Epidemiology of Mycotoxin Producing Fungi

Under the aegis of COST Action 835
'Agriculturally Important Toxigenic Fungi
1998 - 2003', EU Project
(QLK 1-CT-1998-01380)

Edited by
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Bleaching of wheat spikelets caused by Fusarium Head Blight disease.

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Foreword

Mycotoxins continue to be a very serious threat to human health and a major concern for those entrusted with regulating the safety of food and food products. Infection of cereals and other crops, notable grapevine, by *Fusarium*, *Aspergillus* and *Penicillium* are a world-wide problem and recent epidemics in Europe, the USA and Canada have focused attention on this problem. A previous Special Issue of the European Journal of Plant Pathology (108 (7) 2003) was concerned with the nature of these toxins and the fungi that can produce them.

The aim of this publication is to review the environmental factors that influence the success of these fungi as pathogens and as organisms affecting stored products, and to discuss how these factors can also influence the amounts of toxin that accumulate. These reviews were originally presented at a Workshop of the EU COST Action 835 entitled 'Agriculturally Important Toxigenic Fungi' (Chairperson Antonio Logrieco, Bari, Italy), held in East Malling, UK at the Horticultural Research International in September 2002. We hope the information provided will stimulate scientists world-wide to assess the risk of toxins accumulating in plants under a range of environmental conditions and that this will provide an insight into how the accumulation of these toxins can be kept to a minimum.

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Epidemiology of toxigenic fungi and their associated mycotoxins for some Mediterranean crops

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Abstract

Recent data on the epidemiology of the common mycotoxigenic species of *Fusarium*, *Alternaria*, *Aspergillus* and *Penicillium* in infected or colonized plants, and in stored or processed plant products from the Mediterranean area are reviewed. Emphasis is placed on the toxigenicity of the causal fungal species and the natural occurrence of well known mycotoxins (aflatoxins, ochratoxins, fumonisins, trichothecenes, zearalenone, patulin, *Alternaria*-toxins and moniliformin), as well as some more recently described compounds (fusaproliferin, beauvericin) whose toxigenic potential is not yet well understood. Several *Fusarium* species reported from throughout the Mediterranean area are responsible of the formation of mycotoxins in infected plants and in plant products, including: *Fusarium graminearum*, *F. culmorum*, *F. cerealis*, *F. avenaceum*, *F. sporotrichioides* and *F. poae*, which produce deoxynivalenol, nivalenol, fusarenone, zearalenone, moniliformin, and T-2 toxin derivatives in wheat and other small grains affected by head blight or scab, and in maize affected by red ear rot. Moreover, strains of *F. verticillioides*, *F. proliferatum*, and *F. subglutinans*, that form fumonisins, beauvericin, fusaproliferin, and moniliformin, are commonly associated with maize affected by ear rot. Fumonisins, were also associated with *Fusarium* crown and root rot of asparagus and Fusarium endosepsis of figs, caused primarily by *F. proliferatum*. Toxigenic *A. alternata* strains and associated tenuazonic acid and alternariols were commonly found in black mould of tomato, black rot of olive and citrus, black point of small cereals, and black mould of several vegetables. Toxigenic strains of *A. carbonarius* and ochratoxin A were often found associated with black rot of grapes, whereas toxigenic strains of *A. flavus* and/or *P. verrucosum*, forming aflatoxins and ochratoxin A, respectively, were found in moulded plant products from small cereals, peanuts, figs, pea, oilseed rape, sunflower seeds, sesame seeds, pistachios, and almonds. Finally, toxigenic strains of *P. expansum* and patulin were frequently found in apple, pear and other fresh fruits affected by blue mould rot, as well as in derived juices and jams.

