosa* and have been demonstrated by integrating ecological, physiological, biochemical signal transduction, and genomic approaches (Bais et al. 2003). The main points of their argument are summarized as follows:

- *C. maculosa* roots produce an enantiomeric compound, ( $\pm$ )-catechin, with clearly documented phytotoxic properties of only the (-)-form of the chemical.
- ( $\pm$ )-catechin is present in natural soils at concentrations above which affects other plants and its presence is only associated with *C. maculosa* plants.
- greenhouse experiments have demonstrated inhibitory effects of *C. maculosa* roots on the roots and overall growth of a native American grass and activated carbon added as a purification agent ameliorates these inhibitory effects (Ridenour and Callaway, 2001).
- the concentration of (-)-catechin appears to be about twice as high in soils occupied by *C. maculosa* in North America than in similar habitats in Europe (Figure 1).
- experiments show the inhibition of the growth and germination of native species in field soils at natural concentrations of the allelochemical.
- (-)-catechin shows cell-specific targeting against meristematic and elongation zone cells in the roots of target plants as evidenced by cytoplasmic condensation followed by a cascade of cell death proceeding backwards up through the root stele, induction of reactive oxygen species (ROS)-related signaling that leads to rhizotoxicity in susceptible plants, ROS-triggered  $\text{Ca}^{2+}$  signaling cascade leading to cellular pH decrease, and allelochemical-induced genome-wide changes in gene expression patterns.
- germination and growth of European grasses are more resistant to (-)-catechin than that of North American counterparts (Figure 2).

Similar biogeographic patterns have been demonstrated for the effects of the closely related *Centaurea diffusa*, but in more realistic conditions. As noted above, *C. diffusa* does not produce ( $\pm$ )-catechin, but bioassay analyses of chemical profiles in rhizospheres indicate that 8-hydroxyquinoline provides similar allelopathic aggressiveness (Vivanco et al. in review). In highly controlled laboratory experiments, all plant species except *C. diffusa* itself showed 100% mortality after the



*Figure 2.* Effect of different concentrations of (-)-catechin on the germination and total biomass of three North American and European grasses. Values are means + S.D. Reprinted from Bais et al. 2003 supporting online material.

addition of root exudates from *C. diffusa*. The only phytotoxic chemical fraction of *C. diffusa* exudates was 8-hydroxyquinoline. 8-Hydroxyquinoline is well-known as an analytical reagent for metal chelating properties and as a fungistat and antiseptic (Merck Index, 1996); however, 8-hydroxyquinoline was not previously known as a natural product.

Root exudates from *C. diffusa* have very different effects on plant species from communities in which *C. diffusa* is native than communities that the weed has invaded. Completely unaware of what had been proposed nearly twenty years earlier by Rabotnov, two North American researchers, Callaway and Aschehoug (2000), conducted an experiment whose results were remarkably similar to the speculations of the Russian. They compared the inhibitory effects of *C. diffusa* on three bunchgrass species that co-exist with *C. diffusa* in Eurasia to the effects of *C. diffusa* on three

bunchgrass species from North America. Each of the three species from North America was paired with a congener (or a near-congener) from Eurasia of a similar morphology and size. *Centaurea diffusa* had much stronger negative effects on North American species than it had on Eurasian species. Correspondingly, none of the North American grass species (nor all species analyzed collectively) had a significant competitive effect on the biomass of *C. diffusa*, but the Eurasian species *K. laerssenii*, and all Eurasian species analyzed collectively, significantly reduced *C. diffusa* biomass. *Centaurea diffusa* had no effect on the amount of  $^{32}\text{P}$  acquired by Eurasian grass species, but significantly reduced  $^{32}\text{P}$  uptake of all North American species. Correspondingly, North American grasses had no competitive effects on  $^{32}\text{P}$  uptake of *C. diffusa*, but all Eurasian species demonstrated strong negative effects on the amount of  $^{32}\text{P}$  acquired by *C. diffusa*.

More importantly, activated carbon had strikingly different effects on the interactions between *C. diffusa* and the grass species from the different biogeographical regions (Figure 3). When growing with *C. diffusa* the overall effect of carbon on North American species was positive. In contrast, the biomass of all Eurasian grass species growing with *C. diffusa* was reduced dramatically in the presence of activated carbon. Correspondingly, activated carbon put *C. diffusa* at a disadvantage against North American grasses (*Centaurea* biomass decreased) but an advantage when with Eurasian grasses (*Centaurea* biomass increased).  $^{32}\text{P}$  uptake by Eurasian grasses growing with *C. diffusa* decreased in the presence of activated carbon. The effects of activated carbon on  $^{32}\text{P}$  uptake by grasses corresponded with the effects of activated carbon on  $^{32}\text{P}$  uptake by *C. diffusa*. Activated carbon enhanced uptake by *C. diffusa* in the presence of Eurasian grasses but reduced uptake in the presence of North American grasses. Ameliorating effects of activated carbon in general are evidence for allelopathy (Schreiner and Reed, 1907; Mahall and Callaway, 1991; 1992), and the strong effects of the place of origin on the competitive ability of grass species against *C. diffusa*, and the contrasting effects of activated carbon, suggest that *C. diffusa* produces chemicals that long-term and familiar Eurasian neighbors have adapted to, but that *C. diffusa*'s new North American neighbors have not.

Biogeographical differences in the resistance or susceptibility of plant communities to *C. diffusa* were further explored by establishing microcosms in which North American and Eurasian plant communities were established in both North American and Eurasian soils (Vivanco et al. in review). In full support of previous experiments, the regional source of the plant community was by far the most important factor in resistance to *C. diffusa* – Eurasian communities were much more resistant to invasion.

Not only do the effects of bulk root exudates of *C. diffusa* appear to differ between communities of origin and invaded communities, so does the specific effects of 8-hydroxyquinoline. North American plant species were much more susceptible to

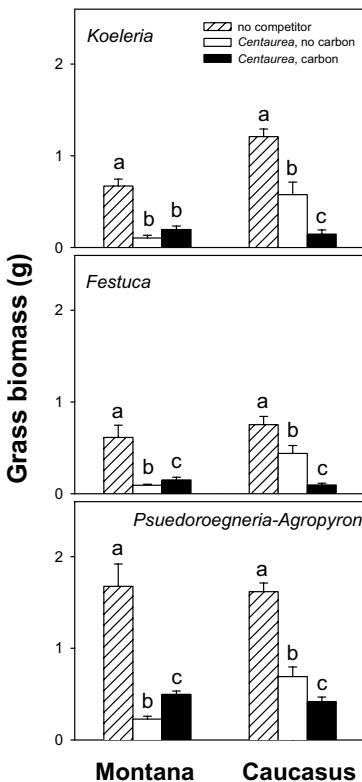


Figure 3. Total biomass of related Eurasian and North American grasses when growing alone or with the invasive *Centaurea diffusa*, either with or without activated carbon added to the soil to ameliorate allelopathic effects. Values are means + 1 S.E. Reprinted from Callaway and Aschehoug 2000.

identical concentrations of 8-hydroxyquinoline than Eurasian species (Vivanco et al. in review).

Using a similar biogeographic approach and methodology, Prati and Bossdorf (in press) tested allelopathic effects of *A. petiolata* (garlic mustard), an aggressive invader of the understory of forests in North America, on germination of two congeneric species that co-occur with *Alliaria* in the field – the American *Geum laciniatum* (new neighbor) and the European *G. urbanum* (old neighbor). In addition, they investigated whether the allelopathic potential of *A. petiolata* varied between native European and exotic North American populations of the weed. In support of the ‘novel weapons’ hypothesis, Prati and Bossdorf found that invasive North American populations of

*A. petiolata* significantly reduced the germination of ‘native’ North American *G. laciniatum* seeds, but it had no effects on ‘experienced’ European *G. urbanum* seeds. Native European *A. petiolata*, on the other hand, significantly reduced seed germination of both North American *G. laciniatum* and European *G. urbanum* in similar proportions, a result that does not fully support the ‘novel weapons’ hypothesis. Contrasting inhibitory effects between *A. petiolata* populations from Europe and North America on European *G. urbanum* suggest that North American *A. petiolata* has lost its detrimental effects on a former neighbor, as well as warn about the possibility that allelopathic effects of an invader can greatly vary according to the origin of its populations (i.e., native versus introduced regions).

The general picture that emerges from research on invasive *Centaurea* species and *A. petiolata* is that chemicals exuding from their roots disrupt native communities in ways that do not occur in their communities of origin. This interpretation suggests that natural biological communities are not individualistic, but evolve in some way as functionally organized units (Goodnight, 1990; Wilson, 1992), and that some exotic invasive plants may disrupt inherent, co-evolved interactions among long-associated native species. This is consistent with the proposal by Lortie et al. (*in review*) to formally recognize plant communities as “integrated”, showing both individualistic and interdependent properties along continua of other factors. The implications of these biogeographic differences in the allelopathic effects of *Centaurea* species and *A. petiolata* are consistent with Rabotnov’s hypothesis that “disturbed homeostasis” takes place when interactions occur among species without an evolutionary history.

## **EVOLUTIONARY CONSEQUENCES OF PLANT-PLANT INTERACTIONS**

Rabotnov’s hypothesis and the general idea that plant species (or genotypes) can “get used to one another”, or at least that the ecological effects of plant-plant interactions have consequences for evolution, also finds support from studies of competition in pasture communities (see review by Turkington and Mehrhoff 1990). In one of the first of such studies that we are aware of, Martin and Harding (1981) tested the hypothesis that a history of coexistence could affect the fitness of individuals when in interspecific competition. They addressed this hypothesis by collecting seeds from *Erodium cicutarium* and *E. obtusipratum* in grasslands where they coexisted (sympatric sites) and grasslands where they did not co-occur (allopatric sites), and planted either “sympatric” individuals or “allopatric” individuals together. A better performance of the mix of individuals from sympatric sites when compared to that of allopatric sites would suggest that coevolution between competitors has occurred. In agreement with this prediction, the total seed output and reproductive rates of the two species when grown together were higher when seeds of the individuals were collected

from the same grassland (sympatric) than when collected from different grasslands. Similarly, Evans et al. (1985) designed greenhouse experiments in which ecotypes ("genets") of *Lolium perenne* and *Trifolium repens* that had been collected from fields in Switzerland, Italy, France, and England were grown either with the coexisting ecotype or with genotypes from a distant field. They found that each species grew larger when paired with a "familiar" genotype of the other species, the genotype that co-occurred naturally in the same field (Figure 4). Joy and Laitinen (1980) found a similar response in experiments with *Phleum pratense* and *T. pratense*.

In an effort to examine evolutionary consequences of competitive relations under more natural conditions, Turkington and Mehrhoff (1990) conducted reciprocal transplants of *T. repens* and *L. perenne* into three fields in British Columbia, Canada that varied in age since clearing; 0 years, 8 years, and 46 years. They found that *T. repens* genets from older fields transplanted into 46-year-old pastures grew much larger either in competition or when competitors had been cleared away, suggesting that selection was occurring over time. In another field experiment involving more species (*Dactylis glomerata*, *Holcus lanatus*, *L. perenne*, and *T. repens*) and using a

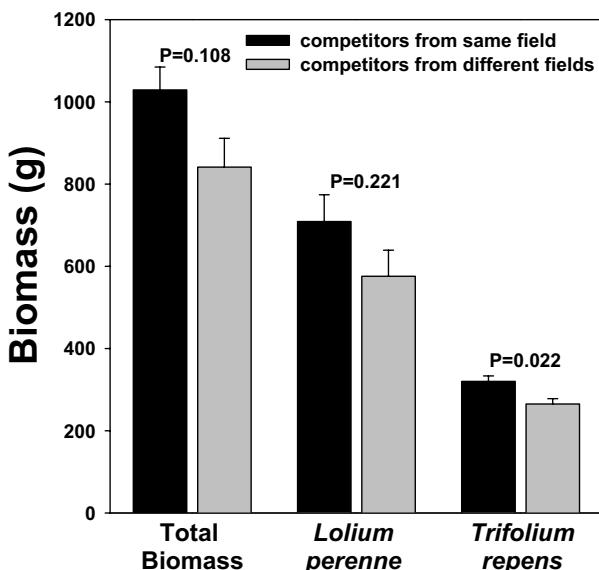


Figure 4. The total biomass of *Lolium perenne* and *Trifolium repens* grown together when competitors where collected from the same fields or from different fields in Europe. Values are means + 1 S.E. Drawn from data presented in Mehrhoff and Turkington (1990).

similar age-based setup, these authors found that total production of two-species mixtures was consistently higher when genets from the older fields were grown together.

In addition, it has been shown that neighbor relationships can even shape the performance of populations within a species. Turkington and Harper (1979), collected ramets of *T. repens* within sites each dominated by one of four different grass species. When ramets were transplanted in all possible combinations with the four grasses, each clover ‘type’ grew best with the grass species from which it had originally been sampled, suggesting the occurrence of a micro-evolutionary change in *T. repens* in response to different constraints imposed by different neighbors. Turkington and Mehrhoff (1990) further investigated the possibility of coevolved interactions between *T. repens* and grasses by using the elegant, although rarely applied, experimental design proposed by Joseph Connell (1980). They focused on the interaction of clover and one of the grass species employed in the former study, *L. perenne*. Results provided strong support for the hypothesis that the population divergence exhibited by *T. repens* when growing in close proximity to *L. perenne* originated due to competition in the past, is maintained by current competition, and may have a genetic basis (i.e., it is probably the result of coevolved interactions between these two neighbors).

The mechanism(s) driving these apparent evolutionary changes is not clear, and there is no direct evidence for chemically mediated selection. Some of the evidence suggests reduction in niche overlap (see Parrish and Bazzaz, 1982). Other experiments suggest that these changes may be microbially mediated. *Trifolium* species are “nitrogen fixers”, they participate in mutualistic relationships with *Rhizobium* bacteria that transform atmospheric N<sub>2</sub> to organic nitrogen that can be used by the host, but can eventually become available to other species. In general, more productive symbioses occur for *Trifolium* with strains of *Rhizobium* that are isolated from the nodules of their own roots than strains isolated from the roots of other cultivars (Mytton, 1975; Turkington et al. 1988). Furthermore, competition between *Trifolium* cultivars can be affected by the particular strain of *Rhizobium* used as the symbiont (Young and Mytton, 1983; Young et al. 1986) and *Trifolium* species will “choose” strains of *Rhizobium* originally isolated from other conspecifics when presented with strains isolated from other species. Finally, Turkington and Mehrhoff (1990) present results that suggest that grasses affect *T. repens*, and perhaps drive coevolutionary responses, by their effects on soil microorganisms. In sum, a substantial body of research indicates that interactions among plant species (perhaps mediated by microbes) drive the population divergence observed for *T. repens* and this divergence may be maintained by current competition. The important aspect of the *Trifolium-Rhizobium*-grass interactions and the apparent consequences of these interactions for evolutionary selection is that they provide an excellent model system for how plants and soil microbes, and perhaps plants and plants, may constitute “homeostatic” communities.

The remarkable transmogrification of some invasive species would seem to require unusually powerful mechanisms to drive them. We suggest that an overlooked mechanism may be that some invaders may wield unusually potent novel weapons; unique chemicals exuded from their roots that do not have the same effect on species in the communities in which they naturally originated. Evidence to date is restricted to studies of species in the genus *Centaurea* and *A. petiolata*, but these studies and others investigating how long-term association with neighbors can modify interaction strengths support Rabotnov's original idea that natural, coevolved plant communities possess some degree of homeostasis. Furthermore, some evidence suggests that community homeostasis may be disrupted by exotic invaders with competitive advantages gained from novel allelopathic weapons.

The 'novel weapons' hypothesis contradicts the widely accepted perception of plant communities as being individualistically organized. Contrasting interactions between invaders and plants from origin and invaded communities suggest that natural plant communities may function as more tightly knit entities than generally thought and evolve as functionally organized units.

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## CHAPTER 18

# ALLELOPATHY IN MARINE ECOSYSTEMS

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## INTRODUCTION

The number of published papers focusing on allelopathy among marine organisms is very small in comparison to those dealing with allelopathy in terrestrial systems. There are no good reasons to expect that this large discrepancy reflects any fundamental difference in the prevalence or ecological significance of allelopathy in these two systems. More likely, it is due to different research traditions in terrestrial plant ecology and marine ecology. There is emerging evidence that chemically mediated interactions among competing marine organisms may be common and important both in planktonic communities and in the benthos. This chapter gives a summary of our current knowledge of allelopathic interactions in the four groups of marine organisms for where most of the research on marine allelopathy has been conducted: phytoplankton, seaweeds, sponges and corals.

## ALLELOPATHY IN PHYTOPLANKTON

### 1. Allelopathy among different phytoplankton groups

The production of allelochemicals by marine phytoplankton seems to be almost exclusively found among the groups where toxin-producing species exist. These groups are: cyanobacteria, dinoflagellates and flagellates. Toxic substances released by

cyanobacteria have been reported to inhibit diatoms (Keating, 1977). Most scientific reports describe inhibition of other co-existing phytoplankton species by substances released by dinoflagellates and flagellates (Pratt, 1966; Tillman and John, 2002). The greatest number of species able to produce and release allelochemical substances is found among the flagellates. These species are mostly known by their ability to kill fishes, as e.g., the microflagellates *Chrysochromulina polylepis* and *Prymnesium parvum*. A bloom of *C. polylepis* in 1988, swept through the waters of the Kattegat, Skagerrack and Southern Norwegian coast killing on its wake wild and cultivated fishes, seaweeds, sea-stars, shellfish and even phytoplankton (Maestrini and Graneli, 1991). Myklestad et al. (1995) reported that a strong negative effect on the diatom *Skeletonema costatum* was caused by the bloom. The flagellate *Prymnesium parvum* has been known to produce toxins able to kill fishes during blooms for more than 40 years (Komarovsky, 1951). Even dinoflagellate species known to produce intracellular toxins, have been shown to be able to produce compounds with hemolytic activity other than their internal toxins (Arzul et al. 1989; Ogata and Kodama, 1986. This is the e.g., the case of *Alexandrium taylori*, which releases proteinaceous hemolytic compounds with a molecular weight of more than 10 000 Dalton (Arzul et al, 1999; Oda et al. 2003; Fistarol et al. 2004).

## 2. Factors influencing allelopathy

Some studies have shown that the action of the allelopathic compounds is of a short duration. E.g. Graneli and Johansson (2003) found a short-time negative effect of cell-free filtrates of *Prymnesium parvum* cultures on the growth of *Thalassiosira weissflogii*, *Prorocentrum minimum* and *Rhodomonas cf. baltica*. All three species were initially subjected to severe cell loss when exposed to cell-free filtrates of *P. parvum* cultures. However, after some days the 3 algae were able to start increasing their cell numbers again. This suggests that at this point, the influence of the allelopathic agents had disappeared. However, if the phytoplankton species are subjected to continuous exposition to the allelopathic compounds recovery is not possible. By adding new cell-free filtrates of *P. parvum* cultures every 2 days to the same algal species above (and a natural occurring plankton community) Fistarol et al. (2003) could kill most of the targeted species without these been able of recovery, corroborating the finding of Graneli and Johansson (2003). *P. parvum* toxins/allelopathic substances seems to be sensitive to high light, temperature, high pH and N and P deficiency (Shilo, 1967; Skulberg et al. 1993; Johansson and Granéli, 1999; Hansen, 2002).

It seems that photoxidation can cause rapid degradation of allelopathic compounds (Legrand et al. 2003; Fistarol et al. 2003). The toxins are quickly inactivated by visible light in the 400-510 nm range and by ultraviolet light in the

225 nm range (Collins, 1978; Skulberg et al. 1993). Simonsen and Moestrup (1997) showed that the toxic effect on *Artemia salina* of the supernatant from *Chrysochromulina polylepis* cultures ceased within 15 hours when exposed to a photon flux density of 50  $\mu\text{mol m}^{-2}\text{s}^{-1}$ . Reversely, Hagström and Granéli (2005), could not detect degradation of the allelopathic substances/toxins of *P. parvum* toxins when the experiments were performed in darkness. The same levels of toxicity could be found 72 hours after the start of their experiments.

Interestingly, Granéli and Johansson (2003) when the allelopathic influence ceased after 36 h the inhibited cultures of each species showed a higher growth rate than the corresponding control. A possible explanation for this observation is that *P. parvum* toxins have a positive effect on the growth rate of other algae at low concentrations, but a negative influence at high concentrations. Pratt (1966) reported a similar result when investigating blooms of *Skeletonema costatum* and *Olithodiscus luteus*. Growth of *Skeletonema* was reduced by high concentrations of *Olithodiscus* conditioned medium, whereas growth was stimulated at low concentrations.

The allelopathic effect seems to increase with higher pH. The allelopathic effect from the flagellate *C. polylepis* filtrates on the dinoflagellate *Heterocapsa triquetra* is enhancend when the pH is high (above 9) but decrease when pH is low (Schmidt and Hansen, 2001; Hansen, 2002).

#### *Allelopathy dependance on external nutrient conditions*

The surrounding nutrient concentrations seem to play a major role on the amount of allelopathic substances being produced by phytoplankton. Several workers (Pratt, 1966; Keating, 1977; McCrackan et al. 1979; Sharp et al. 1979; Tillmann and John, 2002) have reported the presence of toxic metabolites in culture filtrates of various algae. Myklestad et al. (1995) showed that cell-free filtrates of phosphorus-deficient *Chrysochromulina polylepis* cultures strongly inhibited the growth of the diatom *Skeletonema costatum*. The addition of filtrates from *P. Parvum* cultures grown under nutrient-deficient conditions (N or P), had a strong negative effect on other phytoplankton species compared to a positive growth when exposed to cell-free filtrates from non deficient cultures (Granéli and Johansson, 2003; Tillmann, 2003; Skovgaard and Hansen, 2003; Fistarol et al. 2003, et al. 2004). In these studies, analyses of *P. parvum* or *C. polylepi* filtrates showed higher toxicity in the filtrates of nutrient-deficient cultures (Johansson and Granéli, 1999). Thus the decline in cell densities of the phytoplankton species subjected to the action of these filtrates can be accounted by the toxicity of the excreted toxic substances (Granéli and Johansson 2003, Tillmann 2003, Skovgaard and Hansen, 2003, et al 2003; Fistarol et al. 2003, et al. 2004). This effect is even more accentuated if the targeted cells are nutrient deficient themselves (Granéli and Johansson, 2003). This is what we might expect to occur in nature, where

both the allelopathic and the target species grow together under identical nutrient conditions.

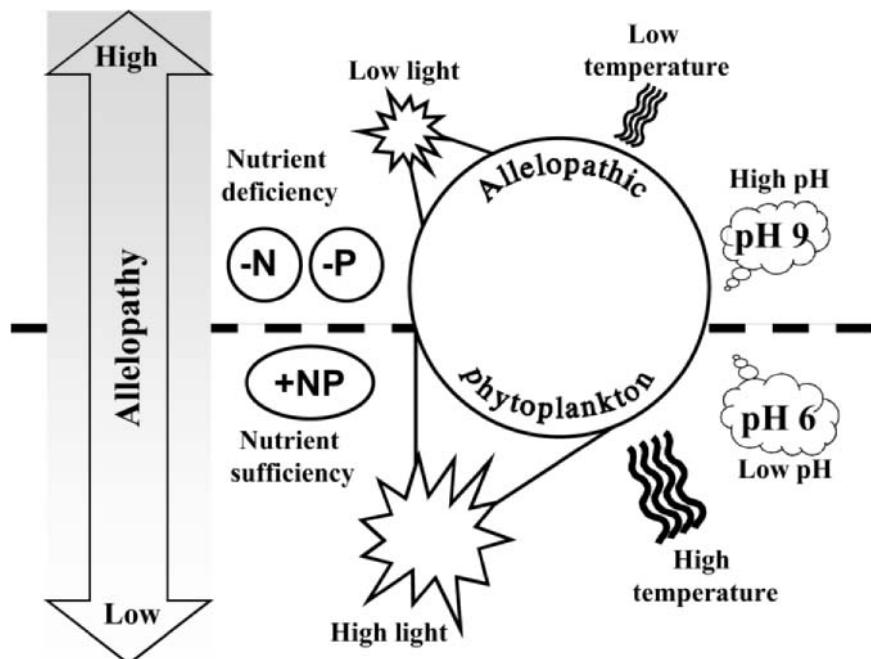


Figure 1. Physical and chemical factors known to affect the production of allelopathic compounds in marine phytoplankton species. Increase production: above the dashed line, decrease: bellow the line.

*Prymnesium patelliferum* known to produce prymnesins in the same way as *P. parvum* has been shown to not be inhibited by cell-free filtrates from the latter (Granéli and Johansson, 2003). Instead, *P. patelliferum* showed a positive growth for all treatments. The insensitivity of *P. patelliferum* to excreted prymnesins suggests specific adaptations to defend itself, however, the mechanisms behind this adaptation are not known. In a similar vein, Windust et al. (1996) showed that micromolar concentrations of the dinoflagellate toxins okadaic acid (OA) and dinophysistoxin-1 (DTX-1) effectively inhibited the growth of several microalgae, but did not affect the growth of the algae producing the toxin. These results suggest that toxin production might be a strategy to repress or exclude algal competitors and thereby play an allelopathic role in several phytoplankton species.

However, the concept of microalgal allelopathy is not a generally accepted phenomenon. The most common criticism of the existing evidence concerns the use of unnaturally dense populations in culture in conjunction with randomly paired taxa that do not occur together in nature. However, the more recent studies do not user higher cell densities of the allelopathic algae than what is found in nature during blooms, i.e. densities between  $10\text{--}100 \times 10^3$  cells ml<sup>-1</sup> for *P. parvum* e.g. (Edvardsen and Paasche, 1998). In addition, the target species are always species that usually are found to co-occur with the allelopathic algae (see e.g., Granéli and Johansson, 2003).

### 3. Ecological significance of allelopathy for marine phytoplankton

Allelopathic phytoplankton such as the haptophytes *Prymnesium parvum* and *Chrysotrichomonas polylepis* have been responsible for toxic incidents with severe ecological impact in many parts of the world (Moestrup, 1994; Edvardsen and Paasche, 1998). These highly potent exotoxins (Shilo, 1981; Igarashi et al. 1996) have been shown to have several biological effects, including ichthyotoxic, neurotoxic, cytotoxic, hepatotoxic and hemolytic activity towards a range of marine organisms. Blooms of *P. parvum* develop in inshore localities and brackish-waters and are usually mono-specific suggesting a mechanism to outcompete other phytoplankton species. The complete dominance of *P. parvum* can not be explained by virtue of its own growth rate, as *P. parvum* is known to have a moderate growth rate under a range of environmental conditions (Holdway et al. 1978; Brand, 1984; Larsen and Bryant, 1998). Nevertheless, the total absence of coexisting species observed in relation to certain blooms is indicative of a high competitive ability of *P. parvum*.

As shown for terrestrial plants, the ability of allelopathy in phytoplankton is closely associated with competition for resources in these systems. For marine environments, limiting amounts of nutrients not only increase the production of allelochemicals but also accentuate their action (Einhellig, 1995). This is a very similar mode of action as the elevated extracellular release of organic compounds occurs under conditions of nutrient limitation (Myklestad, 1977, 1995; Soeder and Bolze, 1981; von Elert and Jüttner, 1997). In marine and coastal waters nitrogen and phosphorus are very seldom in high enough concentrations to sustain the growth of the array of phytoplankton species co-existing in time and space. The nutrient that is found at the lowest concentration in relation to the algal need (limiting nutrient) is the nutrient which will set stop for their growth. Thus the ability to compete for the limiting nutrient is crucial for the proliferation of a specific species. The species which is able to compete successfully for the available growth limiting nutrient has the potential to become dominant by increasing its biomass, ultimately forming blooms.

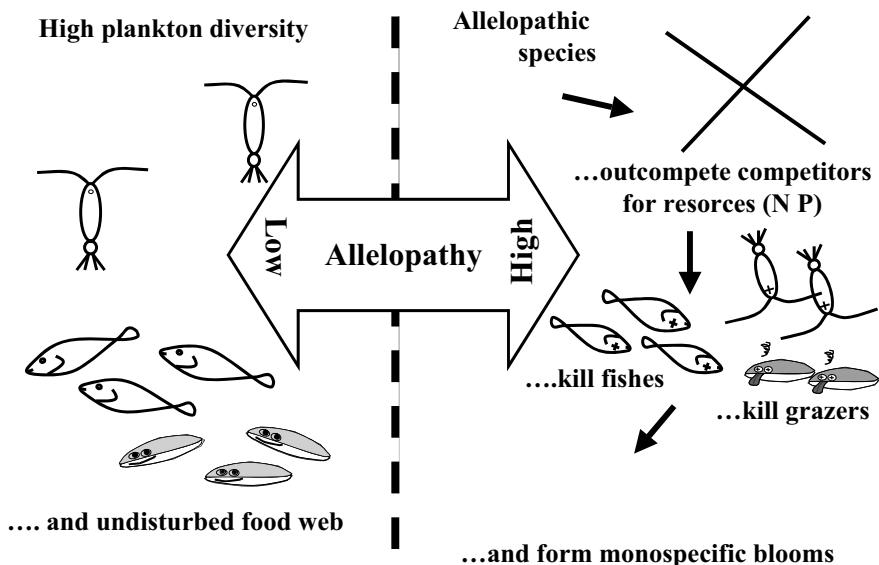


Figure 2. The pathways in which the increase in the production of allelochemicals by phytoplankton can alter the entire marine food web (right side).

## ALLELOPATHIC INTERACTIONS AMONG BENTHIC MARINE MACROORGANISMS

Space is a limiting factor in most benthic marine habitats, in particular on hard substrates in the photic zone (Dayton, 1971; Jackson, 1977). Given that the proportion of sessile organisms, both algae and invertebrates, is high in these habitats, and that many sessile marine species are known to produce a rich variety of secondary metabolites (Harper et al. 2001), the potential for the evolution of allelopathic interactions between competing organisms is immense. Still, there are remarkably few well-established examples of allelopathy in marine macro-organisms that have been reported in the literature. In contrast to the large number of marine studies on chemical defences against herbivore and biofouling, few experiments on chemical interactions between competing species of seaweeds and marine invertebrates have been conducted. Chemical defences against fouling organisms are sometimes included in discussions of allelopathy in marine macroorganisms (e.g., Gross, 2003). However, without attempting to make any definite arguments for or against the inclusion of chemical mediation of living surfaces in the term marine allelopathy, this phenomenon has been excluded from the present review (see Steinberg et al. 2001 for an excellent 420

recent review on chemical mediation of marine biofouling). The majority of fouling organisms are not primarily competitors to their hosts, although they may reduce the resource availability (e.g., Hurd et al. 2000). In this sense, the chemical mediation of surface colonisation of living marine organisms may commonly be regarded as a chemical defence against an attacker, similar to an attacking herbivore, rather than a chemically mediated effect on a competitor.

In accordance with the prevailing tradition in marine chemical ecology (e.g., Kittredge et al. 1974; Thacker et al. 1998; Engel and Pawlik, 2000), the release of metabolites from sessile invertebrates that affect their potential competitors, both other invertebrates and seaweeds, has been defined as allelopathy in this review. This may be considered inconsistent with the common, more narrow definition in terrestrial ecology, i.e. that allelopathy is restricted to situations where a primary producer releases compounds that affect other primary producers (Molisch, 1937; Romeo, 2000). However, many marine invertebrates are sessile, modular organisms which means that the prerequisites for the evolution of allelopathic activity are similar to those of plants. Therefore, it is reasonable that the use of secondary metabolites by invertebrates as a way of affecting the fitness (in this review restricted to negative effects) of competing organisms is defined as allelopathy.

Finally, this review has been restricted to cover only ecologically relevant studies of inhibitory, allelopathic interactions among seaweeds, sponges and corals. This means that the study species should co-occur and be potential competitors in natural populations. Stimulatory (positive) effects as well as effects on microorganisms have not been included. More importantly, we have tried to put extra emphasis on the few published studies that we think can serve as good examples for future experiments on allelopathy among benthic marine macroorganisms. These are studies that provide ecologically relevant experimental information on both the ecological processes and the underlying chemical mechanisms.

## 1. Seaweeds

In comparison to the numerous studies that have explored plant allelopathy in terrestrial and freshwater systems (see Gross, 2003 for a review), remarkably few investigations have looked at allelopathic interactions among seaweeds and their potential competitors. The only rigorously demonstrated example of the capability of seaweeds to chemically affect a natural competitor is the study by de Nys et al. (1991) on the interaction between the tropical red alga *Plocamium hamatum* and the soft coral *Sinularia cruciata*. Through a commendable combination of field observations and manipulative field experiments, using both intact organisms as well as artificial seaweeds coated with extracted lipophilic compounds, it was shown that *P. hamatum* causes tissue necrosis in the coral and that this effect was due to the monoterpenes

chloromertensene produced by the alga. It was further concluded that chloromertensene concentrations in the surrounding water column was too low to affect neighbouring organisms and that direct contact with the alga was required for tissue necrosis in corals to occur. The allelopathic potential of *P. hamatum* was further demonstrated in a relocation experiment by Leone et al. (1995) where all colonies of the soft coral *Lobophytum compactum* that were in direct contact with *P. hamatum* suffered tissue necrosis within two weeks. In a later study, the bioactivity of various monoterpenes from *P. hamatum* was further explored using fungi, bacteria and microalgae as test organism (König et al. 1999). Although a relatively strong antialgal effects of one of the monoterpene compounds was detected, this can hardly be classified as an example of marine allelopathy since the test organism was the non-marine green alga *Chlorella fusca*.

Other seaweed species than *P. hamatum* have also been suggested be allelopathic to corals. Littler and Littler (1997) reported indications that the red alga *Dasyopsis spinuligera* caused the death of corals in the Caribbean through the use of allelochemicals. More recent studies from Australia have shown that filamentous, turf-forming red seaweeds may outcompete corals (*Porites* spp) by causing severe tissue damage on neighbouring corals (Jompa and McCook, 2003a,b). For one the algal species, *Corallophila huyssmansii*, coral tissue mortality occurred also at some distance from the algal filaments, suggesting that the allelochemical involved in this interaction is a rather hydrophilic compound. However, spatial correlation between the growth of one competitor and the decline of another does not provide strong, causal evidence of allelopathic interactions. This will require manipulative experiments using live organisms as well as extracted compounds. In none of the latter examples have allelopathic compounds been isolated and identified. Consequently, it has not been possible to experimentally test the hypothesis that the observed effects can be attributed to the production of algal allelochemicals, as was done by de Nys et al. (1991).

## 2. Sponges

There are a number of studies implying allelopathic activity of sponges towards potential competitors, either through experiments or observations in the field, or through bioassays in the laboratory using crude extracts or purified compounds (e.g., Goodbody, 1961; Jackson and Buss, 1975; Sullivan et al. 1983; Thompson et al. 1985; Turon et al. 1996). Many of these studies are highly ecologically relevant in terms of the interacting organisms and their fitness effects on each other. Alternatively, they may demonstrate interesting bioactivity of unique secondary metabolites under laboratory conditions. However, they are also open to the critique that they may reflect competitive interactions that does not have a chemical basis, or that they produce

results on bioactivity in laboratory, still-water assays that are not meaningful under natural conditions of water flow.

The pioneering work by Porter and Targett (1988) was one of the first studies to convincingly demonstrate the effect of sponge allelochemicals under natural conditions. They conducted a manipulative field experiment with sponge extracts coated onto cellulose pads to show that the tissue necrosis that occurred in the coral *Agaricia lamarckii* as a response to direct contact with living tissue of the sponge *Plakortis halichondroides* had a chemical basis, although they did not characterize the active compounds. During recent years, a few studies on allelopathic interactions among sponges have progressed this field by developing new methodology and by providing examples of more complete experimental approaches that are worth following. Thacker et al. (1998) used a similar approach to that of Porter and Targett (1988) to examine if the observations that the sponge *Dysidea* sp. overgrows the sponge *Cacospongia* sp. and causes tissue necrosis could be explained by allelopathic mechanisms. Thacker et al. (1998) took things further by testing for allelopathic as well as anti-predatory effects of both crude extracts and pure compounds in manipulative field experiments. Furthermore, they presented the first attempt to test for the possible occurrence of inducible allelochemical production in sponges. It was shown that both crude extracts and pure secondary compound (7-deacetoxyoleopupuane) from *Dysidea* sp. causes tissue necrosis in a sponge of the genus *Cacospongia*. The response was stronger for the crude extract than for the pure metabolite, implying that other compounds (probably sesquiterpenoid compounds) have additive or interactive allelopathic effects together with 7-deacetoxyoleopupuane. The production of 7-deacetoxyoleopupuane was not induced by the presence of competitors, which could be due to the fact that this compound also deters predators and that the sponge is at a more or less constant threat of predation (Thacker et al., 1998). The large individual variation in secondary metabolite content of *Dysidea* that was found does, however, not support this reasoning. It also means that the few replicates ( $n = 4$ ) that were used to investigate differences in chemical content of *Dysidea* in the presence and absence of competitors provide a very weak observational test of the hypothesis that the production of allelochemicals is inducible. Manipulative, well-replicated field experiment where sponges are reciprocally transplanted, with proper controls, is required to produce more thorough tests of allelochemical induction.

Engel and Pawlik (2000) developed a technique based on hard, stable Phytigel™ gels incorporated with estimated natural concentration of extracts that enabled them to perform the first *in situ* screening of overgrowth inhibition by sponge extracts. Extracts from twenty species of Caribbean sponges were tested using three species of overgrowth sponges and it was found that extracts from six of the species significantly inhibited the growth of potential competitors. The method used by Engel and Pawlik (2000) represent a progress in experimental research on allelopathy among marine

invertebrates (and seaweeds). However, as recognised by the authors, the stable gels with incorporated extracts are not perfect mimics of living sponges and they probably provide a conservative test of the occurrence of sponge allelopathy.

The stable gel approach developed by Engel and Pawlik (2000) was used in a recent excellent study on multiple defensive and allelopathic roles of triterpene glycosides from the Caribbean sponges *Erylus formosus* and *Ectyoplaxia ferox* (Kubanek et al. 2002). This study take full advantage of the earlier development of approaches and techniques in marine chemical ecology at large and provide a convincing example of allelopathy among sponges, although basic field observations was not included, nor experimental data on the effects of living *E. ferox* on natural competitors. Kubanek et al. (2002) found that both crude extracts as well as natural concentrations of the purified triterpene glycosides from *E. ferox* significantly inhibited overgrowth by the sponge *Tedania ignis*. This result was in accordance with those of Engel and Pawlik (2000), which showed that crude extracts of *E. ferox* prevented overgrowth by three sponge species, including *T. ignis*. Exudation of triterpene glycosides into the water column was not detectable which led the authors to conclude that the allelopathic effects of this compound occurs through direct contact between *E. ferox* and its competitors. In contrast to what was found by (Thacker et al. 1998) for the purified allelochemical (7-deacetoxyoleopupuane) in *Dysidea*, the allelopathic effect of the triterpene glycosides was greater than the effect of crude sponge extracts, suggesting that these compounds are responsible for most or all of the demonstrated allelopathic activity in *E. ferox* (Kubanek et al. 2002). However, to rigorously demonstrate this, a step-wise, bioassay guided fractionation should be required, something that has not been done for any allelopathic interaction among sponges.

### **3. Corals**

Spatial competition between soft alcyonacean corals and hard scleractinian corals is probably a common and important type of interaction on coral reefs, especially in the Indo-West-Pacific (Sammarco et al. 1985). A set of different mechanisms are used by alcyonacean and sceleractinian corals in this competition, including the use of spines, specialized sweeper tentacles, mesenterial filaments and allelopathy (see Koh and Sweatman, 2000 for references). The early use of well-designed, manipulative field experiment provided this area of research with convincing indications that competition between corals can be mediated by allelopathic agents (e.g., Sammarco et al. 1983; Sammarco et al. 1985; La Barre et al. 1986). Especially the alcyonacean corals are known to produce a variety of secondary metabolites, with a strong dominance of terpenoids, primarily diterpenoids. Coll et al. (1982) used a submersible sampling apparatus to show that exuded terpenoids are present in the sea water

surrounding alcyonacean coral colonies, implying that these compounds could have allelopathic activity also on competing organisms that are not in direct contact with the corals. The ability of diterpenes from soft corals to damage scleractinian corals was demonstrated in laboratory experiments with purified compounds from the soft corals *Sinularia flexibilis* and *Lobophytum hedleyi* (Aceret et al. 1995a). It was found that some diterpenes cause severe tissue damage in the scleractinian corals *Acropora formosa* and *Porites cylindrica* through a stepwise process that starts with expulsion of zooxanthellae followed by release of nematocysts, inhibition of polyp activity, necrosis and death of polyps. The results also showed that generalisations about coral diterpenes as allelopathic agents against scleractinian corals should be made with caution since some compounds had no effect on competing corals and the active diterpenes had stronger effect on *A. formosa* than on *P. cylindrica* (Aceret et al. 1995a). The use of allelopathy in coral competition is not restricted to soft corals. The mutual ability of scleractinian and alcyonacean corals to chemically induce necrosis in their competitors was suggested already by the relocation experiments in natural coral populations conducted by Sammarco et al. (1985) on the Great Barrier Reef in Australia. More recent studies imply that allelopathy against larvae of potential coral competitors occur in both soft and hard corals (Aceret et al. 1995b; Maida et al. 1995 a,b; Koh and Sweatman, 2000).

During recent years, much focus have been placed on competitive interactions between scleractinian corals and seaweeds as an important process in the reported world-wide decline of coral reefs (Szmant, 2001). Nevertheless, there is still limited evidence that corals and algae do compete, and even less is known about the mechanisms of their interactions (McCook et al. 2001). De Ruyter van Steveninck et al. (1988) provided indirect evidence of coral allelopathy against seaweeds. They demonstrated reduction in blade growth rate in the brown alga *Lobophora variegata* as a consequence of contact or proximity to scleractinian corals, but they did not provide any direct evidence that this effect was chemically mediated. We know of no later study that has addressed the issue of allelopathic activity of sponges against seaweeds in a more rigorous way, although this should definitely be feasible.

Overall, there is much good experimental data implying that allelopathic activity against competitors may be common and important in corals. Furthermore, the corals are a chemically prolific group where a large number of secondary metabolites, primarily terpenoids, have been described. Many of these metabolites have been bioassayed in the laboratory, including assays with ecologically relevant test organisms (e.g., Aceret et al. 1995a; Koh and Sweatman, 2000). Furthermore, the first direct *in situ* isolation of waterborne secondary metabolites released from a marine organism was done for potential allelochemicals from corals (Coll et al. 1982). Nevertheless, the research field of coral allelopathy still waits for the first direct evidence that secondary metabolites from a coral can inhibit a natural competitor at

ecologically relevant concentrations under natural conditions (*cf.* De Nys et al. 1991; Thacker et al. 1998; Kubanek et al. 2002).

## CONCLUSIONS

In conclusion, it can be said that for marine phytoplankton species producing allelopathic compounds: (1) most producers are species belonging to dinoflagellates, flagellates or cyanobacteria; (2) the allelopathic substances are found in exudates of these algae; (3) under N or P deficient conditions the production of allelopathic compounds is higher than when the cells are growing under N and P sufficient conditions (in the same way as toxin production changes with nutrient status); (4) among the fish-killer species the allelopathic substances seem to be the same as their known toxins (e.g., prymnesins for *P. Parvum*, *P. patelliferum*) (5) species belonging to the same genus as the species producing allelopathic substances seem not to be inhibited by latter, suggesting that the target has specific adaptations to protect itself from allelopathic substances released by a closely related species. Finally, the findings, of Granéli and co-workers demonstrating that *Prymnesium* toxins inhibit the growth of several phytoplankton species, support the hypothesis that *Prymnesium* toxins, by killing other species when the availability of N or P is low in the water, will allow *Prymnesium* to freely utilise the scarce nutrient resource. This will thus change the structure of the phytoplankton communities towards a dominance of *Prymnesium parvum*. Production of allelopathic compounds by *P. parvum* seems to be one of the most important factors contributing to the development of the monospecific blooms of this species in coastal areas.

There is much indirect, experimental evidence that allelopathy is an important aspect also in the competition among seaweeds, sponges and corals. Furthermore, these benthic organisms have been shown to contain a rich variety of unique secondary metabolites. Many of these compounds are highly bioactive in laboratory assays. Still, there is less than a handful of rigorously demonstrated examples of allelopathy among benthic marine macroorganisms, i.e. studies where both ecologically relevant effects on a competitor and the underlying chemical mechanisms have been evaluated under natural conditions. Even in these examples, almost nothing is known about the physiological processes that are affected by the allelopathic compounds or about the factors that regulates the production and release of allelochemicals. Allelopathy in the marine benthos undoubtedly an area of chemical ecology that is largely unexplored and that awaits new players and exciting discoveries.

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## CHAPTER 19

# ALLELOPATHY IN AQUATIC ENVIRONMENTS

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## INTRODUCTION

Like in terrestrial ecosystems, primary producers in aquatic habitats compete with each other for many different resources, such as nutrients, CO<sub>2</sub> and light. All are consumable and their availability influences the structure and composition of the whole ecosystem.

Nutrients may play an important role in the competition between algae or cyanobacteria directly adjacent to each other (Keating, 1999) and in the composition and succession of phytoplankton communities (Keating, 1977, 1978). CO<sub>2</sub> is often limited in macrophyte stands due to high photosynthetic activity and due to its extremely reduced diffusion in water.

Among all resources, light is the most important one because all photoautotrophs depend on it. The growth of submersed macrophytes for example is mainly limited by the availability of light (Phillips et al. 1978) and not by nutrients, since macrophytes obtain their major nutrients (nitrogen and phosphorus) mainly from the (nutrient rich) sediments (Bristow and Whitcombe, 1971; Toetz, 1974; Carignan and Kalff, 1980)<sup>1</sup>. Light is always reduced under water and can not be substituted. This means that it can not be replaced by another resource, as it is the case for e.g., CO<sub>2</sub>, which can be

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<sup>1</sup> Exceptions are e.g., rootless higher plants, like *Ceratophyllum demersum*, or plant fragments (Ozimek et al. 1993) drifting in the water until they resettle in the sediment. Some plants, like the water soldier *Stratiotes aloides*, can selectively take up nutrients - in this case calcium and potassium, which are in general not limiting - from the open water and thus hamper the development of phytoplankton (Brammer and Wetzel, 1984).

replaced through  $\text{HCO}_3^-$  by some plants, like *Elodea* and *Hydrilla*, when it becomes limited. Light can furthermore not be stored (like some algae and cyanobacteria can store phosphorus) and used when it is not present in the surrounding water. Light conditions under water are very peculiar and differ extremely from those ashore. First, the intensity of photosynthetically active radiation (PAR) decreases exponentially with increasing water depth. This is a normal process and means that light conditions at low depths are already significantly worse than on the water surface, even in clear water bodies.

Besides this reduction in light quantity, also light quality (i.e. the wavelength) changes. Both phenomena lead to a vertical zonation and many submersed photoautotrophs have thus evolved morphological and physiological adaptations to low light conditions. Algae and cyanobacteria bear special pigments; typical adaptations in macrophytes are finely dissected leaves, a thin cuticle or an increased number of chloroplasts in the epidermal layer. Low light compensation points and/or low respiration rates permit an efficient photosynthesis at only 1 to 3% of the surface irradiation (Wetzel, 2001). However, these adaptations may not be sufficient when light is further reduced by its consumption through competing photoautotrophs. Light competition may have severe, negative consequences for submersed living primary producers. Phytoplankton in the water column and epiphytes on the leaves of macrophytes further reduce the inherently low light conditions under water. The decrease of the macrophyte *Potamogeton filiformis* in Loch Leven during the summer months has been traced back to the high phytoplankton abundance in this lake (Jupp and Spence, 1977). In fact, phytoplankton at its maximum density can cause up to 70% of the vertical light attenuation (Tilzer, 1983). Lemnids that freely float on the water surface cause immense shading of the vegetation below and according to Den Hartog & Van der Velde (1988), only few plants (*Potamogeton pectinatus*, *Elodea nuttallii*, *Ceratophyllum* and *Utricularia*) are able to tolerate this situation for a considerable time. Epiphytes can account for up to 86% of the total light attenuation (Sand-Jensen and Søndergaard, 1981) and thus cause light limitation for underwater vegetation. To prevent this limitation and their detrimental consequences, adaptations beyond low light adaptation are necessary.

Fast growth, as observed for the eelgrass *Zostera marina*, is a counteracting strategy to avoid light limitation. The leaves of *Z. marina* grow up to 6 cm per day and remain uncovered for nearly one week which enables maximum photosynthetic activity (Wium-Andersen and Borum, 1980). Several plants form so called canopies that lead to severe shading of phytoplankton, competing plants and benthic algae and cyanobacteria (Barko and Smart, 1981). However, canopy formation is not only induced by low light intensities but also by an increase in water temperature. A powerful strategy against competing primary producers is the secretion of chemicals that inhibit growth of algae, cyanobacteria or higher plants. Whereas fast growth

should only be possible at excellent conditions, allelopathy could act alternatively or at least supplementary (Wium-Andersen, 1987).

## Allelopathy in aquatic systems

Although from this background allelopathy should be considered important in aquatic ecosystems, it has been neglected for long time. However, hints for allelopathic interactions between aquatic organisms exist for nearly 100 years and at the beginning, mostly autotoxic interactions were described.

The first report of biochemical interactions between aquatic primary producers was that of Harder (1917) who noticed a putative negative interaction between cells of the blue-green alga, *Nostoc punctiforme*. Similar observations of autoinhibition were made with *Chlorella* cultures (Pratt and Fong, 1940). During that time also negative relations between submersed macrophytes and phytoplankton were observed (Schreiter, 1928; cited in Rice, 1984). This was confirmed by the mesocosm experiments performed by (Hasler and Jones, 1949) who noticed, that ponds containing *Elodea* plants had lower phytoplankton densities than ponds without the plants. Low phytoplankton densities are often found in lakes dominated by macrophytic vegetation (Jeppesen, 1998; Wetzel, 2001), not only by *Elodea*, and some plants are less covered with epiphytes than others (Wium-Andersen, 1987).