Introduction

The Mediterranean basin is a large geographical region with a temperate climate and a diversified agricultural system that includes cereals (maize, rice, wheat and other small grains), legumes (beans, peas, peanuts), several kind of vegetables (cucurbits, solanaceous, brassicas), citrus fruits (oranges, mandarin, lemons, grapefruits and limes), pome fruits (apples and pears), stone fruits (peaches, nectarines, apricots, plums and cherries), nuts (almond, hazelnuts, pistachios, walnuts) olive, grapes, soft fruits (figs, strawberries, raspberries), tubers and roots from temperate and sub-tropical plants. On these host plants, pathogenic fungi may induce plant disease and both pathogens and saprophytes can synthesise toxic secondary metabolites, which may lead to the accumulation of mycotoxins in the colonised tissues. Mycotoxin formation may begin in preharvest infected plants standing in the field and be continued or initiated in postharvest and stored products. Mycotoxins in plant products and in processed food and feed have a significant economic impact and pose a serious problem for animal and human health (IARC, 1993). Mycotoxigenic fungi

belong mainly to *Fusarium*, *Alternaria*, *Aspergillus* and *Penicillium* genera. The toxigenic *Fusarium* and *Alternaria* species are often classified as 'field fungi', because they require very high moisture content in the substrate for growth and mycotoxin synthesis (>20%). The 'storage fungi', primarily species of *Aspergillus* and *Penicillium* also grow well at lower moisture contents. Thus, *Fusarium* and *Alternaria* usually represent a high mycotoxicological risk in preharvested or freshly harvested plant that are drying, whereas toxigenic species of *Aspergillus* and *Penicillium* represent a higher risk for products in storage or being used in food and feed processing. The mycotoxicological aspects review here are related primarily to field and storage fungal diseases of important Mediterranean crops and to the mycotoxins synthesised by strains of *Fusarium* (fumonisins, trichothecenes, zearalenones, moniliformin, beauvericin and fusaproliferin), *Alternaria* (alternaric acid, alternariols, altertoxins), *Aspergillus* (aflatoxins, ochratoxins) and *Penicillium* (ochratoxins, citrinin, patulin).

FUSARIUM DISEASES

Fusarium species are distributed worldwide as important plant pathogens, as well as opportunistic colonisers of plants and agricultural commodities, or as saprophytes on debris and cellulosic plant materials. Several species cause a range of plant diseases, such as vascular wilt, root and stem rot, seedling blight, cereal ear rot, and fruit rot. *Fusarium* species are also the major cause of storage rot of fruits and vegetables and are frequently associated with cereal and legume grains, which they usually colonize before harvest. Some *Fusarium* strains can synthesise several mycotoxins, which may accumulate in infected plants before harvest or in stored or processed agricultural commodities. The occurrence of *Fusarium* mycotoxins, particularly in cereal grain is of great concern in all cereal-growing areas, and their occurrence in processed feeds and foods is often associated with mycotoxicoses in humans or domesticated animals. The most common *Fusarium* mycotoxins are trichothecenes, zearalenones, and fumonisins. In addition, moniliformin, beauvericin, and fusaproliferin may occasionally present problems.