In recent years, aquatic allelopathy has attracted more and more attention among scientists which is reflected by the increasing number of articles that review or at least mention allelopathy in the different types of micro- and macrophytes present in aquatic systems (e.g., Rice, 1984; Gopal and Goel, 1993; Inderjit and Dakshini, 1994; Elakovich and Wooten, 1995; Gross, 1999; Neori et al. 2000; Gross, 2003). Allelopathy is now even subject in ecological and mathematical models (Scheffer et al. 1993; Mukhopadhyay et al. 1998). However, the reason for this raise in attention might be the possible application of allelopathic plants or allelochemicals in the control of undesired weeds and algal blooms. Accordingly, a lot of aquatic primary producers have yet been investigated for their allelopathic potential and many were found to be allelopathically active. The trend is thereby shifting from descriptive studies (e.g., Hasler and Jones, 1949; McNaughton, 1968; Fitzgerald, 1969) to analytical studies that focus on the isolation and identification of active compounds (e.g., Della Greca et al. 1989; Aliotta et al. 1991; Aliotta et al. 1992). Allelochemicals are identified for some algae and macrophytes and they exhibit a broad spectrum of different chemical characteristics. Among them are sulfur compounds, fatty acid derivatives, polyacetylenes and polyphenols (summarized in Gross, 1999). Since allelopathy studies might become more frequent in the future, it is likely that more types of allelopathically active secondary metabolites will be discovered.

The intention of this article is to give an overview on the current knowledge on allelopathy in freshwater ecosystems – with focus on submersed macrophytes – and to discuss it under an ecological point of view. Furthermore, other possible functions of plant secondary metabolites besides allelopathic effects are presented.

## **ALLELOPATHICALLY ACTIVE ORGANISMS IN FRESHWATER ECOSYSTEMS**

All photoautotrophs from aquatic environments have been included in allelopathy research, but the most frequently investigated organisms are algae/cyanobacteria and submersed macrophytes. The latter consist of angiosperms and mosses, but also macroalgae are included. Whilst in marine ecosystems macroalgae are widespread, freshwaters contain very few macroalgae. These are mainly charophytes within the genera *Chara*, *Nitella* and *Nitellopsis*. Some authors include large filamentous green algae, like *Cladophora*, in their definition of macrophytes, but in this article those are treated as ‘algae’, following the definition of freshwater macrophytes *sensu* Cook (1974). Wetland plants are also aquatic plants. Since they grow at the water-land interface, they compete also with terrestrial plants and thus represent a special case in aquatic systems. Considering wetland plants, it is not always easy to distinguish between aquatic and terrestrial allelopathic interactions. The most common investigated helophytes in allelopathy are emergent aquatic plants such as *Typha*, *Phragmites* or *Juncus* and their accompanying species. They are here regarded as aquatic plants, whereas grasses, shrubs and ferns, that occur in humid areas but are generally not found within the water are excluded (see also Cook, 1974).

### **Aquatic macrophytes**

Research on allelopathy in freshwater plants began around 1980 although first observations hinting at allelopathy were already made many years before (Schreiter, 1928; Hasler and Jones, 1949). Since then, numerous studies have been undertaken demonstrating that many aquatic plants exhibit an allelopathic potential (see Gopal and Goel, 1993; Elakovich and Wooten, 1995; Gross, 2003). In most cases, negative effects of plants against target organisms have been considered.

Many allelopathic species have been found in the genus *Eleocharis*, as summarized in Elakovich and Wooten (1995). *Eleocharis coloradoensis* is able to displace many other aquatic plants, such as *Potamogeton* spp., *Najas guadalupensis* or *Elodea* spp., and a phytotoxic compound, a dihydroactinidiolide, was found to inhibit root elongation of watercress (*Nasturtium officinale*) seedlings and to reduce germination in radish seeds (Stevens and Merril, 1980). Later on, Ashton et al. (1985) tested the culture medium of *E. coloradoensis* against *Hydrilla verticillata* and lettuce

and concluded from the results that active (i.e. root growth inhibiting) substances must have been present in the medium. Other allelopathically active *Eleocharis* species are *E. acicularis*, *E. cellulosa*, *E. equisetoides*, *E. flavescens*, *E. interstincta*, *E. montana*, *E. obtusa*, *E. palustris*, *E. quadrangulata* and *E. tuberculosa* (Szczepanski, 1977; Elakovich and Wooten, 1995). In *E. microcarpa*, a trihydroxycyclopentenyl-fatty acid and some other fatty acids could be determined as allelochemicals (van Aller et al. 1985). Allelopathy in other helophytes has been studied by Szczepanski (1977). The most active plant in his study was *Typha latifolia*, which suppressed the development of the related *T. angustifolia* by 64%. Other associated plants were less affected. Former observations on *T. latifolia* revealed an autoinhibitory activity of the plant that prevented the germination of seeds of this species (McNaughton, 1968). The suggestion that cattail populations may preclude invasion by alien *Typha* genotypes (McNaughton, 1968) could thus be supported by the results of Szczepanski (1977). *Typha latifolia* contains three steroids and three fatty acids, among them linoleic and  $\alpha$ -linolenic acid, inhibiting the growth of some microalgae (Aliotta et al. 1990). Most organisms were affected by  $\alpha$ -linolenic acid and the most sensitive organisms were the cyanobacteria *Synechococcus leopoliensis* and *Anabaena flos-aquae*, that were susceptible to all compounds except one steroid ( $\beta$ -sitosterol). Five bioactive compounds were isolated from lipophilic extracts of *T. domingensis*, besides three common phenolic acids also linoleic and  $\alpha$ -linolenic acid. Both fatty acids have been found in the leachates of *T. domingensis* as well, indicating a release of allelochemicals through the roots (Gallardo-Williams et al. 2002). Whether these different allelochemicals can cause the growth reductions observed in associated emergent macrophytes (McNaughton, 1968; Szczepanski, 1977) has not been investigated.

Fatty acids exhibiting allelopathic activity and other lipophilic allelochemicals (sterols, phenylpropanoids and  $\alpha$ -asarone) have also been isolated from *Pistia stratiotes*, with  $\alpha$ -asarone being the most active compound against algae and cyanobacteria (Aliotta et al. 1991). The substance was shown to alter the physiology and ultrastructure of *Selenastrum capricornutum* (Pollio et al. 1993).

Elakovich and Wooten (1989) tested 17 extracts of aquatic and wetland plants against lettuce and common duckweed, *Lemna minor*. All plants significantly reduced lettuce seedling growth and many plants had negative effects on *Lemna* frond growth. Fragrant water lily, *Nymphaea odorata*, and *Brasenia schreberi* turned out to be the most active plants in this study. Even more inhibitory is *Nuphar lutea* (Elakovich and Wooten, 1995). *Nuphar lutea* seedlings release considerable amounts of resorcinol which is supposed to play a role as allelochemical, but no effects on green algae or cyanobacteria could be observed; instead, resorcinol had negative effects on zooplankton (Sütfeld et al. 1996). Another floating leaved macrophyte with allelopathic potential is the pondweed *Potamogeton natans*. This species was found to contain diterpene lactones with antialgal activity (Cangiano et al. 2001; Della Greca et al. 2001). Most submersed pondweeds, however, seem to exhibit no allelopathic

activity, such as *P. crispus* (Nakai et al. 1999), *P. perfoliatus* and *P. pectinatus* (Körner and Nicklisch, 2002).

The most prominent example for allelopathy in submersed freshwater angiosperms is the genus *Myriophyllum*. The first species of this genus tested for allelopathic activity was *M. verticillatum*, but no effect could be observed when aqueous extracts were applied on *Lepidium sativum* (Kleiven and Szczepanska, 1988). The absence of any effects may well be due to the target species used in the assay (see discussion below) and effects might have been underestimated. Aliotta et al. (1992) isolated three different phenylpropanoid glucosides from *M. verticillatum* which inhibited the growth of *Synechococcus leopoliensis*. Synergistic effects exerted by hydrolysis products (gallic, *p*-coumaric and sinapic acid) of the major compound may enhance the bioactivity. Similar compounds are present in the related species, *M. spicatum* and *M. brasiliense*. Here, tellimagrandin II is the main allelochemical (Saito et al. 1989; Gross et al. 1996). Allelopathic activity of *M. spicatum* was already suggested before. The plant inhibited the growth of another submersed macrophyte, *Najas marina* (Agami and Waisel, 1985), but no substances had been isolated. *M. spicatum* was also inhibitory on the growth of algae and cyanobacteria as could be revealed in several coexistence assays and investigations with plant extracts (Nakai et al. 1996; Nakai et al. 1999). Tellimagrandin II prevented the development of different cyanobacteria and some green algae in the agar diffusion assay (Gross et al. 1996). Amounts of 5 µg (5.3 nmol) lead to a significant effect when tested against several strains of *Anabaena* sp., *Synechococcus* sp., *Synechocystis* sp., *Trichormus* (*Anabaena*) *variabilis* and *Nannochloris* sp. Cultures of *Scenedesmus falcatus* and *Stigeoclonium tenue* were less sensitive. Culture medium of *M. spicatum* decreased growth of *Microcystis aeruginosa* after quasi-continuous addition (Nakai et al. 1999) and four bioactive (poly)phenols could be detected in the growth medium of the plant (Nakai et al. 2000). These are distinct from the compounds found earlier by Gross et al. (1996), who detected hydrolyzable polyphenols in the culture medium, among them tellimagrandin II and ellagic acid. The mode of action of *M. spicatum* allelochemicals is well investigated and not restricted to a single target site in the affected organisms. Polyphenols often interact with proteins and tellimagrandin II deactivates the exoenzyme alkaline phosphatase which is synthesized by many algae (Gross et al. 1996). Extracts of plant material as well as purified tellimagrandin II were furthermore found to affect photosystem (PS) II of cyanobacteria and algae (Körner and Nicklisch, 2002; Leu et al. 2002) and the site of action seems to be the non-heme iron between Q<sub>A</sub> and Q<sub>B</sub> (Leu et al. 2002).

Another submersed macrophyte with allelochemicals probably altering photosynthetic activity in target species is *Ceratophyllum demersum*. Labile sulfur compounds or even elemental sulfur inhibiting photosynthesis in the diatom *Nitzschia palea* have been extracted from this plant with lipophilic solvents (Wium-Andersen et al. 1983). Aqueous extracts of *C. demersum* caused a shift from cyanobacteria to

chlorophytes in growth assays with natural phytoplankton (Jasser, 1995), indicating that more allelochemicals than lipophilic sulfur compounds might be involved. Inhibition of cyanobacteria could also be observed in coexistence experiments (Nakai et al. 1999) and exudation assays using dialysis membranes (Jasser, 1995). In similar experiments, interference with PS II activity was shown to cause growth inhibition in several phytoplankton species (Körner and Nicklisch, 2002). All these results are further supported by a recent study of Gross et al. (2003) who found cyanobactericidal activity in extracts and exudates of *C. demersum*. They could not elucidate the main inhibitor, but there seem to be more hydrophilic and only slightly lipophilic compounds present. Although allelopathically active, *Ceratophyllum* itself might be affected by allelochemicals. This could explain why *C. demersum* and *C. muricatum* never coexist with *Hydrilla verticillata* in the same waters. Plant extracts of *Hydrilla* and *Hygrorhiza* severely reduced the biomass of *C. muricatum*, whereas other plants had no or even positive effects (Kulshreshtha and Gopal, 1983). As *Hydrilla*, *Elodea* is a member of the family Hydrocharitaceae. *Elodea* species are highly competitive and invasive neophytes in Europe, thereby often displacing endemic aquatic macrophytes. Whether allelopathy is one factor in this process, has to be elucidated, but *Elodea* spp. synthesizes chemical substances with allelopathic activity. Aqueous extracts of *E. nuttallii* caused growth reduction in several terrestrial indicator plants as determined in germination assays (El-Ghazal and Riemer, 1986). Methanolic extracts and exudates of *E. nuttallii* exhibited cyanobactericidal and antialgal properties in agar diffusion assays as well as in tests with liquid cultures (Erhard, 2001; Erhard and Gross, in prep.). *E. canadensis* seem to have the same allelopathic potential against algae and cyanobacteria (Erhard and Gross, in prep.). Extracts of *E. canadensis* should have an effect on photosynthesis of *Nitzschia palea* (Wium-Andersen, 1987), but unfortunately, extraction procedures used in the study have not been reported. No effects on photosynthetic activity, however, could be demonstrated with the active extracts used in the study of Erhard (2001), indicating another mode of action (Leu, 2001).

Recently, Gross et al. (2003) demonstrated allelopathic activity for *Najas marina* ssp. *intermedia* for the first time. Otherwise, allelopathy in *Najas* had only been reported for the related *N. guadalupensis* (Elakovich and Wooten, 1995).

## ECOLOGICAL RELEVANCE OF ALLELOCHEMICALS

Many freshwater autotrophs exhibit a high allelopathic potential, indicating that allelopathy impacts the biocenoses in natural ecosystems. However, opinions diverge whether allelopathy is really that important in nature. One hypothesis is that allelopathy occurs only between plants that have not co-evolved and thus should be most likely in artificial assemblages ("Rabotnov's Hypothesis", Rabotnov, 1974; cited in Willis, 1985). This suggests that influence of allelopathic interactions may be limited to special situations e.g., when a neophyte invades an established plant

community (Reigosa et al. 1999). On the other hand, allelopathy is considered to play a role in controlling the distribution of probably all plants (Rice, 1984, chapter 5) and seems to be a fine-tuning factor during algal blooms (Keating, 1977). With respect to aquatic systems it is often difficult to decide whether a given water body represents a natural or an artificial/disturbed system. Processes like eutrophication can lead to changes in lakes, accompanied by cyanobacterial blooms. Other systems are young and thus co-evolution might still take place. A third group of scientists, however, even doubt the phenomenon at all which might be due to methodological difficulties (Qasem and Hill, 1989).

To examine whether allelopathy might be operative, six points in research have to be considered: (1) a pattern of inhibition must be shown, (2) the putative aggressor plant must produce a toxin, (3) the toxin must be released in the environment, (4) this toxin has to be transported or must accumulate in the environment, (5) the target plant must take up the toxin, and (6) the pattern of inhibition can not solely be explained by physical or other biotic factors (Willis, 1985). The same should be valid for stimulating allelopathic interactions. Willis doubted that any study had ever fully satisfied all of these criteria. The initial criteria in allelopathic research are points (1-3), but many studies in aquatic allelopathy research even failed to fulfil point (1), when they simply screened plants for allelochemicals or used terrestrial plants as target organisms. It is difficult to transfer the results from these artificial test systems onto naturally interacting organisms. Even if (1) is fulfilled, an observed negative effect is not inevitably a result of allelopathic interference but might result from nutrient competition or shading. An example for this is the water soldier *Stratiotes aloides*. Waters inhabited by this plant have low phytoplankton densities, but the phenomenon was suggested to be caused by competition for essential nutrients and changes in the ionic composition rather than allelopathy (Brammer, 1979). In fact, *Stratiotes* diminishes calcium and potassium contents in the water which leads to the decline in phytoplankton (Brammer and Wetzel, 1984). Another aspect concerning the ecological relevance of allelopathy is the question, whether allelochemicals are really present in the surrounding water to come in contact with the target organism. The least studies cover this important requirement. Since in most studies extracts were applied to target organisms, point (2) is the most fulfilled criterion, although not all allelochemicals have been identified.

## **Activity against potential competitors**

Many allelochemicals or extracts from aquatic plants have been tested against standard target organisms which are often radish or lettuce seedlings. For example, El-Ghazal and Riemer (1986) applied extracts from *Elodea nuttallii* to seedlings of three different land plants and many other aquatic plants have been tested against lettuce (see e.g., Elakovich and Wooten, 1995). Of course such experiments show that the

tested plants bear a certain allelopathic potential, but from an ecological point of view they are at least questionable. Aquatic plants would never compete with lettuce in their natural habitat. It is thus reasonable to investigate a plant's allelopathic potential (and activity) against organisms from the same habitat, i.e. aquatic primary producers. Potential target organisms when considering aquatic allelopathy are algae, cyanobacteria and aquatic macrophytes. One could argue that most algae and cyanobacteria are commercially available from culture collections and do not necessarily represent natural occurring competitors. However, since those cultures derive from aquatic habitats, they are at least probable target organisms. The same is valid for *Lemna* bioassays. They represent artificial systems and the reason for the use of duckweed assays may not always be an ecological but a technical one. Nevertheless, *Lemna* is a potential target organism as it might cause light limitation for many submerged living species (den Hartog and van der Velde, 1988). Considering the use of test organisms, many studies on allelopathy did not have an ecological background and thus interpretation of the results should be cautious. Elakovich and Wooten (1995) listed 97 plant species with allelopathic potential, but at least 38 of them had only been tested against irrelevant organisms or were not even aquatic at all. The most frequently used target plant was lettuce, listed 52 times. Fortunately, recent studies on allelopathy of higher freshwater plants increasingly consider algae, cyanobacteria or aquatic macrophytes as target organisms instead of land plants. Examples are the studies of Nakai and co-workers on several aquatic plants (Nakai et al. 1996; Nakai et al. 1999) or the most recent publications on *C. demersum* (Körner and Nicklisch, 2002; Gross et al. 2003).

## Exudation of allelochemicals

Inhibitory effects of plant extracts towards test organisms in laboratory bioassays clearly hint on allelopathic activity of the plant, but they do not prove allelopathy *in situ*. Allelopathy can only be relevant in natural systems when active substances are released in the environment and reach their target organisms. Thus, it is indispensable to detect – in the water or surrounding culture medium – the allelochemicals that are also found in the extracts.

Allelochemicals might be released either actively by secretion or by passive means, e.g., leaching from plant tissue/algae cells, wounds in plant tissue or decaying material. By such means epiphytes as well as phytoplankton in the vicinity of the donor plant would be addressed. Another possibility of allelochemical release is the presentation of active substance(s) on the cell surface so that the transfer of them would be attained by direct cell-cell contact. By this mainly epiphytes or organisms within a biofilm (e.g., benthic cyanobacteria) would be affected. However, one would not expect the latter to be common in submersed plants, since they lack a considerable cuticle which could act as anchoring matrix.

In many experiments on algal allelopathy, live cells, cell-free culture media or lake water rather than extracts have been used for laboratory assays. It is therefore most likely that the allelopathically active compounds have been released in the surrounding medium and are ecologically relevant although many substances remain to be elucidated. This is not the case in allelopathic studies on macrophytes. Although many potential allelochemicals from macrophytes are identified, a proof for their release is often missing. Wium-Andersen et al. (1982) discovered two sulfur compounds from *Chara* that were allelopathically active against the epiphytic diatom *Nitzschia palea*. The impact of these allelochemicals *in situ*, however, is doubted (Forsberg et al. 1990), since no difference in the biomass relative to phosphorus between *Chara*-rich lakes and lakes without *Chara* could be observed. Furthermore, the reported epiphyte covers on charophytes should indicate the absence of allelopathy in stoneworts (Forsberg et al. 1990). The lipophilic allelochemicals from *Sium erectum* could not be detected analytically in the culture medium either (Wium-Andersen et al. 1987).

Nevertheless, the observations that waters containing considerable vegetation appear to be more transparent than others without vegetation might indicate a release of allelochemicals. And also in the case of *Chara* the discussion is still going on (Van Donk and Van de Bund, 2002). A summary on this special case can be found in (Gross, 2003).

*M. spicatum* is the only submersed macrophyte in which exudation of allelochemicals has been proven and the active compounds were detected and identified. Gross et al. (1996) found the main allelochemical tellimagrandin II in the culture medium, but it is likely that the plant exudes more phenolic compounds with allelopathic activity (Nakai et al. 2000).

Other studies show that more submersed macrophytes may exude their allelochemicals, although they have not yet been isolated and determined. The experiments with dialysis membranes indicated a release of allelochemicals by *Ceratophyllum demersum* (Jasser, 1995; Körner and Nicklisch, 2002). Gross et al. (2003) performed exudation assays with *Ceratophyllum demersum* and *Najas marina* ssp. *intermedia* and concentrated the exudate by solid phase extraction. The exudates of both plants inhibited the growth of cyanobacteria. According to the analysis performed, the active compounds are only slightly lipophilic but still unknown. Similar results can be obtained with incubation water and exudates from *Elodea nuttallii* both inhibiting cyanobacteria and epiphytic algae formerly isolated from submersed macrophytes (Erhard, 2001; Erhard and Gross, unpublished results).

## Probable impact of allelopathic interactions

The variety of different allelochemicals present in aquatic systems offers a broad array of distinct modes of action that cause many diverse effects in the target organisms. Allelochemicals often target different sites, even within the same organism. Tellimagrandin II, the main allelopathic compound in *Myriophyllum spicatum*, can lead to growth inhibition by two different effects. It inactivates the alkaline phosphatase (Gross et al. 1996), an exoenzyme of many algae and bacteria which is synthesized under low phosphorus conditions. The inhibition through allelochemicals prevents the phosphorus supply of these target organisms and thus may lead to phosphorus limitation in algae and bacteria. The second target site of tellimagrandin II is photosystem II (Leu et al. 2002). Allelopathy thus offers the opportunity to reach several potential competitors at once by synthesizing only one or few substances.

The impact of allelopathic interactions especially for submersed macrophytes can be immense. Although many submersed plants are adapted to low light conditions, shading by epiphytes or phytoplankton may lead to negative consequences for the plants. *Najas marina*, for example, possesses a very low light compensation point of  $5 \mu\text{E}/\text{s}\cdot\text{m}^2$  (Agami et al. 1980) but for a complete lifecycle ending with seed production, such intensities are not high enough. According to (Agami et al. 1984) reproduction affords at least  $250 \mu\text{E}/\text{s}\cdot\text{m}^2$ . Since *Najas* is an annual plant, seed production is indispensable for the maintenance of the plant and its return in the next season. Allelopathy would thus be a successful trait in addition to other adaptations to prevent severe shading and to obtain enough energy for metabolic activity and reproduction (Gross et al. 2003). If allelopathy increases the competitive vigour of aquatic plants and allows the establishment of stable macrophytic communities, an indirect effect on the whole littoral community is expected (Gopal and Goel, 1993). Aquatic macrophytes are not only important primary producers. They serve also as substrate for spawn of snails, fish, or amphibia or act as shelter and refuge for juvenile fish and invertebrates (Cook, 1974). In dense stands of macrophytes flow velocity and water turbulence are often slowed down (Sand-Jensen and Pedersen, 1999) leading to stabilisation of sediments and reduced resuspension of nutrients stored therein. Thus, vegetation positively influences water quality, and the reduction of nutrient input results in a negative feed-back regulation of phytoplankton and epiphytic algae and by this in a positive feed-back for macrophytic growth. Due to these positive effects allelopathy was considered as one important factor in the model of alternating stable states in shallow eutrophic lakes (Scheffer et al. 1993). This model predicts that lakes can switch between two alternative states, a macrophyte dominated and a phytoplankton dominated one. For the transition from one state to the other a threshold value has to be overcome. This threshold might be light intensity that can be reduced either by algal growth due to eutrophication or as a result of other processes leading to increased turbidity. Then, the release of antialgal compounds by the plants into the

water should be decreased resulting in the dominance of phytoplankton and a collapse of macrophytic vegetation (Phillips et al. 1978). However, the situation might be even more complex in nature. Scheffer et al. (1993) did not consider allelopathic effects of algae/cyanobacteria on higher plants which are suggested by other authors (Van Vierssen and Prins, 1985; Pflugmacher, 2002).

## ADDITIONAL FUNCTIONS OF ALLELOCHEMICALS AND OTHER PLANT SECONDARY METABOLITES

Photoautotrophs compete not only with each other; they are also food source for many consumers. This is well known for terrestrial plants. In contrast, plant damage and loss of biomass of live macrophytes in aquatic systems due to invertebrate herbivory have been considered negligible (Hutchinson, 1975; Wetzel, 1983). However, there is evidence that the extent might be as high as in terrestrial systems (Newman, 1991). Among the invertebrate herbivores in freshwater systems, lepidopterans, weevils, and chrysomelid beetles show best evidence for direct effects of consumption. These insects are also common pest herbivores in terrestrial systems. Differences exist in the extent of loss due to direct herbivory between different plants. E.g., the loss of leaf area as well as consumption preference is lower in *Elodea* compared to *Potamogeton* spp. (Soszka, 1975; Newman, 1991). Also, *Myriophyllum spicatum* should be less preferred by herbivores (Soszka, 1975). Since he did not find any evidence that such aquatic macrophytes have lower nitrogen or protein contents than terrestrial plants and since structural defences or physical resistance were also unlikely, Newman (1991) concluded, that the presence of defensive or deterrent secondary compounds protect freshwater macrophytes against herbivory. Although aquatic macrophytes contain less secondary metabolites than their terrestrial ancestors, they synthesize a broad array of alkaloids, phenolic acids, polyphenols or flavonoids (Bate-Smith, 1968; McClure, 1970; Ostrofsky and Zettler, 1986). All these classes of compounds are involved in the defence against herbivores or pathogens in land plants. Furthermore, many allelochemicals of aquatic plants belong to these classes of substances. It is thus indeed likely that they protect also freshwater macrophytes.

Important herbivores feeding on freshwater macrophytes are the larvae of the moth *Acentria ephemerella*. They are found feeding on pondweeds like *Potamogeton perfoliatus*, *P. pectinatus* and *P. lucens*, but also on *Ceratophyllum demersum* and *Myriophyllum spicatum* (Gross et al. 2002). During this study, larvae were never found on *Chara* spp., *Najas marina* ssp. *intermedia* or *Elodea nuttallii*. In no-choice assays, larvae grow well with *Potamogeton* as food source, but larvae feeding on *M. spicatum* grow slower and remain smaller than larvae fed with *Potamogeton* (Choi et al. 2002). It is suggested that this effect is due to the high content (5-9%) of polyphenols in *M. spicatum* interfering with some of the gut bacteria in *Acentria* larvae (Walenciak et al. 2002). Whereas biomass of *Myriophyllum* might be significantly

reduced by herbivory of *Acentria* larvae, *Elodea* biomass seems not to be affected (Gross et al. 2001). *Elodea* plants are described to be avoided by invertebrate herbivores (Newman, 1991) and also *Acentria* preferred *Myriophyllum* over *Elodea canadensis* in choice experiments (Gross et al. 2001). Recent laboratory growth experiments indicate that *Acentria* larvae do not grow significantly when fed with another *Elodea* species, *E. nuttallii*, but grow significantly with *Potamogeton perfoliatus* as food source (Erhard and Gross, unpubl.). In contrast to *Myriophyllum*, *Elodea* spp. are lacking tannins but contain simple phenolic acids and flavonoids (Bate-Smith, 1968; McClure, 1970; Mues, 1983). Whether these compounds are involved in the defence against herbivores is currently under investigation. Flavonoids are also discussed as defence against herbivorous snails and tadpoles in *Azolla pinnata*, and might influence the competition between this species and a related one. In coexistence growth experiments, *A. filiculoides* grew better than *A. pinnata* when herbivores were absent. In the presence of herbivores (snails, tadpoles), *A. filiculoides* was the preferred food plant and *A. pinnata* grew better (Cohen et al. 2002). This might be the result of the lower deoxyanthocyanin content in *A. filiculoides*. Chemical defence can also be directed against larger consumers. In *Saururus cernuus*, a complex mixture of natural products, among them lignoid metabolites, deters the crayfish *Procambarus clarkii* that feeds on many aquatic plants (Kubanek et al. 2001).

## CONCLUSIONS

Allelopathy is a widespread phenomenon not only in terrestrial but also in aquatic ecosystems. The exudation of growth inhibiting substances offers a good opportunity to avoid the detrimental effects of light limitation for underwater vegetation. The chemical nature of known allelochemicals suggests that they often locate different targets. One compound might affect not only several different targets within one single cell but also different organisms at once. The submersed macrophyte *Myriophyllum* is an example for such multi targeting. It can suppress growth of algae and cyanobacteria by inhibition of PS II and the exoenzyme alkaline phosphatase. Furthermore, the same allelochemical, tellimagrandin II, might play a role in the defence against herbivores. Similar observations on allelopathy and feeding deterrence can be made with other aquatic plants. This shows that the interactions between plant species and their competitors are much more complex than currently known, and much more work is needed to understand how allelopathy shapes aquatic ecosystems.

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## CHAPTER 20

# FOREST ECOSYSTEMS AND ALLELOPATHY

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## INTRODUCTION

A forest ecosystem is a dynamic set of living organisms: plants, animals and microorganisms, that are characterized by a predominance of trees, all interacting among themselves, with the environment in which they live (soil, climate, water and light) and ecological cycles (energy, water, carbon and nutrients) with which they are closely associated. Plants set up relations in order to compete for resources and many plants have adopted strategies of chemical usage to acquire a greater proportion of the available resources. Some of those relations involve allelopathic interactions, higher plants cannot change the location since germination, but they adapt themselves to the surrounding environment, and they are provided with different mechanisms to promote its own growth. These mechanisms have been obtained during evolution process, and here chemical substances play an important role (Nishimura and Mizutani, 1995) that vary in their relative importance depending on the ecological context in which they are studied (Hierro and Callaway, 2003): exotic plant invasion, tree regeneration, chemical information between plants, inhibition of seedling understory, impact on physico-chemical and biological characteristics of soil.

The production of chemical compounds by trees that are released in the environment, has been widely investigated in forest ecosystems (Souto et al. 1994; Souto et al. 1995; González et al. 1995; Gallet and Pellissier, 1997; Sivagurunathan et al. 1997; Peñuelas and Llusia, 1998; Rawat et al. 1998; Reigosa et al. 2000; Souto et al. 2001; Harris et al. 2003), especially when adverse impacts of understory species on tree species are suspected (Pellissier, 1993; Mallik, 1987; Mallik, 2001; Mallik, 2003)

but we will focus this review on different ecological contexts into the forest ecosystem

## **EXOTIC PLANT INVASION**

Some examples of strong allelopathic effects can be found in the genus *Acacia*, *Ailanthus*, *Eucalyptus*, *Juglans*, *Leucaena* and some *Quercus* species. Other tree species have been studied more deeply than the previous list (*Acer*, *Kalmia*, *Picea*, *Pinus* and *Prunus*) but they produce moderate effects on other species. Most examples of allelopathy in trees are related to exotic species that act as invaders or become dominant; usually they are not as competitive at home.

Allelochemicals are secondary metabolites. The importance of the distribution and concentration of secondary components to plant relationship should not be dismissed because they are produced in plants not necessarily for competition (Berenbaum, 1995). Allelopathy was probably not developed as a specific competition mechanism between plants. If most of allelochemicals are secondary exudates from plants, they would be more likely to be effective in competition with plants outside of the native community (Rabotnov, 1982; Reigosa et al. 1999a).

Introduced species that disperse but do not fill the place of autochthonous species are thought to exploit unfilled niches in the community (Elton, 1958). Exotic species that act as aggressive plant invaders and reduce the abundance or species diversity require different explanations (Rich, 2004) and the reasons why many exotic tree species competitively exclude and eliminate their neighbors in resident communities but coexist in relative peace with neighbors in their native habitat, that usually is species-diverse system, remain one of the most important mysteries in plant ecophysiology (Hierro and Callaway, 2003).

Several hypotheses have been proposed to explain intrusiveness in plants in general (Rejmanek, 2000), all of them related to plant biology of the invader and particular characteristics of the resident community (Levine et al. 2003). The leading theory for the exceptional success of invasive plants is their escape from the natural enemies that hold them in check, freeing them to utilize their full potential for resource competition (Keane and Crawley, 2002). There are often complex interactions between the traits of the invader and resident community attributes (Shea and Chesson, 2002 and Suding et al. 2004), then could be better concur in processes responsible for plant invasions that have been tested experimentally, or are testable, using woodland species: release from natural enemies, broad tolerance limits, more efficient use of resources, hybrid vigor and allelopathic processes (Zedler and Kercher, 2004). These hypotheses are not shutting out one another. They have a good connection to exotic species released from pathogens (Mitchell and Power, 2003), predict that invasive

species have broader tolerance limits or tolerate extreme environmental conditions better than noninvasive species (Kercher and Zedler, 2004a), state that invasive species compete better because of more efficient or complete use of light, and nutrients resources (Kercher and Zedler, 2004b), focus the invasive phenomena on invaders with different species as parents (Ellstrand and Schierenbeck, 2000) and predicts that some plants become reach profit through the release of biochemical compounds that inhibit the growth and germination of species in the area of introduction (Bais et al. 2003 and Hierro and Callaway, 2003).

Despite a much larger body of evidence for allelopathy as an important plant interaction in agroecosystems (Narwal and Tauro, 1994), still some skepticism remains. For a critical evaluation of ecological significance of allelopathy in forest see Wardle et al. 1998; Hierro and Callaway, 2003; Zedler and Kercher, 2004. Disruption of native communities by odd allelochemicals or the release of chemicals at higher concentrations or in different period of the year by an exotic plant would suggest that invasions disrupt co-evolved interactions among long-associated native species. If allelopathy is more important in exotic invasion than in natural communities it is possible that interactions among plant species may drive natural selection in communities. This positive statement implies that natural communities may gradually change (evolutionary time) and, in some way, act as functionally organized units (Wilson, 1997).

Allelopathy seems to be more intense in poor soils (Inderjit and Callaway, 2003, Beltz and Hurle, 2004 and Suding et al. 2004). The hypothesis that allelopathy increases the invasive potential of exotic plants in environments with low resource availability awaits evaluation but its more likely (Hierro and Callaway, 2003). This supposition gives to us a reason to predict that some plants become invasive monotypes through the release of allelochemicals that inhibit the growth and germination of species in the area of introduction. The overall effect of one plant on another is the result of several interacting mechanisms (Ridenour and Callaway, 2001 and Zedler and Kercher, 2004) but the balance of competition in the broader context of interference shifted when allelopathy was ameliorated indicating the important role of allelopathy in exotic plant invasion.

## TREE REGENERATION

One of the greatest challenges for plant ecophysiolologists today is restoring natural and crop forests. This restoration will require understanding complex processes that shape successional pathways, including biochemical interactions between trees and other plants.

Shrub species often quickly invade disturbed woody lands (Duncan and Chapman 2003) and form a dense understory that can alter natural regeneration of trees after removal of canopy trees by forest harvesting (Mallik and Prescott, 2001). This remark has been attributed to allelopathy, competition, and soil nutrient imbalance (Smith et al. 2002 and Mallik, 2003) but its role in forest succession is unclear (Duncan and Chapman 2003).

Usually some understory plants as ericaceous or compositae perpetuate in the community after the disturbance related to forest harvesting, thanks to their stress-tolerating strategies. Different mechanisms have been proposed to explain failure of natural regeneration of trees at the ecosystem level (Mallik, 2003) but habitat degradation by allelochemicals from understory species has earned great significance in the last years. Sometimes this inhibition includes planted tree seedlings that exhibit stunted growth in these forest areas (Mallik, 1992 and Pellissier, 1993) with serious ecological, economic, and social consequences. Unfortunately, to date, the study of disturbance in woodlands by forest management has been primarily anecdotal and resistant to generalization.

Coevolution plays a role in the manifestation of allelopathy (Rabotnov, 1982, Reigosa et al. 1999a). Additionally, there may be genetic and evolutionary dynamics that alter the competitive ability of plant species (Ellstrand and Schierenbeck, 2000; Siemann and Rogers, 2001). Delayed germination in treatments with shrub leachates and humus supports Rabotnov's hypothesis if we accept the tendency of the understory system after release of trees to safeguard internal stability (Mallik and Pellissier, 2000). They found that germination and primary root growth of an exotic spruce was more affected by humus and its allelochemicals than a native spruce thus strengthening the idea of a coevolutionary process in allelopathy.

It is not clear if allelopathy exert a primary role in forest succession after disturbance. The negative influence of some shrubs on tree growth was through root competition for nutrients rather than allelopathic effects of litter leachates (Mallik and Prescott, 2001). Tree harvesting and non adequate management of the forest can cause a long term occupancy of a site by understory species with irreversible habitat degradation, converting conifer forests into ericaceous heath. Different experiments suggest that tree inhibition phenomenon in this area is more than just a case of nutrient deficiency (Mallik, 2001). In other way, the presence of plants has been shown to influence small-scale patterns of nutrient availability in various ecosystems by changing the quantity and quality of organic matter in the nearby soil (Chen and Stark, 2000). These effects are species specific since plants differ in litter production quantity and litter chemical composition. Ericaceous litter has a high content of phenolic compounds (Gallet et al. 1999) and phenolic compounds may play a dominant role in controlling many aspects of plant-plant interactions through soil, especially those related to organic matter dynamics and nutrient cycling (Northup et al. 1998). One of

the most characteristic properties of phenolic compounds is their capacity to constitute recalcitrant complexes with proteins and thus to modify nutrients availability (Hättenschwiler and Vitousek, 2000). Significant amounts of phenolic compounds can be released by rainfall from green foliage and decomposing litter (Gallet and Pellissier, 1997), thus affecting nutrient cycling. The ecological relevance of phenolic compounds in degraded forest can be of especial interest, especially in N-limited systems with slow decomposition, such as boreal ecosystems, where slow growing species with high concentrations of carbon-based secondary compounds predominate (Castells et al. 2003). Then, the poor tree seedling growth that is observed in some tree crops or natural forest may have resulted from the adverse effects of understory litter, causing allelopathy and nutrient imbalance. However, it is still not clear whether changes in N cycling related to plant phenolic compounds (Souto et al. 2001) can be found in natural conditions since these effects have been mainly tested in laboratory experiments with individual compounds or soil samples. Castells et al. (2003) support the hypothesis that potentially negative interactions among plants could be caused by changes in nutrient dynamics although allelopathic effects cannot be excluded.

In a forest ecosystem, allelochemicals can be released to the soil by several ways. Decaying of residues is usually predominant, but in forest crop systems the biomass of stump-roots left in the cutting area is really important (Huang et al. 2000) and the content of allelopathic compounds in roots was thought to be the highest among all parts of the tree (Bertin et al. 2003). Phenols from stump-roots exert an allelopathic influence on tree seed germination and seedling growth (Huang et al. 2000).

## CHEMICAL INFORMATION BETWEEN PLANTS

Plant-plant signaling is the flow of information between individuals. An interaction is considered to be informational when it involves the exchange of an insignificant amount of matter or energy (Aphalo et al. 1999). To exploit the common environmental conditions to maximize reproductive success, organisms can take advantage of information (Dicke and Bruun, 2001) and an important form of information consists of chemical signals (Bais et al. 2004). We consider the mechanisms by which a plant could signal to a neighboring plant, and thus elicit a physiological response.

There is detailed information on how plants respond to environmental stimuli and regulate their internal physiology in response to several stressing conditions, including damage by insects or infection by pathogens (Hunter, 2000). Plants are subjected to multiples interactions in their natural environments and often respond inducing defensive pathways that usually increase the production of secondary metabolites, and in evolutionary sense, can enhance plant fitness (O'Reilly-Wapstra et al. 2004) but many secondary metabolites do not enhance the fitness of the producer plant (Firn and

Jones, 2000). Recent assays provide ample evidence that, for any biological target, most chemicals are inactive unless tested at high concentrations (Firn and Jones, 1999) but it could be due to the result of using inappropriate screening methodologies. If the proper target species or physiological parameter were used, a very high frequency of biological activity would be found (Berenbaum and Zangerl, 1996). The main problem deals with the definition of 'biological activity' (Firn and Jones, 2000). When studied at a molecular level, biological activity can have a different meaning than if it is studied at a whole organism or ecosystem level. Evolution of secondary metabolism is better understood if studied at ecosystem level.

It has been well established that plants obtain ecological profit from their relation with soil microorganisms. Permanent organization of many forest ecosystems depend on the ability of roots to communicate with microbes. Associations between plants and many bacteria and fungi are often regulated by root exudates (Bais et al. 2004). The mechanism by which proteins are secreted is not completely understood, but it has been proposed that proteins are actively secreted from the root epidermal cells (Flores et al. 1999).

Communication between plants is related to plasticity that is essential for plant survival (Aphalo et al. 1999). We can recognize plasticity in the vast range of secondary metabolites present in plants. However, apart from mechanistic questions, evolutionary questions should be addressed asking why plants exploit their neighbor's information and whether their strategy (modulating their developmental programme) is affected by physical, biological or previous experience.

## **INHIBITION OF SEEDLING UNDERSTORY**

Trees modify their environment for more effective and efficient interference. Competition for resources in trees and subcanopy species is a constant in woodland. In order to compete for these resources, many plants have adopted strategies of chemical usage to attain benefit over others. The interaction between exploitative and interference competition remains largely unexplored for species that exploit dynamic resources (Amarasekare, 2002). In this sense the role of allelopathy in the interaction between forest trees and their understory is very interesting.

Allelochemicals are released into the environment by trees and ecologically modify growing sites (Sánchez-Moreiras et al. 2003) affecting the development of the understory. The way these organic chemicals escape the tree are through aboveground parts, roots exudation, and the action of many soil microorganisms on litter (González et al. 1995).

The plant diversity and richness of species found beneath allelopathic trees is significantly lower than in adjacent plots without the cover plant (Souto et al. 1995).

Some tree crops have a sparse understory growth around the trees, with a denser and more varied growth in areas not strongly populated by the dominant tree species or compared with natural woodland (Souto et al. 1992 and Souto et al. 1994). The allelopathic activity of trees is thought to play an important role as leaf decomposition of these plants inhibit the soil microorganisms development (Souto et al. 2001) and germination of target seeds (Souto et al. 1994).

Most of the experiments and interaction processes in forest are related with different types of organic chemicals as terpenes (Paavolainen et al. 1998 and Abraham et al. 2000), phenolics (Souto et al. 1995, Souto et al. 2000 and Huang et al. 2000) and alkaloids (Wink and Latz-Brüning, 1995 and El-Khawas and Shehata, 2005) but the chemistry of interaction is too complex and it has been demonstrated that allelochemicals released by trees can affect plant growth (Durán-Serantes et al. 2002), absorption of water and mineral nutrients (Booker et al. 1992), ion uptake (Yu and Matsui, 1997), membrane permeability and cell cycle (Sánchez-Moreiras et al. 2003) plant water relationship (Barkosky and Einhellig, 2003), protein biosynthesis (Wink, 2003) leaf area expansion (Hane et al. 2003), respiration (Abrahim et al. 2003), and photosynthesis (Kagan et al. 2003) and many other physiologica processes.

Most forest are managed for timber production. In such forests the fate of indigenous understory plant communities, and thus of plant diversity in general, is a function of silvicultural practices, that promote rapid decomposition of plant material Roth et al. 2000), designed with the primary intent of maximizing the value of the dominant tree crop (Thomas et al. 1999). Silvicultural practices can change the physico-chemical conditions of the soil or the biotic relations into the soil solution (Leckie et al. 2004) and therefore, change the allelopathic tree-understory relationships. This should be kept in mind if we are interested in diversifying farm income and reducing environmental impacts of agriculture (Jose and Gillespie, 1998).

## **IMPACT ON PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERISTICS OF SOIL.**

Our understanding of the effects of tree species on soils remains very incomplete (Augusto et al. 2002). Composition of the overstory has an impact on soil structure and the effect on forest communities and soil environment having different tree species in the canopy is significant. Secondary metabolites released into the soil solution by plants can affect abiotic and biotic soil processes that affect other plants and it is difficult to distinguish of direct allelopathy (Inderjit and Weiner, 2001).

There is a massive interface between plant roots and soils where chemical exchanges are highly probable, mainly carbon-containing compounds (Uren, 2000).

Allelopathy influences the ecology of the tree-soil interface and modifies relationships between organisms in forest (Bertin et al. 2003).

The total amount of root exudates produced in a tree ecosystem varies with the plant species, cultivar, age, and stress factors (Uren, 2000). Tree roots exude high molecular weight organic compounds such as flavonoids, enzymes, fatty acids, growth regulators, nucleotides, tannins, carbohydrates, steroids, terpenoids, alkaloids, polyacetylenes and vitamins (Rovira, 1969) that provide a suitable environment for the growth and development of microorganisms through establishment of mucigel (Bertin et al. 2003). Soil microflora seems to be strongly influenced by the plants of the ecosystem, allelochemicals influence directly the development of microorganism colonies (Reigosa et al. 1998), microorganisms have a large impact on the total allelochemical load on a site (Reigosa et al. 2000) and fungi and bacteria can transform exudates into inactive molecules or process them into allelopathic agents modify and interfere with growth regulation signals surrounding fungal growth and infection in mycorrhizal associations affecting indirectly resource competition (Reigosa et al. 1999a). Interactions are too complex to generalize (Pellissier and Souto, 1999). Tree roots secrete many different low molecular weight substances, including sugars and simple polysaccharides, amino acids, organic acids and phenolic compounds (Bertin et al. 2003) with allelopathic activity (Reigosa et al. 1998 and Reigosa et al. 1999b). Then, root exudates play important roles in a number of plant-microbial relations (Inderjit and Weston, 2003) and they have also important function in the determination of microbial community structure in the tree rhizosphere (Grayston et al. 1994).

Allelochemicals that reach soil solution interact with organic and inorganic substances regulating not only their bioavailability in the soil environment, but also their transport (Bertin et al. 2003). The action of those allelochemicals alter soil microflora, which subsequently alter nutrient status through decomposition and mineralization of organic substances, and through the formation of soil organic matter influencing competition for nutrients (Hodge et al. 2000).

Tree composition significantly influences the physical, chemical and biological characteristics of top-soil. By modifying the fluxes of matter or energy, allelochemicals secreted by forest species have the potential to affect strongly soil characteristics (Augusto et al. 2002). More studies are needed to understand the effects of allelochemicals on organic matter, reactive mineral surfaces, ion exchange capacity, inorganic ions and specific microbes.

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## CHAPTER 21

# ALLELOPATHIC INTERACTIONS IN AGROECOSYSTEMS

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## SUMMARY

Allelopathy plays a significant role in the agroecosystems leading to a wide array of interactions among crops, weeds and trees. Generally, these interactions are deleterious to the receiver plants but may also provide a selective advantage to the donor. In agroecosystems, it leads to the problem of soil sickness or causes the autotoxicity that adversely affect the crops and thus their yield. The allelochemicals released from plant and their residues accumulate in the fields and add to further problems. However, if properly understood and managed, allelopathy and allelochemicals - the chemicals involved, can be used for practical weed and pest management. In fact, it is now being viewed as an important tool in sustainable agriculture. In this direction, a number of strategies like use of cover or smother or green manure crops for weed management, and direct use of allelochemicals as natural herbicides and pesticides are followed. Efforts are being made to improve present day crop cultivars for better competitive ability by transferring genes through cultivar selection and from other sources. The purified allelochemicals and/or their derivatives can directly be used as novel agrochemicals for sustainable management in an eco-friendly manner.

## INTRODUCTION AND BRIEF HISTORICAL BACKGROUND

Plants are the storehouse of a myriad of chemicals synthesized as secondary metabolites. Apart from several physiological functions within plants, these are also responsible for orderly plant-plant interactions including allelopathy. Though Hans

Molisch first used term allelopathy in 1937, yet knowledge about the phenomenon is very old. There are several classical references in the literature that indicate the inhibitory effects of one plant on the other through the release of chemical substances in the environment (Theophrastus, 300 BC; Plinius Secundus II, 1 AD; Culpeper, 1633; Young, 1804; de Candolle, 1832). In fact, the first statement related to it came from Theophrastus (300 BC), who observed that chickpea plants exert adverse effect on the others by exhausting the soil. In the beginning of the 19<sup>th</sup> Century, de Candolle, a well-known plant taxonomist, generated some interest in this field when he observed that root exudations of some plants are the cause of *Soil Sickness* and that this can be overcome by the suitable crop rotation (de Candolle, 1832). However, all such references were mere statements/anecdotes without any planned experimentation lacking scientific proofs and thus failed to attract the attention of scientists until the beginning of 20<sup>th</sup> century. During that time Schreiner and his co-workers in USA (Schreiner and Reed, 1907, 1908) and Pickering and his team in UK conducted some experiments and generated interest in the field (Willis, 1997).

Since the phenomenon of allelopathy is mediated through chemicals, these are generally referred to as *Allelochemicals* or *Allelochemics*. These being toxic in nature are generally localized and sequestered in certain specialized organs that may be glandular or sub-epidermal (Duke et al. 1999). Allelochemicals are released from the plants through leachation, volatilization, root exudation and the death and decay of the fallen plant parts either through biotic or abiotic means. Upon release, these are involved in a number of metabolic and physico-chemical processes (Rice, 1984; Einhellig, 1996). Their toxicity, however, depends upon concentration, flux rates, age and metabolic stage of the plant, prevailing climate and season, and environmental conditions (Rice, 1984; Einhellig, 1996). Under natural conditions, the released allelochemicals act synergistically and exert inhibitory effect as complex mixtures and therefore the inhibitory effects are observed at concentrations well below their individual inhibitory levels (Blum, 1996).

In 1960s, Muller and his co-workers did some classic and pioneer work on the Californian Chaparral that provided a sound and solid footing to this science (Muller et al. 1964; Muller, 1965, 1966; del Moral and Muller, 1969; Chou and Muller, 1972). Later, Elroy Rice did pioneering work in this field as evidenced by his excellent and exhaustive writings (Rice, 1984, 1995). Nowadays, as a result of better techniques and methodologies, collaborative approach among the scientists and availability of suitable bioassays, this science has picked up the momentum during the last three decades. Since 1980's, the science of allelopathy has attracted a number of scientists from the diverse fields and there has been a spurt in the number of research papers, reports, articles, reviews and books in this field (Kohli et al. 2001; Singh et al. 2001 and references therein).

## ALLELOPATHIC INTERACTIONS IN AGRO-ECOSYSTEMS

An agro-ecosystem, a man-made solar powered distinct unit in agricultural fields, is regulated by ecological principles where all the biotic and abiotic factors play an important role. These ecosystems are based on lots of external inputs or auxiliary energy in the form of fertilizers, pesticides, animal and human labour and fossil fuels. Crops, weeds and even the trees are the integral visible biotic components of agroecosystems. In addition, a diversity of invisible microbes also plays an important role. Agroecosystems have evolved from the simple and traditional ones to modern where high yielding crop cultivars and opportunistic weeds and pests (despite their removal by sophisticated methods) are flourishing in an environment with residues of fertilizers and pesticides. The phenomenon of allelopathy is very common in agroecosystems (Kohli et al. 1998a; Singh et al. 2001; Weston and Duke, 2003). In fact, first few reports on allelopathy were observed in crops, e.g., chickpea (Theophrastus, 300BC). A number of crops, weeds and trees are known to be allelopathic playing an important role in agroecosystems (Kohli et al. 1998a; Rizvi et al. 1999; Batish et al. 2001; Qasem and Foy, 2001).

### Allelopathic weeds

Weeds are an integral and ecologically important components of agroecosystems. Despite their large-scale removal from these systems, weeds continue to evolve and pose threat to crops. Weeds, in fact, have co-evolved with the crop plants. They have a number of physiological, agronomic and reproductive characteristics, which make them successful compared to the other plants (Kohli et al. 2004). In the agroecosystems, they compete with the crop plants for resources, interfere in crop handling, reduce crop yield and deteriorate their quality and thus resulting in huge financial loss. Nearly 12% of the total loss of crop yields has been attributed to the weeds alone (Anaya, 1999). A number of weeds especially found in the agroecosystems are also known to possess allelopathic properties that further make them competitively stronger and thus adversely affecting crops (Putnam and Weston, 1986; Kohli et al. 1998a; Weston and Duke, 2003). Qasem and Foy (2001) reported nearly 240 weeds to be allelopathic.

Of the several allelopathic weeds, *Parthenium hysterophorus* commonly known as congress grass or ragweed parthenium has caused much harm to plants in India, Australia and other parts of the world (Kohli and Rani, 1994; Evans, 1997). It is an invasive weed possessing several characteristics including allelopathy that favour its fast spread (Kohli and Rani, 1994). All parts of this weed including the dried residues are allelopathic thus contributing a major fraction of allelochemicals in the environment (Kanchan and Jayachandra, 1980; Mersie and Singh, 1987; Kohli and

Batish, 1994; Batish et al. 2002; Singh et al. 2003c). In the fields, it is present along the field boundaries, pathways, space between crop lines and the whole field during fallow periods. Some of the allelochemicals of *P. hysterophorus* are water-soluble while others are water-insoluble and are mainly released through leachation, microbial decomposition and root exudates. Chemically, they are both terpenoids (sesquiterpene lactones) as well as phenolics (phenolics acids) (Kanchan, 1975; Kanchan and Jayachandra, 1980; Kohli and Batish, 1994). Among the sesquiterpene lactones, parthenin is very common and found in greater amount and probably remains sequestered in the trichomes – the hairy structure found all over the weed to avoid intra plant toxicity. In fact, parthenin provides several properties, including allelopathy, to the weed and can also help in weed management by suppressing growth of the noxious weeds (Batish et al. 1997; Batish et al. 2002; Singh et al. 2002d). Among the phenolic acids, caffeic, ferulic, *p*-coumaric, *p*-hydroxybenzoic and anisic acid have been reported in the soil from areas infested by it. The weed causes a number of harms such as health problems in human beings and cattle, fodder scarcity in grasslands, ecological and environmental problems besides loss of crop yield in agricultural systems (Kohli and Rani, 1994).

*Ageratum conyzoides* L – commonly known as billy-goat weed – is another such weed that is becoming a nuisance in agroecosystems. It is an annual invasive weed native to tropical America and has now invaded and become naturalized in various parts of Southeast Asia including India. It is a weed of agricultural land and severely infests the crop fields thereby greatly hampering the growth and reducing the grain yield (Kohli, 1997). It is found to seriously affect the crops of maize, wheat and rice. The aqueous extracts prepared from leaves, stem, roots or whole above ground part and residues of the weed suppressed the germination and growth of crops plants such as wheat, radish and mustard (Kohli, 1997). Even the soil collected from the *Ageratum* infested area was found to be phytotoxic to crops and this was attributed to the presence of water soluble phenolics in it released from the weed (Singh et al. 2003a). The allelopathic properties of the weed have been attributed to the release of volatile essential oils rich in mono- and sesquiterpenes besides phenolics and a few flavonoids identified in aqueous extracts (Kong et al. 1999, 2002; Okunade, 2002; Singh et al. 2003a).

## Allelopathic crops

A number of crops have also been observed to exhibit allelopathic effects on other crops and weeds, besides being autotoxic (Batish et al. 2001). Of these interactions, crop autotoxicity in agroecosystems is significant (Singh et al. 1999b; Batish et al. 2001). This problem is commonly known as of *Soil Sickness* or *Soil Fatigue* (Yu, 1999a). The principal causes of crop autotoxicity include the deliberate leaving of crop

residues or old roots in soil releasing phytotoxins which may directly affect the succeeding crops, cause microbial imbalance, change organic matter of soil, increase ion leakage, disturb nutrient uptake and immobilization (Yu and Matsui, 1997). Some of the highly worked out important crops exhibiting autotoxicity include rice, wheat, maize, sugarcane, alfalfa and vegetable crops like, cucumber, carrot, fennel, watermelon, eggplant, tomato and pea (Singh et al. 1999b; Yu, 1999a; Batish et al. 2001). The problem of crop autotoxicity is particularly acute in croplands where tillage is not practiced.

Besides, residues of preceding crops also affect the performance of other crops through the release of allelochemicals (Singh et al. 2001; Batish et al. 2001). A variety of water-soluble or insoluble or volatile phytotoxins/allelochemicals are released by the crops and their residues. These accumulate in the soil and affect the germinating propagules of other crops growing in vicinity leading to serious repercussions on the quality and quantity of crop yield (Batish et al. 2001). Due to this reason, a number of traditional cropping practices like cover cropping, companion cropping, polyculture and green manuring, etc. need to be thoroughly revised. Besides, the crops are also known to affect growth and establishment through allelopathy that could be tapped for sustainable weed management (Singh et al. 2003b). In fact, a number of cover and smother crops and green manure crops grown in rotation or otherwise hold a great promise for the future weed management programmes (Foley, 1999).

A diversity of allelochemicals have been identified from crops that are released into environment (Table 1). The concentration of allelochemicals varies with age, cultivar, and plant organ, and their amount is often enhanced by various biotic and abiotic stress factors (Einhellig, 1996). Mechanism of action of crop allelochemicals is least understood because of their complex nature and origin, and synergistic action. However, some work has been done with sorgoleone, which is known to inhibit respiration (Rasmussen et al. 1992) and photosynthesis by acting as PSII inhibitor (Gonzalez et al. 1997). However, crops especially modern cultivars possess less quantity of allelochemicals compared to their wild relatives or accessions and co-existing weeds because they are highly specialized for yields rather than developing endogenous chemicals for defense purposes (Lovett, 1985). Thus the process of cultivation has eroded several useful genes such as those encoding allelopathic substances. However, molecular-genetic approaches and conventional breeding techniques could be useful in re-introducing them back (Foley, 1999). Crop allelopathy may be beneficial, if mechanism of crop-weed interference is well understood, traditional beneficial practices are revived and crops are improved for allelopathic property for selective management of weeds (Anaya, 1999; Foley, 1999; Batish et al. 2001).

Table 1. Allelochemicals identified from crops with allelopathic and weed suppressing ability.

Cover crop	Allelochemicals	Reference(s)
<i>Avena sativa</i> L. (Oat)	Scopoletin, Phenolic acids	Guenzi and McCalla, 1966; Rice, 1984
<i>Brassica</i> sp. (Turnip, Mustard, Radish, Rape etc.)	Glucosinolates and their breakdown products like isothiocyanates, nitriles, epithinitriles, ionic thiocyanates	Al-Khatib and Boydston, 1999; Brown and Morra, 1995
<i>Fagopyrum esculentum</i> Moench. (Buckwheat)	Fatty acids (Palmitic, Stearic, Arachidic, and Behenic acid), Fagomine, 4-Pipedone and 2-piperdinemethanol	Tsuzuki et al. 1987; Eskelsen and Crabtree, 1995; Iqbal et al. 2002
<i>Hordeum vulgare</i> L. (Barley)	Gramine, Hordenine	Liu and Lovett, 1993a,b; Hanson et al. 1981
<i>Ipomoea tricolor</i> Cav. (Pearly Gates Morning glory)	Tricolorin A	Anaya et al. 1990
<i>Medicago sativa</i> L. (Alfalfa)	Medicarpin, 4-methoxy medicarpin, sativan, 5-methoxy sativan, canavanine, saponins and phenolic acids like ferulic, chlorogenic and salicylic acid	Miller, 1983; Miller et al. 1988; Dornbos et al. 1990; Gorski et al. 1991; Wyman-Simpson et al. 1991; Waller et al. 1995
<i>Mucuna pruriens</i> (L.) DC (Velvetbean)	L-DOPA (L-3,4-dihydroxyphenylalanine)	Fujii, 1999a,b
<i>Secale cereale</i> L. (Rye)	2,4-dihydroxy-1,4 (2H)-benzoxazin-3-one (DIBOA) and 2(3H)-benzoxazolinone (BOA), β-hydroxybutyric acid, β-phenyllactic acid, phenolics acids (ferulic, <i>p</i> -hydroxybenzoic, salicylic, <i>o</i> -coumaric, and succinic)	Patrick, 1971; Chou and Patrick, 1976; Barnes et al. 1987; Shilling et al. 1985

Cover crop	Allelochemicals	Reference(s)
<i>Sorghum</i> sp. (Sorghum)	Sorgoleone ( <i>p</i> -benzoquinone), dhurrin (cyanogenic glycoside), breakdown products such as <i>p</i> -hydroxybenzoic acid and <i>p</i> -hydroxybenzaldehyde	Guenzi and McCalla, 1966; Nicollier et al. 1983; Netzley et al. 1988; Weston, 1996
<i>Trifolium</i> sp. (Clover)	Phenolics acids	Ohno and Doolan, 2001; Breland, 1996; Rice, 1995
<i>Triticum aestivum</i> L. (Wheat)	DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) and phenolic acids like syringic, vanillic, <i>trans</i> -ferulic, <i>cis</i> -ferulic, <i>trans</i> - <i>p</i> -coumaric, <i>cis</i> - <i>p</i> -coumaric, and <i>p</i> -hydroxybenzoic acid	Guenzi and McCalla, 1966; Shilling et al. 1985; Lodhi et al. 1987; Wu et al. 2000
<i>Vicia villosa</i> Roth. (Hairy vetch)	Cyanamide	Fujii, 1999a

### Allelopathic agroforestry trees

In addition to weeds and crops, trees are also an integral part of the agriculture under various intensive and extensive agroforestry programmes. In fact, agroforestry is now being practiced in a variety of climatic conditions. Several studies show that this practice increases productivity, improves soil quality, microclimate and nutrient cycling, conserves soil, manages weeds and increases overall sustainability (Nair, 1993; Young, 1989), though a number of negative interactions (including allelopathic) have also been recognized (Ong, 1991; Rao et al. 1997; Kohli et al. 1998a; Kohli et al. 2000). Rizvi et al. (1999) have listed over 80 trees grown under agroforestry programme that exhibit allelopathy. Some such tree species are *Acacia* spp., *Albizia lebbeck*, *Eucalyptus* spp., *Grewia optiva*, *Gliricidia sepium*, *Leucaena leucocephala*, *Moringa oleifera*, and *Populus deltoides* that affect the crop performance through the phenomenon of allelopathy (Kohli et al. 1998; Rizvi et al. 1999; Singh et al. 2001). In most of the instances, the litter from the tree interferes with the growth and establishment of the adjoining crop plants. Unfortunately, very little has been done to understand allelopathic implications in agroforestry systems, though it is an important factor in determining the success of trees, e.g., *Eucalyptus* sp., *Populus deltoides* and *Leucaena leucocephala* extensively grown under agroforestry programmes in India.

*Eucalyptus* sp. because of its fast growth, adaptability to varied environmental conditions and commercial value are grown world wide including India. However, a dramatic fall in the popularity of the tree has been seen in India because of its several adverse effects on the ecology and environment of the area, particularly if planted indiscriminately. Several studies have indicated that monoculture plantations of *Eucalyptus* to support little or almost negligible understorey vegetation (del Moral and Muller, 1969; Kohli, 1990). Allelopathy has been demonstrated to be the reason for depletion in floral diversity under *Eucalyptus* trees. Likewise, in agroecosystems *Eucalyptus* grown along field boundaries as windbreaks or shelterbelts or also as alleys is known to affect crop yield especially near the tree line (Jensen, 1983; Onywotu, 1985; Igbanugo, 1988; Kohli, 1990; Kohli et al. 1990; Singh and Kohli, 1992).

Kohli and his co-workers have observed a significant reduction in the growth and establishment of crops grown close to the tree line. They pointed out that up to 11m on the southern side from the tree line of *Eucalyptus*, a significant reduction in crop density, root and shoot length and biomass are visible and this is due to allelopathic effect of the tree (Kohli et al. 1990; Singh and Kohli, 1992). A number of volatile and non-volatile allelochemicals have been identified from the tree (Kohli, 1990). Among the volatile oils, various monoterpenes like cineole, limonene, citronellol, citronellal, grandinol and  $\alpha$ -pinene have been identified and found to be very toxic to the germination and growth of other plants (Kohli, 1990). The volatile oils rich in monoterpenes diffuse through the leaves, travel downwards as they are heavier than air and get adsorbed to the soil particles and affect vegetation. Among the non-volatile allelochemicals various phenolics acids have been identified from the tree especially from its bark, which are phytotoxic (Kohli, 1990). Under natural conditions the allelochemicals of *Eucalyptus* are continuously being added to the environment and thus affect the other plants including crops.

*Populus* is another fast growing tree gaining a lot of importance in India especially after the controversy of *Eucalyptus*. However, various species of *Populus* are also known to exert allelopathic effect on other plants including crop plants (Kohli et al. 2000). Among the various species *P. deltoides* is the most common one that is being grown in India. Several reports are available that indicate adverse effects of *P. deltoides* on the crops including wheat especially the loss of yield (Ralhan et al. 1992; Singh et al. 1998, 1999c). Allelopathy has been demonstrated to be the possible reason for this observed adverse effect (Singh, 1996; Kohli et al. 1997; Singh et al. 1998, 1999c). A number of allelochemicals including phenolics acid and salicin (a phenolic glucoside) were detected in the aqueous leaf extracts and soil collected from under the canopy of mature trees of *P. deltoides* (Singh, 1996).

*Leucaena leucocephala* is another fast growing nitrogen-fixing multipurpose tree that has been widely recommended for plantation under various agroforestry

programmes especially as hedgerows and under alley cropping systems. However, the plantations of *L. leucocephala* harbour very little understorey vegetation (Chou and Kuo, 1986). The aqueous extracts prepared from the leaves of the tree and litter mulched in soil are observed to have deleterious effects on a number of plant species including crops like sorghum, cowpea and sunflower (Suresh and Rai, 1987). Singh et al. (1999a) observed that the litter collected from A and A<sub>0</sub> horizon under the trees of *L. leucocephala*, when mulched into the soil severely affects the growth and development of the maize and it was attributed to the allelopathic interference of the plant. Various studies have shown that mimosine – a non-protein amino acid, and a number of phenolic acids impart allelopathic property to this tree (Kuo et al. 1983; Chou and Kuo, 1986).

From the above discussion it is clear that allelopathy (especially of trees) plays a significant role in agroforestry systems. However, if properly understood and the nature of chemicals involved therein is elucidated, such mechanisms can be effectively exploited to enhance the crop productivity through management of weeds, nematodes, pathogens and insects (Kohli et al. 1998; Rizvi et al. 1999). Allelochemicals from agroforestry trees can be used for controlling the weeds, e.g., volatile oils from *Eucalyptus* sp., mimosine from *Leucaena* spp. (Kohli et al. 1998a,b). Agroforestry can, therefore, be manipulated to make agroecosystems sustainable through proper management and / or mulching of the litter of the trees growing in agroecosystems to improve the soil quality, conserve moisture and bring about the cooling effect.

## **Microbes and allelopathy in agroecosystems**

Microbes have a greater effect on the allelopathic activity of higher plants as they are known to alter and/or transform the amount of allelochemicals, particularly the phenolics. However, their role depends upon the available carbon source and other environmental factors (Blum et al. 1999). The microbes may affect allelochemicals by addition or deletion of side groups, polymerization, production of other organic molecules and/or incorporation of carbon from other phenolic compounds into microbial biomass (Martin and Haider, 1976; Blum et al. 1999). This results in change in activity of allelochemicals. Further, in the soil the preferential utilization of carbon sources may also affect the plant-microbe-soil system and the allelopathic phenomenon (Pue et al. 1995). The allelochemicals from microorganisms are generally non-specific and inhibit the growth of several annual and perennial species (Cutler, 1988; Hoagland, 1990). They may be effective at very low concentration, i.e. cycloheximide at 1 µg/l (Heisey et al. 1988) and have variable effect on the different cultivars (Alstrom, 1991). Alternatively, allelochemicals may influence the growth of microbes positively or negatively and, thereby indirectly interfering with the availability of nutrients, particularly nitrogen and phosphorus, in the soil (Blum et al.

1999; Anaya, 1999). Besides, phenolic compounds released in soil from decomposing residues may cause microbial imbalance (Chou, 1999). In orchards microbes play an important role in replant problem besides the autotoxicity (Singh et al. 1999b). Presence of *Penicillium expansum* in apple orchards facilitates the release of allelochemicals (Berestetsky, 1972). Vesicular Arbuscular Mycorrhizae (VAM) also helps in changing rhizosphere microflora and increasing biomass of apple seedling (Catska, 1994). In peach orchards, nematodes are known to play an important role in releasing and hydrolyzing amygdalin – a cyanogenic compound causing autotoxicity and replant problem. In *Asparagus* it has been observed that allelochemicals synergist with fungal pathogens thereby markedly increasing the disease incidence (Peirce and Colby, 1987).

## **ALLELOPATHY FOR LONG-TERM SUSTAINABILITY OF AGROECOSYSTEMS**

### **Use of cover and smother crops**

Indiscriminate use of synthetic chemicals has deteriorated the quality of modern agroecosystems. This has not only resulted in residual problem in soil environment but also the human health is at stake. Allelopathy can play an important role in restoring their natural balance by managing weeds and pests in a sustainable manner (Weston, 1996; Anaya, 1999; Chou, 1999; Wu et al. 1999; Batish et al. 2001; Singh et al. 2001; Singh et al. 2003b). Several strategies such as use of cover and smother crops, use of green manure crops and use of allelochemicals directly for the management of pests and weeds are followed. Some of the important cover and smother crops exhibiting allelopathy along with their allelochemicals are given in Table 1. If suitably manipulated, these allelopathic cover and smother crops can considerably reduce the weed species in the croplands and thus may provide a non-herbicidal method of weed control (Weston, 1996; Batish et al. 2001; Singh et al. 2003b). Besides, the cover crops also help in enhancing the biomass. Genetic approaches should, therefore, be explored for crop improvement and development of molecular markers to aid in breeding cover crops with allelopathic traits so as to bring about weed suppression (Foley, 1999).

### **Green manure crops for weed management**

*Green manure crops* not only improve soil through addition of organic matter but can also be used for the purpose of weed management, for example, some legumes and cruciferous plants (Al-Khatib and Boydston, 1999). Allelopathy is known to play an important role in the weed suppression by green manure crops. Both leguminous and

non-leguminous green manure crops are known; however, the leguminous ones additionally improve the soil nitrogen. Among the legumes *Trifolium* sp. are best known as green manure crops (Table 2). Among non-legumes, several members of family Brassicaceae viz. field mustard (*Brassica campestris* L.), white or yellow mustard (*Brassica hirta* Moench), brown/Indian mustard (*B. juncea* [L.] Czern.), rapeseed/oilseed rape/canola (*B. napus* L.), black mustard (*B. nigra* [L.] Koch.), and garden cress (*Lepidium sativum* L.) are excellent source of green manure and effectively control weeds (Boydston et al. 1994; Al-Khatib and Boydston, 1999). This is attributed to the presence of volatile glucosinolates in them and their breakdown products such as isothiocyanates, nitriles, epithinitriles, and ionic thiocyanates (Vaughan and Boydston, 1997). These are the most common and potent germination inhibitors and can be used as promising bioherbicides (Brown and Morra, 1995, 1999; Vaughan, 1999). However, the extent of weed suppression by crucifers depends upon species, cultivar, and the seed size of the target species (Al-Khatib and Boydston, 1999). Even the glucosinolate content varies with the species and cultivar type (Eberlein et al. 1998).

Table 2. Green manure crops showing weed-suppressing ability.

Green Manure Crop	Weeds	Reference
<i>Brassica napus</i>	Common lambsquarters ( <i>Chenopodium album</i> L.), redroot pigweed ( <i>Amaranthus retroflexus</i> L.), hairy nightshade ( <i>Solanum sarachoides</i> Sendtner), longspine sandbur ( <i>Cenchrus longispinus</i> [Hack.] Fern.), shepherd's purse ( <i>Capsella bursa-pastoris</i> [L.] Medic.), kochia ( <i>Kochia scoparia</i> [L.] Schrad.) and grassy weeds like barnyard grass ( <i>Echinochloa crus-galli</i> [L.] P. Beauv.) and green foxtail ( <i>Setaria viridis</i> [L.] Beauv.)	Boydston and Hang, 1995; Al-Khatib et al. 1997
<i>B. hirta</i> and <i>B. juncea</i>	Shepherd's purse, Kochia, redroot pigweed, and green foxtail	Krishnan et al. 1998
<i>B. rapa</i> , <i>B. napus</i>	Scentless mayweed ( <i>Matricaria inodora</i> L.) and spiny sowthistle ( <i>Sonchus asper</i> [L.] Hill.)	Petersen et al. 2001

Green Manure Crop	Weeds	Reference
<i>Trifolium incarnatum</i> L. and <i>T. pratense</i> L.	Common lambsquarters, pitted morning glory ( <i>Ipomoea lacunosa</i> L.) and wild mustard ( <i>Sinapis alba</i> L.)	White et al. 1989; Dyck and Liebman, 1994; Ohno and Doolan, 2000

## Allelopathic crop residues and weed control

Residues of several crops like wheat, rice, sorghum, barley, and sunflower are also known to suppress weeds through their allelopathic effects. The presence of crop residues on the soil surface as mulch suppresses weeds and reduces reliance on herbicides (Weston, 1996; Batish et al. 2001). Generally, the effect of crop residues for weed management declines after 4-6 weeks owing to loss of residue mass and breakdown of allelochemicals (Kimber, 1973; Smeda and Weller, 1996). Jones et al. (1999) reported that residues of crops such as barley, wheat, and canola reduce the germination, growth and biomass of several weeds and among them barley was most effective.

In case of **wheat**, residues mulched into the soil suppress the growth of redroot pigweed, spiny amaranth (*Amaranthus spinosus* L.), tall morningglory (*Ipomoea purpurea* [L.] Roth.), and barnyard grass in the next season crop (Banks and Robinson, 1980; Thilsted and Murray, 1980; AlSaadawi, 2001). Weed control by wheat residues, however, depends upon the amount of soil covered by the residues. A soil cover of 60% with wheat residues was found to be optimum for suppressing the next-season weeds (Bilalis et al. 2003). Inhibitory activity of wheat mulch is due to the allelopathic interference (Worsham, 1984) and the weed suppressing effect persists up to 6 weeks (AlSaadawi, 2001). A number of allelochemicals such as DIMBOA and phenolic acids have been identified from the wheat (Table 1). Likewise, residues of **rice** when incorporated into the soil reduce the incidence of both grassy and broad-leaved weeds (Khan and Vaishya, 1992; Lin et al. 1992). The inhibitory effect of rice residues is due to the release of allelochemicals from them. A number of allelochemicals including phenolic acids have been identified from rice residues under flooded, upland and under waterlogged conditions (Mattice et al. 1998; Chou and Lin, 1976; Chou et al. 1981), and momilactone from the root exudates (Kato-Noguchi et al. 2002). Even the residues from *Sorghum* spp. are observed to reduce the incidence and growth of a number of weed species (Putnam and DeFrank, 1983; Panasiuk et al. 1986; Hoffman et al. 1996; Cheema and Khalil (2000)) Sorghum residues release a number of allelochemicals such as sorgoleone, cyanogenic glycosides – dhurrin, and a number of breakdown products that bring about weed suppression (Table 1). Residues from alfalfa and their aqueous extracts inhibit the germination and growth of a

number of broad-leaved and grassy weeds (Chung and Miller, 1995; Xuan et al. 2001, 2003). Though crop residues hold a great potential as weed management strategy, yet there have been reports that allelochemicals released from them may also affect the growing crops (Rice, 1984, 1995; Batish et al. 2001).

## **ALLELOCHEMICALS FROM HIGHER PLANTS AS SOURCE OF HERBICIDES**

A number of allelochemicals found in plants and microbes have a potential to be used as herbicides either directly or indirectly by serving as lead compounds for the synthesis of new classes of herbicides (Duke et al. 2002; Singh et al. 2003b). The most promising ones include volatile monoterpenes, sesquiterpene lactones, benzoxazinones, and sorgoleone. Among these, monoterpenes possessing isoprenoid ring have been extensively explored for weed management programmes (Vaughan and Spencer, 1993; Singh et al. 2002a,c; Singh et al. 2003b; Singh et al. 2004). Cineoles (both 1,4- and 1,8-) inhibit germination and growth of weeds like sicklepod (*Cassia obtusifolia* L.) and barnyard grass (*Echinochloa crus-galli* [L.] Beauv.) with through different modes of action (Romagni et al. 2000). Cineole and citronellal have been reported to suppress germination, growth, chlorophyll content, and respiratory activity of billy goat weed (Singh et al. 2002c). Among sesquiterpene lactones, artemisinin and parthenin and their derivatives have been shown to possess herbicidal activity exhibiting phytotoxicity against a number of weedy species (Dayan et al. 1999; Batish et al. 2002; Singh et al. 2002d). Parthenin suppresses the growth of weeds like billy goat weed, wild oat (*Avena fatua* L.), hairy beggars tick (*Bidens pilosa* L.), and a number of aquatic weeds (Pandey, 1996; Batish et al. 1997, 2002). Singh et al. (2002d) have shown that besides reducing the growth of billy goat weed, parthenin also adversely affects the content of various macromolecules and impairs the activities of enzymes. Thus, parthenin can provide a useful template for the synthesis of novel herbicide.

Benzoxazinoids—cyclic hydroxamic acids such as DIBOA and DIMBOA—present in a number of cereal crops like wheat, maize and rye are known to inhibit weeds like barnyard grass, crabgrass (*Digitaria* spp.), prosomillet (*Panicum miliaceum*), and wild oat (Barnes and Putnam, 1987). Sorgoleone found in the root exudates of *Sorghum* species is another important chemical belonging to the class of benzoquinones. It can be used as both pre- and post-emergent herbicide and is phytotoxic towards a number of weedy species (Weston and Czarnota, 2001).

## PHYTOTOXINS FROM MICROBES WITH WEED SUPPRESSING ABILITY

Both pathogenic as well as non-pathogenic microbes are also source of potential herbicides and a large number of microbial isolates are being screened for this purpose (Hoagland, 2001; Mallik, 2001; Singh et al. 2003b). Phytotoxins of microbial origin belonging to both host selective and non-host selective species have earlier been reviewed (Lynch, 1976; Omura, 1986; Hoagland, 2001; Duke et al. 2002; Mallik, 2001). Unfortunately, little attention has been paid to them mainly owing to their cultural problems and host specificity. Some of the potent sources of microbial phytotoxins with herbicidal activity are *Streptomyces* spp. (particularly *S. hygroscopicus*, and *S. viridochromogenes*), *Alternaria alternata*, *Fusarium* spp., and *Dreschlera* spp., (Hoagland, 2001). The most successful example of microbial products with herbicidal potential is bialaphos and its breakdown product or herbicidal moiety phosphinothricin produced by *Streptomyces viridochromogenes* and *S. hygroscopicus*. Bialaphos is a proherbicide, which gets break down into phosphinothricin in the target species. Only L-isomer – the natural form of phosphinothricin, is inhibitory. Both bialaphos and phosphinothricin have short environment half-life of 2-3 days and 4-7 days, respectively, in soil. The herbicidal activity of bialaphos can be enhanced in combination of nitrogen fertilizers. Studies have shown that phosphinothricin does not have any genotoxic, carcinogenic or any other toxicological hazards towards non-target species (Hoagland, 2001). Bialaphos is effective in controlling a number of dicot and monocot weeds faster than glyphosate but slower than paraquat (Duke, 1986). The commercial herbicidal formulation of phosphinothricin is known as glufosinate, which is its ammonium salt. It is produced and marketed by several companies globally and is a most successful natural herbicide. Since it is non-selective in nature, several crop plants have been made resistant to it transgenically. The general symptoms of glufosinate are chlorosis and wilting within 3-5 days after application. Phosphinothricin is a potent inhibitor of glutamine synthetase. Both bialaphos and phosphinothricin are unique in being antibiotic and herbicidal.

Hundreds of potent microbial phytotoxins have been patented, yet only a few have been commercialized/marketed owing to their mammalian toxicity and other toxicological implications. For example, AAL-toxin was earlier known to be host specific and pathogenic only to recessive genotypes of tomato, but later it was found to be effective against a number of weed species exhibiting a strong herbicidal activity (Abbas et al. 1995). However, it is also toxic to animals, hence not used as a herbicide.

Lichens though not microbes are another source of secondary metabolites, particularly usnic acid and anthraquinones. These are unique chemicals exhibiting new

molecular target sites and herbicidal activity (Dayan and Romagni, 2001). Usnic acid inhibits p-hydroxyphenylpyruvatedioxygenase (HPPD) – an enzyme of carotenoid biosynthesis. In vitro activity of usnic acid is found to be superior to any other inhibitor of this molecular target site. Among lichen derived anthraquinones, emodin and rhodocladonic acid are highly active against seedlings of higher plants, particularly grasses. Because of their simple chemical nature and easy synthesis these compounds are ideal candidates for the production of new herbicides (Dayan and Romagni, 2001).

## **ALLELOPATHY AND CROP IMPROVEMENT**

From the above discussion it is clear that in agroecosystems a multitude of allelopathic interactions occur between crops, weeds, trees and microbes. A number of crop plants when used as cover/green manure/smother crops suppress the weeds in the same or in the next season crops. Different accessions of crops, even varieties, differ in their allelochemical content providing them varying level of selective advantage against weeds (Wu et al. 1999; Singh et al. 2003b).

Even the wild accessions of present day modern cultivars contain more allelochemical, which were lost due to selection pressure. However, these can be reintroduced using various modern day recombinant DNA technologies. As a result the wild accessions and cultivars of a number of crops (mainly rice, wheat, sorghum and rye) are being screened with a view to select those with higher allelochemical content vis-à-vis weed suppressing ability (Singh et al. 2003b). Among these majority of work has been done in rice. In USA, Dilday et al. (2001) screened 17,279 rice accessions collected from 110 countries for their weed suppressing ability against major weeds of rice. Of these, 145 accessions of rice exhibited allelopathy against redstem (*Ammania coccinea* Rottb.), 412 against ducksalad (*Heteranthera limosa* [Sw.] Willd.) and 94 against barnyard grass. Such accessions with higher allelopathic potential can be used for breeding purposes. In wheat, accessions have been screened for their activity against weeds especially ryegrass (*Lolium* spp.) and weed suppressing ability is being correlated with the amount of allelochemical content in them particularly phenolics acids (Wu et al. 2002). Likewise, the accessions are being screened for higher content of hordenine and gramine in barley and sorgoleone in *Sorghum* (Singh et al. 2003b). Based on the screening various crop improvement programmes have been initiated using molecular-genetic techniques but little success has been achieved probably due to the quantitative characters of allelopathic traits and polygenic nature of their inheritance. In this direction, Duke et al. (2001) and Scheffler et al. (2001) have suggested two approaches: increasing allelopathic potential of a

particular crop, and producing allelopathic transgenics by inserting genes encoding for a particular allelochemical. For the first approach, particular genotypes are selected with the help of DNA markers such as PCR, RFLP, RAPD, and AFLP. It has been successfully used in rice for phenotype selection (Rimando et al. 2001), and for production of glucosinolates in rapeseed (Toroser et al. 1995). Even various plant genomic and proteomic techniques can be utilized for enhancing their weed suppressing ability (Birkett et al. 2001).

## ALLELOPATHY AND PEST MANAGEMENT

Besides weed management, allelopathic interactions and the allelochemicals (in pure or crude form) can also be utilized for the management of various pests such as diseases, nematodes, fungi and insects (Rice, 1995; Cutler, 1999; Rizvi et al. 1999; Singh et al. 2001). A number of wild plants and weed species are reported to have antifungal activity against phytopathogenic fungi (Al-Abed et al. 1993; Qasem and Abu-Blan, 1995; Qasem, 1996). In fact, natural plant products and allelochemicals offer one of the best environmentally sustainable method of plant disease control. Allelochemicals released from the residues of allelopathic vegetable crops can greatly reduce the incidence of soil-borne pathogens (Yu, 1999b). Gaspar et al. (1999) reported that triterpenoids and other allelochemicals released from the wheat straw play an important role in plant defense by acting as signal transducers.

DIMBOA and DIBOA - the allelochemicals in wheat, maize and rye, also play an important role against the harmful predators such as insects, fungi and even bacteria (Friebe, 2001). Allelochemicals from the weeds such as *Ranunculus asiaticus* also inhibit the growth and development of many plant pathogenic fungi and bacterial species (Qasem, 1996). *Brassica* species are widely known for their antipesticidal properties as they to inhibit the growth and development of a large number of soil-borne phytopathogenic fungi, and this is attributed to the presence of glucosinolates and their breakdown products (Olivier et al. 1999; Vaughn, 1999). Since the breakdown products of *Brassica* are volatile in nature they have also been used as potential biofumigants. Likewise, saponins present in the roots of higher plants like alfalfa, unripe berries of pokeweed (*Phytolacca dodecandra* L'Herit), and fruits of snake bean (*Swartzia madagascariensis* Desv.) possess fungicidal properties against a number of phytopathogenic fungi (Oleszek, 1999; Waller, 1999). Likewise, the fungicidal property of a number of flavonoids has been reported against a number of seed borne fungi (Weidenborner et al. 1992; Dixon, 1999). Further, a number of allelopathic compounds from microbes are found to possess fungicidal potential against a number of phytopathogens (Hoagland, 2001). Several bacterial secondary metabolites are also known to suppress the diseases of roots. Of late attempt are being made to transfer genes synthesizing such metabolites into plants through DNA

recombinant technology to produce transgenic plants that will have natural protection against pathogens and this method is environmentally safe (Weller and Thomashow, 1999). Neem (*Azadirachta indica* A. Juss.) a well-known Indian tree possesses a number of active principles like azadirachtin that have a potential to kill pathogens (Ghewande, 1989). Its seed cake, seed and fruit extract, seed kernel powder and seed oil have been reported to control a wide spectrum of fungal pathogens (Gunasekaran et al. 1986; Jeyarajan et al. 1987; Srivastava et al. 1997). Water and ethanol extracts from the leaves and oil extracts from the seeds of neem significantly reduced the radial growth of *Pyricularia oryzae* causing blast disease of rice under *in vitro* conditions and the effect was comparable to commercial fungicide cabendazim at the concentration of 0.1% used under *in vivo* conditions (Amadioha, 2000).

Allelopathy can also be exploited for the control of harmful insects (Anaya, 1999). Allelochemicals deter insects by acting as metabolic poisons or as feeding deterrents (Brattsten, 1986). Rice extracts have been reported to be active against brown planthopper *Nilaparvata lugens* (Zhang et al. 1999). Azadirachtin is known to be effective against nearly 300 insect pests (Devakumar and Parmar, 1993). It acts as a phage and oviposition deterrent, repellent, anti-feedant, growth retardant, etc. (Schumutterer, 1995). Remarkably, it is relatively non-toxic to warm-blooded animals including human beings.

Likewise, allelochemicals from the plants have a great potential to combat the nematodes. Some of the important ones include neem (Alam and Khan, 1974), *Coffea* species (Tronocorn et al. 1986), *Leucaena leucocephala* (Jain and Hassan, 1985), and velvet bean (Reddy et al. 1986). Green manure of rapeseed, extracts from black mustard and glucosinolates (mainly AITC) have been reported to be effective against a number of nematodes like *Meloidogyne chitwoodi*, *Caenorhabditis elegans*, *Heterodera schachtii*, and *H. rostochiensis* (Mojtahedi et al. 1991, 1993; Lazzeri et al. 1993; Vaughan, 1999).

## CONCLUSION AND FUTURE DIRECTIONS

From the above discussion, it is clear that allelopathic interactions among crops, weeds, trees and microbes play an important role in the agroecosystems thereby resulting in a decline in crop productivity, problem of soil sickness and depletion of biodiversity. However, the phenomenon and the chemicals involved in it (allelochemicals) can also be exploited or manipulated for enhancing crop production, if its mechanism is well understood. Some of the major approaches by which the allelopathy in agroecosystems can be utilized are:

- Changing the cropping pattern and cultural practices such as selection of crops with divergent life cycle, and selective allelopathic potential in rotational sequence to check the build-up of weeds in the fields.
- Breeding of cover and smother crops with more allelopathic potential and content so as to have a sustainable weed management.
- Molecular genetic approaches for the improvement of allelopathic crops (transgenically as well as non-transgenically) can further expand their use and enhance efficiency (Foley, 1999; Scheffler *et al.* 2001; Duke *et al.* 2002; Singh *et al.* 2003b). In this direction efforts can be made to transgenically design cover crops that can undergo self-destruction and thus reduce the need for use of herbicides.
- Use of allelochemicals directly as herbicides is another useful strategy that can solve some of the problems associated with the use of synthetic herbicides or at least can reduce their usage. Although this seems to be lucrative endeavour, yet a number of difficulties could be anticipated. The very fact that natural plant products are safer from toxicological point of view seems paradoxical when the most toxic compounds to human beings are from the plants, e.g., toxins like brevitoxin, AAL-toxin, and fumonisin, and ricin.  $\alpha$ -terthienyl, a natural compound found in *Tagetes* sp., although possesses weed-suppressing properties but has mammalian toxicity. Care should therefore be taken in screening the natural plant products. Further, the extraction and isolation of allelochemicals is a complicated task. In spite of best available technology and informatics the extraction of allelochemicals remains lengthy and costly. Besides, the quality of allelochemicals with short half-life becomes a bane if the time is too short to bring about the desired effect. There is also a need to develop proper bioassay and dose-response curves. Nevertheless, these problems can be taken care of by employing various molecular genetic techniques. Lastly, there are practical difficulties in marketing, patenting, and the intellectual property rights associated with the use of rare organisms brought from other places and these involve huge costs (Duke *et al.* 2002).

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## CHAPTER 22

# PLAYING WITH CHEMISTRY: STUDIES ON *OROBANCHE* spp. GERMINATION STIMULANTS

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## INTRODUCTION

The genus Orobanche was first described by Linnaeus in 1753 and comprises up to 60 different species spread out of the Mediterranean countries, east Africa, and part of Asia (Thieret, 1971). Broomrapes (*Orobanche* spp.) are common parasitic weeds affecting many important crops (Table 1). These holoparasites depend of their hosts to get the nutrients and to complete their life cycle, as they are not able to fix carbon through photosynthesis. The host-parasite relationship is very specific and the recognition mechanism yet is not well understood. Two well-differentiated phases can be distinguished in their life cycle. The independent phase covers seed dispersion, the latent phase, and the seed-conditioning period. During this period the plant does not need the presence of any host in the vicinity. However, this turns to be necessary during seed germination and the development of the plant.

Among broomrapes, *O. cernua* and *O. cumana* are species of major interest due to economical reasons. *O. cernua* was described by Linnaeus in 1758 on materials collected in Spain while *O. cumana* was cited one century later (1825) on plants collected on desert areas of south-western Asia and south-eastern Europe. Initially, *O. cumana* was considered as a variety of *O. cernua* and their main distribution areas are coincident to the Mediterranean countries and central Asia, both being most frequently found parasiting plants of the genus *Artemisia*. After the introduction

sunflower cultivation in Europe, plants of *O. cumana* were detected parasitizing sunflower in central-Europe (Encheneva and Sindrova, 1994), wide spreading later to other countries and being reported in the Iberian Peninsula in 1958 (Díaz-Celayeta, 1974).

Table 1. Main *Orobanche* species and their common hosts

<b><i>Orobanche</i> species</b>		<b>Host</b>
<i>O. cumana</i>	Compositae	<i>Helianthus annuus</i> (sunflower)
<i>O. cernua</i>	Compositae	<i>Artemisia</i> spp.
	Solanaceae	<i>Lycopersicon esculentum</i> (tomato)
	Compositae	<i>Nicotiana tabacum</i> (tobacco)
<i>O. crenata</i>	Compositae	<i>Carthamus tinctoris</i> (safflower)
	Leguminosae	<i>Vicia faba</i> (broad bean )
		<i>Pisum sativum</i> (green pea)
		<i>Lens culinaris</i> (lentil)
	Umbelliferae	<i>Cicer arietinum</i> (chickpea)
		<i>Daucus carota</i> (carrot)
		<i>Apium graveolens</i> (celery)
<i>O. ramosa/O. aegyptiaca</i>	Alliaceae	<i>Allium cepa</i>
	Compositae	<i>Lactuca sativa</i> (lettuce)
		<i>Helianthus annuus</i> (sunflower)
	Cruciferae	<i>Brassica napus</i> (colza, canola)
	Cucurbitaceae	<i>Cucurbita pepo</i> (pumpkin)
		<i>Cucumis melo</i> (melon)
	Leguminosae	<i>Vicia faba</i> (broad bean )
		<i>Pisum sativum</i> (green pea)
		<i>Lens culinaris</i> (lentil)
		<i>Cicer arietinum</i> (chickpea)
	Solanaceae	<i>Lycopersicon esculentum</i> (tomato)
		<i>Nicotiana tabacum</i> (tobacco)
		<i>Solanum tuberosum</i> (potato)
		<i>Solanum melongena</i> (eggplant)
	Umbelliferae	<i>Daucus carota</i> (carrot)
		<i>Apium graveolens</i> (celery)
<i>O. minor</i>	Compositae	<i>Carthamus tinctoris</i> (safflower)
		<i>Lactuca sativa</i> (lettuce)
	Leguminosae	<i>Vicia faba</i> (broad bean )
	Solanaceae	<i>Nicotiana tabacum</i> (tobacco)
	Umbelliferae	<i>Daucus carota</i> (carrot)
		<i>Apium graveolens</i> (celery)

These two species have been recently described as two differentiated species based on their respective seed fatty acid contents (Pujadas-Salvá and Velasco, 2000).

*O. cumana* is a serious threat to sunflower plantations, causing enormous damages and almost the total lost of the crop. In fact, *O. cumana* is found in the Iberian Peninsula parasitizing sunflower exclusively in cultivated lands, while *O. cernua* is typical of arid scrub areas, parasitizing *Artemisia* spp. At present time, there are no satisfactory methods available to control this weed. On the other hand their areas of distribution are increasing every year, especially in Eastern Europe, the Iberian Peninsula, and other important sunflower cultivation areas. Thus, the development of broomrape control methods are in great need and constitutes a subject of interest for many research groups (Joel, 2001).

## Life cycle

Probably, the most interesting part of the parasitic weeds life cycle for allelopathy researchers is the parasitic phase. During this period, and prior to germinate, the seed needs to “know” if there is any available host in its vicinity; once it germinates, the radicle has to grow in the appropriate direction to get in contact with the host root; and when the root of the parasite gets in touch with its host, the broomrape develops the haustorium. This organ allows the weed to attach to the host root tissue, and to penetrate trying to connect to the vascular tissue. If this process succeeds, the parasite starts to get the nutrients from its host, thus allowing its development.

One important feature of the host-parasite interaction is its high specificity. Each parasite recognizes only its host(s) and its success depends on the ability of the parasite to recognize and attack the host plant and to breakdown their defense mechanisms. The interaction between the host and the parasite is chemically mediated and represents a clear example of allelopathy: it is well known that plants exude many different kinds of compounds for several purposes (self-defense, excretion ...) and the parasite might recognize certain chemicals from this pool, using them as chemical clues. Consequently, germination takes place when the receptors in the seed detect the presence of certain chemicals, and this triggers the cascade of events that lead to germination. The growth of the radicle follows a concentration gradient being another example of chemotropism (Press and Graves, 1995 and Eastbroo and Yoder, 1998).

When the radicle of the pathogen gets in touch with the host root the parasite develops a new organ called haustorium. The role of this new structure is to facilitate the penetration through the host tissues and it seems to be also chemically regulated in the case of *Striga* spp. (Lynn et al. 1981 and Steffens et al. 1982). However, this has

not yet proven in the case of *Orobanche* spp. The excretion of hydrolases and other enzymes allows the parasite to penetrate through the cortex and connect with the vascular system in the central section of the root (Joel and Losner-Goshen, 1994 and Loshner-Goshen et al. 1998).

## GERMINATION STIMULANTS

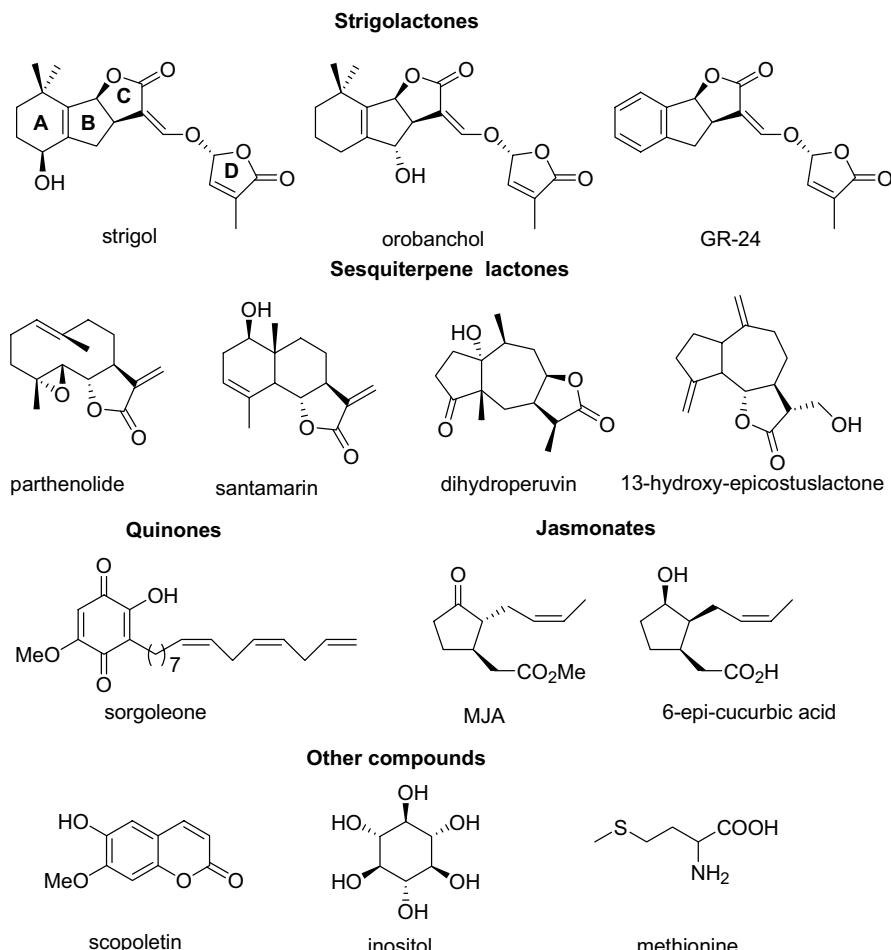


Figure 1. Germination stimulants of broomrape and witchweed species.

The germination inductors of *Striga* spp. and *Orobanche* spp. isolated from host and non-host sources have been recently reviewed (Galindo et al. 2003). They can be broadly classified into five structural groups: strigolactones, sesquiterpene lactones, quinones, jasmones, and other compounds (Figure 1).

Among these compounds, only strigolactones and the sorgoleone-related quinones have been isolated and characterized from host plants. All strigolactones induce the germination of *Orobanche* and *Striga* spp. indistinctly. The rest of compounds, including the synthetic strigolactones analogues named as GR, have not been obtained from host sources and many of them are synthetic – especially many sesquiterpene lactones. Consequently, it seems to be a good starting point to hypothesize that strigolactones should match target protein requirements as the chemical signals.

A classic hypothesis to explain the chemical mechanism by which strigolactones induce germination proposes a Michael addition of a nucleophile present in the receptors' cavity to the lactone-enol- $\gamma$ -lactone moiety (rings C and D, Figure 1) followed by a retro Michael reaction, liberating 4-carboxy-2-methyl-2-butenoic acid (Figure 2) (Mangnus and Zwanenburg, 1992b). Whether this acid plays or not a role in the sequence of events leading to germination is a point that has not been addressed yet.

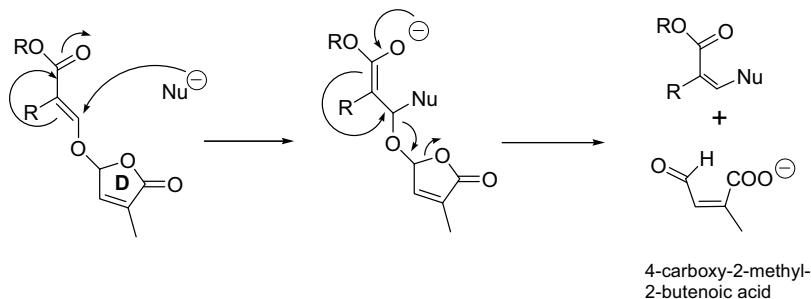


Figure 2. Mechanism proposed to explain the activity of strigolactones.