Trichothecenes. Based on the functional group at the position in the molecule C-8, the *Fusarium*-trichothecenes may be divided into two types, A and B. Type-A trichothecenes have a functional group at C-8, other than a keto, and include: T-2 toxin (T2) and its derivatives (HT-2 toxin, T-2 triol, T-2 tetraol), produced by strains of *F. sporotrichioides*, *F. acuminatum*, and *F. poae*; diacetoxyscirpenol (DAS) and monoacetoxyscirpenol (MAS), produced by strains of *F. poae*, *F. equiseti* (syn. *F. scirpi*) (*Gibberella intricans*), *F. sambucinum* and *F. sporotrichioides*; and neosolaniol (NEO), produced by strains of *F. sporotrichioides*, *F. poae* and *F. acuminatum.* The type B-trichothecenes are characterised by the presence of a carbonyl group at C-8 and include: deoxynivalenol or vomitoxin (DON) and their mono-(3-AcDON, 15-AcDON) and di-acetylated derivatives (3,15-AcDON), produced by strains of *F. graminearum* (*Gibberella zeae*), and *F. culmorum*; nivalenol (NIV) and its monoacetylated derivative fusarenone X (FUS) and the di-acetylated derivative (4,15-AcNIV), produced by strains of *F. cerealis* (syn. *F. crookwellense*), *F. poae*, *F. graminearum* and *F. culmorum*. Trichothecenes can cause a variety of toxic effects in laboratory and farm animals including skin inflammation, digestive disorders, haemorrhagic syndrome in internal organs, blood disorders, haemolytic imbalance and depletion of the bone marrow, immunosuppression (leukopenia) and disturbance of the nervous system (IARC, 1993). Trichothecenes are responsible for several mycotoxicoses in livestock, including haemorrhagic syndrome caused by A type trichothecenes (T2, DAS and MAS), and emetic and feed refusal syndromes associated with the occurrence of B type trichothecenes (DON, NIV and FUS). T2 and DON also have been implicated in human toxicoses (ATA). However, they have not been proved to be genotoxic and IARC (1993) included the trichothecenes formed by *F. graminearum*, *F. culmorum*, *F. cerealis* and *F. sporotrichioides* amongst the compounds not yet classified for their carcinogenicity in animals or humans.

Zearalenones. Zearalenone (ZEA), produced by *F. graminearum*, *F. culmorum*, *F. cerealis*, *F. equiseti* and *F. semitectum* (syn. *F. pallidoroseum*, *F. incarnatum*), is found associated with zearalenols (ZOH) (α- and β-zearalenol isomers). ZEA is among the most widely distributed *Fusarium* mycotoxins in agricultural commodities and is often found at relatively high concentrations, especially in maize. ZEA is both uterotrophic and estrogenic, and may cause reproductive disorders in farm animals, particularly swine. ZEA is responsible for recurring toxicoses in livestock,

characterised by hyperestrogenism in swine, infertility and poor performance in cattle and poultry, and there is a possible impact on human health. The preliminary scanty evidence of the genotoxicity of ZEA, is limited to mice and cultured mammalian and human cells, and it is not classified as human carcinogen (IARC, 1993).

Fumonisins. Fumonisins were first isolated from *F. verticillioides* (syn. *F. moniliforme*) and then also found in cultures of *F. proliferatum* and a few other *Fusarium* species with unclear ecological distributions. Amongst the characterised compounds, fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) present the greatest mycotoxicological concern. Feeds contaminated by FB_1 cause leukoencephalomalacia in horses, pulmonary oedema in swine, poor performance in poultry, and altered hepatic and immune function in cattle. Moreover, home-grown maize contaminated by FB_1 has been associated with oesophageal cancer of humans in Africa, China, and the United States. The structural similarity with sphingosines suggests a role for fumonisins as depletion agents of the complex sphingolipids from biological membranes. This that could account for their toxicity and, perhaps, their carcinogenicity. However, the evidence that cultures of *F. verticillioides* and samples of FB_1 can promote liver cancer in rats, led to the classification of fumonisins as carcinogenic to animals and possibly to humans (Group 2B) (IARC, 1993).

Moniliformin. Moniliformin (MON) has been purified from cultures of several *Fusarium* species, including: *F. proliferatum*, *F. subglutinans*, *F. avenaceum* (*G. avenacea*), and *F. tricinctum*. Diets containing material naturally contaminated with MON can cause reduced performance, haematologic disorders, and mortality in rodents, chicks, ducklings, and pigs. Its significance as a contaminant of maize and other cereal grains has not been clarified. At present, MON is regarded as cytotoxic but not genotoxic, and has yet to be associated with a natural disease outbreak in domesticated animals. MON is suspected as the cause of Keshan disease, a human heart problem that occurs in rural regions of China and South Africa (Transkei) in which maize constitutes a large portion of the diet.