In this scheme, the lactone-enol- $\gamma$ -lactone moiety is proposed as the bioactiphore. Structure-Activity Relationship (SAR) studies reveal this chemical system as necessary for the activity (Mangnus and Zwanenburg, 1992a). The introduction of changes such as the substitution of the oxygen atom in the enol system by a carbon atom leads to the loss of the activity (Thuring et al. 1997a). However, if the enol ether moiety is maintained, changes in the stereochemistry of the double bond or addition of small groups do not adversely affect the activity (Thuring et al. 1997b).

## **PLAYING WITH CHEMISTRY: THE SPECIFIC INTERACTION OF SESQUITERPENE LACTONES AND *O. CUMANA***

One of the questions that remain unsolved is the specificity of the host-parasite interaction. Few natural germination inducers have been isolated from hosts so far and, with the only exception of sorgoleone, all of them are sesquiterpenes of the strigolactone type (Figure 3). In almost all cases, the parasitic weed species studied belong to the families *Striga* and *Orobanche* (Galindo et al. 2003). It is also true that many other natural and synthetic compounds present positive activities as germination inducers of *Striga* and/or *Orobanche* species (Figure 1). However, the question is which are the structural or molecular clues that trigger the germination cascade of events? Are these structural requirements the same for all *Striga* and *Orobanche* species?

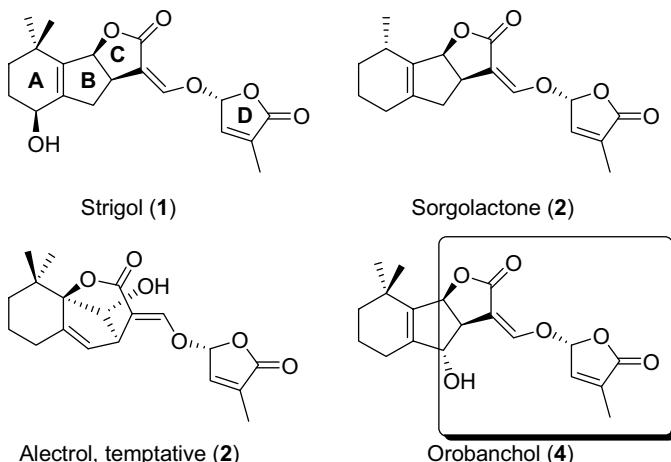


Figure 3. Natural strigolactones. The lactone-enol- $\gamma$ -lactone moiety is highlighted.

Model studies with witchweed (*Striga asiatica*) reported that the  $\gamma$ -butyrolactone (ring D, Figure 3) and the enol- $\gamma$ -lactone (rings C and D, Figure 3) moieties are crucial for the activity in strigolactones (Mangnus and Zwanenburg, 1992a). However, studies with sesquiterpene lactones showed these compounds (Figure 4) to effectively induce the germination of *S. hermonthica* and *S. asiatica* (Fisher et al. 1989, Fisher et al. 1990 and Rugutt and Rugutt, 1997).

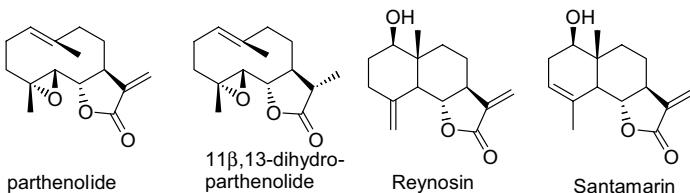


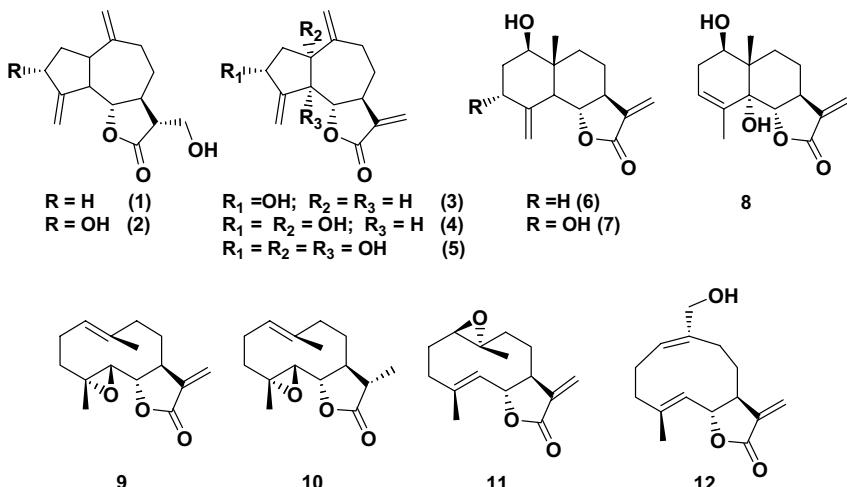
Figure 4. Sesquiterpene lactones that induce germination of witchweed.

Sesquiterpene lactones do not present the second  $\gamma$ -butyrolactone moiety; also, many of them do not have the unsaturated carbonyl system in the lactone ring but just the intramolecular ester. The first consequence of these differences is that the bionucleophile Michael addition model do not apply in this case. However, parthenolide and dihydroparthenolide easily undergo transanular cyclization through the epoxide moiety and it has been hypothesized that the resulting carbocation could be trapped by the thiol or amino group in the bionucleophile (Rugutt and Rugutt, 1997). In this case, the final compound is the target protein attached to the germination inductor – as strigolactones does, but not any butenoic acid is released.

With these data in hand, we initially tested a wide array of sesquiterpene lactones (Figure 5) on sunflower (*Orobanche cumana*) and tobacco (*O. ramosa* and *O. aegyptiaca*) broomrapes. Compounds tested present guianolide (**1-5**), eudesmanolide (**6-8**), *trans,trans*-germacranolide (**9-11**) and melampolide (**12**) backbones (Pérez de Luque, 2000). Surprisingly, compounds tested –when active – specifically stimulate germination of sunflower broomrape but they were inactive with the other two species.

Such a high specificity was initially linked with the high content in sesquiterpene lactones of sunflower, and then we hypothesized that the recognition chemical signals for the sunflower - *O. cumana* system were sesquiterpene lactones. This idea is further supported by the fact that guianolides, heliangolides, and *trans,trans*-germacranolides are common constituents of sunflower (Gershenson and Mabry, 1984 and Vyvyan, 2002). Consequently, we decided to introduce several modifications in the guianolide and germacranolide backbones to perform SAR studies. Michael addition of a hydroxyl group under conditions developed by our group (Macías et al. 2000b) lead to compounds **1** and **2**; allylic oxidation using selenium dioxide under controlled conditions yield compounds **3-5** (Macías et al. 2000a); cyclization of costunolide under mild acid conditions led to reynosin (**6**) and allylic oxidation of the resulting product with  $\text{SeO}_2$  provided compound **7**; reduction of the double bond of parthenolide (**9**) with sodium borohydride afforded compound **10**; epoxidation in a buffered media

with MCPBA gave **11**; and allylic oxidation of costunolide led to the melampolide **12** (Macías et al. 1992). Bioassays showed these compounds to be very active inductors of *O. cumana* germination but inactive with the other broomrape species tested (*O. crenata* – green pea parasite, and *O. ramosa* – tobacco parasite) (Galindo et al. 2002).



*Figure 5.* Compounds tested on *O. cumana*, *O. ramosa*, *O. aegyptiaca*, and *O. crenata*. Compounds **1-6** and **9-12** were active with *O. cumana*. Note that none of these compounds were able to induce germination in the other *Orobanche* species.

The activities obtained were higher or at the same level than those of the synthetic strigolactone GR-24, used as internal standard for means of comparisons.

Noteworthy, some of these active compounds do not have an unsaturated lactone system (**1**, **2**, and **9**) and others – the guaianolides **1** and **2** – present an oxygen atom in the lactone ring (C13) with the same disposition than rings C and D of strigolactones. (Figure 7). Molecular modeling of sesquiterpene lactones, natural strigolactones, and synthetic strigolactones allowed us to calculate several theoretical molecular characteristics for all of them. Steric demands are mostly the same for both families as total volume values calculated are similar (GR-24:  $352.8 \text{ \AA}^3$ ; strigol:  $435.6 \text{ \AA}^3$ ;  $309.0 \text{ \AA}^3 < V_{\text{sesq. lac.}} < 333.0 \text{ \AA}^3$ ). Consequently, sesquiterpene lactones fit into the hypothetical receptor's cavity of strigolactones, thus explaining the activity observed by Fischer et al. in witchweed (Galindo et al. 2002).

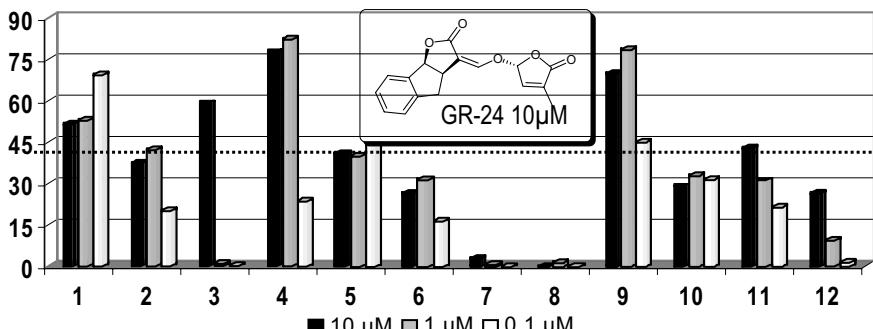


Figure 6. Percentage of germination obtained with sesquiterpene lactones **1-12** on *O. cumana* var. 21. Dashed line represents the percentage of germination obtained with GR-24. Guianolides: **1-5**; eudesmanolides: **6-8**; *trans,trans*-germacranolides: **9-11**; melampolides: **12**. (With permission of J. Agric. Food Chem.).

Values of dipole moment were similar within the strigolactones group ( $\mu \approx 6$  Db) and higher than those of sesquiterpene lactones ( $2.6 < \mu < 5.6$  Db). Sesquiterpenolides showed activities comparable with strigolactones and dipolar interactions could resemble differences in Van der Waals interactions between polar and apolar areas in the receptor's site of broomrape and witchweed. However, such differences, if produced, should be more related with the spatial disposition of these areas rather than with the total surface, as both families present similar percentages of polar and unsaturated areas (Galindo et al. 2002). Consequently, differences in molecule recognition could come from different interactions between polar or apolar areas of the chemical and the receptor's active site.

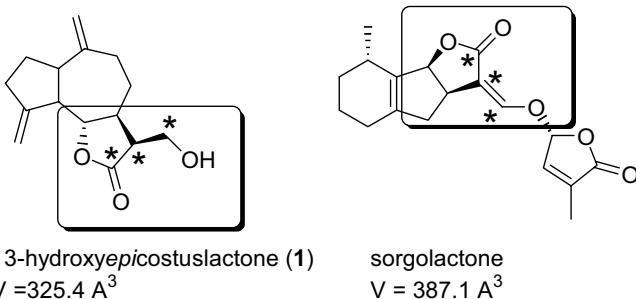


Figure 7. Comparison of compound **1** and sorgolactone illustrates structural similarities among sesquiterpene lactones and strigolactones. Marked atoms are used for structure overlapping comparisons.

GR-24 and strigolactones are able to induce germination in all witchweed and broomrape species tested. The same is also true for Nijmegen-1, another synthetic germination stimulant. However, comparative studies usually show higher levels of induction in witchweed than in broomrape (Thuring et al. 1997a and Thuring et al. 1997b). Such differences should resemble particularities of each species site of action.

On the other hand, it is remarkable that sesquiterpene lactones are able to induce witchweed germination (Fischer et al. 1989 and Fischer et al. 1990) but they are not active with all broomrape species. In fact, they induce germination only in *O. cumana*, the specific weed parasite of sunflower (Pérez de Luque et al. 2000 and Galindo et al. 2002). Up to date, no natural germination inductors of weedy parasites have been yet isolated and/or characterized from sunflower. However, the high content in sesquiterpene lactones of sunflower and their presence in several parts of the plant included the roots makes feasible the hypothesis of their role as specific chemical signals for sunflower broomrape. Molecular modeling clearly shows that all strigolactones present similar shapes and overlapping of their backbones led to a perfect overlay of rings C and D, corresponding to the lactone-enol- $\gamma$ -lactone moiety (MOPAC. Ver. 7.0.0). At the same time, the rest of the carbon skeleton ring is similar in all strigolactones (Figure 8). These similarities in the spatial disposition could also reflect the similarity between target sites in all broomrape and witchweed species and explain why strigolactones are able to induce germination in all of these parasites.

Now the question is, which are the differences between the target site of *O. cumana* and the rest of broomrape species that makes of sesquiterpene lactones specific inductors of sunflower broomrape? And second, what should it happen if we incorporate a system similar to that of rings C and D of strigolactones into the sesquiterpene lactones backbone?

Regarding the first question overlay of minimum energy conformers of strigol and sesquiterpene lactone 5-hydroxyisozaluzanin C through the lactone rings shows that rings A and B of sesquiterpene lactones present a different spatial disposition when compared with sorgolactones (Figure 9). This could be indicative of different accommodations of each compound in the active site and different Van der Waals interactions between rings A and B and the receptor's cavity, or of different target sites for each family of compounds.

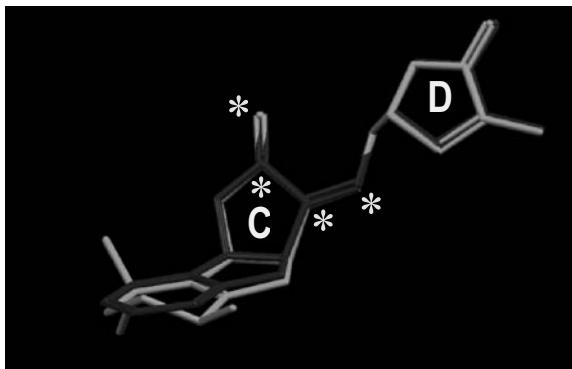


Figure 8. Overlapping minimum energy conformers of GR-24 (blue) and strigol (green) as obtained from PM3 calculations; marked atoms were used for overlay. Note the perfect overlapping of rings C and D and the good coincidence in the rest of the backbone.

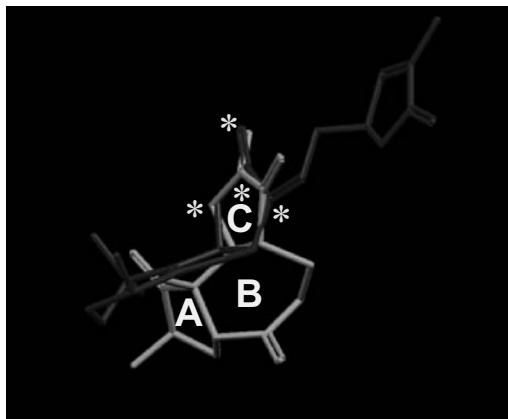


Figure 9. Overlapping minimum energy conformers of strigol (blue) and 5-hydroxyisozaluzanin C (green) as obtained from PM3 calculations; marked atoms were used for overlay. Note how rings A and B in strigolactone and sesquiterpene lactone present a different spatial disposition.

With respect to the mechanism of action by which sesquiterpene lactones induce germination, Michael nucleophilic addition to the  $\alpha,\beta$ -unsaturated carbonyl system could work on compounds **3-9**, **11**, and **12** (Figure 5). Also, it has been already mentioned that bionucleophiles could also trap the carbocation resulting of cyclization of *trans,trans*-germacranolides **9-11**. However, this mechanism should not explain why guaianolides **1** and **2** are active, unless the activity relays in the lactone ring itself. In this case, the reaction should proceed through lactone ring opening (Figure 10).

To answer the second question, we have synthesized new guaiane sesquiterpenolides incorporating a second lactone ring into the skeleton. These new part is binded to the rest of the molecule through an oxygen bridge, in a similar way as rings C and D are connected in strigolactones (Figure 11). These compounds loss their specificity towards *O. cumana* showing activity with all broomrape species tested (unpublished results).

These compounds might undergo nucleophilic attacks in a similar way as in the retro-Michael set of reactions proposed for strigolactones (Figure 2), as they present the proper functionalization in rings C and D. These fact. should certainly explain the new activities found for them and the loss of their specificity.

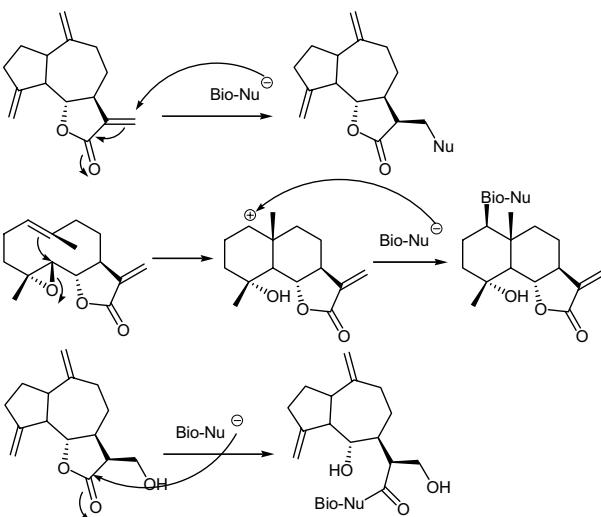


Figure 10. Possible mechanism of action by which SL could react with the bionucleophile proposed for strigolactones. **A:** Michael addition to the unsaturated carbonyl system of the lactone ring; **B:** trapping of the carbocation resulting from a cyclization reaction of *trans,trans*-germacranolides; **C:** lactone ring cleavage.

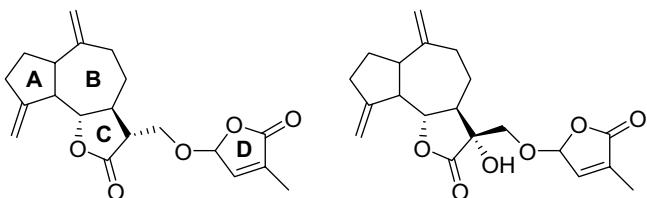


Figure 11. Guaianolides incorporating the additional ring D. The structure mimics that of strigolactones.

Finally, we have checked out several possibilities, like solvation surface, Van der Waals radius, partial charges, and other theoretical molecular features to find clues to explain the specific recognition of SL by sunflower broomrapes (Galindo et al. 2002). Main differences are found in the dipolar moments, which are lower in the sesquiterpene lactones. Also, when partial charges are compared, strigol and GR-24 presents a similar disposition of positive and negative partial charges in C and D rings – which otherwise is logic, and different from that in sesquiterpene lactones (Figure 12). However, the charge disposition varies too much between the different SL depending on the substituents, so as it is difficult to find out a similar pattern for all of them that might be recognized.

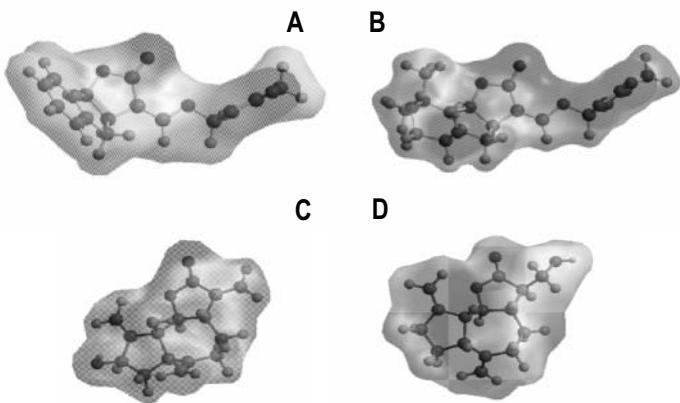


Figure 12. Van der Waals volumes and partial surface charges (red: positive partial charge; blue: negative partial charge) as obtained from PM3 calculations (MOPAC. Ver.7.0.0)). **A:** GR-24; **B:** strigol; **C:** 5-hydroxyisozaluzanin C; **D:** 13-hydroxyepicostuslactone.

## CONCLUSIONS

Sesquiterpene lactones are specific inductors of sunflower broomrape (*O. cumana*) germination. However, the reason for such specificity remains still unresolved. Our findings suggest that the lactone-enol- $\gamma$ -lactone moiety is common to all broomrape and witchweed species, while sunflower broomrape is the only one that recognizes sesquiterpene lactones as germination signals. Whether there is a common target site for the two families of compounds with small differences that allow one species to recognize sesquiterpene lactones, or whether there are separate target sites for each type of compounds is a subject that has not been addressed yet. Ongoing research is in progress to elucidate the clues of such specificity and the molecular mechanisms governing the *O. cumana*-sunflower interaction. No doubt the results will be of application in broomrape control.

## ACKNOWLEDGEMENTS

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## CHAPTER 23

# MODES OF ACTION OF PHYTOTOXINS FROM PLANTS

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## INTRODUCTION

Relatively little is known of the mode of action of natural phytotoxins, when compared to our understanding of the mode of action of synthetic herbicides (Devine et al. 1993; Fedtke and Duke, 2005). Furthermore, most of the scientific literature on mode of action of natural phytotoxins focuses on compounds from microbial origins. Relatively little effort has been made to study the mode of action of plant-produced phytotoxins, including allelochemicals involved in plant/plant interactions.

This review will cover phytotoxins produced by higher plants, as well as algae and lichens, even though some of the phytotoxins from lichens may be produced by the fungal component. We will not discuss compounds from microbes nor non-lichen fungi. Where possible, we will discuss molecular target sites, however, much of the literature on mode of action of these compounds does not provide definitive proof of the molecular target site. Those with known molecular target sites will be discussed separately from those for which some mode of action information is available, but the precise target site is unknown. Any strong phytotoxin will eventually affect most physiological processes at sufficient dosage. Indeed, this fact has been the basis for many inconclusive publications. We will try to include mostly publications that report more than physiological responses that are chosen without a good rationale to link them to the mode of action of the phytotoxin.

Likewise, at sufficiently high concentrations, almost any compound will affect an *in vitro* activity of an enzyme or a physiological process such as respiration. If a compound is substantially active *in vitro* only at millimolar concentrations, it is

unlikely to act as a phytotoxins in nature. So, we will focus on compounds that have *in vitro* effects at submillimolar concentrations.

We use the word phytotoxin rather than allelochemical in this chapter, since, for many of the compounds discussed here, there is little or no proof that they act as allelochemicals in plant/plant interactions in nature. This chapter would be very short if we limited ourselves to compounds that are clearly established as allelochemicals in natural environments.

There may be cases in which a phytotoxic molecule affects several molecular target sites. This greatly complicates establishing a mode of action, and may be another reason why the mode of action of relatively few plant-produced phytotoxins is well understood. We have divided this chapter into a section on sites of action for natural products that are apparently well understood and another section on compounds for which a significant amount of mode of action research has been done without fully resolving the molecular target site(s).

## **KNOWN SITES OF ACTION**

### **Photosystem II (PSII)**

Photosystem II (PSII) is localized in the thylakoid membranes of chloroplasts and is involved in the photosynthetic electron transport (PET) system. When illuminated, the water-oxidizing complex of PSII converts a water molecule into  $\frac{1}{2} O_2$  and  $2 H^+$ , and releases 2 electrons. These electrons are transported across a series of electron carriers that ultimately lead to the formation of NADPH. The concurrent accumulation of a proton gradient within the lumen of the thylakoids also leads to the formation of ATP. Most inhibitors of PSII compete for the binding site of plastoquinone (PQ) on the D1 protein of PSII. PQ is a lipophilic benzoquinone that receives two electrons from the D1 protein of PSII and two  $H^+$  from the stroma. The reduced form of PQ (plastohydroquinone) then moves across the thylakoid membrane and donates two electrons to cytochrome  $b_6/f$  complex which ultimately transfers the electrons to photosystem I (PSI), and releases  $2 H^+$  into the lumen. This process is essential to all plants and inhibiting this electron transfer pathway is lethal.

A large number of commercial herbicides, including the triazines and substituted ureas, target PSII by competing for the binding site of PQ on the D1 protein (Draber et al. 1991). PSII-inhibiting *p*-quinones are structural analogues of PQ that compete for the same binding site on PSII (Draber et al. 1995), the QB binding site. Quinones also mimic the electron accepting function of PQ. Upon binding to the QB binding site, these inhibitors interrupt the normal photosynthetic electron flow by hindering PQ from binding. While the interruption of photosynthetic electron transfer would itself be lethal after a period of time, plants exposed to PSII inhibitors die relatively quickly,

primarily because of the oxidative stress associated with the over-energized chlorophyll molecules. The higher the photo flux, the more acute this stress becomes.

Many different natural quinones from plants inhibit PSII. Sorgoleone, the allelochemical produced by the roots of *Sorghum* spp. is a particularly potent inhibitor (Figure 1) (Einhellig et al. 1993; Nimal et al. 1996; Gonzalez et al. 1997).

All of the sorgoleone and resorcinolic lipid (e.g., 1,3-dihydroxy-4,6-dimethoxy-5-alkyl resorcinol) congeners produced by *Sorghum* spp. appear to target PSII as well, suggesting that they may all contribute toward the overall allelochemical potential of the root exudates (Figure 1) (Rimando et al. 1998, Rimando et al. 2003; Kagan et al. 2003).

The structural characteristics and inhibitory activity of quinones from Sorghum suggest that they share the same mechanism of action as other PSII-inhibiting quinones (Draber et al. 1995). Sorgoleone and atrazine compete for the same binding site on PSII (Nimal et al. 1996). However, resistance to quinone-type inhibitors is achieved by point mutation of His-215, whereas resistance to synthetic triazine inhibitors, such as atrazine, is obtained with substitutions at Ser-264 (Draber et al. 1995). Therefore, quinones probably interact with different amino acid residues in the binding pocket than the synthetic compounds.

The herbicidal quinone hydroxydietrichquinone (isolated from *Cyperus javanicus*) is a good inhibitor of PET (Figure 1) (Morimoto et al. 2001). Consistent with the other natural quinones that target PSII, hydroxydietrichquinone has long aliphatic tails that most likely enables them to partition within the thylakoid membrane.

A recent study reported the activity of 2-hydroxy-3-alkyl-*p*-naphthoquinone (Figure 1) on PSII (Jewess et al. 2002). The relationship between the length and lipophilicity of the 3-alkyl side chain pointed to optimum *in vitro* activity with chain length ranging from 9 to 12 carbons. These compounds had log *P* values ranging from 6 to 8. These relatively high values are reflective of the fact that their target site is localized within the thylakoid membranes. Naphthoquinones with shorter 3-alkyl tails were less active, probably because their lipophilicity was not sufficient to partition efficiently in the thylakoid membranes. On the other hand, optimum herbicidal activity was obtained with compounds having 3-alkyl tails ranging from 4 to 8 carbons in length. This is probably due to the fact that the more lipophilic compounds with longer tails do not have the appropriate physico-chemical properties for uptake and translocation. The discrepancy between *in vivo* and *in vitro* data illustrates the difficulties of reconciling whole plant activity to target site activity.

A wide variety of other natural products not related to quinones have also been reported to target PSII. The lichen metabolites lecanorin, gyrophoric acid and usnic acid (Figure 1) inhibited PET (Rojas et al. 2000; Inoue et al. 1987). It was suggested

that these compounds might act as allelopathic agents, providing a competitive advantage to the lichens producing them.

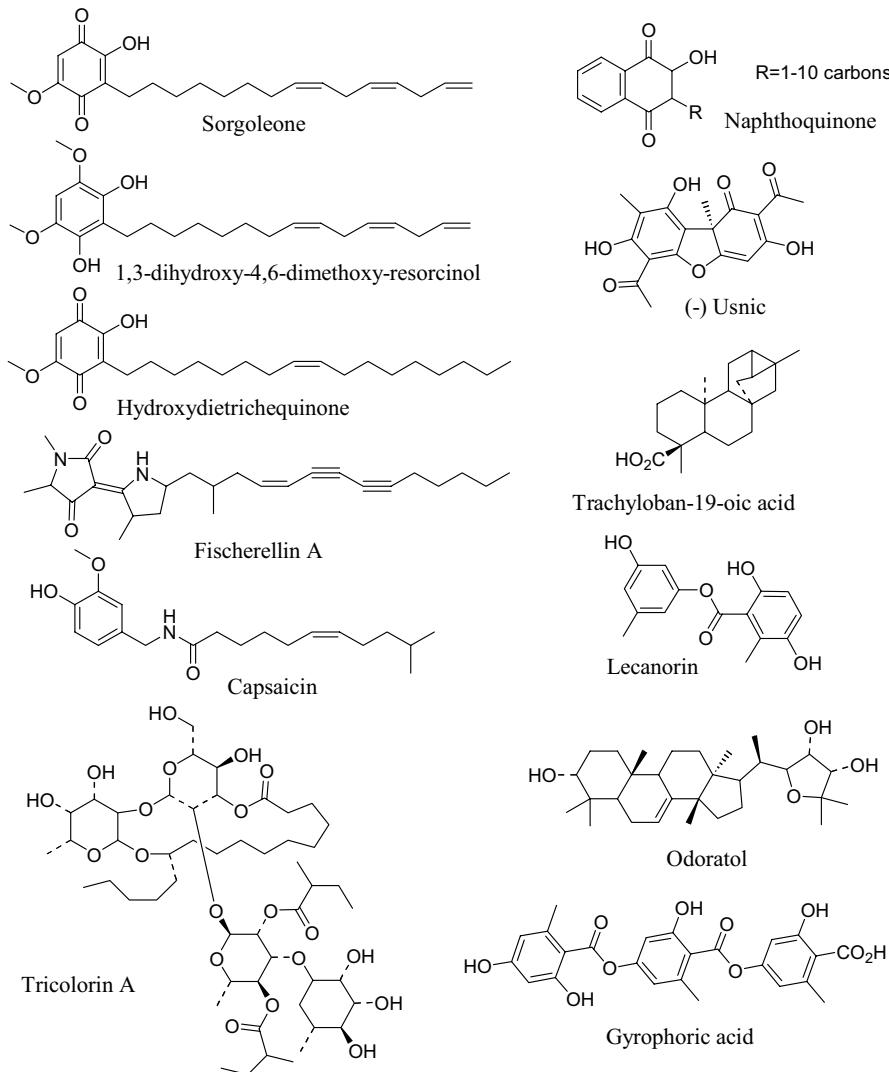


Figure 1. Structures of some of the photosynthetic inhibitors mentioned in the text.

Fischerellin A (Figure 1) (an allelochemical produced by *Fischerella muscicola*) and capsaicin (Figure 1) (isolated from red peppers) are potent inhibitors of PSII (Hagmann and Juettner, 1996; Spyridaki et al. 2000). These non-quinoid compounds have long lipophilic tails not too dissimilar to those found in the benzoquinones and naphthoquinones discussed above, suggesting that they, too, have the ability to partition in the thylakoid membrane. More complex molecules such as the limonoid terpene odoratol (isolated from *Cedrela odorata*) and the tetrasaccharide macrolactone tricolorin A (Figure 1) (isolated from *Ipomoea tricolor*) also inhibit PSII by competing for the binding site of PQ (Achnine et al. 1998; 1999b). Finally, the trachyloban diterpene trachyloban-19-oic acid (Figure 1) produced by *Helianthus annuus* was found to inhibit PSII at the level of QA to QB instead of the typical QB binding domain described above (Hernandez-Terrones et al. 2003).

The cyclic decapeptide, oscillatorin {cyclo [L-arginylglycyl-L-tyrosylglycyl-L-leucyl-1, 2, 3, 3a, 8, 8a-hexahydro-3a-(3-methyl-2-but enyl) pyrrolo [2,3-b] indole-2-carbonyl-L-valyl-L-prolyl-L-asparaginyl-L- $\alpha$ -glutamyl]} from the cyanobacterium *Oscillatoria laetevirens*, is a PSII inhibitor (Shrivastava et al. 2001). The authors concluded that this compound acts more like the commercial herbicide metribuzin than triazines. Algae selected for resistance to oscillatorin were not cross resistant to atrazine (Ray et al. 2003). Oscillatorin is also a potent protease inhibitor (Sano and Kaya, 1996).

The ellagitanin, tellimagrandin II, produced by the aquatic angiosperm *Myriophyllum spicatum*, is a PSII inhibitor (Leu et al. 2002). It is also a potent inhibitor of exoenzymes. The latter mode of action may be more viable, as the uptake and translocation of this compound is problematic due to its physicochemical properties. The heartwood of *Cedrela salvadorensis* L. contains two bioactive epimeric photogedunin acetates that inhibit photosynthetic electron flow (Céspedes et al. 1998). Inhibition requires exposure of the compounds to light. The activity of  $\alpha$ -photogedunin acetate is localized at the QB level, whereas  $\beta$ -photogedunin acetate inhibits PSI electron flow without affecting PSII electron flow, interacting at the b<sub>6</sub>f level and in the span of P700 to F<sub>x</sub>.

The biflavonoid crassifolin and the flavonoids tephrobotin and glabranin from *Tephrosia* spp. are PSII inhibitors (Céspedes et al. 2001). Likewise, two growth-inhibiting dihydro- $\beta$ -agarofuran sesquiterpenes (9 $\beta$ -benzoyloxy-1 $\alpha$ ,2 $\alpha$ ,6 $\beta$ ,8 $\alpha$ ,15-penta-acetoxy-dihydro- $\beta$ -agarofuran from the aerial parts of *Maytenus disticha* and 9 $\beta$ -furoyloxy-1 $\alpha$ ,6 $\beta$ ,8 $\alpha$ -triacetoxy-dihydro- $\beta$ -agarofuran from seeds of *Maytenus boaria*) inhibited chloroplast PET between P680 and QA (Céspedes et al. 2000). The first of these compounds was ten-fold more inhibitory ( $I_{50} = 2.6 \mu\text{M}$ ) than the latter.

Grandinol, homograndinol, and acylphloroglucinol, constituents of *Eucalyptus* spp., are inhibitors of PET (Yoshida et al. 1988; Yoneyama et al. 1989). The

structural requirements for PET inhibition in these compounds are similar, but not identical, to those for the phenol type of inhibitors (Devine et al. 1993).

PSII inhibitors have been, and continue to be, important tools for weed control in agriculture. However, the continued emergence of weeds resistant to commercial PSII inhibitors is cause for concern. It may be possible to develop new classes of PSII-inhibiting herbicides based on the quinone backbone that may help address the problem of herbicide resistance, since quinones do not interact with the same amino acid in the PQ binding domain. Most of the natural PSII inhibitors mentioned in this review have log *P* values of 6 or greater, highlighting the relatively high lipophilicity requirements for these compounds to partition in the thylakoid membrane. Such high lipophilic properties may not be very suitable for the development of herbicides that must be translocated.

## **Photosystem I (PSI)**

The synthetic herbicides paraquat and diquat accept electrons from PSI to form free radicals which interact with molecular oxygen to form levels of superoxide radical that overwhelm the protective mechanisms of the plant (Devine et al. 1993). Some natural products from plants can apparently act in the same way. For example, nostocine A, a violet pigment produced by the freshwater cyanobacterium *Nostoc spongiaeforme* TISTR 8169, is quite phytotoxic (Hirata et al. 2003). By ESR analysis, NaBH<sub>4</sub>-reduced nostocine A was found to be oxidized by air, resulting in superoxide radical formation, indicating that PSI-reduced nostocine A could generate superoxide in the same way as the synthetic herbicide paraquat.

## **Photophosphorylation**

No commercial herbicides exert their effects by inhibiting photophosphorylation. Photophosphorylation, the light-driven formation of ATP, can be inhibited by inhibition of PET, uncoupling, or direct inhibition of CF<sub>1</sub> ATPase. Only the latter two mechanisms will be discussed here, as PET inhibitors are discussed under PSII inhibitors. The first plant-derived inhibitor of CF<sub>1</sub> ATPase discovered was the dihydrochalcone phlorizin (also known as phloridzin) (Figure 2), a compound exclusive to *Malus* species (Izawa et al. 1966). It is a CF<sub>1</sub> ATPase energy transfer inhibitor (Spencer and Wimmer, 1985). An analogue, 4,6'-dihydroxydihydrochalcone 2'-glucoside, which has one less hydroxyl, is equally specific, but about 10-fold more potent than phlorizin (Winget et al. 1969).

The flavonoids quercitin and naringenin strongly inhibit CF<sub>1</sub> ATPase as energy transfer inhibitors (Figure 2) (Shoshan et al. 1980; Moreland and Novitzky, 1987). These compounds have lesser effects as inhibitors of PET. Only high concentrations of

naringinen would displace  $^{14}\text{C}$ -atrazine from the QB binding site. At relatively high concentrations (ca. 0.2 mM), the limonoid 7-oxo-7-deacetoxygedunin from *Guarea grandiflora* inhibits photophosphorylation without affecting proton uptake or PET in spinach thylakoids (Achnine et al. 1999a), suggesting that it is an energy transfer inhibitor. More recently, Céspedes et al. (2001) found the flavonoids tephroleocarpin and methylglabranin to inhibit photophosphorylation by energy transfer inhibition of CF<sub>1</sub> ATPase. 5-O- $\beta$ -D-galactopyranosyl-7-methoxy-3',4'-dihydroxy-4-phenylcoumarin isolated from *Exostema caribaeum* (Rubiaceae) acts as an energy-transfer inhibitor in chloroplasts by blocking the transport of protons through the carrier channel (CF<sub>0</sub>) located in a hydrophobic region at or near the functional binding site for the CF<sub>1</sub> ATPase (Calera et al. 1995).

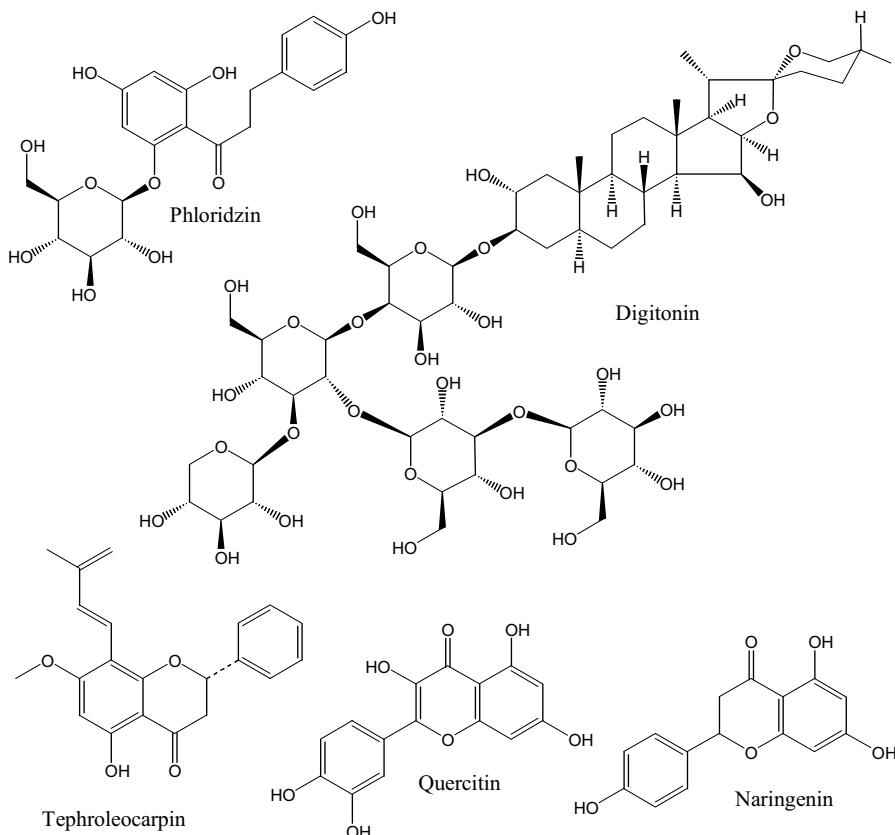


Figure 2. Structures of some photophosphorylation inhibitors mentioned in the text.

Epigallocatechin and epigallocatechin gallate can stimulate photophosphorylation at low concentrations and inhibit at higher concentrations, leading some researchers to speculate that these compounds might be involved in regulation of this process (Tatarintsev et al. 1984). Such a role has also been proposed for quercitin and flavonols in general (Zhestkova et al. 1984).

ATP synthesis and phosphorylating (coupled) electron flow of freshly lysed pea chloroplasts are inhibited by piquerol A and diacetyl piquerol. H<sup>+</sup>-uptake, basal and uncoupled electron transport are not affected by either compound, indicating that they act as energy transfer inhibitors of the ATPase of the CF<sub>1</sub> complex (Mendoza et al. 1994). Digitonin (Figure 2), a cardiac glycoside derived from *Digitalis purpurea*, uncouples CF<sub>1</sub> ATPase as a protonophore (Yagi and Mukohata, 1980). Other uncouplers of photophosphorylation include 4-phenylcoumarins (Calera et al. 1996).

## **Protoporphyrinogen oxidase and other enzymes of chlorophyll synthesis**

Protoporphyrinogen oxidase (Protox) is the target site of a large number of synthetic, commercial herbicides (Dayan and Duke, 2003). These compounds competitively inhibit the enzyme by mimicking half of the substrate, protoporphyrinogen IX (Protogen) (Dayan and Duke, 1997). Protox inhibitors tend to be bicyclic compounds with similar distance between the rings as between the porphyrin rings of Protogen (e.g., diphenyl ethers). Inhibition of Protox leads to accumulation of the product protoporphyrin IX (Proto) in parts of the cell that cannot cope with the singlet oxygen generated by Proto in the presence of light and molecular oxygen. Rapid photooxidative damage occurs. The only plant-derived compound so far known to be a Protox inhibitor is usnic acid (Figure 1) (Romagni et al. 2000b). It is not a particularly good Protox inhibitor, and, thus, most of its phytotoxicity is probably due to its effects on *p*-hydroxyphenyl pyruvate dioxygenase (HPPD) (see below). Considering the wide structural diversity of synthetic Protox inhibitors (Dayan and Duke, 2003), plant-derived Protox inhibitors more potent than usnic acid probably exist.

The phytotoxic alkaloid lycoricidinol (also called narciclasine), produced by bulbs of the family Amaryllidaceae, inhibits synthesis of δ-aminolevulinic (ALA) a precursor of chlorophyll and heme (Miller et al. 1984). This results in phytotoxicity through chlorosis, as well as by dysfunction of enzymes and processes dependent on heme.

## **Singlet oxygen generators**

Plants produce an array of photodynamic compounds that generate singlet oxygen in the presence of molecular oxygen and light. These compounds are generally cytotoxic, but there is little evidence that they are involved in plant/plant interactions.

Some of the more studied photodynamic compounds from plants are: hypericin, certain quinones, porphyrins, furanocoumarins, thiophenes, and polyacetylenes (Dodge and Knox, 1986; Towers and Arnasson, 1988). Similar compounds produced by plant pathogens (e.g., cercosporin) have a role as a phytotoxin in nature.

The porphyrin pathway precursor, ALA, is ubiquitous to plants and animals for both heme and chlorophyll synthesis. When applied to plants, it can cause the accumulation of photodynamic chlorophyll synthesis intermediates, especially when applied with inhibitors of later steps in the chlorophyll synthesis pathway. Accumulation of these photodynamic intermediates results in herbicidal effects, much like those of Protox inhibitors (Rebeiz et al. 1994). So, this is a phytotoxic phytochemical with a known mode of action, but it almost certainly is not an allelochemical.

### HPPD – *p*-hydroxyphenylpyruvate dioxygenase

Studies on the phytotoxicity of the allelochemical leptospermone, a natural triketone isolated from bottlebrush plant (*Calispermum* spp.) (Hellyer, 1968; Knudsen et al. 2000) ultimately led to the synthesis of the triketone herbicides (i.e., Sulcotriione) (Figure 3). Subsequent studies recognized that these compounds were potent inhibitors of enzyme *p*-hydroxyphenylpyruvate dioxygenase (HPPD) (Schulz et al. 1993; Lee et al. 1997; Pallett et al. 1998).

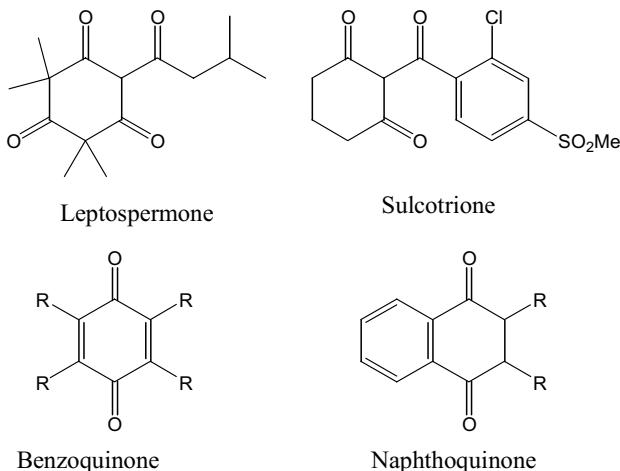


Figure 3. Structures of some of the *p*-hydroxyphenylpyruvate dioxygenase inhibitors mentioned in the text.

Inhibition of HPPD results in loss of carotenoids and chlorophyll (bleaching) of the foliage. These symptoms are identical to those exhibited by plants exposed to phytoene desaturase (PDS) inhibitors (Lee et al. 1997). However, the mechanism of action of HPPD inhibitors is different and more complicated than that of PDS inhibitors. HPPD catalyzes the formation of homogentisate (HGA), a precursor of  $\alpha$ -tocopherol and plastoquinone. Since plastoquinone is an essential cofactor for PDS activity (Norris et al. 1995), inhibition of HPPD indirectly affects PDS activity by reducing the cellular concentration of plastoquinone (Pallett et al. 1998). The subsequent decrease in carotenoid levels causes bleaching of the foliage because the photosynthetic apparatus is no longer protected from photodestruction. Indeed, carotenoids quench excess excitation energy in the photosynthetic apparatus occurring under highlight intensities (Zubay, 1993). In the absence of carotenoids, the excess energy from the chlorophyll in their triplet state is transferred to oxygen, causing formation of singlet oxygen. Singlet oxygen is highly reactive and causes bleaching of pigments and lipid peroxidation of membranes.

Unlike most dioxygenases, HPPD is a non-heme, ferrous containing,  $\alpha$ -keto acid-dependent enzyme (Que and Ho, 1996). The complex reaction mechanism catalyzed by HPPD involves an oxidative decarboxylation of the 2-oxoacid side chain of 4-hydroxyphenylpyruvate (4-HPP), accompanied with hydroxylation of the aromatic ring and a migration of the carboxymethyl group (Crouch et al. 1997; Pascal et al. 1985; Que and Ho, 1996). Commercial HPPD inhibitors mimic a reaction intermediate and tend to be time-dependent (tight-binding) inhibitors of this enzyme. Triketone herbicides bind slowly but very tightly to the catalytic site of HPPD and competitively with respect to 4-HPP. A factor complicating the analysis of kinetic data is that some HPPD inhibitors, such as the diketonitrile isoxaflutole, have to be metabolically bioactivated (Pallett et al. 1998) and sometimes exhibit half-site reactivity where binding to one catalytic site of the dimer completely inhibits the enzyme (Garcia et al. 2000).

The discovery of HPPD inhibitors hinged on the work done with leptospermone, but most of the information available on this molecular target site focuses on synthetic compounds. However, we have recently reported that a natural lichen triketone, and several benzoquinones and naphthoquinones also inhibit HPPD (Romagni et al. 2000b; Meazza et al. 2002). Usnic acid (Figure 1) is a structural analog of synthetic commercial triketone HPPD inhibitors, whereas the benzo- and naphthoquinones are structurally different from the commercial compounds (Figure 3).

(-)Usnic acid was the strongest inhibitor of HPPD, with an apparent  $IC_{50}$  of 70 nM, surpassing the activity obtained with the commercial herbicide sulcotrione (Figure 3). Usnic acid possesses a 2-keto-cyclohexane-1,3-dione common to many triketone herbicides such as sulcotrione. As its synthetic structural analogues, usnic acid bound irreversibly to HPPD.

While benzoquinones and naphthoquinones can inhibit HPPD, the binding kinetics observed with these compounds was different from that observed with the triketone class of natural products, with the lines intersecting at their point of origin, suggesting that the benzoquinones and naphthoquinones do not interact with HPPD in a time-dependent tight-binding manner (Meazza et al. 2002).

The fact that the triketone-type natural products, such as (-)-usnic acid, bind tightly to HPPD can readily be explained by previous research on synthetic triketones. The triketone functionality of the inhibitors mimic the  $\alpha$ -keto acid moiety of 4-HPP and compete for the binding site of the natural substrate (Pascal et al. 1985; Garcia et al. 2000). These intermediates form stable enzyme-complex intermediates in association with the ferrous iron in the catalytic site. On the other hand, benzoquinones and naphthoquinones are structurally more rigid (planar) than the triketones and may not mimic the  $\alpha$ -keto acid moiety of 4-HPP as well. Instead, their backbones may resemble the conformation of one of the later intermediate step in the reaction mechanism of HPPD. The activity of the *p*-benzoquinones was positively affected with the presence of a 2-hydroxyl group and reduced in the methoxy derivatives, suggesting that oxygen atoms may still interact with the metal iron of HPPD.

While it is clear that many natural products have the potential of being excellent HPPD inhibitors, it remains to be demonstrated that their *in vivo* mechanism of action involves inhibition of HPPD.

## Respiration

Much of the early mode of action work with allelochemicals dealt with their effects on respiration, perhaps because the methods for such studies were readily available. Unfortunately, much of this work involves simply examination of effects on oxygen uptake by treated plants, organs, or tissues (e.g., Chispusio et al. 2000). Any phytotoxin will eventually have an effect on respiration, whether the effect is direct or not. In some cases, the effects on respiration have been shown to be indirect. For example, the inhibition of cyanide-insensitive respiration in potato tuber tissue by lycorine was shown to be an indirect effect of lycorine's inhibition of ascorbate synthesis (Arrigoni et al. 1976; see more about lycorine under Others below).

However, some of the early work, such as that by Demos et al. (1975) who found tannic, gentisic, and *p*-coumaric acids to inhibit plant mitochondrial respiration, suggested direct effects on mitochondrial function. They speculated that the plant growth inhibition by these compounds was associated with their effects on respiration. Such a link has not been clearly established in most studies. A survey of some allelochemical effects on respiration is provided in Table 1.

Respiration can be directly affected by more than one mode of action. For example,  $\alpha$ -pinene both uncouples oxidative phosphorylation and inhibits mitochondrial electron transport (Abrahim et al. 2003). Both effects were speculated to be due to disturbances of the inner mitochondrial membrane. Uncouplers generally increase oxygen uptake. It is likely that many of the inhibitors of PET also inhibit mitochondrial electron transport, as this is the case for many synthetic PSII inhibitors (Devine et al. 1993). This is the case for sorgoleone (Rimando et al. 1998; Rasmussen et al. 1992). It is also the case for hydroxydietetrichoquinone from *Cyperus javanicus* (Morimoto et al. 2001).

Table 1. Effects of some allelochemicals on respiration or respiratory function

Allelochemical	Conc. ( $\mu$ M)	Assay	Inhibition (%)	Reference
Butein	250	uncoupling of potato mitochondrial respiration	100	Ravanel et al. 1982
5,7-dihydroxy-isoflavone	30	ATP formation by cucumber mitochondria	61	Stenlid, 1970
10,11-epoxy-4-hydroxy tremeton	17	O <sub>2</sub> uptake by isolated mitochondria	50	Céspedes et al. 2002
juglone	500	maize roots	>90	Koeppe, 1972
kaempferol	243	plant mitochondrial electron transport	50	Tissut et al. 1980
quercitin	20	malate oxidation by mitochondria	50	Moreland & Novitzky, 1987
sorgoleone	0.5-5	soybean and maize mitochondrial respiration	50	Rasmussen et al. 1992
tannic acid	46	bean mitochondrial respiration	94	Demos et al. 1975

Some plant-derived compounds have been shown to inhibit animal mitochondrial respiration, but have not been studied with plant mitochondria. For example, several jatrophane diterpenes from the latex of *Euphorbia obtusifolia* inhibit NADH oxidase of mammalian mitochondrial electron transport chain (Betancur-Galvis et al. 2003). Two naphthoquinones from *Calceolaria andina* inhibit insect mitochondrial respiration, but were safe enough for plants to apply to leaf surfaces to test for insect control (Khambay and Jewess, 2000).

Cyanamide was one of the first synthetic herbicides (Sturkie, 1937). It is apparently a respiration inhibitor (Inoue et al. 1971). Recently, cyanamide was found by bioassay-directed isolation to be a phytotoxic constituent of *Vicia villosa*, possibly explaining the allelopathic properties of this species (Kamo et al. 2003).

In some cases, strong phytotoxins stimulate oxygen consumption of affected tissues (e.g., Galindo et al. 1999). Artemisinin stimulates oxygen consumption by root tips (Dayan et al. 1999), but it inhibits respiration of *Lemna minor* plants (Stiles et al. 1994). In a few carefully conducted studies (e.g., Céspedes et al. 2002), comparison of several phytotoxins, such as 10,11-epoxy-4-hydroxy tremeton, on several physiological parameters indicates that the primary mode of action is probably inhibition of respiration.

There are many other studies of allelochemicals that are only significantly inhibitory at concentrations of 1 mM or higher, and are thus unlikely to be phytotoxic due to effects on respiration. Some compounds that have effects on respiration have been found later to be much more active on other metabolic processes. For example, juglone is a much more potent inhibitor of HPPD than of respiration (Meazza et al. 2002).

### **H<sup>+</sup>-ATPase of the plasma membrane and tonoplast**

The H<sup>+</sup>-ATPase of the plasma and tonoplast membranes is essential to maintain proper water relations of plant cells. Several phytotoxins from plants inhibit this enzyme. For example, the allelochemical diacetyl piquerol inhibits both plasma membrane and tonoplast H<sup>+</sup>-ATPase (Cruz-Ortega et al. 1990). Juglone was recently found to have profound effects on plasma membrane function through inhibition of H<sup>+</sup>-ATPase (Hejl and Koster, 2004). Inhibition was seen at much lower concentrations than those reported to affect respiration or other physiological functions.

### **Mitotic inhibitors**

Several commercial herbicides, such as trifluralin, target primary components of the mitotic process (Devine et al. 1993). A relatively large number of phytochemicals appear to have direct effects on mitosis through interactions with tubulin or other components of the mitotic apparatus. This topic has been reviewed previously (Vaughan and Vaughn, 1988; Vaughn and Vaughan, 1988). The mode of action of several plant-derived mitotic inhibitors is provided in Table 2. The references cited are mostly from the animal literature, but in every case in which the mode of action has been determined in plant tissues, it was the same as in animals (Vaughn and Vaughan, 1988), although relative sensitivities differ.

Table 2. Mode of action of several mitosis-disrupting phytochemicals

Compound	Plant source	Mode of action	Reference
Betulin	several species	Inhibition of topoisomerase	Wada et al. 2001
Camptothecin	Camptotheca acuminata	Inhibition of topoisomerase I	Hsiang et al. 1985
Colchicine	Colchicum autumnale	Inhibited microtubule formation	Iwasaki, 1998
Podophyllotoxin	Podophyllum peltatum	Inhibited microtubule formation	Gordazlia, et al. 2000
Taxol	Taxus spp.	Hyperstabilized microtubules	Leu, et al. 1994
Vinblastine	Vinca spp.	Inhibited microtubule formation	Iwasaki, 1998

Some of these compounds, such as taxol, vinblastine/vincristine, and podophyllotoxin (Figure 4), are better known for their effects on mitosis of animal cells, for which they are used as anti-cancer drugs. However, plant cells have been found to be similarly affected in those cases in which appropriate studies have been made. For example, podophyllotoxin is both an antimitotic compound for human cancer cells (Gordiliza et al. 2000) and for plants (Oliva et al. 2002). Two synthetic derivatives of podophylloxin are inhibitors of topoisomerase II.

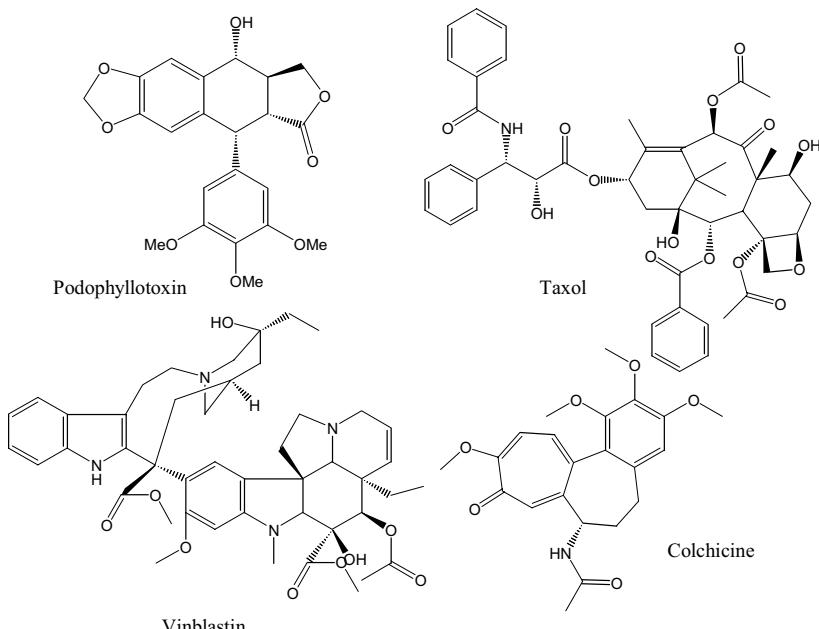


Figure 4. Structures of several mitosis-disrupting phytochemicals

Some mitotic inhibitors may have more than one mode of action. For example, narciclastine is reported to have antimitotic properties similar to colchicine on plant and animal cells (Ceriotti, 1967), yet it has strong effects on some enzymes and on peptide bond formation in eukaryotic ribosomes (see under Other below). The effect on ribosomes is associated with its antimitotic activity (Baez and Vazquez, 1978). Several plant-derived antimitotic compounds (colchicine, taxol, vinblastine; Figure 4) bind to tubulin, either destabilizing or hyperstabilizing microtubules needed for cell division and cell expansion.

## Protein synthesis and non-amino acid antimetabolites

Numerous microbially-produced compounds inhibit protein synthesis directly. However, few phytochemicals have been found to have such a mode of action. Lycoricidinol, a compound from *Lycoris radiata*, inhibits protein synthesis of plants, but not of bacteria (Imaseki and Kang, 1984). It does not inhibit initiation of translation, but inhibits polypeptide elongation. Lycoricidinol also inhibits auxin transport (Sasse et al. 1982). Plants produce many toxic non-protein amino acids. Some of these are phytotoxic and may exert their effect by being incorporated into proteins or by inhibiting enzymes that use protein amino acids as substrates. For example, L-canavanine, a structural analogue of L-arginine is utilized by arginyl-tRNA synthetases of numerous canavanine-free species, resulting in canavanine-containing proteins with loss of essential metabolic function (Rosenthal 1977). Canavanine can also affect regulatory and catalytic reactions of arginine metabolism. Other non-protein amino acids, such as mimosine are quite phytotoxic, but their exact modes of action are unclear (Prasad and Subhashni, 1994).

## Glutamine synthetase

Glutamine synthetase (GS) is a crucial enzyme in amino acid metabolism and photorespiration (Lydon and Duke, 1999). Many microbial compounds are potent inhibitors of GS, and one of them, phosphinotrichin, is synthesized and sold as the highly successful herbicide, glufosinate. The only plant-derived GS inhibitor is methionine sulfoximine (Jeannoda et al. 1985). This compound was known as a highly potent synthetic GS inhibitor before it was found in nature.

## Acetylactate synthase

Acetylactate synthase (ALS, also known as acetohydroxy acid synthase) is a key enzyme of the branched chain amino acid pathway (leucine, isoleucine, and valine). It is the target site of a chemically diverse array of commercial herbicides (Shaner and

Singh, 1997) that apparently bind a vestigial quinone binding site (Schloss et al. 1988). We are aware of no phytochemical that has been identified as an ALS inhibitor, but at least one compound from a microbe, gliotoxin, inhibits ALS (Haraguchi et al. 1992). Flavonins (a category of flavonoid) have been patented as ALS inhibitors (Mido et al. 1999). To obtain good activity, the flavonin had to be chlorinated.

## **Asparagine synthetase**

Asparagine synthetase (AS) is the key enzyme in asparagine synthesis in plants. AS catalyzes an ATP-dependent reaction, where the amine group of glutamine is transferred directly to aspartate (Richards and Schuster, 1998). AS has been identified as a potential herbicide target site when it was discovered that an exogenous supply of asparagine reversed the growth inhibition caused by the natural phytotoxin 1,4-cineole (Romagni et al. 2000a). Root uptake of asparagine increased in the presence of the inhibitors, whereas uptake of aspartate was not affected. The phytotoxic effect of 1,4-cineole was assumed to be due to the *in vivo* inhibition of AS activity, because it was very inhibitory to this enzyme *in vitro*.

## **Others**

The alkaloid lycorine, a product of Amaryllidaceae species, inhibits the synthesis of ascorbate in plants by inhibition of L-galactono- $\gamma$ -lactone dehydrogenase (Arrigoni et al. 1997b). Ascorbate is required in high concentrations in photosynthesizing chloroplasts to prevent peroxidative damage. Lycorine also reduces ascorbate accumulation indirectly by enhancing the *de novo* synthesis of dehydroascorbate reductase (De Tullio et al. 1998). Arrigoni et al. (1997a) found a good positive correlation between level of ascorbate in plant tissues as influenced by lycorine and growth.

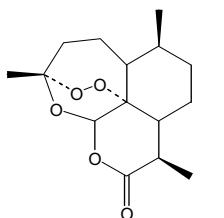
The biphenyl ether compound, cylindol A, isolated from the noxious weed, *Imperata cylindrica*, is an inhibitor of 5-lipoxygenase (Matsunaga et al. 1994). This plant is considered to be allelopathic (e.g., Abdul-Rahman et al. 1989), however, whether this activity is related to cylindol A and its activity on 5-lipoxygenase is unknown.

Narciclasine, a constituent of the mucilage form *Narcissus* spp. bulbs, is highly phytotoxic (Bi et al. 2003). It completely inhibits isocitrate lyase and hydroxypyruvate reductase at 10  $\mu$ M. This mode of action was used to explain its inhibition of development of microbodies and chloroplasts. This compound is also reported to block peptide bond formation in eukaryotic ribosomes (Carrasco et al. 1975).

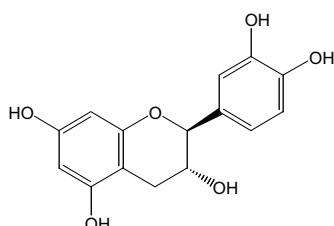
## ALLELOCHEMICALS WITH UNKNOWN SITES OF ACTION

### Artemisinin

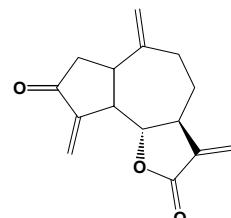
Considerable research has been conducted on the mode of action of the sesquiterpene endoperoxide lactone artemisinin (Figure 5), but no studies with plants have been definitive (summarized by Duke and Oliva, 2003). This compound is highly toxic to both plants and apicomplexans such as the malaria parasite (*Plasmodium* spp.) (Klayman, 1985). Apicomplexans have vestigial plastids (Lang-Unnasch et al. 1998), suggesting that the molecular site of action may be plastid-localized, as is the molecular target site of the majority of commercial herbicides. Meshnick (2002) hypothesized that the mechanism of action of artemisinin appears to involve a heme-mediated decomposition of the endoperoxide bridge to produce carbon-centered free radicals. He further elaborates that the involvement of heme explains why it is selectively toxic to malaria parasites. However, it is not selectively toxic to malaria parasites. It is also quite toxic to the other category of plastid-containing organisms (plants). Furthermore, the symptoms of phytotoxicity are not those of oxidative stress.



Artemisinin



(-)-Catechin



Dihydrozaluzanin C

Figure 5. Structures of some of the phytotoxins with unknown modes of action mentioned in the text.

More recently, Eckstein-Ludwig et al. (2003) reported that artemisinin targets sarco-endoplasmic reticulum ATPases (SERCA). Activated artemisinin can form adducts with a variety of proteins, but it may be much more likely to interact with particular proteins. These scientists found that artemisinin inhibits SERCA with similar potency to thapsigargin (another sesquiterpene lactone and highly specific SERCA inhibitor). Thapsigargin antagonized the parasiticidal activity of artemisinin. However, artemisinin can form adducts with other proteins (Meshnick, 2002) and can react with other compounds such as glutathione (Wang and Wu, 2000) (see discussion of dehydrozaluzanin C below). Another compound with sulfhydryl groups, cysteine, is a good antidote for the phytotoxic activity of artemisinin (Duke et al. 1988). Reduced glutathione is essential to the function of the cell cycle, so depletion of glutathione

levels by artemisinin could inhibit mitosis. Indeed, artemisinin inhibits all stages of plant cell division, causing a low incidence of abnormal mitotic figures (Dayan et al. 1999). Structure activity comparisons of artemisinin and eleven analogues suggested that the mode of action in plants is different than in *Plasmodium* spp., but differences in uptake, compartmentation, and metabolic degradation can confound such comparisons. In conclusion, the mode of action of artemisinin as both a phytotoxin and an antimalarial is still unknown, but the volume of research on this important drug should soon reveal its mode of action as a pharmaceutical. This may provide the needed clue to determination of its target site in plants.

### (*-*)-Catechin

Recently, (*-*)-catechin (Figure 5) was found to be an allelopathic component of root exudates of *Centaurea maculosa* (Bais et al. 2002, 2003a; Veluri et al. 2004). The plant generates a racemic mixture of catechins (( $\pm$ )-catechin), but only (*-*)-catechin was found to be phytotoxic. These authors found (*-*)-catechin to cause an increase in reactive oxygen species (ROS), and inhibition of the formation of ROS with ascorbate reduced the phytotoxicity of (*-*)-catechin (Bais et al. 2003b). This seems inconsistent with the fact that catechins are considered strong antioxidants (Higdon and Frei, 2003). Bais et al. (2003b) provided evidence that elevated ROS caused  $\text{Ca}^{2+}$ -dependent programmed cell death (apoptosis). ROS damage has been linked to apoptosis before, but the relationship in plants is unclear. Pelargonic acid (nonanoic acid), a phytochemical sold as a herbicide (Bradley and Hagood, 2002), is reported to cause apoptosis in some types of animal cells (Foursey et al. 1998). Whether this is the mode of action in plants is doubtful, considering the rapidity of this contact herbicide.

Microarray data on the effects of (*-*)-catechin on gene transduction within 10 minutes of plant cell exposure did not clearly indicate what the molecular target site of (*-*)-catechin might be. At concentrations greater than 50  $\mu\text{M}$ , catechins inhibit cyclic and pseudocyclic energy transfer processes of plant photophosphorylation (Muzafarov et al. 1986).

### Dehydrozaluzanin C

Dehydrozaluzanin C (DHZ) (Figure 5) is a sesquiterpene lactone that is generally cytotoxic, being a good fungicide (Wedge et al. 2000), herbicide (Galindo et al. 1999; Macías et al. 2000), antiprotozoal compound (Fournet et al. 1993), and general toxin of mammalian cells and bacteria (Galindo, pers. communication). It is both a strong growth inhibitor and inducer of cellular leakage of plants (Galindo et al. 1999). Glutathione is a good antidote for both activities because of the covalent bonding of DHZ to the sulphydryl groups of this compound (Galindo et al. 1999). However,

*isozaluzanin C* (IC), the 3 $\alpha$ -hydroxy derivative of DHZ inhibits growth, but does not cause membrane leakage.

It was proposed that the growth inhibition is due to the exomethylene- $\gamma$ -lactone moiety possessed by both DHZ and IC, whereas the effects on membrane function is due to the  $\alpha,\beta$ -unsaturated carbonyl of the cyclopentanone portion of DHZ. Whether the exomethylene- $\gamma$ -lactone moiety of DHZ is also needed for membrane disruption is unknown. DHZ had no effect on the plasma membrane enzyme NADH oxidase (Galindo et al. 1999). It was concluded that DHZ may have several modes of action, but the molecular sites have yet been determined. Selective reaction of DHZ with sulphydryl groups of particular functional proteins could cause either effect.

## CONCLUSIONS

The amount of information about the mode of action of phytotoxins from plants is increasing rapidly, yet we have much more to learn. In previous reviews of the modes of action of phytotoxins from all natural products, we have stated that there is little overlap between the modes of action of natural products and those of synthetic herbicides (e.g., Duke et al. 2000). However, if only phytotoxic phytochemicals are considered, the information available at this time indicates that most phytotoxic compounds from plants target molecular sites that have or are targets of commercial herbicides. The implications of the fact that most phytotoxins from plants seem to have modes of actions similar those of synthetic herbicides are not clear from a chemical ecology perspective. Understanding the mechanisms of resistance to phytotoxins of the producing plant might help in understanding why compounds with these modes of action have evolved.

The modes of action of most phytotoxins produced by plants are still unknown or unclear. New methods of probing modes of action, such as analysis of whole genome gene expression (transcriptional profiling with DNA microarrays) in response to a toxicant (e.g., Agarwal et al. 2003), may improve our understanding to how these phytotoxins exert their effects.

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## CHAPTER 24

# ALLELOPATHY IN ECOLOGICAL SUSTAINABLE AGRICULTURE

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## INTRODUCTION

The indiscriminate use of new agricultural technology [agrochemicals (fertilizers, pesticides etc.) and multiple cropping in irrigated areas], for the success of modern agriculture has (a) made our soils sick, (b) caused environmental pollution, (c) development of resistance in pest (weeds insects, pathogens etc) and (d) toxic residues in our food. The new technology in a short span of 35–40 years has caused these major problems, which have severely deteriorated our soil health, human and livestock health, environment and quality of life. This indicates the new technology is not sustainable over long periods. Modern agriculture is exploitative of growth resources and has caused various problems such as environmental pollution through (a) contamination of underground drinking water resources with pesticides and nitrates, (b) contamination of food and fodder with residues of pesticides, nitrates and antibiotics, (c) both 'a' and 'b' cause harm to farm workers (Pretty 1995), (d) poor soil health and soil productivity and (e) poor quality of rural life. There is growing evidence that grain yields of cereals in cereal based rotations in modern agriculture cannot be sustained at the current levels e.g. rice-wheat rotation. In the last 16 years, there is a decline of 25–30% in the grain yields of both rice and wheat in Philippines, Indonesia, India and Pakistan despite the use of recommended cultural practices (Pretty 1995). Therefore, the recent emphasis in agriculture has shifted from a primary goal of maximizing yields over the short term, to a sustainable productivity over long periods of time. The knowledge of ecological interactions occurring within an agroecosystem and the sustainable functioning of the system as a whole has become the overall approach. Sustainability can be achieved in an agriculture that is ecologically sound, resource conserving and not environmentally degrading.

Therefore, several definitions of sustainable/ecological/ecofriendly/organic agriculture are available, but for this chapter, the ecological sustainable agriculture means that “farmer grow the crops with the resources available on the farm, reduces dependence on off-farm inputs and maintains soil productivity and clean environment over a long period of time”. Hence, farmer conserves the resource base to minimize artificial inputs from outside the farm and manage pests (weeds, insects, nematodes, pathogens) through internal regulating mechanisms based on ecological principles and processes (Stinner and House, 1987). Thus, ecological sustainable agriculture strives for the integrated use of a wide range of pests, nutrients and soil management technologies (Pretty 1995). In the last few years, some good books on similar aspects have been released (Franco and Ponte, 1988; Gliessman, 1990a; Narwal, 1998; National Research Council, 1989 and Pretty 1995).

All plant spp. and their residues produce secondary metabolites called allelochemicals. Allelopathy means any process involving secondary metabolites produced by plants, microorganisms, viruses, fungi that influence the growth and development of agricultural and biological systems (International Allelopathy Society, 1996). Although allelochemicals are produced by all plant parts, but the leaves and roots are mainly responsible for their production and release. The ecological agriculture maintains diversity of plant spp. on the farm through various types of multiple cropping systems viz., mixed cropping, crop rotations etc., hence, allelopathy assumes great significance. Allelopathy may be used to increase crop production through avoidance of negative impacts, exploitation of stimulatory effects, management and development of allelopathic crops and varieties to suppress pests (weeds, insects, nematodes, pathogens) and use of allelochemicals as pesticides and growth regulators (Einhellig, 1985).

The productivity of monoculture declines after few years mainly due to build up of pests and soil sickness etc. These problems could be overcome through the adoption of crop rotations and intercropping systems, which exert detrimental effects on the pest through various chemical interactions (Allelopathy) and cause physical hindrances to restrict movement of pests. Likewise, the bio-diversity provided by crop mixtures has a smothering effect on pest and diseases. These indirect synergistic effects also contribute to higher productivity in crop rotations and intercropping systems. If these effects are utilized properly it is possible to reduce the use of chemical pesticides and herbicides.

This chapter reviews allelopathic effects in crop production and possible use of multiple cropping systems, plant residues, plants and varieties rich in allelochemicals to control pests and supplement nitrogen requirement through Biological Nitrogen Fixation by legumes in ecological sustainable agriculture.

## SOIL FERTILITY

In tropical and sub-tropical countries of the World, climate is suitable for round the year cropping. Therefore, in assured irrigated or high rainfall areas, farmers grow 2-4 crops in a calendar year in multiple cropping systems (MCS). The properly planned MCS (crop rotations, crop mixtures, intercropping) aims at making ecological sustainable agriculture successful, has little harmful impact on environment conditions and maintains soil productivity over a long period of time. They maintain soil fertility, keep the pest under check and reduce soil sickness problem. Both the crop rotations and intercropping systems or crop mixtures through inclusion of legumes maintains or improves soil fertility.

### Crop rotation

A well planned crop rotation maintains and even improves the soil fertility, prevents the build up of pest and soil sickness as compared to monoculture (Arnon, 1972). If scientifically sound crop rotations are followed, they provide sustainability to agriculture through reducing the requirement of chemical nitrogenous fertilizers and thereby decreasing environmental pollution by substituting them with biologically fixed nitrogen of legumes.

### Biological nitrogen fixation

#### Legumes

In the past when chemical fertilizers were not available, biological nitrogen fixation (BNF), fallowing and application of farmyard manure were the only practical steps to maintain soil fertility and to provide essential nutrients to the crops. Even today in dryland areas receiving less than 300 mm annual rainfall, the farmers do not apply chemical fertilizers or manures due to uncertainty of crop production. In such regions, legumes are the major component of cropping systems, which through BNF maintains soil fertility and also provide protein rich diet to the people and nutritious feed to the livestock. In irrigated areas, continuous cropping of cereals necessitates the use of large quantities of nitrogenous fertilizers resulting in the development of undesirable soil properties and contamination of underground water resources. Under such conditions, the inclusion of legumes in the cropping system and the use of Blue green algae (Azolla) is imperative, as organic nitrogen from BNF may be more suitable than fertilizer nitrogen, because it is released gradually to become available at a time when conditions are favourable both for microbial activity and plant growth. In ecological sustainable farming, legumes should alternate nitrogen demanding crops-

ideally it should be possible to meet the farm's nitrogen requirements from well designed crop rotations (Lampkin, 1992) as they fixed upto 450 kg N/ha/yr (Peoples and Herridge, 1990). In sequential cropping, the legumes are generally used as green manure, forage and for grains. As such, their nitrogen contribution varies and follows the order: green manure > forage legume > grain legume, provided that factors governing BNF are similar. However, the allelopathic weeds reduce the  $N_2$  fixation in legumes, hence, weeds must be controlled for more BNF.

The beneficial effect of preceding legumes on the succeeding non-legumes is exerted through the transfer of nitrogen and other nutrients. In crop rotation, nitrogen transfer takes place through decomposition of legumes residues and mineralization of organic nitrogen. The mineralised forms of nitrogen are then absorbed by the succeeding crop. The quantity of nitrogen transferred depends upon the total quantity fixed and the end use of legume i.e. green manure, forage or grain crop, residues removed/added to soil and other factors.

In tropical and subtropical countries, green manuring is generally known to farmers and is practiced wherever, facilities are available. However, green manuring is getting less and less popular with the advent of modern farming. In the irrigated belts of India, continuous cropping of cereals is adopted and green manure crops seldom find a place in the cropping system. This has caused serious soil health problems. For green manuring, fast growing legume crops are grown and incorporated *in situ* before flowering for quicker decomposition and mineralisation of plant nutrients, so that these becomes easily available to the succeeding crop. Two types of practices are followed: (a) growing of legumes especially for green manuring and (b) incorporating residues of grain legumes after the harvest of seeds at maturity. This difference causes variation in the beneficial effects of green manuring.

Therefore to reduce environmental pollution from nitrogenous chemical fertilizers, the inclusion of legumes (grain legumes, forage legumes, green manure crops) in annual crop rotations is necessary. Since forage and green manure legumes provide more nitrogen to the crop than grain legumes, hence, these should be preferably included in crop rotations.

### **Blue green algae**

In rice fields, a small fern called Azolla (*Anabena azollae*) covers the water surface but does not harm the crop growth. It fixes upto 120 kg atmospheric N/ ha in the leaves and the nitrogen becomes available to the rice crop after its soil incorporation and decomposition. In Philippines, 100 days old azolla produces 57 t fresh weight/ha, which yields more than 120 kg N/ha (Kohli and Mitra, 1987). In one whole year, it may fix upto 400 kg N/ ha i.e. more than tropical and subtropical legumes and thus offers opportunity to substitute inorganic fertilizers.

## Crop mixtures or intercropping

In terms of land use, growing of crops in mixtures is more productive than growing them separately (Willey, 1979); hence, it is practised traditionally in parts of Asia, Africa and Latin America. Interest in cereal-legume intercropping system is also developing in temperate and warm regions of Australia and U.S.A. because of higher grain yields, greater land use efficiency per unit land area and the improvement of soil fertility through BNF and nitrogen excretion from the legume component (Willey, 1979). In South American countries like Mexico etc., the interplanting of maize, bean and squash in the same planting hole is very ancient practice (Gliessman, 1990b). In such soils of poor fertility, the cultivation of cereals + legumes together improves both total yields and reduces the nitrogen requirement of cereal component. In maize + cowpea mixture, 30% nitrogen taken up by the maize is obtained from the legume (Aggarwal and Garrity, 1987). Besides, the legume biomass may be used as mulch/green manure. Many legume spp. such as velvet bean (*Mucuna pruriens*), *Sesbania* spp. and *Tephrosia* spp. are used in many countries to conserve soil, improve soil fertility through BNF and as green manure crops. Hence, it also offers scope for developing energy efficient ecological agriculture (Papendick et al. 1976).

### Nitrogen transfer

It has been shown that N fixation is the source of transferred N. In intercropping systems, the transfer of nitrogen from legume to non-legume component occurs through root exudates (current transfer), via VAM (vesicular-arbuscular mycorrhizae) connections or through decomposition and mineralisation of N from fallen leaves and dead roots and nodules (residual transfer). The root exudates of legumes contain various nitrogenous components which are directly absorbed from the rhizosphere by intermingled roots of non-legume component. The residual transfer also occurs in intercropping systems, provided that non legume component grows for more than 80 days after the harvest of legume, so that nitrogen is mineralised from legume residues e.g., cowpea/greengram/clusterbean/blackgram + sugarcane intercropping etc.

The direct/current transfer of nitrogen from forage legumes to companion grasses occurs in mixed pasture swards (Ofori and Stern, 1987). Eaglesham et al. (1981) confirmed the current transfer of nitrogen from cowpea to maize in cowpea + maize intercropping system using  $N_{15}$ . Using replacement series designs, Patra et al. (1986) have reported substantial transfer of nitrogen from legume component to the associated cereal in wheat + chickpea and maize + cowpea intercropping systems both in green house and field studies.

Estimates of residual transfer of nitrogen from legumes + sugarcane intercropping system are not available. However, results of studies on residual effects of legumes + cereal intercropping on succeeding crops are available. Nair et al. (1979) found a mean wheat yield increase of about 30% after a maize + soybean intercropping and after maize + cowpea the yield increase was 34% when compared to wheat after sole maize. Singh et al. (1983) estimated the nitrogen benefits to wheat of various preceding legume intercrops. When comparing wheat after sole sorghum, with wheat after intercrops, he obtained nitrogen fertilizer equivalents of 3, 31, 46, 40 and 54 with soybean, greengram, cowpea (grain), groundnut cowpea (fodder), respectively.

Besides VAM mediated transfer of phosphorus from a non-legume to the legume crop has also been documented.

## **Biomass**

Soil fertility management in ecological sustainable agriculture gives much reliance on the use of biomass (crop residues and other organic wastes) to maintain the status of organic matter in the soil and to meet the nutrients requirement of the crops. The crop residues release allelochemicals through volatiles, leaching and during microbial decomposition. The production of allelochemicals in soil affects germination, growth and yield of crops depending on plant residue type, amount, depth of placement and length of decomposing period. The allelochemicals may either be inhibitory or stimulatory to the succeeding crops (Rice, 1984; Waller, 1987).

## **Crops residues**

Plant residues when recycled, improve the physical, chemical and biological properties of the soil. A surface mulch of plant residues ameliorates the microclimate, reduces runoff and soil erosion and makes tillage easier. In Asia, the availability of plant residues has, of late, increased substantially due to adoption of multiple cropping, reduced tillage, stubble mulch agriculture and combine harvesting of grain crops.

Few studies on stimulatory effect of legumes crop residues have been reported. For example, chopped alfalfa added to soil stimulated the growth of tomato, cucumber, lettuce and several other plants (Ries et al. 1977). The stimulatory allelochemical was identified as triacontanol. Gill et al. (Gill et al. 1994) have also reported stimulatory effect of mungbean, sesame and soybean residues on wheat, chickpea and lentil at lower concentration. Improvement in corn yields following legume crops has also been reported (Phetchawee et al. 1985; Sukhthumrong et al. 1985).

## **Tree litter**

Trees form a major component of integrated ecological farming. They perform productive and protective functions in the agroecosystem. Most of the mature trees produce substantial quantity of litter, whose proper management is essential in organic agriculture. Trees due to their deep root system have the capability to improve physico-chemical condition of soil and extract nutrients from deep layers and return them to the surface through litter fall. In general, most of the litter falls underneath tree canopy.

Like crop residues, the litter of some tree species also stimulates the growth of associated crops. For example, corn, bean, black raspberry and quince (*Cydonia oblonga*) grew better within the root zone of the walnut trees than outside (Davis, 1928). *P. roxburghii* field soil increased the growth of blackgram (Singh and Verma, 1928) and dried residues of *Glyricidia maculata* significantly promoted the growth of tomato seedlings (Chandersona et al. 1989). Likewise, a marked increase in the productivity of pigeonpea, sesame, castor and sorghum under leucaena tree (Singh, 1983) and beneficial effects of eucalyptus on sorghum (Igboanugo, 1988) were also reported.

## **WEED MANAGEMENT**

There are about 250 major weed species in agriculture and many of them have allelopathic properties, which reduce crop growth and yield (Patterson, 1986). Continuous use of herbicides for weed control has created many problems including their persistence in soil, contamination of environment, crop injury, increase in herbicide-resistant weed population, etc. Hence, non-chemical methods of weed control are preferred. in ecological agriculture. Among different non-chemical methods of weed control, allelopathic suppression of weeds through the use of allelopathic plants in crop rotations and of phytotoxic mulches in soil fertility management is very effective (Narwal, 1994).

### **Cover crops and residue mulches**

For weed management in ecological sustainable agriculture, the use of phytotoxic mulches and cover crops is very effective. Allelochemicals significantly contribute to weed suppression when planted no-till into residues of cover crops or previous crop residues. Cover crops of wheat, barley, oats, rye, grain sorghum and Sudan grass have been used effectively to suppress broad leaf weeds (Liebl and Worsham, 1983; Shilling et al. 1986; Shilling et al. 1985).

The suppressive effect of allelochemicals of rye mulch on weeds in the field is outstanding. Chou and Patrick (Chou and Patrick, 1976) identified nine acids from ether extract of decaying rye residues in soil. Phenylacetic, 4-phenylbutyric, vanillic, ferulic, *p*-coumaric, *p*-hydroxybenzoic, *o*-coumaric and salicylic acids, all inhibited the growth of bioassay plants. Shilling et al. (1986a, 1986b) found that  $\beta$ -phenyllacetic acid (PLA) and  $\beta$ -hydroxybutyric acid (HBA) from rye residues provided 20 to 60% inhibition of common lambsquarters and red root pigweed in no-till planted soybean, sunflower and tobacco. Barnes et al. (Barnes et al. 1986) isolated two hydroxamic acids, 2-4 dihydroxy-1,4 (2H)-benzoxazine-3-one (DIBOA) and 2(3H)-benzoxazolinone (BOA) with phytotoxicity on a large number of weed test plants. Further, they also reported that a mulch of 40 days old spring planted rye reduced 69% weed biomass. Muraleedhaeran et al. (1989) isolated a microbially transformed allelochemical, 2-2- epidoxy-1, 1-azobenzene (2, "2-oxo-1- 1" azobenzene) (AZOB) from a soil supplemented with 2 (3H<sup>+</sup>)-benzoxazolinone (BOA). AZOB was more toxic to curly cress and barnyard grass than DIBOA or BOA. Although there were no detectable amounts of the biotransformation product in soil under rye residues, the implications of such phytotoxic biomagnification of allelochemicals may explain allelopathic weed suppression under field conditions.

Discoveries concerning microbial transformation of certain allelochemicals from wheat and rye residues may be significant in increasing phytotoxicity of such materials to weeds. Liebel and Worsham (1983) reported that ferulic acid released during the decomposition of wheat and rye residues in the presence of prickly sida seed carpels was decorboxylated by a bacterium living on the seeds to a styrene derivative, 2-methoxy-4-ethenylphenol. The styrene was more phytotoxic to prickly sida than ferulic acid and controls this weed in natural conditions under wheat and rye mulch.

In field experiments, residues of sorghum, sunflower, rapeseed, wheat and pea at five tonnes ha<sup>-1</sup> selectively toxified broad-leaved and grass weeds. The response of wild oats was of particular interest. Field pea and wheat residues significantly stimulated wild oat germination and growth. The germination and growth of other grass weeds was, however, significantly inhibited by all types of residues (1985). It is possible that the stimulatory compound(s) produced from wheat residues could be employed to counter the discontinuity of germination in wild oat, facilitating a more complete kill by subsequent adoption of an appropriate cultural practice.

Putnam and DeFrank (1983) tested residues of several fall and spring planted crops for weed control in Michigan, USA. The plants were desiccated by the herbicides or by freezing. Wheat and rye residues reduced weed growth by upto 88%. Mulches of sorghum or Sudan grass applied to apple and cherry orchards in early spring reduced weed biomass by 90% and 85%, respectively. In a three years series of field experiments, sorghum residues reduced population of common purslane by 70% and smooth crabgrass by 98%.

Sunflower has a pronounced allelopathic effect on germination and growth of many other plants. Consequently, it strongly influences the patterning of the surrounding vegetation (Rice, 1984). It was observed that decomposition of sunflower residues significantly reduced the total number of weeds especially the dicotyledonous ones. In a 5-years field study with oats and sunflower grown in rotation, the weed density was significantly less than in control plots with oats only (Leather, 1983). In sunflower-wheat rotation field trials, sunflower decreased the density and dry weight of wild oat and *Cirsium arvense* in the following wheat crop (Cernusko and Borkey, 1992). Similarly, sunflower reduced the population of associated weed *Trianthema portulacastrum* by 75% at flowering and 96% at maturity stage and of *Parthenium hysterophorus* by 56 and 84%, respectively. The indication was that allelopathic material was released by the roots of sunflower. The maximum inhibition was in BSH-1 variety followed by MSFH-1, Co-2 and EC-68415 in descending order (Dharamraj and Sheriff, 1994). Likewise, preceding crop of sunflower reduced the population of broad leaf weeds such as *Cleome viscosa* and *Corchorus trilocularis* and sedges like *Cyperus iria* in succeeding crop (Prusty et al. 1994).

Narwal et al. (1992) observed suppression by pearl millet of the weed density and growth in the succeeding sorghum crop. *Brassica campestris* also reduced the weed density in the same field in the following year owing to inhibitory effect of its residues and volatile excretions from the leaves on the germination and growth of other plants. This crop is used for weed control by the Tarahumara Indians in North Mexico (Chacon and Gliessman, 1982).

Residual effects on weeds have also been reported for *Tagetes patula* L., beans, corn, cassava (Altieri and Doll, 1978); sunflower, sweet potato, sorghum and soybean (Einhellig and Leather, 1988); forage sorghum, Sudangrass hybrid (Forney et al. 1985); sorghum, barley, oats, wheat and rye (Putnam, 1988); crimson clover and hairy vetch (White et al. 1989), fescue (Peters, 1968), alfalfa (Abdul-Rahman and Habib, 1989) and cucumber (Lockerman and Putnam, 1979).

## Intercropping

The mixing or intercropping of plant species with different growth habits and morphology e.g., melons + plantains provides effective weed control (Obiefuna, 1989). Likewise, undersowing of wide row crops like maize with clover or other species with spreading nature control weeds (Werner, 1988). Bantilan et al. (1974) reported that in maize crop, mungbean provides more weed suppression than peanut and ascribed it to more rapid early growth and uniform canopy structure in mungbean. Among mungbean cultivars, the more prostrate ones were more suppressive.

The farmers in south eastern Mexico, interplant squash in maize/cowpea fields for effective weed control (Letourneau et al. 1956). The squash plant suppresses weeds through shade effect and selective allelochemical inhibition. Here the peasants also grow *Stizolobium pruriens* legume with maize to control weeds (Gliessman and García, 1979). Grechkanov and Rodionov (1971) reported benefits from mixing 1-2 kg seed ha<sup>-1</sup> of wild heliotrope (*Heliotropium europaeum* L.) with several legumes. This plant not only reduced weeds by 30 to 70% but also controlled other pests.

Expression of allelopathy in fields through plant to plant interactions has also been observed. Joshi and Mahadevappa (1986) and Mahadevappa and Kulkarni (1995) found that *Cassia sericea*, a leguminous plant, effectively controlled the parthenium weed in the fields through allelopathic activity. Bansal (1994) showed that buttercup's (*Ranunculus* sp.) weed species, which cause severe infestation and suppression of wheat in mid-hill conditions of Himachal Pradesh, India could be effectively controlled by planting linseed with wheat.

In a three-year field study, barley, rye and *Vicia faba* were planted in monoculture after the harvest of summer crop. These crops grew during the winter season and were ploughed in the soil in the month of March or early April. Thereafter, summer vegetables were planted by the end of May. Barley and *V. faba* and rye + *V. faba* offered almost complete weed control and the latter was most effective. It was attributed to the release of allelochemicals from root exudates during crop growth and from decomposing crop residues (Gliessman, 1989).

## **Crop rotations**

Some weeds species are specific to some crops. Such species are: wild oat and downy brome in wheat; barnyardgrass, foxtail, sandbur and fall panicum in corn/sorghum; cocklebur and velvetleaf in soybean; field bindweed, prickly sida, spurred amoda, sicklepod and silvershade in cotton (Unger and McCalla, 1980). In these cases, crop rotation is the most effective and economical control method. Only those crops which can compete effectively against weeds are included in such rotations. Fields with summer annual weed problems are rotated with winter grain crops; likewise, fields with troublesome winter annual weeds are rotated with spring or summer crops (Wiese et al. 1989). In common poppy infested fields, rotation with wheat was less effective, while rotation with oat effectively controlled its population possibly due to allelopathic effect of oat. The poppy seeds germinated in oat field but failed to reach maturity (Lampkin, 1992).

## Phytotoxic varieties

There is variation in the content of allelochemicals in various crops. In addition, the crop varieties also differ in the exudation or excretion of allelochemicals which may affect the degree of weed control. Fay and Duke (1988) screened 3000 accessions of oat germplasm for their ability to exude scopoletin, a compound with growth inhibiting properties. Twenty five accessions exuded more scopoletin from their roots than a standard oat cultivar 'Garry'. Four accessions exuded up to three times as much scopoletin as 'Garry' oats. When one of these was grown in sand culture for 16 days with a wild mustard, growth of the mustard was significantly less than when the weed was grown with 'Garry' oats. Moreover, plants grown in close association with toxic accessions exhibited severe chlorosis, stunting and twisting indicative of chemical effects rather than competition.

Dilday (1992) reported that 347 accessions of rice out of 16134 from 99 countries showed allelopathic activity against five aquatic weeds viz. ducksalad, signal grass, redstem, flatsedge and barnyard grass. Some of the accessions repelled weeds and maintained weed free area upto a radius of 12-25 cm from their base. Since rice is planted in rows at spacings of 10-25 cm, allelopathic activity would overlap the space between the rice plants or rows leading to control of these problem weeds in rice. Dilday et al. (1992) further reported that out of the tested accessions, 347, 161 and 6 accessions demonstrated allelopathic activity to ducksalad, purple ammannia and broadleaf signal grass, respectively. Some accessions from India and Bangladesh also exhibited allelopathic activity to barnyard grass and *Cyperus iria*.

Narwal et al. (1992) screened 13 genotypes of pearl millet and found that HHB-67 and 8800 4A x 833-2 had greatest suppression effect on weeds particularly *T. portulacastrum*, the major weed of irrigated crops in Haryana, India. Sarmah et al. (1992) determined the suppression effect of 11, 10 and 8 accessions of *Brassica juncea*, *B. napus* and *B. carinata*, respectively, on winter weeds of north-west India under field conditions. They observed that RH-8689, RH-8605 and RH-8693 of *B. juncea*, HNS-8902 and HNS-11-1 of *B. napus* and BCCN-5 genotype of *B. carinata* had more smothering effect on weeds compared to their other genotypes.

Superior genotypes for weed control have also been reported in cucumber (Putnam and Duke, 1974), oats (Fay and Duke, 1977; Leather, 1983), sunflower (Leather, 1983) and soybean (Massantini et al. 1977). It appears possible, therefore, to breed allelopathic genes into standard cultivars to aid in weed control.

## Natural herbicides

Growing awareness about the environmental and public health problems linked with the excessive use of plant protection chemicals in agriculture has stimulated interest in the search for new selective, easily degradable and environmentally safe herbicides. The ability of some natural plant compounds to effectively inhibit the development of other plants has suggested that they may be used as herbicides. Besides, recent advances in the microbial and plant biochemistry have stimulated scientific interest in the possible role of secondary plant metabolites and microbial toxins as herbicides.

Among the plant products as herbicides, juglone, isolated from walnut tree has been found effective against redroot pigweed, velvetleaf and barnyard grass (Shettler and Abalke, 1983; Spelce and Muselman, 1981 and Weston et al. 1987). Caffeine derived from coffee showed considerable selectivity in inhibiting germination of *Amaranthus spinosus* L. at a concentration that has no effect on blackgram (Rizvi et al. 1980 and Rizvi et al. 1981). Strigol, a sesquiterpenoid derivative from cotton roots is a potent germination stimulant of witchweed (*Striga asiatica* L. Kuntz), an obligate parasite of maize, sorghum (Cock et al. 1966) and *Orobanche minor* (Spelce and Muselman, 1981).

Dhurrin (sorghum); gallic acid (spurge); Phlorizin (apple root); trimethylxanthene (coffee) and cinch (eucalyptus) are some other important plant products having promising herbicidal activity. The commercialization and marketing of “Herbiacae” the herbicide from microbial natural product bialaphos in Japan (Hatzios, 1987) has opened up a new era in weed management. Other microbial phytotoxins found to suppress weed growth include anisomycin, tentoxin, biopoloroxin, herbimycin etc.

## Tree farming

Very little information is available on this aspect, but it offers scope owing to availability of large quantity of tree litter during leaf fall. It may play a major role in agroforestry systems. It has been observed that in poplar (*Populus deltoides*) based agrisilviculture system, the field remains almost free from weeds during the winter season. It may be due to physical barrier on account of leaf fall at the time of germination of winter weed seeds and the release of catechol and benzoic acid inhibitors during the fast decomposition of th; leaf litter. About 30% less weed population was recorded in wheat grown in the alleys of *Dalbergia sissoo* as compared to control plots (Nandal and Bisla, 1994).

Allelochemicals from eucalyptus could be successfully exploited for the control of noxious weeds. Oils from *Eucalyptus citriodora* and *E. globulus* completely

reduced the germination of *Parthenium hysterophorus* seeds and affected the growth of mature weed plants. Similarly, the oils and other chemicals of eucalyptus checked the rooting potential of *Lantana camara* weed (Kolhe, 1994).

## NEMATODE MANAGEMENT

The root-knot nematode (*Meloidogyne*) is ranked as number one among the ten most important phytoparasitic nematode genera with wide geographical distribution, phytophagous food habit and infecting over 2200 plant species (Sasser, 1989). Owing to the high cost, uncertain availability, problems of application and phytotoxicity of plant protection chemicals as well as the environmental and health hazards associated with the use, other approaches to nematode management, including allelopathy, appear to be potential alternatives for ecological agriculture.

### Plant material as nematocides

Chopped mature dried residues of lespedeza, alfalfa, oats and flax when incorporated @ 25 tonnes ha<sup>-1</sup> into fields infested with *Meloidogyne incognita*, significantly reduced the incidence of rootknot in tomato (Johnson et al. 1967). The ploughing of rye crop in the infested soil effectively checked the *Pratylenchus penetrans* and the effect was identical to D.D. fumigation (Mountain and Elliott, 1962). The soil amended with cotton waste, lucerne pellets and lucerne hay showed reduced incidence of *Tylenchulus semipenetrans* (79). Rice straw @ 22.5 or 44.75 tonnes ha<sup>-1</sup> reduced the population of *Belonolaimus longicaudatus* and other plant parasitic nematodes (Tomerlin and Smart, 1969). Johnson (1972) observed 75-90% reduction in root-knot incidence in potted tomato grown in soil mulched with flax, lucerne or orchard grass residues. Prasad et al. (1972) found that wheat straw and neem cake + NPK gave maximum reduction in the plant parasitic nematodes associated with wheat and mungbean. Mishra and Prasad (1978) reported good reduction in *M. incognita* incidence in tomato through application of wheat straw and paddy husk.

The oil extracts from the seeds of *Argemone mexicana* weed, when applied @ 0.2% as soil drench or foliar spray, reduced root-knot nematode infestation of okra and increased plant growth. The oil showed systematic effect and the foliar spray proved more effective than soil drench (Das and Sukul, 1988). *Azolla pinnata*, a biofertilizer, suppressed the infestation of *M. incognita* in okra (Thakar et al. 1987). Species of polygonum weed have also been found effective in controlling nematodes (Ruelo, 1983).

Sukul (1994) has recommended the planting of some trees like *Anthocephalus cadamba* Mig., *Azadirachta indica* A. Juss., *Eucalyptus*, *Tectona grandis* L. and *Pongamia glabra* Vent. along roads, river banks and forests for the collection and field application of their leaves to control nematodes. Mishra and Mojumdar (1994) have reported that neem (*Azadirachta indica*) seed kernel is more toxic followed by its seed and seed coat. They found that addition of decomposed *A. indica* seed, seed kernel and seed coat drastically reduced the root-knot nematode population in soil and increased the yield of mungbean. Mulching with green leaves of *Pongamia* and *A. indica* reduced root-knot nematode infestation of mulberry plants in field experiments (Govindaialu et al. 1989). The leaves of *Ieucaena* and *A. indica* after a fixed period of degradation also control root-knot nematodes (Jain and Bhatti, 1988 and Pruthi et al. 1987).