Beauvericin and enniatins. Beauvericin (BEA) is a cyclic hexadepsipeptide first reported to be produced by some entomopathogenic fungi and then found in cultures of *F. semitectum*, *F. subglutinans*, and *F. proliferatum* that were isolated from maize and maize-based feed. Some of these samples were associated with animal toxicoses. In addition to its high toxicity to insects, BEA is cytotoxic to mammalian cell tissues, can cause apoptosis in both murine and human cell lines and reduce the contractility of guinea pigs smooth muscle. However, the biological activity of BEA, as found more in generally for the enniatins, seems mediated by the ability of ionophoric compounds to affect the ion transport across membranes, leading to disruption of the ionic balance of cell wall. Such activity by BEA could increase the toxicity of other *Fusarium* mycotoxins that co-occur with BEA in contaminated cereals.

Fusaproliferin. Fusaproliferin (FUP) is a novel sesterterpene produced by strains of *F. proliferatum* and *F. subglutinans* isolated from maize ear rot and is often found in naturally infected maize. FUP is lethal to *Artemia salina* larvae and cytotoxic to insect and human cell lines. FUP also caused high mortality in broiler chicks fed with maize cultures of *F. proliferatum* and had severe teratogenic effects in chick embryo bioassays.

Head blight of wheat and other small cereals

Causal agents and symptoms. The species predominantly found associated with head blight of wheat and other small-grain cereals in the Mediterranean region are *F. graminearum* and its widespread teleomorph *G. zeae*, *F. culmorum*, and *F. avenaceum*. Less frequently isolated species are *F. poae*, *F. cerealis*, *F. equiseti*, *F. sporotrichioides*, and *F. tricinctum.* Other species encountered sporadically include *F. acuminatum*, *F. subglutinans*, *F. solani*, *F. oxysporum*, *F. semitectum*, *F. verticillioides*, and *F. proliferatum.*

Fusarium pathogens on wheat, barley and other small-grain cereals, are responsible for two forms of disease, a 'foot rot', which affects roots and crowns, and may cause seedling blight at early stages; and a 'head blight' (FHB), which affects individual kernels, single ear spikelets or entire heads, and results in 'scab' of the kernels. Infected spikelets first appear water soaked, then lose their chlorophyll and become straw coloured. In warm, humid weather, pinkish-red mycelium, and conidia develop abundantly in infected spikelets and

the infection can spread to adjacent spikelets or through the entire head. Infected kernels become shrivelled and discoloured with a white, pink, or light-brown scaly appearance as a result of mycelial outgrowths from the pericarp. From the mycotoxicological point of view, FHB is of greatest concern, because of the potential accumulation of mycotoxins in scabby grains intended for foods and feeds. However, the risk associated with the consumption of contaminated forage and straw by domesticated animals should not be underestimated. FHB causes severe damage to wheat and the other cereals, especially in areas with warm temperatures and high relative humidity or frequent precipitation during the heading and blossoming periods. In addition to favourable environmental conditions, other factors involved in determining FHB severity, include agronomic factors (e.g. farming systems, soil management and tillage, crop rotation and preceding crop, and fungicide applied) and host genotype, e.g. varietal disease escape and varietal genetic resistance to the pathogen or the toxins it produces. The etiological characteristic of FHB is the co-occurrence, or quick succession, of several *Fusarium* species usually referred to as a 'complex'. It is not uncommon to isolate up to nine different *Fusarium* species from a single fragment of infected tissue, or to recover as many as seventeen different *Fusarium* species from freshly harvested grain samples collected in a limited geographic area. However, only a few species are regarded as pathogenic and generally only a limited number dominate in any particular host-agroclimatic system. Strains of the less-pathogenic or opportunistic *Fusarium* species can also produce considerable amounts of mycotoxins. Thus, the toxigenic profile of a contaminated crop is affected not only by the predominant pathogenic *Fusarium* species, but also by the opportunistic species making up the 'complex'. The *Fusarium* species (Table 1) most frequently isolated from 105 samples of cereals grain (wheat, barley, rice, oat, rye, and maize) collected from Mediterranean countries (Egypt, France, Greece, Lebanon, Turkey, and Yugoslavia) were *F. culmorum* and *F. graminearum* from wheat, *F. verticillioides* and *F. proliferatum* from maize, and *F. semitectum* from rice (Logrieco et al., 1990c).