### **Soil seed cakes and by-products**

It is a common practice among vegetable and fruit growers to use oil seed cakes as a source of plant nutrients and to control nematodes. The application of neem, castor, mustard and rocket salad oil seed cakes suppressed the population of root-knot nematode and the reniform nematode (*Rotylenchulus reniformis*) in okra and improved the plant growth and water absorption capacity of its roots (Anver and Alam, 1994). Mainpueira, a sub product in the production of cassava flour, proved effective against *M. incognita* under green house conditions as well as in the field (Franco and Ponte, 1988 and Ponte et al. 1987). The citrus fruits canning factory waste used as soil amendment gave good control of tomato nematodes (Babatola, 1989). The use of cassava root peeling and locust bean husk as soil amendments reduced root-knot of sugarcane and increased its growth (Salawu, 1988).

### **Nematicidal allelopathic compounds**

Bhatti and Nandal (1994) have reviewed the information on nematicidal substances (allelochemicals). Only a few allelochemicals have been isolated, elucidated and characterized against plant nematodes. Terthienyl isolated from marigold exhibited strong nematicidal properties under laboratory conditions. It, however, failed to suppress nematode population under field conditions even at 200 ppm (Uhlenbroek and Bijloo, 1960). Cucurbitacin that accumulate in bitter cucumber genotypes repelled more juveniles of *M. incognita* from infecting them than did the non-bitter genotype and the cucurbitacins were implicated with such repellent actions (Haynes and Jores, 1976).

The compounds isolated from tobacco (nicotine), jackbean (phytolectins) and *Ocimum sanctum* (eugenol) have been found to reduce *M. incognita* infestation of host plants including tomato and okra (Al-Sayed and Montasser, 1986; Al-Sayed and Thomson, 1988; Das and Sukul, 1988 and Davis et al. 1989). Juices from numerous plants or their parts and extracts with organic solvents or root exudates contain nematicidal compounds (Devakaumar, 1994; Gommers, 1973 and Rice, 1984).

## INSECT PEST MANAGEMENT

Most pests spp. are naturally regulated by various ecological processes viz., competition for food or by predation and parasitism by natural enemies. Their population is stable and the damage caused is relatively insignificant in most cases. Conversely modern high input farms are planted with uniform varieties, well watered and fertilized i.e. providing ideal conditions for pests attack, for which farmers use pesticides. Pesticides may be dangerous to human and livestock health and damage the natural resources, cause pest resurgence by killing the natural enemies of target pests, can produce new pest, which were not pest in the past e.g. whitefly in cotton. Pests become resistant to pesticides so necessitating their further applications and lastly they do not provide lasting control, hence, have to be repeatedly applied. To overcome such problems, farmers are advised to use wide range of technologies based on ecological processes of predation, competition and parasitism to control pests more effectively than pesticides alone. Besides the use of Integrated Pest Management practices, not only reduces the pests population to satisfactory level, but also are sustainable and non-polluting. In ecological sustainable agriculture, insect control with non-chemicals can be achieved through the use of cropping systems, insect pest resistant varieties and antibiotic allelochemicals of plant origin.

### Cropping systems

#### Crop rotation

In crop rotations, maximum use of those crops which reduce pests infestation should be encouraged. The approach is to rotate non-host crops with susceptible crops in sequence. Non-host crops reduce the pests population to very low level and then susceptible crops may be grown. The non-host crops provides a break, disrupting the relationship between a pest or pathogen and its host.

In crop rotation, the best results are usually achieved when botanically unrelated crops are rotated. For example, in upland rice-corn-cowpea-fallow rotation, corn and cowpea are unrelated, have different growth habits and attract different pests. Soil

insects such as wire worm (*Elateridae*) and white grubs usually have a wide range of hosts, which restricts the choice of crops in rotation on the same land. Rotation of about 4 years duration, under host free conditions is usually necessary to reduce the pest population to a tolerance level (Gibson et al. 1958).

### **Crop mixture/intercropping**

When different crops are grown together as in mixed cropping or intercropping, the pests recognise a suitable crop either by sight or smell. In such a situation, it is possible to confuse the pests by adopting suitable structuring of the crop components.

The crop mixtures/intercropping are also very useful to reduce the pests infestation through (a) release of repellent allelochemicals as volatiles, (b) different plant spp. confuse the insect pests and (c) the various plant spp. provide a physical barrier to the movement of insect pests etc. A large number of small farmers in tropical and sub-tropical countries rely on this system. In Latin America, 60% maize is grown with beans and generally rice, cotton, beans and cassava are grown in mixtures. Generally, more diverse the agroecosystem, the less will be abundance of herbivore pests. Mixtures of cabbage + tomato, reduces the colonization of diamond backmoth, while maize + beans/squash mixtures have the same effect on chrysomelid beetles. Besides, the odours of some plants can also disrupt the searching behaviour of pests. Grass borders repel leaf hoppers from beans and the chemical stimuli from onions prevent carrot fly from finding the carrots. Alternatively, one crop in mixture may act as a trap or decoy crop the “fly paper effect”. Strips of alfalfa interspersed in cotton fields in California attract and trap *Lygus* bugs. The loss in alfalfa yield, offsets the cost of alternative control methods for cotton. Similarly, crucifers interplanted with beans, grass, clover or spinach are damaged less by cabbage maggot and cabbage aphids. There is less egg laying on the crucifers and the pests are subjected to increased predation (Pretty, 1995).

### **Resistant varieties**

The presence of antibiotic metabolites in some plants makes them comparatively more resistant to the common insect pests. The resistant varieties identified in rice, wheat, maize, sorghum, cotton and alfalfa are being used for breeding commercial varieties. Pathak and Dale (1983) have isolated some antibiotic metabolites such as saponins, phenolic acids, 6-MBOA, DIMBOA, gossypol, quercetin and 2-tridecanone. Rao (1994) while screening 17 chilli genotypes, found that the pod borer resistant variety ‘Loc’ had the highest content of total phenols (0.61%) while the susceptible genotypes ‘Tetraold’ and ‘Lanka-l’ had the least amount (0.31%).

## INSECTICIDAL ALLELOCHEMICALS

Many locally available plants have pesticidal properties and are used to repel, deter or poison pests due to presence of pesticidal allelochemicals. Many of these kill only pests and not the predators degrade rapidly so do not contaminate environment. Increasingly scientists are determining the mechanisms of such practices. The role of plant allelochemicals in plant-insect interactions has received considerable attention in recent years. The allelochemicals are used in pest control as repellents, antifeedants, growth disrupters and toxicants. Allelochemicals commonly found in plants are toxic amino acids, protease inhibitors, alkaloids, cyanogenic glycosides, phenols, tannins, lignins, flavonoids, toxic lipids, glucosinolates, terpenoids, saponins and phytohaemagglutinins.

Among tree species, neem (*A. indica*) has received substantial attention during the past decade particularly for insect pest control. It has been an age old practice in rural India to mix dried neem leaves with stored grains or to place them among warm clothes to repel-insects. Butterwoth and Morgan (1968) isolated a substance 'azadirachtin' from neem seed that inhibited feeding in desert locust. Since then many azadirachtin based insecticides have been formulated and found effective against insects. The azadirachtin based formulations viz. Azadirachtin, Neemark, Achook, Margoside, Nimbicidine, Repelin, Parasmani, Jawan, Sukrima, Neem oil, Neem gold, Nocilneem, Neemata-2100 and Neemrich-I and II have been found effective against cotton bollworm (Dilday, 1992 and Panchabhavi et al. 1994); castor semilooper (Ivani et al. 1994); white fly of cotton (Simawat and Dhawan, 1994) and rice hispa (*Dicladispa armigera* (Dhaliwal et al. 1994).

## DISEASE MANAGEMENT

### Cropping systems

Continuous cultivation of same or related crops leads to perpetuation and build up of soil pathogens which gradually increase the disease intensity. Crop rotation is one of the natural methods of disease prevention especially when botanically unrelated crops are included as they are affected by different pathogens. Crop rotation helps to control many soil borne diseases such as mosaic, wilt of legume crops (pigeonpea, pea, chickpea) and linseed, red rot and wilt in sugarcane, ergot and smut in pearl millet, leaf smut and bunt in rice, bunt and molya disease in wheat and barley and root rot in vegetable crops. Chohan (1968) observed that crop rotation was particularly effective in lowering the population of soil borne pathogens.

The mixtures of different crop species provide buffer against losses from diseases by delaying the onset of disease, reducing spore dissemination and/or modifying microenvironmental conditions. In soil borne pathogens some plant conditions may enhance soil fungistasis and antibiosis through indirect effects on soil organic matter content. The presence of immune or resistant plants in mixed cropping systems impedes the spread of pathogens and increases the separation between susceptible plants. Larios and Moreno (1977) documented evidence of disease buffering in various intercropping systems.

## **Crop residues**

Many plants produce chemicals either prior to or after infection by certain pathogens which render the plants resistant to diseases (Rice, 1984). The first scientific report on the benefits of plant and other organic materials in disease control appeared 50 years ago. They included reports on control of potato scab, wheat take all and root rot of cotton with various organics (Cook and Watson, 1969). In Texas, grain sorghum is a popular alternate crop with cotton. Sorghum provides 20-25 tonnes ha<sup>-1</sup> of residue. This residue on incorporation in soil, controls root-rot in the subsequent crop of cotton. Green manuring with clover also controls this fungus (Lyle et al. 1948). Soybean residue incorporated in soil before planting of potatoes, controlled the potato scab due to antibiotic production by *Bacillus subtilis*, a bacterium antagonistic to *Streptomyces scabies* which cause potato scab. Volatile compounds released during decomposition of crop residues also deserve attention because of their influence on plant pathogens (Linderman and Gilbert, 1975). Various aldehydes from decomposing alfalfa may stimulate the germination of micro sclerotia *Verticillium dahliae* and *Sclerotium rolfsii*, followed by lysis causing a net reduction in the population of these two fungi. Crucifer residues emit sulphur containing volatiles during decomposition that are inhibitory to *Aphanomyces euteiches* and may reduce the root rot of peas caused by this fungus (Lewis and Papavizas, 1975).

Although residues incorporated in adequate amounts and at the right time are generally suppressive to root diseases, they may also increase some diseases. Plant residues may be colonized by a pathogen which uses them as energy source. Residues may also produce certain phytotoxic decomposition products that may predispose roots to infections. Such phytotoxins have been reported to increase the susceptibility of certain tobacco cultivars to black root-rot (Patric and Koch, 1963); cotton plants to root rot caused by *Theilaviopsis basicola* (Linderman and Toussoun, 1968), bean to root-rot (Toussoun and Patrick, 1963); sugarcane to pythium root-rot (Rands and Doppe, 1938) or cause injury to plant roots and thereby open the way for secondary root decay (Carley and Watson, 1967 and Cochrane, 1948).

## Organic amendments etc

Among the common organic amendments, neem seed and cake have been found effective against many plant diseases like rice bacterial blight (Eswaramurthy et al. 1994); rhizome rot of ginger (Dohroo, 1994) and other diseases caused by *Rhizoctonia solani*, *Macrophomina phaseolina*, *Fusarium solani* and *Phytophthora capsici* (Singh, 1994). Addition of cotton cake @ 15 9 kg-1 soil reduced the incidence of seedling blight of eucalyptus from 80 to 27% owing to release of inhibitor chemicals (Kaushik et al. 1994).

Some neem derivatives like Neemta-2100, Neemark, Nimbicidine, Sukrina, Neemoil and Jawan have been found effective against yellow mosaic virus of horsegram. Powdery mildew fungi has also shown sensitivity to Neemta-2100, Neemark and Nimbicidine (Joshi et al. 1994).

## ALLELOCHEMICALS AS GROWTH REGULATORS

Growth regulators are exogenous non-nutrient substances that manipulate growth, development and composition of plants and functions by interaction with the endogenous phytohormones groups. Their action include growth retardation, flower induction, hastening of maturity or senescence and stimulating biomass production etc. Allelochemicals provide a promising source for new growth regulating compounds viz., agrostemin, triacontanol and brassinolide, which have received maximum attention.

Bioprodukt (1984) summarized Yugoslavian work showing that 100 g agrostemin per hectare through seed treatment or foliar spray hastened germination and increased yields of wheat, maize, sunflower and sugarbeet by 10, 15, 15 and 10%, respectively. It also enhanced the oil content of sunflower by 4%. It has proved beneficial to vegetables, flowers, fruits, pastures and forest species.

Triacontanol, a 30-carbon primary alcohol, was isolated as a growth promoting compound from alfalfa. Its foliar applications increased the yields in cucumber, carrot, rice, corn, soybean and others. Inconsistent results, perhaps due to accumulation problems and to method, rate and time of application, reduced its efficacy (Laughlin et al. 1983). Extensive work has been done on evaluation of brassinolide, a steroid isolated from rape (*Brassica napus* L.) pollen as a yield stimulant. Brassinolide and several analogues have been synthesized (Maugh, 1981) but they are too expensive for use in field crops.

All the bioregulators have shown increase in yields of major crops. However, inconsistency in performance between the locations, genotypes and spraying dates, besides difficulties in formulations, has hindered their commercial use.

## CONCLUSIONS

The crop, weed and tree residues constitute the major source of organic matter in the soil. They also release numerous organic and inorganic compounds in the soil. These compounds (allelochemicals) are generally inhibitory to the growth of other crops/trees depending on the residue amount, length of decomposition period and type of residue. However, some plant residues especially legume residues like alfalfa, mungbean, soybean and leucaena stimulate the growth of crops. In addition to this, plant residues through the release of allelochemicals and biotransformation products decrease the incidence of pests (weeds, nematodes, insect pests and diseases). Intercropping and use of phytotoxic crop varieties and natural herbicides are effective methods of non- chemical weed control in ecological sustainable agriculture. Plant diseases including those caused by nematodes and infestation by insect pests in agriculture could be successfully controlled through the use of organic amendments, allelopathic compounds and crop varieties rich in allelochemicals content. In view of the practical significance of allelopathy in ecological sustainable agriculture, research efforts are needed to make use of the inhibitory allelopathic effects of plants for natural control of crop pests and diseases.

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## CHAPTER 25

# PARASITIC WEEDS AND ALLELOPATHY: FROM THE HYPOTHESIS TO THE PROOF

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## ABSTRACT

Parasitic weeds represent a main component of weed problems facing agriculture and negatively impact agroecosystem and environment. They belong to different plant families and are either hemi- or holo- root or shoot parasites. They attack plant species of different botanical families and growth forms and distributed in different geographical regions in the world, cause great yield losses and under sever infestation they lead to complete crop loss. Parasitic weeds represent a highly ecological, biological and physiological tolerant species to both internal and external environmental changes. They adapt themselves to disperse by different common agents and to survive and tolerate severe environmental condition under which long-term ecophysiological dormancy has been very well demonstrated and developed in many of the parasitic species in absence of suitable conditions for germination mainly the availability of host plants. The relationship between these parasites and their hosts is highly complicated, both sometimes work jointly for successful attachment and complete parasitism, while in contrast, antagonistic interactions are most common between both at which different mechanisms may be involved in the resistance of host to parasite attachment, germination, penetration and development which is termed as tolerant/resistance mechanism or sometimes described as a defense mechanism. The parasite may be also very well prepared for positive response to host signals and showed ability to attach itself to the host cell surface and to penetrate with aid of enzymes or other chemical secretions that allow dissolvent and separation of host cells. In the central of these complicated chemical reactions between parasite and host plants

allelochemicals are involved. Both partners can use these chemicals to negate the effect of the other either by stimulation of parasite and host growth, and thus in some cases facilitate this association or each may use them as harmful natural chemicals to achieve their final goal. The nature of parasite/host relationships, the allelochemicals role in parasitism process, their chemical nature either as stimulants or inhibitors and the development and utilization of these secondary metabolites as new tools for parasitic weed control and their possible practical application are reviewed and discussed. The most important parasitic species were considered and their relations with host plants from the allelopathy point of view and the role of this mechanism in parasitism process were thoroughly reviewed.

## INTRODUCTION

Parasitic weeds represent a group of plants of unique life forms, growth habits and habitats; they depend partially or totally on host plants for almost the entire life cycle. Generally, these species lack normal root system or for certain species roots were just lost shortly after emergence. These parasites represent a real problem to agriculturalist, ecologist as well as to scientists since they cause great yield losses due to their direct sucking habit of food and/or water from host plants which can lead to complete crop failure under sever infestation. Parasitic species attack plants of different growth forms and botanical families. Variations in host range within and among species are evident (Parker and Riches, 1993), depending on their chosen co-evolutionary pathway (Musselman, 1987), while host preference for the same species or between species of the same or different genera has been also documented (Parker and Riches, 1993; Qasem and Kasrawi, 1996; Hudu and Gworgwor, 1998). Parasitic species were reported to attack most vegetables, forage plants, shrubs, and fruit and forest trees, reflecting high physiological/chemical tolerance and/or a wide variation in the mechanism they adopt to dissolve plant tissues and food materials and to facilitates penetration and insures food and water availability for their peruse.

Parasitic habit is a wide spread phenomenon among angiosperms. Kuijt (1969) reported 17 plant families with parasitic species, and later Musselmann (1982) mentioned the presence of at least five orders of parasitic plants that have evolved independently in 20 plant families (Musselmann, 1987) and comprising 3000-5000 species (Musselmann, 1987; Sauerborn, 2001) However, six families were considered of great economic importance including Scrophulariaceae, Orobanchaceae, Santalaceae, Cuscutaceae, Viscaceae and Loranthaceae. Approximately 30 genera of parasitic angiosperms have been reported to negatively impact cultivated crops, while only about 11% of all genera have members that could be considered pathogenic. It is generally considered that *Cuscuta*, *Arceuthobium*, *Orobanche*, and *Striga* cause most of the damage to economic host crops.

Parasitic species are either root or shoot parasites, widely varied in their degree of dependence on host plants. Holo-parasites totally lack chlorophyll (or nearly so); completely dependent on their hosts, most are root parasites, while certain stem parasites (e.g. *C. europaea*) of these have lost rubisco, thylakoids, chlorophyll and light-dependent CO<sub>2</sub> fixation (Machado and Zetsche, 1990). On the other hand, hemi-parasites, do photosynthesis (at least during some portion of the life cycle), but in addition they parasitize other plant species. They can be further divided into two groups, facultative (do not require a host to complete life cycle), and obligate that completely host- dependent.

Once the parasite became in contact with host plant it starts forming and developing a sucker like structures (haustoria), inserted into host tissues (Figure 1).

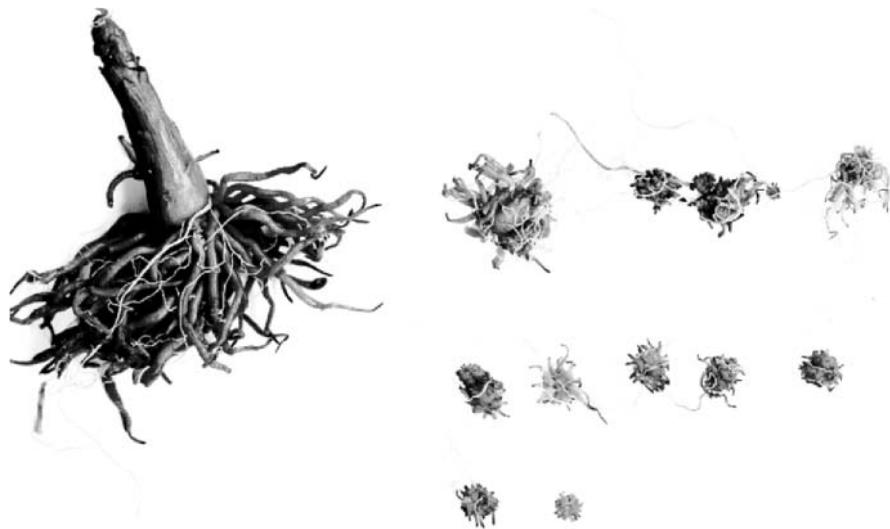


Figure 1. *Orobanche ramosa* haustorium at different development stages

Haustoria are varied in size, length and number per individual parasitic plant depending on the species and range in number between one (single connection point) such as for *Orobanche*, *Striga* and *Viscum* to many in *Cuscuta* and *Loranthus* (Figure 2). The haustorium is a bridge of tissue connecting host and parasite, it is usually a swollen mass consisting of both host and parasite tissues and act as a conduit for the flow of water and nutrients from host to parasite.



*L. acacea*



*C. tubulosa*

Figure 2. *Cistanche tubulosa* (single connection point) and *Loranthus acacea* (many connection points)

Differences between parasitic species in seed production, longevity period, germination, dispensability and dispersing agents, attachment to host plants, morphology and growth habit, physiological and anatomical relationships between parasites and their hosts all challenged control measures. They are very well adapted to short and long dispersal distance. Selective chemical control with herbicides is far from success, difficult to achieve in most cases, and depends on differences in tolerance between parasite and host plants. The direct internal contact of parasites to host plants greatly limits herbicide application without serious injuries to crop plants in 568

most cases. In addition, certain parasitic species are not visible before emergence (root parasites); the stage at which most damage to host plants occurs. However, different methods of control are recommended and applied against many parasitic species, while some other promising strategies such as allelopathy have been recently emphasized.

## PARASITE/HOST RELATIONSHIPS

The relationship between parasitic species and their hosts can be described as a kind of compatible/antagonistic one since both involve chemicals that stimulate germination and attachment, and others incompatible, phytotoxic or prevent germination, attachment, growth or development of the parasite. The first group can be classified as synergistic chemicals or stimulants while the second termed antagonistic or inhibitory one. Mallet (1973) suggested that root extract of broad bean contains both inhibitory and stimulatory substances to *Orobanche crenata* seed germination, and Whitney (1978) came to the same conclusion and clearly showed that certain nonhost species to *O. crenata* release compounds that inhibit parasite germination even in the presence of suitable stimulant, and the possibility that different stimulants may interact together in inducing seed germination of the same parasite is also existing. Both types of chemicals however, were considered as allelochemicals based on the most recent definition of Allelopathy proposed by the International Allelopathy Society (IAS), and therefore both stimulation and inhibition processes are involved in plant-plant interactions and the whole phenomenon was classified and laid under the term allelopathy (Rice, 1984). The wide differences between species in host range they attack proved differences in their ability to produce different tissue-dissolving enzymes, or their fitness and compatibility to indigenous chemicals that host plants release in form of phytoalexins as a defense mechanism against parasite attack and invasion. Differences between host plants in production of these defense chemicals or in stimulating parasite attachment to their tissue surface explain in part the susceptibility or tolerance level that these species or cultivars show in response to parasite attack. Joel and Goldman-Guez (2001) reported pectin in host cell walls deesterify, presumably by enzymes that derived from the host itself, indicating that some steps in penetration are facilitated by the host, through a mechanism that are induced by the parasite. It has been also suggested that *Striga* lactase activity releases quinones from the host root that stimulate haustoria initiation (Stewart and Press, 1990). In this regard, different studies tried to quantify this relationship between host species and their parasites and its reflection on their general ecological responses. In a study tried to analyze, the interaction between *Cuscuta epithymum* and its host plant *Hormathophylla spinosa*, Gomez (1994) found that parasitized plants were visited by fewer pollinators than were non-parasitized plants and the diversity of the pollinator assemblage also differed. Even the relationship between an individual dodder and an individual host plant is neutral, due to the array of direct and indirect, positive and

negative effects appearing between *C. epithymum* and *H. spinosa* during the successive phases of the reproductive cycle of the host plant. Exchange of chemicals between host and parasite was also documented and is a good evident on the strong relationship between the parasite and its prey, which should be also considered. Export of abscisic acid (ABA) from leaf petiole of *Pelargonium zonale* to *Cuscuta reflexa* shoot was reported by Bock and Fer (1992), while ABA was found to rapidly accumulate in haustorium. The authors suggested that the very fast ABA uptake might have involved facilitated diffusion in addition to the diffusion of undissociated acid, and the high concentration of ABA in *C. reflexa*, especially in haustoria, could be explained by ABA import from host tissues. The authors concluded that ABA might have a central role in the host-parasite relationship by enhancing sucrose transfer. Kumar et al. (1993) studied *Cuscuta* sp. grown separately in pots and with host crops and indicated that the parasite did not require any stimulus for germination. Among the crops tested for host specificity, pulses in general were more seriously infested than oilseeds. The cereals were never infested by the parasite, which may be due to mechanical resistance. Baumel et al. (1994) reported exchange of quinolizidine alkaloids between various host plants of the Fabaceae to parasitizing *Cuscuta* species.

Allelochemicals were reported to play a significant role in the host/parasite interaction at least during the germination phase (Perez-de-Luque et al. 2001). It is known that a number of host plants produce and excrete chemicals, which induce parasitic plant seed germination, while other inhibit or kill germinated seeds (Jorrin et al. 1999). However, both parasite and host plant may release phytotoxic materials against each other. The former may use these chemicals to break down any defense mechanism shown by host plants or to counteract the effect of host chemicals and facilitate dissolvent or separation of its cells and tissues and thus enhance parasite attachment and haustoria embedment in host organ. In contrast, host phytotoxic chemicals may be released to prevent attachment and act as repellent to the parasite or mask its ability to successfully attach itself to the host plant or to prevent its development. Sinebo and Drennan (2001) were to suggest that sorghum released resistance-conferring substances to the infection points of *Striga hermonthica* after sensing infection. When infection points are widely distributed as in fully infected sorghum, less of such substances appear to render the host more vulnerable. Mohamed et al. (2001) suggested the potential release of germination inhibitors from sorghum genotype P78 since *Striga* seeds near its roots consistently showed lower germination after ethylene treatment than those further out on the Petri-dish. Pigeon pea (*Cajanus cajan*) a trap species for *Orobanche* spp., was reported to affect different weed species through an allelopathy mechanism (Semidey and Bosques-Vega, 1999). In contrast, Bell-Lelong et al. (1994) suggested that *Striga* shows allelopathic activity on its host by means of a toxin and this was proved by the presence of high levels of oxidizable phenols and oxidative enzymes in *Striga hermonthica* shoot extracts. On the other hand, Perez-de-Luque et al. (2001) found that susceptible variety of sunflower to

*O. cumana* excreted a higher amount of secondary metabolites inducing parasite germination than the resistant one or more specific compounds.

Genetics play an integral role in allelochemical production, and an allelochemical-control gene is mainly involved in this processes. In a study carried out by Yoder et al. (2001) they reported that when parasitic plants are exposed to host root exudates, genetic pathways for both allelochemical detoxification and developmental signaling are invoked. A xenognostic organogenesis mechanism is also invoked and thus parasitic plants may have evolved the ability to use host defense molecules (allelopathic molecules) that act against neighboring plants. Different parasitic plants mainly *Orobanche* and *Striga* depend on chemical signals received in the root exudates of their hosts in order to germinate, this however, suggests expression of several parasite genes (Atanasova et al. 2001). The adaptation of *Striga* to parasitism includes also dependence for developmental signals and therefore parasite and host development are highly integrated. It was reported that early host-derived chemical signals that *Striga* requires are mainly exuded from host roots into the soil (Butler, 1995).

Host plants were found to respond in different ways to parasite attack (Westwood et al. 1996), among which lignifications for the parasite or host cell wall at the point of parasite penetration was reported by different authors (Arnaud et al. 1998 and Hood et al. 1998). Among the defense responses used by plants are phytoalexins which can be attributed in part to altered regulation of the isoprenoid biosynthetic pathway (Griffiths et al. 2001) leading to production of many compounds necessary for general metabolism and growth.

In contrast, different parasitic species were reported to contain different forms of allelochemicals of various inhibitory properties. Dini et al. (1995) reported phenolic metabolites from dried and aerial parts of *Orobanche speciosa* that gave this species antimicrobial properties. Saadoun et al. (2001) showed that spike tissues of different *Orobanche* species contain both phenols and manitol and may act in the defense mechanism of these parasites against bacterial infection. These authors concluded that *Orobanche* tissues could be a potential source for antipathogenic bacterial agents. These allelochemicals may be also used against host plants to facilitate parasite invasion. However, failure of host plants from defending themselves against parasite attack despite the induction of defense-related genes was in part addressed by Bell-Lelong et al. (1994) by that defense compounds produced may be degraded by the parasite or phytoalexins produced by the host could be not specifically toxic to parasite.

## BIOLOGY OF PARASITIC WEEDS AND ALLELOPATHY ROLE

Different workers have mentioned that the stage from seed germination to attachment is the most sensitive stage in the life cycle of parasitic weeds and control should be applied during this phase (Salle et al. 1984; Dhanapal, 1996b). Parasitic species differ in their requirements for chemical stimulants for seed germination. Certain species require seed preconditioning period and host stimulant/s for germination to start (*Orobanche*, *Striga*, *Cistanche*), while others do not (*Cuscuta*, *Thesium*, *Osyris*, *Viscum* and *Loranthus*). Conditioning period may increase the responses of dormant seeds to germination stimulants of host species, however, differences in parasite requirements for chemical stimulants created wide differences in their hosts. Certain parasites are more confined to specific host growth form and appeared highly specialized in woody shrubs, fruit trees or forestry species (*Loranthus*, *Viscum*, *Osyris*, *Cistanche*), others are more restricted to herbaceous and field crops (*Thesium*, *Striga*), while some can attack both woody and herbaceous species (certain species of *Orobanche*, *Cistanche* and *Cuscuta*). Differences between species of the same genus in their host range have been already noted (Parker and Riches, 1993). Seeds of certain species can't germinate without passing a pre-conditioning period and receive host stimulants, others do not require such conditions but appeared of a limited and specific host range, and not able to attack other plant species, which may reflect host preference/specifity or certain host mechanical, physiological or chemical resistance of the non-host species.

The chemical mechanism behind failure of these parasites from attacking any plant species is generally unknown and could be utilized as another method of their control. However, different workers mentioned that host preference in *Striga* spp. is due to different germination stimulants in the host root exudates (Bharathalakshmi and Jayachandra, 1984 and Bharathalakshmi et al. 1990). The ability of host and nonhost plant species to stimulate parasite seed germination encouraged studies on possible use of these as catch or trap species to reduce parasite infestation. Stimulants of root parasites are mainly natural chemicals (Alleochemicals) released through host root system into the surrounding environment and received by parasite seeds. These may encourage parasite seed germination by affecting internal hormonal balance and thus overcome the special seed chemical-based dormancy. They may also act as hormones (e.g. strigols and their analogs of GA-like chemicals) affecting the embryo, germination and growth of the parasite germination tube. Other chemical stimulants produced by host plants could encourage stem parasites to penetrate and to grow inside host tissues (Joel and Goldman-Guez, 2001). It is worth mentioning that both susceptible and tolerant or resistant species produce germination stimulants but differences are mainly in the amount of stimulants received by parasite seeds. However, some of the chemicals exuded from roots of resistant, tolerant or non-host species may contain phytotoxic materials that also prevent seed germination or harm germinated parasite seedling or prevent attachment. In this regard, it has been reported

that xenognostic allelochemicals that trigger haustoria development are commonly quinones, phenolic acids, or flavonoids derived from the phenylpropanoid pathway (Riopel and Timko, 1991 and Albrecht et al. 1999).

### **Orobanche**

*Orobanche* species are holo-obligate root parasites, lack chlorophyll and depend completely upon their hosts for water and nutrients. They produced a huge number of seeds varied between species, exhibit dormancy, and in general require a period of after ripening in warm, dry storage, followed by conditioning in a warm, moist environment, before respond to germination stimulants. Seeds of certain *Orobanche* species (e.g. *O. crenata*) in particular must undergo after ripening dormancy, which may extend from 18-24 months of storage. However, the length and effectiveness of the after ripening period may vary with temperature, humidity, and other environmental factors. The nature of the changes occurring in the seeds during after ripening is unknown. Various structural and metabolic changes in the seed coat and embryo may occur, and changes in the respiratory substrate are also involved. Mature seeds require conditioning period between 1-3 weeks during which seed coat permeability may increase, water absorption occurs and/or changes in the levels or activities of endogenous germination promoters or inhibitors may also occur. Joel et al. (1995a) provided evidence that gibberellin synthesis occurs during this period and that hormone sensitizes the seed to germination stimulants during this period. The duration of conditioning is dependent on certain factors like temperature, origin, and age of the seeds. The period of time necessary for conditioning generally ranges from several days up to several weeks. However, seeds germinate when in contact with host roots, triggered by chemical recognition, hence seeds should receive germination stimulants with root exudates of host plants while host roots should be within few mm of germinated seeds, and in general germination occurs in the immediate vicinity of the host root. It appears that the chemical stimulant secreted by the host root system can cause germination up to a distance of 1 cm from the root but only seeds within 2-3 mm of the root can actually infest the host. On the other hand, changes in pH or presence of calcium or phosphate ions that modify the effect of pH had a significant effect on germination stimulants while inactivation of germination stimulants in the soil was found to be due to microorganisms and the effect varied with soil type. Exudates concentration is another factor affecting germination in which high concentration reduces germination due to components rather than stimulants. It seems that plant exudates contain more than one stimulant some trigger particular species to germinate but not others. This explains variation in host range of different *Romance* species and also differences in the degree of specificity shown by seeds of the same species but of different origin. Radicle movement toward the host root system is in a chemotropic response, or by bending movement in response to inhibitory chemical substances in the

root exudates of host plants. This germination feature probably is highly evolved and acts to enhance seedling survival.

Different materials were found to enhance *Orobanche* seed germination which will be reviewed later in this chapter. However, life cycle of *Orobanche* species consists of two main stages, the hypogeal that occurs below the soil level and the epigeal stage when the parasite appears above the soil. The first is responsible on most of the damage that parasite cause to host plant, while the second stage mainly occurs for flowering and seed production and it is relatively short.

### ***Striga***

*Striga* spp. are holo-root parasites turned to green color after emerge which enable them to photosynthesize and therefore sometimes regarded as a hemi-parasitic species by different authors. However, most species are obligate parasites, host specific although differences between species in the extent of their host range are evident (Parker and Riches, 1993). *Striga* species are mainly spread in the tropical and subtropical regions of the world. All require host stimulant/s for germination to start. *Striga* seeds produced in a huge number per plant varied per capsule from about 700 in *S. hermonthica* to 800 in *S. asiatica*. Numbers of capsules are also differing per plant, but average between 60-70 in *S. hermonthica* and *S. asiatica*. However, healthy and well-developed plant may produce up to 500000 seeds can stay dormant in the soil for 10-14 years. Seeds are minute and range in size from 0.15 x 0.31 mm and weighed from 3 - 15 µg characterized by the non smooth seed coat which may play a role in the uptake of germination stimulants.

Like *Orobanche*, *Striga* seeds require after-ripening period for at least 6 months for *S. asiatica* and *S. hermonthica* before germinate, during which germination rates are very low or zero. Seeds are known to possess a physiological dormancy and they should be adapted to semi-arid conditions that enhance dormancy breaking (Parker and Riches, 1993). The after-ripened seeds fail to germinate even in the presence of host stimulants unless they receive a period of conditioning, during which imbibitions occurs for a period of 10 to 21 days before exposed to a germination stimulant for germination to start. Conditioning temperature range between 20-40°C, but optimum temperatures are between 25-35°C, depending on the species. Other requirement for this phase is the adaptation of parasite to the exact time of germination. However, optimum conditioning period should be strict and only for 2 to 3 weeks otherwise, germination declines and eventually becomes zero. Long conditioning force seeds to enter secondary dormancy (wet dormancy) which acts as a survival strategy for these species. During conditioning main metabolic changes occur with a characteristic pattern of respiration and synthesis of DNA proteins and hormones. This period was also thought to be necessary for removal of phenolic compounds that inhibit

germination (Musselman, 1980). In contrast, excessive washing or prolonged conditioning period may leach a water soluble unknown germination stimulants and lowered germination response (Joel et al. 1995b).

It has been also reported that conditioned seeds of *S. asiatica* release ethylene, which elicits germination. Non-dormant seeds were found produced 3000 etaL of ethylene/600 seeds/24 h after 12 days of conditioning (Mohamed et al. 2001). However, Involvement of ethylene biosynthesis in *Striga* seed germination is well documented (Babiker et al. 2000), and ethylene initiates the biochemical reaction lead to seed germination (Logan and Stewart, 1991). Seeds germination however, occurs in response to specific germination stimulants exuded by host plant and in a region of 3 to 6 mm from the root apex. Furthermore, germination of *Striga* seeds only occurs when they found not more than 2 cm away from the root surface (Kuiper, 1997). Suitable environmental conditions for almost a week should be available before and after seeds receive the host germination stimulants.

Once the *Striga* seeds have germinated, they have to make contact with the root of the host to parasitize it. Parasite seedling should locate the host roots within few days after germination before exhaustion of the limited-stored food, or they will soon die. Therefore for successful attachment, parasitic species have evolved complicated host recognition strategies (Musselman, 1980; Calder and Berhardt, 1983), while germination must take place within 3 to 4 mm of the host root since *Striga* radicles can manage only 2-4 mm (Ramaiah et al. 1991). *Striga* radicle growth move towards the host root in a chemotropism response and attachment to the host root surface takes place as soon as the parasite meets a host root. This is facilitated by the secretion of an adhesive substance by the parasite. When the germ tube contacts the host root the elongation of the radicle stops and the development of the so-called haustorium begins. Parasite seedling develops the haustoria, which attach itself through haustorial hairs to the host root, and represent the physiological connection between host and parasite. Afterwards, haustorium penetrates host vascular tissue establishing a connection that allows absorption of water, minerals and carbohydrates necessary for its development and therefore the parasite affects the photosynthetic activity of its hosts (Oliver, 1995). The penetration of the host tissue occurs by intrusive cells, developed at the root tip, which penetrate the cortex of the host root. Very shortly after the penetration an open connection between the parasite xylem and the host xylem is formed allowing the absorption of water and nutrients. As the seedling grows, adventitious roots develop from the base of the elongation stem. Where these roots make contact with other host roots and secondary haustoria are formed. This usually occurs already within two weeks after attachment of *Striga* to host roots, and thus it may be assumed that these secondary haustoria (there may be many hundred to a single *Striga* plant) are very important in supplying the parasite with resources (Kuiper, 1997; Parker and Riches, 1993). The most destructive effect occurs to host plant while the parasite still hidden below the soil surface which extend approximately four to five weeks during which

most physiological and morphological disturbance to host plant occurs. However, more detailed information on biology, physiology and growth of parasitic angiosperms can be found elsewhere (Musselman, 1987; Keyes et al. 2001).

### ***Cuscuta***

*Cuscuta* species are holo-obligate-stem parasites, greatly differ in morphology and host range but all do not require any host stimulants for germination. However, chlorophyll and other pigments are present but their function in photosynthesis is greatly limited and appeared not satisfying the actual need of these parasites. *Cuscuta* species are generally prolific seed producers, seed capsule is about 1/8<sup>th</sup> inch in diameter, with thin papery walls and contain 1 to 4 seeds yellow to brown or black, nearly round and have a fine rough surface with one round and two flat sides.

*Cuscuta* seed dormancy is mainly due to the seed coat itself. However, part of the seeds may germinate directly after maturity on the mother plant. Dormant seeds called hard coated seeds require sulfuric acid treatment to break dormancy (Zaki et al. 1998); in addition, potassium nitrate, and mechanical scarification are also effective for dormancy breaking of all species. The removal of hard seed coat by sulfuric acid and seed conditioning were found to promote *C. trifolii* germination (Lados, 1998).

Far-red and blue light is also important treatment for seed germination. Furuhashi et al. (1995) found that far-red light induced mutual and self-parasitism of *Cuscuta japonica* and seedlings irradiation with far-red or blue light of *C. japonica* grown with *Vigna radiata*, allowed host seedling to be parasitized (Tada et al. 1996). However, the coordinated effects of two physical signals, Far-red light and appropriate tactile pressure control the parasitism of this parasitic species, and seed treatment of *C. trifolii* and *C. campestris* was effective in increasing germination rate. However, soft seeds of different *Cuscuta* species are ready to germinate if favorable environmental conditions are available. The parasite, does not require any host stimulation or the presence of host for seed germination to occur and this was confirmed through an anatomical study of *Cuscuta* spp. grown separately in pot with host crops including mungbean (*Vigna radiata*), *Vigna mungo*, pigeon peas (*Cajanus cajan*), sunflower (*H. annuus*), sesame (*Sesamum indicum*), castor bean (*Ricinus communis*), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*) and maize (*Zea mays*) (Kumar et al. 1993). Germination occurs (permeability of seed testa provided) when temperature and moisture conditions are favorable, even immediately after harvest. However, seedling emergence of *Cuscuta* spp. depends on temperature, rainfall, weed population, cultivation and cropping system. The optimal temperature ranges from 20-26° C. Sand soil with sufficient moisture and acidic pH range is important for germination, but there is a variation among species in response to pH. Emergence of *C. chinensis* was found depending on temperature, rainfall; weed population, cultivation and cropping system. The optimal temperature range for germination of *C. trifolii* in Petri-dishes under controlled conditions was 20-26°C. A higher germination ratio for this species was found in

acidic pH range, around 5.5, while changes in pH had no effect on *C. campestris* (Lados, 1998). However, optimum conditions for seed germination were found better in sandy than in clay soils (Zaki et al. 1998).

*Cuscuta* is a parasitic weed that grows only by penetrating tissues of host plants to obtain water and nutrients. Seedling usually emerges from shallow soil depths as a leafless shoot with a swollen, stubby terminal appendage. At the time of germination, the root has no apical meristem and the vascular tissue is exposed at the apex of the seedling. Nodes and small scale-like leaves are also present and branching occurs at the nodes.

By three days of emergence, many of the root cells, with the exception of the vascular tissue, began to lose their cellular integrity, with wall loosening and swelling of the cytoplasmic component that crushes neighboring cells. Immunocytochemical investigations indicate that both cellulose and xyloglucans in the cell wall are being degraded, leaving just the middle lamellae holding cells together. By five days of growth, even more dramatic loss of cellular integrity occurs, progressing from the radicle-end. The shoot-end of the plant, however, sustains normal growth. *Cuscuta* roots undergo a rapid senescence/apoptosis, perhaps shunting the metabolites to the shoot, sustaining growth there until a successful parasitism occurs. The parasite loses its root tissue within 3-5 days after emergence.

Seedling grows upright for 2 to 4 cm before attaching to the host (Ristau, 2001). It emerges as an arch and the tip extends and straightens upward after emergence. The entire seedling looks like a yellow thread with no stem-root differences. To survive, *Cuscuta* seedlings must attach to a suitable host within a few days of germination or they die. The epicotyl (3-6 cm long) search for a host in a slow counter-clockwise rotation occurs after extension; it is even able to move for some distance in order to reach a host and to establish contact to it. The stem entwines around any elongated objects within 1 to 3 inches of the seedling. Stems grow and branch up to 3 inches per day. It is sensitive to touch, and yellowish stem gropes in the air until it makes contact with a plant. The contact is made firm by one or more coils around the stem. The degree of twining, branching and attachment varies among species. Some species have tendrils for twining while shading can inhibit initial growth and attachment (Ristau, 2001). The yellow or orange vine strands entwine around stems and leaves of plants. The growing tips reach out and attack adjacent plants, forming a gradually enlarging circle of infestation. A single plant of *Cuscuta* can form patches of 3 m in diameter. Upon flowering clusters of white, pink or yellowish flowers are produced, which soon form large amount of seeds.

The parasite is more likely to coil on host of high nutritional status and to grow away from hosts of poor quality than *vice versa*. They do this before taking up any food from the host to dissociate active choice from the negative effect of growth and mortality. If this host happens to contain foods suitable to *Cuscuta* then secondary

stimulus is aroused which causes root-like branches (haustoria) to form and penetrate the stem. Ihl and Wiese (2000) found that induction of haustoria formation in *Cuscuta reflexa* proved to be independent of the presence of a suitable potential host. The induction, caused by mechanical pressure, was also possible in non-twining stems of the isolated parasite without applying any exogenous plant growth regulators. They suggested that an interaction of IAA and cytokinin, possibly involved in the regulation of these processes.

The basal part of the parasite soon shrivels away so that no soil connection exists (Swift, 2001), then a dense mat will develop over the host as *Cuscuta* spread. *Cuscuta* achieve greater growth volume when simultaneously infected two hosts of different species than one species, which depends on the order in which the parasite encounter the hosts. This relationship is parasitic, not symbiotic.

Xylem bridge between the host and *Cuscuta* extends throughout the length of the haustorium. Haustorial treachery elements in contact with host bundles form only after cells extended from the front of the haustrium becomes attached to the xylem tissue in all the dicots examined and also interfascicular tissues (Tsiovion, 1980). The role of allelopathy in the attachment process should be taken into account in which Chatterjee and Sanwal (1999) identified a high molecular weight protein from *Lantana camara* leaf and petiole as cellulase stimulator (Cs) in *Cuscuta reflexa* and exhibited activation effect both in terms of saccharifying and liquefying activity.

*Cuscuta* stems, nodes, tendrils, and even haustoria also have regenerative cells. These cells are capable of developing new shoots. Twenty or more shoots can develop from one tendril attached to a host. Even when all stems are removed from the host, the haustoria can renew the infestation from the host tissue. *Cuscuta* disseminate by seeds and mainly with contaminated crop seeds, soil, hay, machines, water, animals and even by man. A single plant produces thousands of hard seeds which can stay dormant in the soil for years due to their hard seed coat.

### ***Loranthus* and *Viscum* (Mistletoes)**

Mistletoe plants are hemi-obligate-shoot parasites able to carry on photosynthesis but can't germinate and grow in absence of host plants. Plants of these are either female or male. The berries of the female plant are small, sticky, and whitish for *V. album* but turn red for *V. cruciatum* and *Loranthus acaciae*; they are very attractive to birds that feed on and digest their pulps, excreting the living seeds that stick tightly to any branch on which they land. But these parasites appeared also highly specific since are not able to attack any plant species in the vicinity of their infested area. This host specificity may be due to compatibility/incompatibility between these parasites and their hosts which reflect the advanced complicated host recognition strategy they adopted. In most cases, the initial infestation occurs on larger or older trees. A heavy buildup of

mistletoe often occurs within an infested tree. In addition, seeds may fall from mistletoe plants in the upper part of the tree, creating new infestations on the lower branches. The rapidity with which mistletoe spreads is directly related to the proximity and severity of established infestations, and newly planted trees can be quickly infested if they are growing near old, heavily infested trees. Hawksworth et al. (1991) reported that *V. album* spreads at a rate of approximately 0.35 km per year.

Contrary to other parasitic genera, *Viscum* or *Loranthus* seeds do not require any special germination stimulant from the host. After seed germinates, the parasite radicle grows through the bark to the cambial layer and into the tree's water-conducting tissues, where root-like structures called haustoria develop. The haustoria gradually extend up and down within the branch as the mistletoe grows. The mistletoe produces tough green leaves on woody, forked stem and seems to rely upon its host plant for the water and salts which normal plants derive from the soil through their roots.

On germination a disc-like structure is formed first, and from this there grows out a fine pin like structure, which penetrates by way of crack in the bark and grows through the tissues of the host until finally reaches the surface of the wood. From the disc also the leafy shoot is produced, but this at first shows very little growth. The pin formed by the sucker develops a core at lignified wood and in following year as the host-stem undergoes radial growth this peg becomes buried in the host wood and each year from the neck of the original sucker other branches are formed which row along the surface at the wood for some distance and become buried by radial growth of the host. The neck of the sucker itself does not become buried, and it has a growing region, which is active about the same level as the cambium at the host. From the original sucker the mistletoe continually makes new unions with the wood of the host. There is thus built up a sort of 'woody crown' in the position of the original sucker, from which there arises the woody shoot externally and internally (Karim, 1978).

Initially, the parasitic plant grows slowly; it may take years before the plant blooms and produces seeds. Broadleaf mistletoes have succulent, fragile stems that become woody at the base. Old, mature mistletoe plants may be several feet in diameter, and on some host species, large swollen areas develop at the point of parasite attachment on the infected branches where the mistletoe penetrates swollen points are varied from moderate swelling to the gall formation. If the visible portion of the mistletoe is removed, new plants often re-vegetate from the haustoria.

Dwarf mistletoes are smaller plants than broadleaf mistletoes, with mature stems less than 6 to 8 inches long. Their shoots are non-woody, segmented, and have small scale-like leaves. While birds disperse broadleaf mistletoe seeds, seeds of dwarf mistletoe are spread mostly by their forcible discharge from fruit, which can propel seeds horizontally into trees up to 30 to 40 feet away. Dwarf mistletoes can weaken or kill trees by sapping their resources and making them easy targets for marauding

insects such as bark beetles. Also, the mistletoe's growth compounds can send a tree into a tizzy of sprouting weird, ultra-dense foliage called witches' brooms (Geils, undated).

Mistletoes absorb water, mineral nutrients and may be some photosynthate from host trees. Under moisture stress, sever damage may be resulted to host plants due to competition between the host and the parasite for water. However, healthy host trees can tolerate a few mistletoe branch infections, but individual branches may be weakened or sometimes killed. Heavily infested trees may be reduced in vigor (Figure 3), stunted, or even killed, especially if they are stressed by other factors such as drought or disease.



Figure 3. *Loaranthus acacea* heavily infesting *Casuarina equisetifolia*.

It has been mentioned that *Viscum album* plants are able to photosynthesize all year-round, but at slower rates than their hosts (Garcia-Plazaola et al. 2001); in addition, mistletoes are able to change their photochemical efficiency daily depending on the climatic conditions as other temperate evergreen plants do (Adams and Demming-Adams, 1995).

## ALLELOPATHY IN HOST RESISTANCE TO PARASITE

Allelochemicals are secondary metabolites produced in plant cells and mostly have no recognized specific function in plants. However, many of these byproducts are well

known to produce in response to external factors such as wounding, injuries and stresses. The role of these as natural products was very well recognized when dealing with allelopathy mechanism since many are either inhibitors to different living organisms such as plant pathogens, nematode, insects, plants and even animals. Many of these are involved in the defense mechanism that plants express under stress conditions and act as repellents to animals or produced and secreted in enough amounts to negatively affect or harm other plants in the same environment and thus giving an advantage for allelopathic species over others in the chemical war occurs between plants in nature. On the other hand, many of these products have stimulatory action on other species and can enhance seed germination, plant growth and physiological reactions. These however, are specific in their action and the main factor control this effect is their concentration, time of application and prevailing environmental conditions. With great increase concern in environmental pollution and protection, many of these find their ways to scientific laboratories and industry to be used in different purposes as safe, non-residual natural products and as an excellent replacement to synthetic chemicals in fields of drug industry and agricultural pesticides. Below is a review on the most recent findings on the role of these allelochemicals in the defense mechanism that host crops or parasitic weeds show in response to each other.

Neumann et al. (2001) pointed out that resistance mechanism can exist even in susceptible host species/cultivars but they do not prevent the penetration of haustorial cells. However, some host plants develop hypersensitive reactions that block haustorial penetration of various parasitic plants, such as cowpea (*Vigna unguiculata*) resistant to *Striga* (Lane and Bailey, 1992), cotton (*Gossypium hirsutum*) to *Cuscuta lupuliformis* (Capdepon et al. 1985), poplar to *Viscum album* (Salle et al. 1991), sunflower (*Helianthus annuus*) to *O. cumana* (Dorr et al. 1994) and *Vicia thompsonii* to *O. aegyptiaca* (Goldwasser et al. 1997). The physiological basis of this mechanism of resistance was explained by Osbourn (1996) in that cellular decomposition may lead to a release of toxic substances that are stored in the vacuole. Alternatively, the levels of induced phytoalexines, which usually are rapidly turned over; in plant cells may accumulate in inhibitory concentration. Another reaction reported is the death of *Orobanche* tubercle after penetration into the host before the start of shoot development (Dorr et al. 1994) or the necrosis of the parasite. Incompatibility response of *Sorghum* plant to *Striga* invasions was characterized by either purpling or withering of attached *Striga* seedlings. Such a host apparently produces essential signals for germination and haustorial formation leading to attachment but perhaps possesses certain metabolic inhibitors that discourage the parasite from further growth and development (Ejeta et al. 2000).

Different researchers have accepted the accumulation of defensive materials from the host such as phenolic compounds, suberin and lignin (Perez-de-Luque et al. 2001), while others suggested that at least some substances that were found in the host xylem

could originate from the parasite, and may secrete it in the reaction to difficulties in penetrating the resistant host (Joel et al. 1996). The parasite tends to secrete higher amounts of these substances in order to obtain a better anchoring and dissolve the middle lamella between neighboring host cells.

### ***Orobanche* spp.**

Research on crop resistance to *Orobanche* has not received much attention (Cubero, 1991). However, significant number of studies was carried out on this aspect in last decade. Good levels of resistance have been found in several host/parasite systems as sunflower to *O. cernua*, broad bean and common vetch to *O. crenata*, eggplant to *O. aegyptiaca* and several cucurbits to *O. ramosa* and *O. aegyptiaca*. Observations on collection of peas and chickpeas also indicate the existance of strong levels of resistance (Alonso, 1998). It has been reported that once the parasite contacts and adheres to a host root, lytic enzymes such as pectin methyl esterase and polygalacturonase act to loosen the adhesion between host cells (Ben-Hod et al. 1993; Joel and Losner-Goshen, 1994). In response to that host plants may show opposite reaction to prevent parasite attachment to its tissue surface. Antonova and Borg (1996) showed that the extracellular peroxidase in *O. cumana* races C and D reacts with phenolic compounds which are lignin precursor of the host sunflower resulting in host resistance due to the formation of lignin layers in sunflower plants possessing the Or3 gene for resistance. However, the absence of extracellular peroxidase in parasite race D prevents lignin formation and enable the parasite to attach to the host vascular system. Wegmann et al. (1991) reported that phytoalexines are almost certainly involved in the resistance response to *Orobanche cumana*, cv. 81-14 showed twice as much scopoletin in its roots as did a susceptible line Giganta sunflower, however, the researchers couldn't confirm that phytoalexines played role in the resistance of broad bean. Ish-Shalom-Gordon et al. (1993) noticed phenolic materials in roots of resistant sunflower in response to haustorial penetration. Secretion of inhibitors (phytoalexins) by the resistant sunflower cultivars to *Orobanche cumana* infestation has been also suggested by Jorrin et al. (1996). It has been postulated by Eizenberg et al. (2001) that under summer conditions *O. cumana* penetrates and forms attachments on roots of a resistant cultivar indicating possible involvement of phytoalexines in the resistance reaction. These researchers reported that methanolic crude extract exhibited the strongest phytotoxic activity. They assumed that the resistance mechanism(s) of the resistant Amber cultivar is associated with phytotoxic substances produced by the host, and the amount produced at low temperatures is not sufficient to prevent parasitism. They further hypothesized that these phytotoxic substances inhibit broomrape development after penetration and establishment in host roots.

Different weed species showed different resistant levels including endodermic barrier, encapsulation layer, thickening of host vessels, vessels occlusion and cell wall

polyphenolic impregnation in *Anagallis arvensis* and *Solanum nigrum* (Boulet et al. 2001) and lead to death of *Orobanche ramosa*. Other workers reported vessels of host plants to be completely filled up by phenolic compounds, gel or gum-like substances in combinations of LR1 sunflower/*O. cumana* and Palaiseau carrot/*O. ramosa*. Cell wall polyphenolic impregnation was also noted in *Helianthus rigidus/O. cumana* and *Anagallis arvensis/O. ramosa* (Labrousse et al. 2001). Sillero et al. (2001b) reported wild legumes of *Vicia*, *Pisum*, *Lathyrus*, *Cicer* and *Lens* all induced a lower germination of *O. crenata* seeds compared with the susceptible control, being particularly low in *Cicer* and *Lathyrus*. The authors suggested that low germination of the parasite is due to low excretion of germination stimulants instead of an excretion of germination inhibitors. However, it is well established that wild varieties are good sources of allelochemicals and have greater effect through this mechanism than cultivated species or cultivars (Rice 1974; Putnam and Duke, 1978; Lovett et al. 1982).

### ***Cuscuta* spp.**

Although *Cuscuta* seeds do not require any host stimulants to start germination, but this parasite also showed preference among hosts and not all plant species are attacked. In addition *Cuscuta* species are widely differ in their host range, certain plant species are well known as preferable hosts, heavily attacked by certain *Cuscuta* species, others are less so or preferred by other parasite species, while many are less infested, immune or not attacked at all. Among crop cultivars showed tolerance/resistance to one or more species of *Cuscuta*, were bean cultivars namely Bulgarian and Tenderette, which showed very high degree of resistance to *C. campestris* (Tsiovion, 1979). *Vigna mungo* cv. T9 and *V. radiata* cv. M2 were found tolerant to *Cuscuta* infestation due to their early vigour, hard and woody stem (Kumar and Kondap, 1993). Other two cultivars of *V. mungo* (cv. LBG-628 and AKU-3) showed some tolerance while *V. radiata* cv. PDM-85-182, PDM-85-186, RMG-131, RMG-146 and Lam-M2 showed considerable resistance to *C. chinensis* (Rao and Rao, 1991).

Variations between plant species or cultivars in their resistance to *Cuscuta* reflecting differences in their defense mechanism adopted against these parasites. Differences in resistance or susceptibility to *Cuscuta* attack were noticed on all levels of plant categories. Mishra and Sanwal (1992) reported differences between *Brassica juncea* cultivars in their susceptibility to *Cuscuta reflexa*. The occurrence of a polygalacturonase was reported in tissue of *C. campestris* to breakdown pectin in host tissues (Bar-Nun et al. 1999). The enzyme activity requires the presence of a cofactor, which appears to be a low molecular weight peptide. Al-Menoufi and Ashton (1991) studied the susceptibility and resistance of some *Lycopersicon* species to *Cuscuta campestris* infection and found that plants of *L. chilense* and *L. esculentum* cv. VF 14578 x 79 were highly susceptible whereas plants of *L. chmielewskii* and *L. hirsutum*

were less susceptible. The epidermal, cortical and pith cells of the infected host plants increased in size and some regulated their meristematic ability. Plants of *L. esculentum* showed a hypersensitive reaction to *C. campestris*, with necrosis of host cells occurring immediately after the threads of the parasite coiled around the stem. Such a reaction is considered as a type of resistance. Field observations by Kelly and Horning (1999) showed that *Cuscuta attenuata* grows more vigorously in patches of mixed host species than in monospecific host patches. Field experiments with naturally occurring host individuals demonstrated that the parasite achieved greater volume when simultaneously infesting two hosts of differing species rather than two hosts of the same species, and that this effect depends on the order in which the parasite encounters those hosts. Goldwasser et al. (2001) reported *Cuscuta pentagona* to have the most diverse and numerous host ranges among the *Cuscuta* genus and is a major weed problem in tomato production in certain parts of the world. They found those Heinz varieties '9492', '9553', and '9992' of commercial hybrid tomato exhibited tolerance to the parasite and in most cases, haustoria failed to penetrate into the stem, eventually leading to the death of the parasite. Under field conditions, *Cuscuta pentagona* attachments were 75% less on tolerant varieties, and dodder growth was reduced by more than 70%. The resistance mechanism of Graminae and tomato was found different to *C. campestris*. There was a mechanical barrier in Graminae, but in tomato infection is partially successful. On the other hand, there was a reaction between beans and tomato towards *C. campestris* infection. In tomato, if the haustrium manages to penetrate the outer layers of the undamaged cortex, a functional connection with the transport element is achieved, whereas in beans haustorial cells damaged inside the host stem thereby preventing their connection with host transport elements (Tsiovion, 1979). Two bands of vinyl group compounds were found to exist in green bean extracts. The haustoria of *Cuscuta* did not succeed in entering the beans resulting in the death of the parasite (Zaki et al. 1998).

Many workers on different plant species and their reactions with *Cuscuta* spp. have proposed different resistance mechanisms. Rao and Rao (1991) studied the tolerance of black gram and green gram varieties to *Cuscuta chinensis* and found more genetic sources of *C. chinensis* resistance in green gram than in black gram. This resistance could be due to a hypersensitive reaction of the host cells to parasite infestation resulting in isolation of the haustorial channel from the host vascular system. Nagar et al. (1984) reported that in non-susceptible species of sweet potato and potato the inhibition mechanism is related to different cations found in the host cell wall. From the divalent ions tested  $\text{Ca}^{2+}$  was the most potent inhibitor of *Cuscuta* cellulase, followed by  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Fe}^{2+}$ . Five to 10 folds Ca content was found in non-susceptible plants (Nagar and Sanwal, 1984). Xylanase and cellulase activity of the host increased while cellulase activity of the parasite decreased as the result of infection. Tsiovion (1981) reported competition between the sink of the host and *C. campestris* for common pool of photosynthate produced by the host, which reduced the shoot length of the host. In such competition conditions, the less efficient sink is

weakened. He concluded that the parasite influence the host through the xylem. In addition to reduction in height, infection of the parasite reduced the chloroplast content by 24% but increased carotenoid by 44 %. It was suggested that chlorophyll acts as a reactive site of the host-parasite interaction in case of angiosperm parasites. Loffler et al. (1999) reported that during infection with *Cuscuta reflexa*, the incompatible host plant *Lycopersicon esculentum* shows characteristic anatomical tissue modifications at the infection sites that are exclusively provoked following contact with parasite-striking cell elongation is observed in the hypodermis and collenchyma of the host plant. They found that tissue modifications in the host plant, as well as cell elongation in *C. reflexa* tissue leading to the formation of an adhesive-secretory epithelium, are correlated with increasing IAA levels in the respective tissues. Both anatomical modifications can also be induced artificially by injection of IAA into control tissue. They hypothesized that during the parasite attack; IAA is accumulated in the haustoria-bearing regions of *C. reflexa* and exuded from the epithelial cells. Due to the close contact between host and parasite, IAA probably enters the host plant tissue causing the observed anatomical reactions. There was high concentration of cytokinin in the haustorium-bearing region in *C. reflexa*, which might be due to a high RNase activity in the region or translocation of RNase from host to the parasite or both (Gupta and Singh, 1985). The induction of haustorial formation in *C. reflexa* proved to be independent from the presence of a suitable host. During haustorial development, the growth rates of this parasite are retarded, an interaction of IAA and cytokinins, possibly involved in the regulation of these processes (Ihl and Wiese, 2000). *Cuscuta* species haustoria fused with vascular bundle of *Digitalis purpurea*, but avoided contact with the host of *Digitalis dalanta*, although both species could be infected, *Cardenolia* concentration decreased from the haustorium regions to the shoot tip (Rothe et al. 1999).

Lignifications or suberizations are other mechanisms of resistance reported by different authors. In *Gossypium hirsutum*, resistance based on the formation of a suberized layers of cells from a secondary meristem resulting in isolation of the haustorium development of secondary parenchyma and expulsion of the parasite (Capderon et al. 1985). Different crop species including *Abelmoschus esculentus*, *Beta vulgaris* subsp. *Cicla*, *Clitoria ternatea*, *Cucumis melo*, *Gossypium barbadense*, *Lathyrus sativus*, *Lycopersicon esculentum*, *Mentha piperita*, *Phaseolus vulgaris* and *Solanum tuberosum* were reported to resist *C. campestris* (Farah, 2001). Both lignin and subarin could be also considered as allelochemicals and produced in response to external influence on host plants. Therefore this kind of resistant mechanism is included under allelopathy role in this reaction between host and its parasite. These allelochemicals may act directly against parasite or indirectly by degrading parasite enzymes dissolve plant cells and facilitate its attachment and penetration. Singh and Singh (1997). Reported that host resistance to *Cuscuta reflexa* infection was considered at two levels: at the surface of the host and during the haustorial bursting

and its connection with the vascular bundles of the host. Peelings of the two resistant hosts sweet potatoes (*Ipomoea batatas*) and tomatoes (*Lycopersicon esculentum*) contained strong inhibitors of cell-wall degrading enzymes (cellulase, polygalacturonase and xylanase) were studied in the haustoria bearing region of *C. reflexa* and in different parts (peelings, peeled and unpeeled) of the stem/petiole of resistant hosts. Peelings showed no detectable activity, the peeled portion showed high activity, while unpeeled tissue occupied an intermediate position. Mixed enzyme preparations of the haustoria-bearing region of *C. reflexa* and those of different parts of the stem/petiole of resistant hosts indicated that the peelings contained strong inhibitor(s) of cell-wall degrading enzymes of *C. reflexa*, providing a defense mechanism to the host against haustorial penetration. Although haustorial penetration in the peeled host tissue becomes comparatively easy, the haustorial bursting inside the host tissue and subsequent connection with the host vascular bundle is prevented. Thus, the defense mechanism appears to be spread throughout the resistant host tissue. These results were also confirmed by Archana and Madhav (1997).

The direct role of allelochemicals was very well demonstrated through different studies. Sahm et al. (1994) investigated the role of phenylpropanoid derivatives for the pronounced resistance of tomato towards *C. reflexa*. Infection site of tomato stems revealed the formation of necrotic tissue surrounding the prehaustoria and suberization of cell walls of living cells adjacent to the necrotic tissue. Extracts from infection sites on tomato stems showed an enhanced accumulation of chlorogenic acid and unknown hydroxycinnamic acid derivative which known as allelochemicals. In another study, Sahm et al. (1995) reported that both anatomical and chemical defense reactions were shown during the incompatible reaction of *Lycopersicon esculentum* against *Cuscuta reflexa*. Tomato stem revealed the elongation of epidermal, hypodermal and collenchymatic cells beneath the parasitic prehaustorium. The elongated cells had collapsed later, forming a visible brownish plaque at the tomato stem, followed by scariform tissue with lignified and suberized cell walls. Concomitantly, an enhanced accumulation of soluble phenolic compounds (chlorogenic acid and an unidentified hydroxycinnamic acid derivative), as well as a stimulation of peroxidases, was observed. Arnaud et al. (1998) studied crop resistance to parasitic weeds using both a root parasite (*Striga hermonthica*) and a stem parasite (*Cuscuta reflexa*), in both cases, resistance was late and due to several mechanisms. They found that in and around the haustorium developed by *Cuscuta* on *Phaseolus vulgaris*, both cellular proliferation and polyphenolic compound accumulation occurred. Ethylene production in response to *Cuscuta* attack might be the alarm signal inducing host defense, during which an increase in polyacetolactase (PAL) and peroxidases activities was also observed, which did not exhibit morphological and histological differences. Delayed polyphenol compound accumulation was also observed in the tissues surrounding the *Striga* haustorium on *Sorghum* cv. Framida. Dhopte (1998) reported inhibition of *Cuscuta* sp. growing on *Parthenium hysterophorus* and the parasite was unable to survive on this species. The author

suggested that *Cuscuta* mortality was due to the presence of toxic sesquiterpene lactones present in *P. hysterophorus*. Infected *P. hysterophorus* reinitiated flowering after 1 week, indicating the allelopathic nature of *P. hysterophorus* to *Cuscuta* sp. It has been also reported that twining parasitic plant *Cuscuta reflexa* is able to attack *Ancistrocladus heyneanus* by invading the stem tissues and forming haustoria that penetrate the vascular bundles of the host. Subsequent reactions by the host, including phytoalexin production and hypersensitive reactions, lead to a degeneration of the parasite's haustoria and eventually to the abortion of parasitic tissues (Bringmann et al. 1999). The acetogenic secondary metabolites produced by the host, the naphthoquinone plumbagin in the first instance, are demonstrated to be the major antipathogenic factors involved in this incompatible relationship.

### ***Striga* spp.**

A huge number and intensive studies were conducted on this parasite and its reaction with host plants, and tried to analyze the internal physioplogical, anatomical and chemical relationships between different *Striga* species and their host plants. It is well known that *Striga* species require stimulants in root exudates of host plants to start germination. Erickson et al. (2001) reported that analyses of root exudates of *Sorghum* composed of fatty acids, resorcinol and a series of structurally related hydroquinones. At least one of the hydroquinones induces germination in *Striga asiatica*, and the resorcinol is thought to stabilize the hydroquinone in the soil. The previously unknown series of hydroquinone offers insight into the possible biosynthesis of the components of the exudate and their possible importance in initiating *Striga* germination.

Studying the defense mechanism of host crop against this parasite, Lane et al. (1997) reported resistance to *Striga hermonthica* in the wild species *Zea diploperennis*. The percentage of progeny resistant to *S. hermonthica* increased only slightly in the derived progeny, suggesting that the genetic basis of resistance is complex. However, It has been suggested that one of the better-understood mechanisms of resistance against *Striga* by *Sorghum bicolor* is the low production of compounds by the host root that *Striga* seeds require as stimulants for germination (Vogler et al. 1996), which was already proposed by Reda et al. (1994), and proved more recently by Haussmann et al. (2001) through their work on *Striga hermonthica* and *Striga asiatica* who found a single recessive gene in the *Sorghum* x *Striga asiatica* interaction controls the trait, but information is lacking for *Striga hermonthica*.

Several wild sorghums were found to display potential *Striga* resistant mechanisms including low germination stimulation. However, some showed no *Striga* tubercle formation outside of 1mm from their roots. The accessions showing little or no germination stimulating and haustorial initiating activity also had germination indices less than one indicating some inhibitory effects of these accessions on *Striga*.

germination (Rich et al. 2001). Several native African savannah grass species were reported to possess a high level of resistance to *Striga hermonthica*, *Striga aspera* and interspecific hybrids of these two *Striga* species (Kuiper et al. 1998). Root exudates of 14-grass species tested induced *Striga* seeds to germinate and attachment to the host roots was always observed. Subsequent development of the *Striga* seedlings, however, was absent or heavily impaired in resistant grasses. Preliminary observations suggested that the parasite could not break the endodermal barrier of the roots.

A good number of studies concentrated on the role of allelopathy and allelochemicals in the interaction between host and its parasite. Neumann et al. (1999) reported that at the margin of contact area of host root cortex and parasite cells, pectins are implicated in sealing the parasite to the attacked host organ. Phenolic substances and/or lignins can be found at the site of penetration of the haustorium into the host root, suggesting at least, a partial defence reaction in the invaded host root tissues. In another study, Neumann et al. (2001) reported that phenolic substances and/or lignins could be found at the site of penetration of the haustorium into the host root. These observations and the fact that anti-hydroxyproline-rich glycoprotein antibodies accumulate at the side of the interface support the view of at least a partial defense reaction in the invaded host root tissue. The resistance of *Sorghum bicolor* cv. Framida to *Striga* was studied by Arnaud et al. (1999) and found it to occur during the establishment of a functional haustorium. At a later stage of infestation, significant accumulation of a coloured material likely to be rich in phenolic compounds was observed in and around Framida conductive tissues. The authors indicated the involvement of at least three steps in the development of resistance in Framida roots including a partial inhibition of development of the young haustorium, the physiological events that decrease nutrient translocation towards the haustoria and the accumulation of a coloured phenolic-like material. The last step was confirmed by the thick endodermal cell walls and extensive layer of polyphenol and lignin found at uninfected roots of *Pennisetum setosum* (species resistant to *S. hermonthica*) compared with that seen on maize-*S. hermonthica* connection point (Mayer et al. 1997). Mohamed et al. (2001) indicated the potential existence of at least four separate mechanisms in *Sorghum bicolor* resistance to *Striga* spp. among which the presence of germination inhibitors and the low production of haustorial initiation factor. Since *Striga* seeds near *Sorghum* roots consistently showed lower germination after ethylene treatment compared with those away from the roots suggesting the potential presence of germination inhibitors and inhibitory effects of host seedlings on *Striga* germination.

### **Other parasitic genera (Mistletoes and *Osyris*)**

Literature on interaction of other parasitic genera with their hosts and the involvement of allelochemicals in the defense mechanism of host species are limited. In a study

carried out by Salle et al. (1991) they pointed out that some tree species appear resistant to broadleaf mistletoe. Bradford flowering pear, Chinese pistache, crape myrtle, eucalyptus, ginkgo, golden rain tree, liquidambar, sycamore, and conifers such as redwood and cedar are rarely infested. The authors concluded that these or other resistant species should be considered when planting in infested areas, or when replacing infested trees (Perry and Elmore, undated). Moreover, varietal resistance has been also noted for certain *Populus nigra* and *Quercus* spp. However, the resistant mechanism and the role of allelopathy are to be investigated. Working on *Osyris* plants, Woldemichael and Wink (2002) detected both pyrrolizidine and quinolizidine alkaloids in the alkaloid extracts of *Osyris alba* and demonstrating the simultaneous presence of two classes of alkaloids in a single parasitic plant. As these alkaloids do not occur in the same host plant, the results indicate that *Osyris* must have trapped more than one host plant concomitantly. Studies showed that both alkaloids serve as defense compounds against herbivores and thus could provide a novel mode of defense of hemiparasites against herbivores. The role of these alkaloids in parasite invasion to host plant tissues need to be clarified.

## ALLELOPATHY AND PARASITIC WEED CONTROL

### *Orobanche* spp.

#### *Trap species*

Different cultivated or wild species including plants producing ethylene or strigoles with great potential to stimulate seed germination of certain parasitic weeds, including different species of *Orobanche* and *Striga*, without being themselves attacked, have been reported. Trap species for different *Orobanche* species are shown in Table 1.

In a study carried out by Chabrolin (1935), 65 nonhost plant species able to stimulate *Orobanche* seed germination were identified. Other *Orobanche* stimulating species reported were sorghum (*Sorghum vulgare* L.), barley (*Hordeum vulgare* L.), and vetch (*Vicia dasycarpa* spp. *villosa*) (Kasasian, 1973; Linke et al. 1991) for *O. crenata*; bean, sorghum, maize, and cucumber for *O. ramosa* (Labrada and Perez, 1988) and sorghum, cowpea, chili, hemp, mung bean, flax, lucerne, soybean and chickpea for *O. cernua* (Krishnamurthy and Rao, 1976; Krishnamurthy et al. 1977). However, flax was the most potent trap crop. Linke et al. (1991) showed significant reduction in *O. crenata* infestation after three years of growing *Vicia dasycarpa* spp. *villosa*. Forage legumes have been recommended as trap crops to decrease the broomrape's seed bank in the soil (Linke et al. 1993 and Saxena et al. 1994) and to replace fallow in areas where cultivation of other food legumes is not possible. Sillero et al. (2001a) reported possible use of *Lathyrus choranthus* as trap crop to reduce broomrape seed bank in the soil as permitted very low emergence of broomrape shoots

but allowed a relatively high establishment of the parasite with low susceptibility to *Orobanche*.

The non-suitable host crops of *O. cernua* including chili, sorghum, cowpea, *Phaseolus aconitifolius* and *Hibiscus sabdariffa* stimulated germination of the parasite at a high level, while sesame stimulated germination but without allowing further growth and development (Krishnamurthy and Chandwani, 1975). Different studies showed that root diffusate of *Linum usitatissimum*, *Capsicum annuum*, *Sesamum indicum* and *Trifolium alexandrinum* all enhanced *Orobanche* seed germination, but the germinated seeds normally failed to attach to the root systems of these crops (Abu-Shakra et al. 1970; Krishnamurthy et al. 1977; Musselman, 1980; Al-Menoufi, 1991). Strong induced germination of *O. cernua* seeds was obtained using trap crops especially *Vigna radiata* and *Crotalaria juncea* (Dhanapal et al. 1998). In addition good inhibition and resistant level to *Orobanche* infestation to different weed species were detected. In a study conducted by Boulet et al. (2001), 30 weed species were tested for their susceptibility to *O. ramosa*, they reported that *Avena sativa* sp. *sterilis*, *Ammi majus*, *Solanum nigrum*, and *Anagallis arvensis* possessed different levels of resistance lead to the death of parasite. The authors suggested to use these species as trap plants. Out of twenty-one tested crops by Rodriguez-Ojeda et al. (2001) seven were found to stimulate germination of *Orobanche cumana* seeds, these were (from the strongest to the weakest) corn, sorghum, millet, cotton, rice, eggplant and cauliflower.

The success of trap crops in exhausting soil seed bank of the parasite may be highly questionable under severe infestation or may requires a fairly long period for effective control which may not be economically feasible under certain growing systems. However, in a short term cropping system it may increase crop yield as found by Zemrag and Bajja (2001) who reported that fenugreek and coriander decreased the number of attached parasites per host plant and disturbed their development. They showed that these trap crops increased broad bean yield by 18.37 and 14.82%, respectively. In general, the use of trap crops may effectively reduce *Orobanche* seed bank, but to a certain extent these crops could be used as a part of integrated *Orobanche* control approach. Crop rotation alone will not solve the problem but it can contribute to reduce the infestation level to a degree that renders control methods like hand pulling. However, good results with effective trap crops may not be efficient, possibly not practicable for ecological/economical reasons. Development of the parasite on weeds needs to be eliminated, while important trap crops to reduce the seed bank for different parasitic weeds are not available.

Table 1. Trap crops for *Orobanche* spp.

Scientific name	Common name	References
<b><i>Orobanche ramosa</i></b>		
<i>Allium sativum</i>	Garlic	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Arabidopsis thaliana</i>	Thale cress	Goldwasser et al. 2000
<i>Brassica</i> sp.	Mustard	Cited by Kasasian, 1973
<i>Brassica napus</i>	Rape	Cited by Kasasian, 1971
<i>Brassica rapa</i>	Turnip	Al-Menoufi & Adam, 1996
<i>Capsicum</i> spp.	Capsicums	Cited by Kasasian, 1971; Sand, 1983
<i>Carum ajowan</i>	Caraway	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Coriandrum sativum</i>	Coriander	Al-Menoufi & Adam, 1996
<i>Cucumis sativus</i>	Cucumber	Labrada & Perez, 1988
<i>Glycine max</i>	Soybean	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Lablab purpureus</i>	Hyacinth bean	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Lathyrus ochrus</i>	Ochrus vetch	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Linum usitatissimum</i>	Linseed	Eplee, 1984; Khalaf, 1992
<i>Lupinus termis</i>	Lupine	Al-Menoufi & Adam, 1996
<i>Medicago sativa</i>	Lucerne	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Pennisetum</i> sp.	Fountain	Cited by Kasasian, 1971
<i>Phaseolus aureus</i>	Green gram	Kleifeld, 1996
<i>Phaseolus vulgaris</i>	French bean	Labrada & Perez, 1988; <a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Pisum sativum</i>	Pea	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Ricinus communis</i>	Castor bean	Cited by Kasasian, 1971; <a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Sesamum indicum</i>	Sesame	Cited by Kasasian, 1971; <a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Setaria</i> sp.	Millets	Cited by Kasasian, 1971
<i>Sorghum bicolor</i>	Sorghum	Labrada & Perez, 1988
<i>Trifolium</i> sp.	Clover	Cited by Kasasian, 1971
<i>Trigonella foenum graecum</i>	Fenugreek	Al-Menoufi & Adam, 1996

<i>Vigna radiata</i>	Green gram	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Vigna unguiculata</i> spp. <i>unguiculata</i>	Cowpea	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Zea mays</i>	Maize	Cited by Kasasian, 1971; Labrada & Perez, 1988

***Orobanche aegyptiaca***

<i>Allium sativum</i>	Garlic	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Arabidopsis thaliana</i>	Thale cress	Goldwasser et al. 2000
<i>Capsicum annuum</i>	Sweet pepper	Hershenson et al. 1996
<i>Carum ajowan</i>	Caraway	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Glycine max</i>	Soybean	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Lablab purpureus</i>	Hyacinth bean	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Lathyrus ochrus</i>	Ochrus vetch	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Linum usitatissimum</i>	Linseed	Kleifeld et al. 1994
<i>Medicago sativa</i>	Alfalfa	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Phaseolus vulgaris</i>	French bean	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Pisum sativum</i>	Pea	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Ricinus communis</i>	Castor bean	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Sesamum indicum</i>	Sesame	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Vigna radiata</i>	Green gram	Kleifeld et al. 1994
<i>Vigna unguiculata</i> spp. <i>unguiculata</i>	Cowpea	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>

***Orobanche cernua***

<i>Amaranthus</i> spp.	Pigweeds	Rao, 1955
<i>Bidens pilosa</i>	Spanish needle	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Cajanus cajan</i>	Pigeon pea	Krishnamurthy & Chandwani, 1975
<i>Capsicum annuum</i>	Sweet chillies	Cited by Kasasian, 1971; <a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Cicer arietinum</i>	Chickpeas	Krishnamurthy & Rao, 1976; Krishnamurthy et al. 1977

<i>Cichorium intybus</i>	Chicory	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Colocasia</i> sp.	Elephant's-ear	Rao, 1955
<i>Crotalaria juncea</i>	Sunhemp	Dhanapal & Struik, 1996; Dhanapal, Mallory-Smith & Ter Borg, 2001
<i>Curcuma domestica</i>	Turmeric	Rao, 1955
<i>Glycine max</i>	Soybean	Krishnamurthy & Rao, 1976; Krishnamurthy et al. 1977
<i>Gossypium</i> spp.	Cotton	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Guizotia abyssinica</i>	Nugcoat buttons	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Hibiscus sabdariffa</i>	Hibiscus	Rao, 1955
<i>Illacrvloma uniflorum</i>	Horsegaram	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Linum usitatissimum</i>	Linseed	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Medicago sativa</i>	Alfalfa	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Pennisetum typhoides</i>	Millet	Rao, 1955
<i>Phaseolus aureus</i>	Green gram	Dhanapal, Mallory-Smith & Ter Borg, 2001
<i>Phaseolus aconitifolius</i>	Moth bean	Rao, 1955
<i>Ricinus communis</i>	Castor bean	Rao, 1955; <a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Sesamum indicum</i>	Sesame	Rao, 1955
<i>Setaria indica</i>	Foxtail millet	Rao, 1955
<i>Sinapis alba</i>	Wild mustard	Cited by Kasasian, 1971
<i>Solanum melongena</i>	Eggplant	Rao, 1955
<i>Sorghum</i> sp.	Sorghum	Krishnamurthy & Chandwani, 1975; Krishnamurthy & Rao, 1976; Krishnamurthy et al. 1977
<i>Tridax procumbens</i>	Buttons	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Vigna mungo</i>	Black gram	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Vigna radiata</i>	Green gram	Dhanapal & Struik, 1996
<i>Cannabis sativa</i>	Hemp	Krishnamurthy & Rao, 1976; Krishnamurthy et al. 1977
<i>Vigna radiata</i>	Mung bean	Krishnamurthy & Rao, 1976; Krishnamurthy et al. 1977

<i>Medicago sativa</i>	Lucerne	Krishnamurthy & Rao, 1976; Krishnamurthy et al. 1977
<i>Vigna aconitifolia</i>	Moth bean	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Vigna unguiculata</i>	Cowpea	Krishnamurthy & Rao, 1976; Krishnamurthy et al. 1977
<i>Vicia dasycarpa</i> spp. <i>villosa</i>	Vetch	Linke et al. 1991
<i>Orobanche cumana</i>		
<i>Bidens pilosa</i>	Spanish needle	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Capsicum annuum</i>	Sweet chillies	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Cichorium intybus</i>	Chirory	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Crotalaria juncea</i>	Sunhemp	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Gossypium</i> spp.	Cotton	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Guizotia abyssinica</i>	Nugcoat	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Illacrvyloma uniflorum</i>	Horsegram	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Linum usitatissimum</i>	Linseed	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Medicago sativa</i>	Alfalfa	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Ricinus communis</i>	Castor bean	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Tridax procumbens</i>	Buttons	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Vigna aconitifolia</i>	Moth bean	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Vigna mungo</i>	Black gram	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Vigna radiata</i>	Green gram	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Orobanche crenata</i>		
<i>Allium sativum</i>	Garlic	Hassan, 1998
<i>Astragalus boeticus</i>	Vetch	Schnell et al. 1994
<i>Brassica rapa</i>	Turnip	Al-Menoufi & Adam, 1996
<i>Capsicum annuum</i>	Pepper	Al-Menoufi & Adam, 1996
<i>Coriandrum sativum</i>	Coriander	Al-Menoufi & Adam, 1996; Zemrag & Bajja, 2001

<i>Glycine max</i>	Soybean	Schnell et al. 1994
<i>Hedysarum coronarium</i>	Sulla	Schnell et al. 1994
<i>Helianthus annuus</i>	Sunflower	<a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Hordeum vulgare</i>	Barley	Kasasian, 1973; Linke et al. 1991; <a href="http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm">http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm</a>
<i>Lablab purpureus</i>	Hyacinth bean	Schnell et al. 1994
<i>Lathyrus ochrus</i>	Ochrus vetch	Schnell et al. 1994
<i>Linum usitatissimum</i>	Linseed	Khalaf, 1992; Abou-Salama, 1995
<i>Lupinus termis</i>	Lupine	Al-Menoufi & Adam, 1996
<i>Phaseolus vulgaris</i>	French bean	Schnell et al. 1994
<i>Pisum sativum</i>	Pea	Hassan, 1998
<i>Saccharum officinarum</i>	Sugarcane	Abou-Salama, 1995
<i>Sesamum indicum</i>	Sesame	Al-Menoufi, 1991
<i>Trifolium alexandrinum</i>	Berseem	Schnell et al. 1994; Al-Menoufi & Adam, 1996
<i>Trigonella foenum-graecum</i>	Fenugreek	Al-Menoufi & Adam, 1996; Zemrag & Bajja, 2001
<i>Vicia dasycarpa</i> spp. <i>villosa</i>	Vetch	Kasasian, 1973; Linke et al. 1991
<i>Vicia narbonensis</i>	Narbonne vetch	Schnell et al. 1994
<i>Vigna radiata</i>	Green gram	Schnell et al. 1994
<i>Vigna unguiculata</i>	Cowpea	Schnell et al. 1994
<b><i>Orobanche minor</i></b>		
<i>Allium sativum</i>	Garlic	Hassan, 1998
<i>Arabidopsis thaliana</i>	Thale cress	Goldwasser et al. 2000
<i>Linum usitatissimum</i>	Linseed	Brown et al. 1952
<i>Pisum sativum</i>	Pea	Hassan, 1998
<i>Sorghum bicolor</i>	Sorghum	Brown et al. 1952
<i>Zea mays</i>	Corn	Brown et al. 1952

### *Catch species*

Catch crops or plant species of *Orobanche* are host plants of parasitic weeds that induce germination and can be parasitized. They are true hosts infected or enhanced germination and attachment but hinder parasite development by mechanical, physiological or chemical factors. However, true hosts should be harvested, converted into the soil or destroyed after 6-8 weeks, before the parasite appears above the soil, or at least before starts flowering and seeding. This method can exhaust the parasite seed

bank in the soil and may be effective on the long run term. Acharya et al. (2002) evaluated the effectiveness of toria (*Brassica campestris* var. *toria*) plant as a catch crop for the reduction of *O. aegyptiaca* seed bank. They found that two successive crops of toria reduced *O. aegyptiaca* seed bank by 20.9% and 26.2% for both crops, respectively. Optimum density of toria plants required for significant reduction of *O. aegyptiaca* seed bank was about 140/m<sup>2</sup>. However, catch crops or plant species are likely to be used as preceding crops and have to be closely planted or sown at high density. This method of control however, is costly due to additional labor, not usable if growing season is short, and needs good mechanization because of possible loss of a growing period. On the other hand, cultivation of all hosts should be easy and inexpensive, germination stimulation should be high, their elimination is not problematic, and should be high yielding as fodder or green manure.

### *Other methods of Allelopathy application*

#### *Plant extract*

The effect of different plant extract in stimulating or inhibiting *Orobanche* seed germination or seedling attachment was investigated by different workers. Eizenberg et al.(2001) reported root extracts of sunflower resistant cultivars to inhibit development of *O. cumana*, while methalonic crude extract exhibited the strongest phytotoxic activity. These results indicated possible involvement of phytoalexines in the resistant reaction (Eizenberg et al. 1999). Sunflower coumarins (ayapin and scopoletin) were found to act as toxic allelochemicals responsible on inhibition of *O. cumana* seed germination and seedling development (Perez-de-Luque et al. 2001). Lopez-Granados and Garcia-Torres (1996) studied the effect of root extract stimulant of broad bean plants on *Orobanche crenata* seed dormancy and germination, and found germination stimulant activity of the root extract from broad bean plants grown during different seasons were varied considerably and gradually decreasing from autumn to summer seasons. This suggests an ecological adaptation of *O. crenata* to its main host crop. Egyptian clover was reported as an effective species in stimulating seed germination of *O. crenata*. Extract of this crop induced parasite germination by 98% followed by chickpea extract (95%) and lupine extract (91%), and seed germination was found better in sandy soils than in clay soils (Zaki et al. 1998).

#### *Intercropping with trap or catch species*

Intercropping was suggested by Al-Menoufi (1991, 1992) and Al-Menoufi et al. (1996) who found significant reduction in *O. crenata* infestation in broad bean intercropped with *Trigonella foenum-graecum*. In later study, Al-Menoufi and Adam (1998) concluded the possible use of *Trigonella foenum-graecum*, *Lupinus termis*, *Coriandrum sativum* and *Brassica rapa* in intercropping with broad bean or tomato to reduce the infection rate of *Orobanche* attached to the host root systems. The use of a

variety of mixtures of plant species is considered as a possible strategy to affect *Orobanche*-host interaction by combining certain host species with different degrees of susceptibility (Bouhatous and Jacquard, 1994).

Intercropping system was followed for *O. crenata* and *O. minor* control in pea by growing in mixture with garlic which resulted in best root system of peas and least *Orobanche* emergence, while higher seed yield was obtained when peas was mixed with onion. However, complete elimination of *Orobanche* emergence was achieved in mixture with black cumin (Hassan, 1998). Maximum percentage of germination of *O. cernua* was observed when the parasite seeds were exposed to green gram, sunhemp and sesame crops followed by black gram and sunflower crops both in incubator and glasshouse conditions (Dhanapal et al. 1998). *Arabidopsis thaliana* was also reported to induce seed germination of *O. aegyptiaca*, *O. minor* and *O. ramosa* at a rate of 87, 72, and 67% of maximum seed germination, respectively (Goldwasser et al. 2000). Root exudates of corn, sorghum, millet, cotton, rice, eggplant and cauliflower to induced seed germination of *O. cumana* under laboratory conditions and none of the crops mentioned was infected with this parasite (Rodriguez-Ojeda et al. 2001) suggesting possible use of some of these crops as trapping species. Certain weeds including *Avena sterilis*, *Conyza canadense*, *Ammi majus*, *Datura stramonium*, *Cichorium endivia*, *Anagallis arvensis* and *Solanum nigrum* were mentioned to support *O. ramosa* attachment but different resistant levels were observed and lead later to the death of parasite (Boulet et al. 2001). The authors suggested using these weeds as trap species for *O. ramosa* control.

#### *Crop rotation with trap or catch species*

Severe infestation and great build-up of *Orobanche* population occur when a host mono-crop is continuously cultivated or when susceptible crops come frequently at short intervals in crop rotation. Therefore rotation with non-host crops especially with trap species is of great advantage (Eltayeb et al. 2000). When *Orobanche* seeds found close to the roots of such species, 13 to 15% of these seeds germinate but do not develop haustorial connections. Suicidal germination of the parasitic seeds triggered by growing trap crops reduced the weed population and the growth of the host plants was hastened due to green manuring effect of trap crops. Therefore, including a trap crop in the rotation may reduce the problem. A well known example is *Capsicum annuum* grown in rotation with tobacco which can reduce *Orobanche* seed infestations in the soil (<http://www.wssa.net/subpages/weed/larrymitich/orobanche.html>). In a study carried out by Hershenhorn et al. (1996) few attached and developed *O. aegyptiaca* shoots were found on sweet pepper. Pepper (*Capsicum annuum*) roots stimulated germination of 22-26% of nodding *Orobanche* seeds but without the formation of parasitic attachments. Four-fold increase in the number of nodding *Orobanche* was observed on tomato roots interplanted with pepper compared with the number of parasites on tomato roots when planted without pepper.

*In vitro*, flax seed exudates induced germination of *O. crenata* and *O. ramosa*, and similar results were obtained on *O. aegyptiaca* with flax root exudates (Matthews et al. 1991). Flax and sugarcane significantly reduced *O. crenata* in a rotation with broad bean (Abou-Salama, 1995). Flax grown in two successive winter seasons or one summer cropping with *Phaseolus aureus* (*Vigna radiata*) reduced early infestation of *O. aegyptiaca* and significantly increased tomato growth and production (Kleifeld et al. 1994). However, flax as trap crop for eliminating *Orobanche* infection in broad bean fields had a limited potential (Khalaf, 1992). Other trap species reported were *Tidax procumbens* and *Bidens pilosa* (<http://www.wssa.net/subpages/weed/larrymitich/orobanche.html>). Sunhemp (*Crotalaria juncea* L.) and green gram (*Vigna radiata* L.) were also reported as promising trap crops in a cropping system containing bidi tobacco in areas where tobacco is grown in a long growing season. The crop rotation is already a current farmer's option in that area and susceptible crops are not cultivated year by year on the same fields. However, different workers reported successful control of different *Orobanche* species following this method. Schnell et al. (1994) mentioned legume crops to cause great reduction in *O. crenata* seed bank, and resistance to *O. crenata* has been found both within cultivated and wild grown legumes (Rubiales et al. 1998, 1999; Sillero et al. 2001b). Resistance to the same parasite has been demonstrated in different species of *Lathyrus* which were recommended as trap crops under field conditions (Sillero et al. 2001a). *Vicia villosa* subsp. *Dasycarpa* in rotation with lentil, chickpea and broad bean kept *O. crenata* infestation at a low level (Schnell et al. 1996).

### Plant oils

Application of plant oils (gigelly, groundnuts, palm, sunflower, safflower, niger, castor, linseed, neem, coconut or tobacco seed oils) at a rate of two or three drops applied to the top of vegetative *O. cernua* killed shoots in 2-4 days (Krishna-Murthy, 1992). However, oil treatment needs to be repeated every 4-5 days and for 3-4 times on emerging shoots. Botanical pesticides from neem (*Azadirachta indica*), *Pongamia*, *Nicotiana gossei* and nicotine sulfate were also recommended for post-emergence control of *O. cernua* with plant oils (Chari et al. 1993). Swabbing natural plant oils killed the bud and stem parts of the parasite by suffocation. The effect was selective on different parasite parts. While neem, coconut and sunflower oils showed quick knockdown effects in killing the bud part., neem oil did not kill the stem part of the parasite, and niger (*Guizotia abyssinica*), castorbean and mustard oils appeared to be somewhat less effective (Dhanapal and Struik, 1998). In another study, *Azadirachta indica*, coconut and sunflower oils affected the buds of *O. cernua* within 3 days; *R. communis* and *G. abyssinica* oils killed buds 3-4 days; and mustard oil took 5 days to kill buds. Coconut and sunflower oils killed *Orobanche* stems more rapidly than niger and castorbean oils with no phytotoxicity on tobacco was observed (Dhanapal et al. 1998). Volatile oils and crude acetone extracts of different plant species were also found effective against *O. ramosa* under greenhouse conditions (Solymosi, 1998).

### Plant residues

Plant residues left in the soil could yield allelochemicals which may be effective against different *Orobanche* spp. It was found that surface and pre-plant incorporated mulches of wheat and barley straw residues used at different rates significantly reduced *O. ramosa* infestation and growth in potato (Haidar et al. 1995). Olive jift (Pomace) added to pea growing soil prevented *O. crenata* infestation to this crop and reduced infection on broad bean and tomato (Ghosheh et al. 1999). Qasem and Foy (2001), reported high allelopathic activity of dried shoot mixtures of certain weed species on seed germination of *Orobanche ramosa* in tomato grown under glasshouse conditions, and Qasem (2002) reported inhibitory effects of dried shoot residues and root exudates of different weed and crop species to *O. ramosa* infestation on tomato plants.

### *Striga* spp.

#### Trap species

*Striga* seed germination can be induced by root exudates of some non-host plants including cotton that stimulate *Striga asiatica* seed germination without serving as a host for this parasite. Table 2 shows trap plant species of different species of *Striga* and below are some details on the effectiveness of certain species reported.

Sugimoto et al. (2001) discussed the induction of *Striga hermonthica* haustorium by several non-host plant tissues with a high potential to produce haustorial-inducing substances. The non-host cotton varieties STAM 18-A and FK 290 were reported to strongly stimulate seed germination of *Striga hermonthica* (Diarra and Traore, 2001). These workers demonstrated through laboratory and outdoor pot studies that cotton, as trap crop is a powerful tool to reduce yield losses caused by *Striga* infestation and the potential of a high stimulant production is cultivar specific. *Faidherbia albida* was reported to effectively control *Striga hermonthica* in millet (*Pennisetum glaucum*)-based cropping system (Gworgwor and Weber, 2001). This agro-forestry tree could completely control the parasite attacking millet under the canopy and no single *Striga* plant was observed under the trees surveyed. In contrast, the adjacent millet crop outside the tree canopy was highly infested. However, the authors attributed this effect on parasite germination to the high fertility level caused by this tree, while the role of allelochemicals in such a case should be also considered. In a pot experiment, Khan et al. (2001) studied the role of allelopathy in *Striga hermonthica* suppression by *Desmodium uncinatum* and the possibility of an allelochemical mechanism involvement in such effect. They found that root exudate of *D. uncinatum* added to maize suppressed *Striga hermonthica*.

Table 2. Trap plant species reported for different *Striga* species.

Scientific name	Common name	References
<i>Striga asiatica</i>		
<i>Arachis hypogaea</i>	Peanut	Prabhakarasetty, 1980
<i>Cajanus cajan</i>	Pigeon pea	Prabhakarasetty, 1980
<i>Gossypium hirsutum</i>	Cotton	Prabhakarasetty, 1980, Norria, 1990
<i>Crotalaria juncea</i>	Sunhemp	Prabhakarasetty, 1980
<i>Phaseolus aureus</i>	Green gram	Prabhakarasetty, 1980
<i>Medicago sativa</i>	Lucerne	Prabhakarasetty, 1980
<i>Helianthus annuus</i>	Sunflower	Prabhakarasetty, 1980
<i>Sesamum indicum</i>	Sesame	Prabhakarasetty, 1980
<i>Striga hermonthica</i>		
<i>Lablab purpureus</i>	Hyacinth bean, Egyptian kidney bean	Bebawi & Mutwali, 1991
<i>Gossypium hirsutum</i>	Cotton	Bebawi & Mutwali, 1991; Jost, 1997
<i>Cyamopsis tetragonoloba</i>	Cluster bean	Bebawi & Mutwali, 1991
<i>Sesamum indicum</i>	Sesame	Bebawi & Mutwali, 1991; Hudu & Gworgwor, 1998
<i>Helianthus annuus</i>	Sunflower	Bebawi & Mutwali, 1991; Hudu & Gworgwor, 1998
<i>Menispermum dauricum</i>	Koumorikazura	Ma et al. 1998
<i>Vigna unguiculata</i>	Cowpea	Schulz et al. 2003
<i>Glycine max</i>	Soybean	Jost, 1997; Kureh et al. 2000; Schulz et al. 2003
<i>Aeschynomene histrix</i>	Porcupine jointvetch	Merkel et al. 2000
<i>Vigna subterranea</i>	Bambara groundnut	Hudu & Gworgwor, 1998
<i>Abelmoschus esculentus</i>	Okra	Hudu & Gworgwor, 1998
<i>Corchorus olitrius</i>	Jute	Parker & Riches, 1993
<i>Cajanus cajan</i>	Pigeon pea	Parker & Riches, 1993
<i>Cicer arietinum</i>	Chickpea	Parker & Riches, 1993
<i>Hibiscus cannabinus</i>	Kenaf	Parker & Riches, 1993
<i>Arachis hypogaea</i>	Groundnut	Parker & Riches, 1993
<i>Striga gesnerioides</i>		
<i>Vigna unguiculata</i>	Cowpea	Wild, 1948
<i>Lablab purpureus</i>	Hyacinth bean, Egyptian kidney bean	Berner & Williams, 1998
<i>Sphenostylis stenocarpa</i>	African yam bean	Berner & Williams, 1998
<i>Cajanus cajan</i>	Pigeon pea	Wild, 1948

<i>Mucuna pruriens</i>	Velvet bean	Wild, 1948; Doggett, 1965
<i>Striga</i> spp.		
<i>Gossypium hirsutum</i>	Cotton	Doggett, 1965
<i>Helianthus annuus</i>	Sunflower	Doggett, 1965
<i>Vigna radiata</i>	Mungbean	Doggett, 1965
<i>Pennisetum glaucum</i>	Pearl millet	Robinson & Dowler, 1966
<i>Pisum sativum</i>	Pea	Robinson & Dowler, 1966
<i>Glycine max</i>	Soybean	Robinson & Dowler, 1966

### *Catch species*

Millet, corn, sorghum and Sudan grass were mentioned as catch crops to *Striga* (Bebawi, 1987; Oswald et al. 1997). Sprich (1994) reported reduction in *S. hermonthica* seed bank by several catch crops, with 43% by maize-sorghum, approx. 30% by sunflowers, soybeans and cotton, and 20% by cowpeas and groundnuts, compared with an average of 12% in fallow land. He concluded that a well-structured crop rotation over several years consisting of a number of trap or catch crops would give effective control of this parasite. Cardwell and Lane (1995) found that predominant cropping systems where *Striga gesnerioides* infestation occurred were sole cowpea; intercrop with millet, and with sorghum. In contrast, cowpea fields in rotation with cotton, or intercropped with vegetables and legumes were always *Striga*-free.

### *Other methods of Allelopathy application*

#### *Intercropping with trap or catch species*

Intercropping system significantly influenced *Striga* infestation (Ariga et al. 2001). Sorghum line P9405 intercropped with cowpea reduced *Striga* number compared to the number observed in pure stands of either P9405 or the susceptible check Pato (Mbawaga et al. 2001). Tenebe and Kamara (2002) reported that growth performance of the intercropped sorghum was significantly better than that of the monoculture. Intercropping of sorghum with groundnut RMP-12 resulted in a significant suppression of *Striga* compared to other groundnut varieties. The dry matter yield of sorghum intercropped with groundnut was significantly higher than that of the sorghum monocrop and was the highest with RMP-12 variety. Intercropping maize with beans resulted in a significant increase in grain yields by 51.2% and 61.4% over farmer's practice. The highest yield (78.6% above yield in pure maize stand) obtained when intercropping maize with four beans in the same hole. Reda and Bayou (2001) reported that sorghum planted after legume crops showed significantly improved yield than the

control. The highest grain yield obtained was from plots of cowpea and haricot bean of the previous year. These authors confirmed that significant grain yield in terms of improved sorghum performance, following crop rotation and fertilizer use, were recorded despite high *Striga* infestation. Gworgwor et al. (2001) reported that growing ICMV-IS-91116 millet variety with sesame in 1:1 hill prevented *Striga hermonthica* infestation compared with millet grown in the same field with sesame and sole millet crop. Sudan grass [*Sorghum sudanense*] intercropped into maize as a *Striga* catch-crop and up-rooted after 30 or 50 days stimulated the germination of high numbers of *Striga* seeds (Oswald et al. 1997). Velvet bean [*Mucuna pruriens*], sunhemp [*Crotalaria juncea*], cowpeas [*Vigna unguiculata*] and dolichos [*Lablab purpureus*] used either in rotation or intercropped with maize could improve soil fertility, reduced *Striga* sp. incidence and increased maize yields (Chibudu, 1997). In one of the recent studies, Chivinge et al. (2001) examined the effectiveness of cowpea cultivars on *Striga asiatica* emergence in a maize/cowpea intercropping system and found that all cultivars reduced *S. asiatica* emergence by at least 40% with IT82D-849 being the most effective. The cowpeas resulted in maize grain increases of 150-290%, and the authors recommended intercropping maize on *S. asiatica*-infested lands with any of the cowpea cultivars. A successful intercropping mixture of peanut, bean, yellow gram, bambara nut and soybean with maize was reported (Oswald, et al. 2002). This combination produced considerable yields and increased the overall productivity of the cropping systems. However, of the most productive intercrops, yellow gram provided the most stable *Striga* control over years and locations comparable only to cowpea. Peanut, bambara nut, bean and soybean were more variable in terms of *Striga* suppression in maize depending on the specific agro-ecological conditions.

### *Crop Rotation*

Jost (1997) reported an improved fallow with *Calopogonium mucunoides* and *Pueraria phaseoloides* lowered *Striga hermonthica* in sorghum seed banks by 48% compared to natural fallows of only 27%. Soybean rotation increased maize yield by approximately 90% (Carsky et al. 2000). However, increasing soybean plant density did not reduce emerged *S. hermonthica* but application of phosphorus associated with high soybean densities resulted in lower emerged *S. hermonthica* on maize and significantly higher maize yield.

### *Plant Residues*

Although literature on the effect of plant residues on *Striga* spp. through production of allelochemicals are limited, but different workers reported significant effect of residues of different plant species on these parasites. Gacheru and Rao (2001) reported reduction in *Striga hermonthica* infestation using organic residues of different plant species which was decomposition rate and N mineralization dependent. With a high tissue concentration of N and low lignin and polyphenols, *Tithonia diversifolia*

biomass rapidly mineralized N and reduced *Striga hermonthica* stand. However, *Sesbania sesban* biomass reduced *Striga hermonthica* only in the fourth season of its residue decomposition, while biomass of *Croton megalocarpus* and *Lantana camara* had limited effect. It was concluded that only the high quality organic residues such as that of *T. diversifolia* can suppress *Striga hermonthica* infestation. Other workers reported shoot residues of *Sesbania sesban* added to the soil-infested *Striga hermonthica* seeds and grown with sorghum significantly reduced *Striga* emergence and biomass. The use of residues from *Gliricidia sepium* or *Vitellaria paradoxa* reduced the emergence of the parasite and *G. sepium* residues increased sorghum yield (Letourneau et al. 2001). From the above reported results on the effect of plant residues on *Striga* spp. it is clearly shown that authors did not separate the effect of increased soil fertility levels from the possible involvement of allelochemicals in parasite suppression. However, in such a work the role of allelopathy could be significant.

#### *Root exudates*

Ma et al. (1996) reported that *Menispermum dauricum* roots cultured in a modified B5 medium produced and exuded into the medium compounds with activity comparable to that of the synthetic strigol analogue GR24. The active components stimulate germination of *Striga hermonthica* by induction of ethylene biosynthesis. Their accumulation in roots and culture medium, as indicated by activity, was at a maximum 6-8 weeks after subculturing and was positively correlated with root growth. Analysis revealed at least two or three compounds, none of which had chromatographic properties consistent with those of strigol. The results indicated the potentials of root cultures as a possible source of novel *Striga* germination stimulants. In more recent work, Ma et al. (1998) reported that germination of *Striga hermonthica* requires an exogenous stimulant produced by the roots of host and some non-host plant species. Root cultures of *Menispermum dauricum*, a non-host broad-leaved herbaceous plant, produced a group of substances that induced parasite germination. Root exudates of sorghum cv. Haygrazer and cowpeas cv. Saunders Upright were found to stimulate seed germination of *S. gesnerioides* and *S. asiatica* when exuded into the medium flowing through plant trys. However, both germination stimulants and inhibitors were detected in exudates of these species (Muller et al. 1993). Root exudates of several crop plants susceptible to *Striga* or *Orobanche* spp. exhibited significant activities on *Orobanche* seed germination. In particular, those of tomato, garden pea, sunflower and soybean showed strong induction of seed germination. All stimulants in these exudates may be structurally related to strigol and some crops including garden pea produce novel germination stimulants-strigolactones (Yoneyama et al. 2001).

Allelopathic influence was clearly demonstrated against *Striga hermonthica* using *Desmodium uncinatum* in maize-based farming system. Dramatic reduction in parasite infestation was found when eluate from *D. uncinatum* roots was introduced into pots of

maize growing in soil inoculated with high levels of *S. hermonthica*. Growth of the parasitic weed was almost completely suppressed whereas extensive infestation occurred with control elute. The allelopathic mechanism was found to be involved in inhibition of development of haustoria of *S. hermonthica* (Khan et al. 2001). In more recent work, Khan et al. (2002) reported both *Desmodium uncinatum* and *D. intortum* reduced infestation of maize by *Striga hermonthica* through a putative allelopathic mechanism. High significant reduction in *S. hermonthica* infestation was obtained when an aqueous solution, eluting from pots of *D. uncinatum* plants was used to irrigate pots of maize planted in soil infested-*S. hermonthica*. Growth of the parasite was almost completely suppressed compared with the control eluate. Water-soluble chemical components exuded from cleaned roots of *D. uncinatum*, was found to contain a germination stimulant for *S. hermonthica* and also an inhibitor for haustorial development.

### ***Cuscuta* spp**

#### *Plant extract*

Generally, reports on the effect of allelopathy on *Cuscuta* spp. are very limited, since these parasites do not require any host germination stimulants. However, in one study on the effect of plant extract against *Cuscuta trifoliae* and *C. campestris*, Lados (1999) reported that host plant (*Medicago sativa* bv. Verko [lucerne]) extract had little effect on seed germination of these species. In contrast, Zaki et al. (1998) found that Green bean (*Phaseolus vulgaris*) extract showed strong seed germination inhibition of different *Cuscuta* spp., while all other plant extracts had stimulatory action. The authors detected two bands of vinyl group compounds and a histological study revealed that parasite haustoria did not succeed in entering bean plants, and thus resulted in death of the parasite.

## **ALLELOCHEMICALS AS NATURAL HERBICIDES IN ACTION**

It is now evident that allelochemicals play an important role in increasing or decreasing parasitic weed problems and can be utilized as promising and effective natural tools in controlling these species. Some of these allelochemicals are seed germination inhibitors that can be used in a pre-planting soil applied treatment to inhibit parasite germination or attachment to host plants, while others are able to stimulate parasite seed germination and thus can be used in a similar way but in absence of host species. However, the amount, concentration and environmental conditions are significant factors determine the effectiveness of these chemicals. Sugimoto (2000) reported different natural stimulants for different parasitic species including strigol from cotton, sorgolactone from sorghum, alectrol from cowpeas, and

orobanchol from red clover [*Trifolium pratense*], while Chittapur et al. (2001) discussed and evaluated the use of catch and trap crops in integrated weed management and emphasized their role in controlling different parasitic species of economic importance through allelopathy mechanism.

### ***Orobanche* spp.**

#### *Stimulants*

Several natural germination stimulants were isolated and identified from host and non-host plant species of *Orobanche*. Structure activity studies of the natural stimulants and their synthetic analogues that induce the induction of *Striga* and *Orobanche* seed germination proceeds via receptor-mediated mechanism (Zwanenburg and Reizelman, 2001) and the germination of root parasites depends on chemical products exuded from the roots of host plants known as germination stimulants. The predominant part of them belongs to sesquiterpenes lactones (Butler, 1995 and Denev et al. 2001). Yokota et al. (1998) isolated three seed germination stimulants of *Orobanche minor*, from the root exudate of its host *Trifolium pratense*. One was identified as alectrol and the other was a novel strigol-related compound, which was named orobanchol and tentatively proposed to be a strigol isomer. Similar findings were reported by Yoneyama et al. (2001) in which at least three different germination stimulants to *O. minor* and to *Striga* spp. produced by red clover including orobanchol and strigolactones indicating that strigolactones are likely to be natural germination stimulants for *Orobanche* spp.

However, the identified natural germination stimulants of host root exudates with known chemical structures are strigol from maize, alectrol from cowpea and sorgolactone from sorghum, and all produced by *Striga* host plants (Wegmann, 1998). Strigol is a powerful germination stimulant of *Striga* seeds (and later *Orobanche* seeds) that was first isolated and identified from the root exudates of cotton plants (a non-host crop) and stimulates seed germination of *Striga* by 85 and 100% at concentrations of  $10^{-4}$  to  $10^{-6}$  M (Hsiao et al. 1981). Other synthetic and natural germination stimulants of *Striga* species are lactonic or contain one or more unsaturated lactone rings (Worsham et al. 1962; Johnson et al. 1976; Musselman, 1980). Some sesquiterpene lactones were tested by Perez-de-Luque et al. (2001) and some were found to induce *O. cumana* seed germination better than GR24 at low concentrations and they were specific in this parasitic species. However, some of these chemicals, such as coumarin-type compounds are absolute allelochemicals (Worsham et al. 1962; Rice, 1984). On the other hand, different species have shown strong ability to stimulate seed germination of different *Orobanche* species by more than 90% (Wegmann, 1998) but the structures of the germination stimulants exuded from these hosts remain unknown although several haustoria-inducing compounds have now been characterized including xenognosin A & B, identified in root exudate of *Astragalus gummifer*, and

soyasapogenol B characterized from roots of *Lespedeza sericea*. More recent studies, reported sesquiterpene lactone group to play an essential role in stimulating seed germination of *Orobanche cumana* (Perez-de-Luque et al. 2001). Ethylene was recently reported by Zehhar et al. (2002) to be involved in the induction of germination of conditioned seeds of *Orobanche ramosa* by GR24, as inhibitors of its synthesis or action, applied to conditioned seeds, also strongly reduced induction of germination by GR24. However, exogenous ethylene did not induce germination of conditioned seeds, but 2-chloroethylphosphonic acid was able to do so. When inhibitors of gibberellin biosynthesis were applied to conditioned seeds in the presence of GR24, parasite germination was inhibited. The same chemicals also strongly inhibited germination of conditioned *Striga hermonthica* seeds in response to GR24; this inhibitory effect was reversed by the addition of 2-chloroethylphosphonic acid. The effect of these inhibitors on *S. hermonthica*, in which ethylene is a necessary mediator of germination induction by GR24, strongly suggests that ethylene synthesis is also required for the induction of *O. ramosa* seed germination by GR24. These growth regulators, which inhibit the two steps of germination in *O. ramosa*, could be useful for the development of methods for early season control of this parasite.

### Inhibitors

Inhibitory allelochemicals may work at different stages of parasite life cycle from germination to growth and development. However, a reverse action may be obtained at low concentration received by the parasite. Sauerborn (2001) suggested that phytotoxines could contribute in improving parasitic weed management. Serghini et al. (2001) reported differences between *O. cernua*-resistant and susceptible sunflower varieties with seed germination of *O. cernua* halved and germinated seeds displayed enhanced browning symptoms in the presence of resistant sunflower roots. Parasite radicles or host-tissue around the contact point turned brown after *O. cernua* attachment especially to roots of the resistant varieties. They suggested possible accumulation of toxic compounds as a defense strategy in the resistant sunflower varieties. Serghini et al. (2001) reported that coumarins affect the normal growth and development of the *Orobanche cumana* seedlings and the effect was more intense from the resistant cultivars than in the susceptible one. Coumarins (ayapin and scopoletin) from sunflower plants were implicated as inhibitory allelochemicals to germination, growth and development of *O. cumana* (Perez-de-Luque et al. 2001) and both excrete by sunflower roots into the environment and could act as a toxic allelochemicals to the parasite (Gutierrez-Mellado et al. 1995). Sunflower 7-hydroxylated simple coumarins may play a defensive role against *O. cernua* parasitism by preventing successful germination, penetration and/or connection to the host vascular system. This hypothesis is supported by that coumarins inhibited the *in vitro* germination of *O. cernua* seeds induced by the strigol analogue GR24 and caused a browning reaction in germinated seeds and by resistant sunflowers accumulated higher levels of coumarins

in roots and excreted greater amounts than susceptible varieties in response to *O. cernua* infection. Bar-Nun and Mayer (2002) reported the presence of large amounts of oleic and linoleic acids and small amounts of stearic and palmitic acids in *O. aegyptiaca* seeds. The authors expected these acids to interfere with sugar metabolism and thus preventing parasite seed germination during the preconditioning period. Pre-plant treatment of crop seeds with these inhibitors may prevent parasite infestation and could be considered as an effective technique in parasitic weed control. Sunflower seeds treated with 40 ppm of benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) for 36 h completely prevented infection of *Orobanche cumana* in root chambers (Sauerborn et al. 2002). In pot studies, at considerable inoculaums of *O. cumana* seeds, the total number of parasite shoots was reduced by almost 90% with 60-ppm BTH. The inhibitory chemical prevented both attachment and penetration of *O. cumana* at the sunflower root. Results showed synthesis of the phytoalexin scopoletin and of hydrogen peroxide in the BTH-treated sunflower roots and demonstrated the importance of allelopathy strategy as an effective component of future plant production systems.

The role of microorganisms (e.g. *Streptomyces*) has been also implicated in *Orobanche* seed germination. Christeva and Naumova (1998) reported different strains of *Streptomyces* to induce germination of *O. ramosa* and concluded that microorganisms may take part in germination processes of this parasite. In another study, Yoneyama et al. (1998) reported stimulation of *O. minor* seed germination by certain fungal metabolites including cotylenins and fusicoccins at concentrations as low as  $10^{-5}$  M and up to more than 50%. Different *Fusarium* spp. were reported by Sauerborn (2001) to infect *Orobanche* and *Striga* seeds and plants, and the author emphasized the role of phytotoxins in the process. He added that members of the genus *Fusarium* produce toxins such as enniatin, fumonisin, fusaric acid, moniliformin and trichothecenes that possess a broad range of biological activities and metabolic effects. However, some of these compounds have been already considered and proposed to be used as natural herbicides (Strobel et al. 1991) and later studies showed fusaric acid and 9,10-dehydrofusaric acid to be active at low doses in reducing *Striga* seed germination (Zonno et al. 1996; Zonno and Vurro, 1999).

Khalaf et al. (1997) studied *Orobanche crenata* control using *Azotobacter* spp. and *Escherichia coli* transformants to break parasite seed dormancy. Using crude DNA preparations from *Vicia faba* roots to prepare transformants of *Azotobacter* spp. and *Escherichia coli* the transformants produced *Vicia faba*-like stimulants, which promoted seed germination of the parasite. Transformants of *A. chroococcum* and *E. coli* were most active. The greatest levels of *O. crenata* germination achieved in the bioassays with transformed colonies of *A. chroococcum* and *E. coli* were 41.8 and 27.3%, respectively. They proposed that such transformants have potential as biological control agents by causing non-host germination of parasitic weed seeds. Zonno and Vurro (1999) found T-2 toxin from *Fusarium* sp. to be the most active

among 14 fungal toxins tested, inhibiting by 100% seed germination at  $10^{-5}$  M and was active down to  $10^{-7}$  M (19% inhibition). Deoxynivalenol [vomitoxin], produced by *Fusarium* spp. was also found very effective, causing 100% and 69% reduction in germination when assayed at  $10^{-4}$  and  $10^{-5}$  M, respectively. Cytochalasin E, tenuazonic acid, fumonisin B1, enniatin and nivalenol, produced by *Aspergillus clavatus*, *Alternaria alternata*, *Fusarium moniliforme* [*Gibberella fujikuroi*], a microbial source and *Fusarium nivale* [*Monographella nivalis*], respectively, all had inhibitory effects of about 50% at concentration  $10^{-4}$  M. Other toxins had lower or no activity. The high activity shown by some fungal toxins suggests that they may have potential as more natural and safe herbicides to suppress *S. hermonthica* seed germination. Amsellem et al. (2001) were to mention that mycotoxins isolated from different *Fusarium* and other fungal species killed *Orobanche*, and are being considered for direct use, or to complement other control strategies. *Fusarium* spp. used have been transformed with gus and/or gfp genes allowing tracing their movement in the environment, and opening the way to future transformations to hypervirulence.

In one of more recent studies, Zonno and Vurro (2002) tested the effect of toxins produced by *Fusarium* species on *O. ramosa* seed germination and found many of them were active at the highest concentration used. Fusarenon-X, nivalenol, deoxynivalenol, T-2 toxin, HT-2 toxin, diacetoxyscirpenol and neosolaniol, were highly active at  $10 \mu\text{M}$ , causing 100% inhibition of seed germination. Many of the toxins tested were active when assayed at a concentration ten times lower, with T-2, HT-2, nivalenol, neosolaniol and diacetoxyscirpenol were still able to cause complete inhibition. Diacetoxyscirpenol was also very active at  $0.1 \mu\text{M}$ , causing more than 90% inhibition. Results showed that the use of toxic secondary metabolites could represent a useful alternative strategy in the management of parasitic weeds, by interfering with the induced germination process, and that fungal culture extracts could be an interesting source of new compounds acting as natural and original herbicides on these parasites.

## ***Striga* spp.**

### *Stimulants*

Chemicals stimulate *Striga* seed germination include those naturally occurring in plant root exudates, different synthetic plant hormones, and a various synthetic compounds. Strigol was the first germination stimulant characterized (Cook et al. 1966, 1972) and isolated from cotton roots, a trap nonhost species. It was active at low concentration against *Striga asiatica* and mentioned as a natural product triggers suicidal germination of parasitic *Striga* species (Kadas et al. 1996). However, natural germination stimulants reported for *Striga* belong to different chemical groups, most of them being sesquiterpenes. The largest group collectively named strigolactones (Butler, 1995)

which have a benzoquinone structure, isolated from sorghum roots (Chang et al. 1986 and Netzly et al. 1988), and was found very effective on *S. asiatica*. Natural stimulants from sorghum were found closely related to strigol and named sorgolactone which was active against different parasitic species including *S. asiatica*, *S. hermonthica*, *Orobanche aegyptiaca* and *Alectra vogelii* (Hauck et al. 1992). Sorgoleone was the first stimulant of these isolated from root exudates of *Sorghum bicolor* (Chang et al. 1986; Netzly et al. 1988). Wigchert and Zwanenburg (1999) findings supported the view that strigolactones induce germination of parasitic weed seeds via a receptor-mediated mechanism. Three active compounds on *S. asiatica* have been also isolated from root exudates of maize, sorghum and *Panicum miliaceum* and a fourth compound was also found in sorghum exudate (Siame et al. 1993). The authors concluded that strigol is the major seed germination stimulate to this parasitic species.

More work was carried out on different hosts to isolate and identify the chemicals responsible on parasitic seed germination using different host species. A large number of sesquiterpene lactones were isolated from sunflower plants (Galindo, 1993), and 11beta, 13-dihydroparthenolide (DHP), a sesquiterpene lactone from *Ambrosia artemisiifolia* (Rugutt and Rugutt, 1997) which was found to induce germination of *Striga hermonthica* *in vitro* suggesting that the mode of action of DHP could be hormonal and some of these chemicals also stimulating *O. cumana* seed germination (Perez -de-Luque et al. 2001) and were better than GR24 compound.

Jasmonates and related compounds were reported by Yoneyama et al. (1998) to elicit seed germination of *Orobanche minor* and *Striga hermonthica*. Esters were more effective than by the corresponding free acids. Among the compounds tested, methyl jasmonate (MJA) was the most active stimulant. Rugutt et al. (2001) isolated deoxyobacunone, the known limonoids obacunone, harrisonin and 12beta-acetoxyharrisonin from the root bark of *Harrisonia abyssinica* and found that 10-3-10-5 M concentrations of these compounds exhibited significant stimulatory activity ranged between 12-98% of conditioned *S. hermonthica* seeds under laboratory conditions. Among other sources of germination stimulants reported to *Striga* were sorghum root residues (Chidley and Drennan, 1987), and aqueous extracts of a range of different *Euphorbia* spp. including *E. acalyphoides* and *E. pilulifera* which were inhibitory at high concentration. Extracts of *E. pilulifera*, *E. aegyptiaca* and *E. hirta* induced haustorial initiation, while those of *E. aegyptiaca* stimulated seed germination and haustorial initiation in a range of *Striga* spp. and strains (Ibrahim et al. 1985). In another study, Rugutt and Berner (1998) reported dichloromethane and water extracts of leaves, stems and roots of different legume cultivars exhibited stimulatory activity to *Striga hermonthica* seed germination at different concentrations. Differences in the activity of different cultivars were also found. Among other chemicals reported were plant hormones including zeatin which found effective under laboratory conditions (Worsham, 1987).

Certainly other stimulants can be also involved in *Striga* seed germination and haustoria development but are difficult to isolate and characterize because the lack of stability of these compounds (Mangnus, 1992) and their rapid changes to non-effective chemicals.

### Inhibitors

Reiss et al. (1995) reported cytokinin dimethylallyaminopurine initiated growth of *Striga gesnerioides* shoot without further tubercle development. The absence of an essential physiological process was thought to be responsible for impaired growth of the parasite on the resistant cowpea line B301. However, cytokinins and L-methionine have already been mentioned as substances cause germination stimulation of *Striga* (Worsham and Egley, 1990), while in contrast cytokinins are well-known haustorial development inhibitors (Babiker et al. 1992). Sorgoleone has shown some herbicidal activity and may function as an allelopathic chemical.

Most of the work on *Striga* seed germination inhibition was carried out using plant pathogens or their chemical assimilates. Ciotoia et al. (1995) reported that an isolate of *Fusarium oxysporum* (M12-4A), grown on sorghum straw and incorporated into pots, successfully prevented emergence of *S. hermonthica*, and resulted in a 400% increase of sorghum dry weight. The fungus inhibited germination and attachment of *S. hermonthica* to sorghum roots in root chambers. In addition, several crop species, including sorghum, inoculated with the fungus showed neither any disease symptoms nor any reduction in biomass. A 10-15% reduction of *S. hermonthica* biomass was found due to *Fusarium nygamai* infection (Sauerborn et al. 1996) although the number of emerged plants was not significantly affected. The higher levels of *S. hermonthica* biomass reduction resulted in a corresponding improvement in maize vigour. Zonno et al. (1996) reported Fusaric and 9,10-dehydrofusaric acids and their corresponding methyl esters to be the main phytotoxins produced by *Fusarium nygamai*, a potential control agent of *Striga hermonthica*. The application of very low concentration of toxins ( $10^{-6}$  M) caused a dramatic reduction of seed germination. The toxic effects of these metabolites were clearly expressed on punctured leaves, on which the toxins caused the appearance of large necrotic spots. The authors suggested that the use of these metabolites against *Striga*, possibly in combination with other cultural and biological methods, may help in controlling this noxious parasite. Fusaric acid and dehydrofusaric acid toxins produced by a strain of *Fusarium oxysporum* (M12-4A) are both used for *Striga hermonthica* control (Savard et al. 1997), and methyl ester of fusaric acid, one of four toxins produced by the fungus *Fusarium nygamai*, could be also used as a natural herbicide against the same parasite (Vischetti and Esposito, 1999). Persistence of these natural herbicides in all soils and at all incubation conditions was long enough to protect crops for the first week of growth.

Czerwenka-Wenkstetten et al. (1997) were able to isolate *Myrothecium roridum*, *Curvularia eragrostidis* [*Cochliobolus eragrostidis*] and *Curvularia lunata* [*C. lunatus*] from *Striga hermonthica* seeds and reported complete seed germination inhibition of the parasite by *Myrothecium roridum* and 48% by *Curvularia eragrostidis* and *Curvularia lunatus*. Ahmed et al. (2001) showed that an isolate of *Fusarium solani* (Sud 96) obtained from infected *Striga hermonthica* plants in Sudan demonstrated high inhibitory activity against parasite seed germination. Aqueous and organic solvent culture extracts, as well as fungus suspension, mixed with GR24 inhibited the germination of conditioned *S. hermonthica* seeds. Filtrates of the same isolate grown on different growing media were different in their inhibitory effects to parasite germination with isolates grown on autoclaved rice, sorghum grains, and potato dextrose agar (PDA), were more effective than that on wheat straw. Chemical analysis indicated the presence of several compounds with high inhibitory activity. Sugimoto et al. (2002) reported inhibition of *S. hermonthica* germination induced by the germination stimulant GR24 with metabolites of *Fusarium solani* (Sud 96). The identified active compounds were trichothecenes, acuminatin, neosolaniol, 8-acetylneosolaniol, and tetraacetoxy T-2 tetraol (neosolaniol diacetate). Inhibitory activity of the four trichothecenes against parasite germination increased with acetylation of the hydroxyl moieties. However, the inhibitor, 8-acetylneosolaniol, completely prevented *S. hermonthica* germination at 24 µM.

Different species of bacteria were also reported to have a significant role in *Striga* germination inhibition. Bouillant et al. (1997) reported two strains of the *Azospirillum brasiliense* out of four strains assayed did significantly inhibit germination of *Striga hermonthica* seeds. However, one strain showed also a plant growth promoting effect indicating different activity. Similar interaction between the same bacteria species and *S. hermonthica* was reported by Miche et al. (2000) who isolated two strains of this bacteria from an African sorghum rhizosphere which prevented *Striga* seed germination although they showed stimulatory action in presence of sorghum roots. However, *Azospirillum* cells suspended in a synthetic germination stimulant (GR24) did not inhibit *Striga* seed germination, but blocked radicle elongation, while lipophilic compounds extracted from the medium of bacteria in the log and stationary growth phases prevented the germination of *Striga* seeds. Berner et al. (1999) compared ethylene-producing strains of *Pseudomonas syringae* pv. *glycinea* 8/83, 16/83 and 19/84 with other germination stimulants [strigol analogue GR24, roots of cowpea (*Vigna unguiculata* cv. IT84S-2246-4) and ethylene gas] to stimulate germination of *Striga aspera*, *S. gesnerioides* and *S. hermonthica* and reported that bacterial strains were more effective stimulators of germination than either cowpea roots or ethylene gas, and gave germination levels in *S. aspera* and *S. gesnerioides* similar to those produced by GR24.

On the other hand, auto- and hetro-phytotoxic effects of different *Striga* species have been also documented. Parker and Riches (1993) indicated involvement of

inhibitors in *Striga* seeds that need to be leached out or degraded during conditioning period and Rugutt et al. (1996) reported extracts of *Striga hermonthica*, *S. aspera* and *S. gesnerioides* to contain tetradecanoic acid, cis, cis-9, 12-octadecadienoic acid, cis-9-octadecenoic acid and sitosterol, in addition to 2,6-dimethoxy-p-benzoquinone(2,6-DMBQ), and several long chain aldehydes and n-hydrocarbons detected in some of *Striga* extracts. However, 2,6-DMBQ was also present in sorghum root extracts but not in its root exudate (Chang and Lynn, 1986). Ransom et al. (1996) emphasized that *Striga* exert a potent phytotoxic influence on its host by inducing enzyme and plant hormone changes, disrupting host-water relations; and reducing carbon fixation below that expected purely by competition on growth factors (Gurney et al. 1995). These results may indicate the possible involvement of allelochemicals in this reaction. Antimicrobial effects of *Striga lutea* (*S. asiatica*) plant extracts was also reported against *Escherichia coli*, *Staphylococcus citreus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Aspergillus niger* and *Candida albicans* (Hiremath et al. 1994). In contrast, Reizelman and Zwanenburg (2002) reported Nijmegen-1 (synthetic analogues of (+)-strigol), as a germination stimulant identified from the seeds of *Striga asiatica*.

### ***Cuscuta* spp.**

Although allelochemicals may have no effect on germination of these parasitic species, they may play an important role in the attachment, haustoria embedment and development and generally in the interaction between the parasite and host plant. Huang and Li (1991) reported that haustorial primordia of *Cuscuta japonica* grown on *Salix purpurea* cv. *multinervis* were rich in calmodulin and the formation of primordia induced by cytokinin application could be inhibited by triflurperazine, a specific inhibitor of calmodulin activation. Based on the results obtained, it is possible that calmodulin participates in the formation of haustoria. Frolisek and Novotny (1992) reported chemical compositions of *C. epithymum* subsp. *trifolii* to include mainly proteins, and bound and free amino acids. Nair and Thirupurasundari (1992) identified coumarins and flavonoids from *Cuscuta reflexa* parasitizing *Bougainvillea spectabilis*. Scoparone (6,7-dimethoxycoumarin) and melanettin (6-hydroxy-7-methoxy-4-(4-hydroxyphenyl)-coumarin) were isolated together with quercetin and hyperoside, from the whole plant. Among other chemicals reported were three ursane-type triterpenes, 3 steroid compounds, palmitic acid and sucrose isolated from whole *C. songaricum* plants (Ma et al. 1993). The triterpenes were identified as acetyl ursolic acid, ursolic acid and a new compound, ursa-12-ene-28-oic acid, 3 beta-propanedioic acid monoester. The steroid compounds were beta-sitosterol palmitate, beta- sitosterol and beta-sitosterol glucoside (daucosterol, 6).

Some well known allelochemicals were detected in different *Cuscuta* spp. Loffler et al. (1995) identified eight major phenolic constituents from *Cuscuta reflexa* and *Cuscuta platyloba* parasitizing different host plants, these were chlorogenic acid,

3,5-dicaffeoyl-quinic acid, 4,5-dicaffeoyl-quinic acid, quercetin, and its O-glycosides quercetin-3-O-beta-galactoside and quercetin-3-O-beta-glucoside as well as kaempferol-3-O-beta-galactoside and kaempferol-3-O-beta-glucoside, at which all are active allelochemicals. *C. reflexa* cultivated in a greenhouse yielded mainly hydroxycinnamic acid derivatives, whereas field-grown plants showed the ability to synthesize flavonoids as well. *C. platyloba* was characterized by the accumulation of flavonoids of the flavonol-type while *C. reflexa* contained mainly caffeic acid depsides. The pattern of phenolic compounds in both *Cuscuta* species was irrespective of the host plants. In another study, phytochemical analysis of nine species of the genus *Cuscuta* yielded characteristic patterns of soluble phenolic constituents which were stable in all *Cuscuta* spp., irrespective of host plant or localities (Loffler et al. 1997).

Perez-Amador et al. (1996) analyzed stems of *Cuscuta americana*, *C. arvensis* [*C. campestris*], *C. corymbosa* var. *stylosa* and *C. tinctoria* and found ergoline alkaloids only during the rainy season in all samples, whereas the glycoresins and the kauranoic glucosides were produced during both seasons and with a different distribution pattern for each species. Long chain esters of oleane series, 3-BETA-hydroxyolean-12 (13)-ene tridecanoate and heptadecanoate were detected from *Cuscuta reflexa* (Anis, et al. 1998) and strong antifungal activity of *C. reflexa* leaf extract against conidial germination and mycelial growth of *Fusarium pallidoroseum* was also reported (Harsh, 1998). The extract was highly effective in controlling the fungus under field conditions.

Haidar et al. (1997) found that irradiation of 4-day-old *Cuscuta campestris* and *Cuscuta indecora* seedlings for 1 minute with red light stimulated coiling and prehaustoria formation in excised upper 4-cm segments of seedlings during a 48-h dark period in which zeatin was applied. Coiling and prehaustoria development were completely reversed when irradiated with 2 minutes of far-red light, applied directly after red light. Zeatin substituted for high irradiance blue light and mechanical stimulation, caused coiling and prehaustoria formation. In another study Haidar et al. (1998) investigated the effect of phytohormones on induction of coiling and prehaustoria in de-etiolated excised dodder seedlings and observed a synergistic effect with zeatin and far-red light (700-800 nm). Application of IAA inhibited zeatin-induced coiling and prehaustoria development under blue (400-500 nm) and a mixture of red plus far-red light while ethylene showed no effect. The authors suggested that zeatin-induced coiling and prehaustoria development might be mediated by phytochrome. Phytochemical analysis of *Cuscuta reflexa* and *Cuscuta platyloba* yielded characteristic patterns of soluble phenolic constituents. *C. reflexa* contained mainly caffeic acid depsides while *C. platyloba* accumulate flavonoids of the flavonol-type. The phenolic patterns proved to be stable in both species, irrespective of different host plants employed (Loffler et al. 1995). Segmentation of growing *Cuscuta* shoots showed different concentrations of phenolic compounds. In contrast to the flavonoids

that gradually declined from the meristematic apex towards the haustoria the hydroxycinnamic acid derivations were found to be enriched in the haustorial region as well as in the stem apex. The high concentration of phenylpropanoids in the apex, as well as the accumulation of caffeic acid depsides in the haustorial regions, was correlated to an enhanced activity of PAL in the respective segments.

Paliyath et al. (1978) reported that 6-benzylaminopurine (BA) or kinetin 0.5% in linolin in ring application around the stem of *C. chinensis* and 1 cm below the apex decreased elongation and induction of coiling preceded radial expansion of the stem and the appearance of haustoria 72 h after cytokinin application. Fresh weight of *C. reflexa* was inhibited and seedlings looked thinner after gebberellic acid treatment compared with the control. Ethylene, cytokinin and high concentration of auxin (IAA) were reported to exhibit a very marked growth inhibition accompanied by swelling of parasite stem (Rajput et al. 1990).

### ***Loranthus* spp.**

WonYung (1996) reported the presence of triterpenoids in Korean native mistletoes of *Korthalsella japonica* parasitizing *Camellia japonica*, *Viscum album* var. *coloratum* on *Quercus acutissima* and *Loranthus yadoriki* on *Neolitsea sericea*. Oleanolic acid and ursolic acid derivatives were found highest in *K. japonica*. Oleanolic acid contents in leaves, and olean-12-en-3beta-ol and lup-20 (29)-en-3-one in twigs of *V. album* were prominent. In *L. yadoriko* both olean-12-en-3beta-ol, lup-20 (29)-en-3-one and urs-12-en-3beta-ol in leaves and lup-20 (29)-en-3-one in twigs were abundant. The author noted that mistletoes tested had three types of triterpenoid, oleanane, lupane, and ursane, irrespective of hosts, sampling positions and species. WonSil and WonYung (2000) thought that phenolic compounds including gallic acid account for antioxidative activity of *Loranthus yadoriki*, *Pseudixus japonicus* and *Viscum album* var. *coloratum*. *Pseudixus japonicus* had the largest number of triterpenoids and showed the greatest biological activity.

### ***Viscum* spp.**

Different allelochemicals were isolated and identified from tissues of different *Viscum* species, subspecies or varieties. The presence of triterpenoids, in *Viscum album* var. *coloratum* grown on *Quercus acutissima* has been already mentioned (WonYung, 1996). Oleanolic acid in leaves of this species was prominent. The toxins viscumin and a mistletoe lectin were previously reported in the extract of *V. album* plants (Tonevitskii et al. 1994).

Phenylpropanoid glycosides (syringin, coniferin and kalopanaxin D), were detected in leaves, stems and twigs of the subspecies of *V. album* (Deliorman et al. 614

1999). Syringin was the major compound in the ethyl acetate and n-butanol extracts, and was highest in *V. album* subsp. *album*. The highest kalopanaxin D content was also found in the n-butanol extract of the same subspecies; but not in subsp. *abietis*. Its cuticular waxes contain oleanolic acid as the main constituent, accompanied by aliphatic compounds such as alkanes, esters and primary alcohols. A number of flavonol aglycones (methyl ethers of quercetin and kaempferol) were also detected (Wollenweber et al. 2000). Bussing (2000) isolated lectins, viscotoxins, oligo-/polysaccharides, flavonoids and alkaloids from *Viscum album* tissues, and Deliorman et al. (2001) identified a new monoterpene diglucoside, 2,6-Dimethylocta-2, 7-diene-1, 6-diol 6-O-[6'-O-beta-D-apiofuranosyl]-beta-D-glucopyran oxide, in leaves and stems of *V. album* subsp. *album*. Recently two new flavonoid glycosides, 5,7-dimethoxy-flavanone-4'-O-[2''-O-(5'''-O-trans-cinnamoyl)-beta-D-apiofuranosyl]-beta-D-glucopyranoside and 2'-hydroxy-4',6'-dimethoxy-chalcone-4-O-2''-O-(5'''-O-trans-cinnamoyl)-beta-D-apiofuranosyl]-beta-D-glucopyranoside in addition to syringin, coniferin, kalopanaxin D, 2'-hydroxy-4', 6'-dimethoxy-chalcone-4-O-beta-D-glucopyranoside, 5,7-dimethoxy-flavanone-4'-O-beta-D-glucopyranoside, 5,7-dimethoxy-lavanone-4'-O-[beta-D-apiofuranosyl(1 right arrow 2)]-beta-D-glucopyranoside were also reported (Orhan et al. 2002).

Allelopathic activity of *Viscum cruciatum* was demonstrated using methanolic extract of this species which showed a mitodepressive effect on the meristematic cells of *Allium cepa* (Gomez et al. 1996). The effect increased with the concentration and time until complete inhibition of cell division was achieved. An attempt to revive the mitotic activity of the meristems was ineffective using methanolic extracts of *V. cruciatum*, but with methanolic extracts from parasitized and non-parasitized *C. monogyna* there was complete recovery. These results confirm the antagonistic interaction between this parasite and its host through production of different allelochemicals of antagonistic activities. Ahumada et al. (2001) carried out a phytochemical study of *Viscum cruciatum* on the host *Crateagus monogyna* and consisted of hexane extracts of the parasite and the host. Ursolic acid, beta-sitosterol and a triterpene fraction were found containing mainly butyrospermol (3beta-lanost 8, 24-dien, 3-ol), 24-methylene-24-dihydrolanosterol (24-methylene-5alpha-lanost-8-en-3beta-ol), cycloartenol (9beta, 19-cyclo-5alpha, 9beta-lanost-24-en-3beta-ol), beta-amyrin (olean-12-en-3beta-ol) and several aliphatic alcohols many of which are allelochemicals. Beta-Amyrin acetate was only isolated from *V. cruciatum* and was not found in *C. monogyna*.

Other *Viscum* species were also studied and found to contain allelochemicals in their tissues. Chou et al. (1999) isolated two new flavonoid glycosides, (2S)-5-hydroxy-7, 3'-dimethoxyflavanone-4'-O-beta- [apiosyl (1 right arrow 2)] glucoside and rhamnazin-4'-O-beta- [apiosyl (1 right arrow 2)] glucoside from *Viscum alniformosanae*, and Lin et al. (2002) reported glycosides from *Viscum angulatum* and viscumneoside and homoeeriodictyol from *Viscum naringenin*.

## ALLELOCHEMICAL ANALOGUE COMPOUNDS FOR PARASITIC WEED CONTROL

### Stimulants

A large number of synthetic substances were reported to stimulate *Striga* species, among these were coumarin derivatives, scopoletin, thiourea, allythiourea, sulphuric acid, sodium hypochlorite and inositol (Worsham, 1987; Worsham and Egley, 1990). Thidiazuron herbicide was also reported to activate ethylene release (Babiker et al. 1992) and thus indirectly enhance *Striga* seed germination, although it is regarded by the same author as an inhibitor of haustorial development. Gabbar et al. (1993) found that seeds of *S. asiatica* treated with cytokinin-1-aminocyclopropane-1-carboxylic acid (ACC) combinations displayed high rates of germination (63-71%) and this was positively correlated with ethylene production. Ethylene was early mentioned by different workers as a germination stimulant of *Striga asiatica* and *Striga hermonthica* seeds (Egley and Dale, 1970; Hsiao et al. 1981). Ethylene and ethephon releasing compound was effective in stimulating *S. asiatica* (Egley and Dale, 1970; Egley, 1982), while under field conditions it was highly efficient against *S. hermonthica* (Babiker and Hamdoun, 1983). Other chemicals have been found to stimulate *Striga* germination *in vitro* including several growth hormones and kinetins (Worsham, 1987).

Willem et al. (1997) were able to synthesize two diastereomers of demethylsorgolactone. The germination stimulatory activity of these compounds is comparable to that of strigol and that there existing significant differences in activity among the individual stereoisomers. A newly designed strigol analogue Nijmegen 1 was prepared starting from N-phthaloylglycine (Nefkens et al. 1997) and was found active at low concentration as a suicidal germination agent for both *Striga* and *Orobanche* (Wigchert et al. 1999). This relatively simple analogue exhibited high bioactivity in the stimulation of germination of seeds of the parasitic weeds *Striga hermonthica* and *Orobanche crenata*. Nijmegen 1 was resolved into its rac and ent enantiomers, which were found to exhibit significant differences in germination activity. Calcium hypochlorite and GA in combination or separately enhanced *O. crenata* seed germination (Pieterse, 1981). Immersion of 6- to 12-month-old seeds of *O. crenata* in 0.5-5.0 N H<sub>2</sub>SO<sub>4</sub> solution for 1-20 minutes increased germination of this parasite by 20% (Lopez Granados and Garcia Torres, 1996). Yoneyyama et al. (1998) reported that jasmonates and related compounds elicited seed germination of *O. minor* and methyl jasmonate was the most active stimulant on several *Striga* and *Orobanche* species. The synthetic germination stimulants, strigol analogues (GR compounds) were found to stimulate seed germination of different *Striga* and *Orobanche* species. Strigol has been reported for *Orobanche* stimulation (Vail et al. 1985), and its analogues GR7 and GR45 stimulated germination of *O. minor*. It was

found effective at  $10^{-10}$  g/seed and GR45 was effective at  $10^{-6}$  g/seed (Spelce and Musselman, 1981). However, the highest activity occurred at concentration of 0.1-1 ppm. GR7, GR 24 and GR 28 at 1ppm were the most active of these compounds in inducing *O. ramosa* seed germination *in vitro*.

Studies on the activity of various strigol analogues in different soils revealed that GR 7, GR 24 and GR 45 at 1ppm were most active. In pot experiment, GR7 applied at 1-3 ppm 6 weeks before transplanting tomatoes resulted in significant reduction in the dry weight and number of shoots of *O. ramosa* plants and increased tomato yield (Saghir et al. 1983). Under field conditions, GR7 at 0.3 Kg/ha resulted in good control of *O. crenata* in broad bean grown in acidic soils and application of 1.5 Kg/ha of the stimulant controlled *O. crenata* in alkaline soils (Saghir, 1994). Strigol and sorgolactone are well known as highly potent germination stimulants of *Orobanche* seeds (Willem et al. 1997). Luque et al. (2000) reported that parthenolide and 3,5-dihydroxydehydrocostus-lactone significantly increased *O. cumana* germination and exhibited higher activity than GR24. Dhanapal (1996) mentioned GR 24 at 1.0 and 0.1 ppm as the standard to assess potential germination and reduction in seed bank of *O. cernua* in tobacco. However, it has been mentioned in general that strigol analogues have stability problem in the soil (Parker and Riches, 1993) and using them under field condition may posses' different complications.

In an experiment conducted by Georgieva and Bozoukov (1995) to test different pesticides for their ability to stimulate seed germination of different *Orobanche* spp., results showed the highly stimulatory effects of the fungicides Topsin-M [thiophanate-methyl] (97%) at 50 ppm and Ambis (90%) at 100 ppm, insecticides Thiodan [endosulfan] (80%) at 100 ppm and Thiofanox (70%) at 10 ppm, biological pesticide Ditrapex [dichloropropane + methyl isothiocyanate] (90%) at 30 ppm, and herbicide Vernam 6E (97.7%) [vernolate] at 200 ppm. At higher than 1000 ppm all compounds showed inhibitory action.

Among other chemicals reported, gibberellic acid at 10 and 20 ppm was most effective. The stimulatory effects of host plants were significant even when GR24 was applied. The positive role of this hormone was further confirmed by Joel (1998) who reported that application of uniconazole as an inhibitor of gibberellin synthesis in pre-planting treatment to sunflower significantly reduced the number of infections by *O. cumana* and allowed the development of normal sunflower heads. Application of NAA and IBA induced increase in root growth of *Solanum melongena* and enabled *O. cernua* to be maintained successfully. NAA at 60 ppm gave best results (Rao et al. 1982) and recent study carried out by Harb (2002) confirmed the role that IAA plays in *Orobanche* maintenance on tomato roots. However, the activity of germination stimulants for suicidal germination of *Orobanche* seeds under field conditions could be substantially enhanced by applying brassinolide (2a, 3a, 22R, 23R-tetrahydroxy-24S-methyl-B-homo-7-oxa-5-a-cholest-6-on) and related compounds to the infested soils (Takeuchi et al. 1995).

## Inhibitors

Ancymidol, uniconazole and paclobutrazol were reported as strong inhibitors of *Orobanche ramosa* germination and CCC, daminozide and prohexadione at  $10^{-5}$  had similar effect (Zehar and Fer, 2001). The inhibitory effect of paclobutrazol and uniconazole could be resulted from the increased level of abscisic acid (ABA). Oxidative metabolism of ABA into phaseic acid and exogenous ABA is a strong inhibitor of *O. ramosa* germination (Pageau et al. 2001, cited by Zehar and Fer, 2001).

Germination of *S. asiatica* was reduced by silver thiosulphate and  $\text{CoCl}_2$ , inhibitors of ethylene action and ACC oxide (Gabbar et al. 1993). Aminoethoxyvinyl glycine reduced cytokinins thidiazuron induced parasite germination. The authors concluded that cytokinins promoted ACC conversion into ethylene and consequently parasite germination by enhancing ACC activity and/or synthesis.

## CONCLUSION AND RECOMMENDATIONS

This review study on allelopathy role in the host-parasite relationships clearly demonstrated that allelochemicals play an integral part in these relations. Many of these can stimulate parasite seed germination, attachment, and development, while in contrast many are also considered to be responsible on the defense mechanism that host plants exert against their parasites. On the other hand certain groups of these allelochemicals are true inhibitors of parasite seed germination and growth on host plants. Allelochemicals were detected in host and parasite tissues of many species and may be exchangeable between them. However, chemical interaction between parasite and host is really complicated, with one partner may stimulate production of allelochemicals by the other. Promising role of allelochemicals in controlling parasitic weed problems appear so far a fruitful mechanism in management strategies of these species. Different techniques may be utilized and followed through the use of natural chemicals in a pre-planting or post planting treatments of host species as stimulatory or inhibitory compounds of parasite seed germination, attachment, growth or development. Other chemicals may be used in a post-emergence treatment of the parasite at which they may be directly sprayed on parasite aerial parts as growth inhibitory materials or natural herbicides to control parasite growth or seeding. Plants yielded allelochemicals are the most practical tools to be used in managing parasitic weed infestation. Trap and catch plant species either cultivated or wild grown are promising solution and a good example and proof on allelopathic action under field conditions. Their extracts, residues or oils can be usefully used for the success of parasitic weed management strategy. With more facilities available to isolate and identify allelochemicals stimulants or inhibitors of parasitic weeds germination or development, a wide door is opened for large-scale industry of these natural allelochemicals or their synthetic analogue compounds for better and cheaper chemical

control methods of these parasites. The future role of allelochemicals in engineering crops resistant to one or more parasitic species is still a wait for rigorous research in this field.

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