Table 1. Toxigenic *Fusarium* species associated with cereals and their mycotoxins

Fusarium species[a]	Mycotoxins[b]
F. acuminatum	**T2**, **MON**, HT2, DAS, MAS, NEO, BEA
F. anthophilum	BEA
F. avenaceum	**MON, EN, BEA**
F. cerealis (F. crookwellense)	**NIV**, **FUS**, **ZEA**, ZOH
F. chlamydosporum	**MON**
F. culmorum	**DON**, **ZEA**, **NIV**, FUS, ZOH, AcDON
F. equiseti	**ZEA**, **ZOH**, MAS, DAS, NIV, DAcNIV, FUS, FUC, BEA
F. graminearum	**DON**, **ZEA**, **NIV**, **FUS**, **AcDON**, DAcDON, DAcNIV
F. heterosporum	**ZEA**, ZOH.
F. nygamai	**FB_1**, **BEA**, FB_2
F. oxysporum	**MON**, EN, BEA
F. poae	**DAS**, **NIV**, **FUS**, MAS, T2, HT2, NEO, BEA
F. proliferatum	**FB_1**, **BEA**, **MON**, **FUP**, FB_2
F. sambucinum	**DAS**, T2, NEO, MAS, BEA
F. semitectum	**BEA**
F. sporotrichioides	**T2**, **HT2**, **NEO**, MAS, DAS
F. subglutinans	**BEA**, **MON**, FUP
F. tricinctum	**MON**, BEA
F. verticillioides (F. moniliforme)	**FB_1**, FB_2, FB_3

[a] AcDON = mono-acetyldeoxynivalenols (3-AcDON, 15-AcDON); AcNIV = mono-acetylnivalenol (15-AcNIV); BEA = beauvericin; DiAcDON = di-acetyldeoxynivalenol (3,15-AcDON); DAcNIV = diacetylnivalenol (4,15-AcNIV); DAS = diacetoxyscirpenol; DON = deoxynivalenol (Vomitoxin); EN = enniatins; FB_1 = fumonisin B_1; FB_2 = fumonisin B_2; FB_3 = fumonisin B_3; FUP = fusaproliferin; FUS = fusarenone-X (=4-Acetyl-NIV); FUC = fusarochromanone; HT2 = HT-2 toxin; MAS = monoacetoxyscirpenol; MON = moniliformin; NEO = neosolaniol; NIV = nivalenol; T2 = T-2 toxin; ZEA = zearalenone; ZOH = zearalenols (α and β isomers).

[b] Bold letters indicate the main mycotoxin produced.

The incidence FHB on wheat in Italy is exemplified by the data from the 2000 crop in which the incidence of the disease in the northern region averaged 4% on both soft and hard wheat, 35% and 25% on soft and hard wheat in central region and was completely absent in the southern region. The higher incidence of FHB in the central region may be attributable to warmer maritime weather (Pasquini et al., 2001). The dominant FHB species on both soft and hard wheat in Italy are *F. graminearum*, *F. culmorum*, and *F. avenaceum*. *F. graminearum* usually dominates when weather conditions near flowering are wetter and warmer than normal, while drier weather appears to be more conducive to *F. culmorum.* Similar results also have been reported from Spain and France, where the dominant species from wheat were *F. culmorum* and *F. graminearum* followed by *F. poae* (Assemat et al., 1995). Investigations on the toxigenic mycoflora associated with freshly harvested wheat grains collected from different localities in Egypt, the most common *Fusarium* species on small grains are *F. semitectum* and *F. acuminatum*, which are associated with the occurrence of ZEA, DON, NIV, and T2 (El-Kady and El-Maraghy, 1990; Atalla et al., 1999). Severe *Fusarium* head blight also occurs on barley, with significant reductions in yield and quality in several Mediterranean countries including Italy where the dominant species is *F. graminearum* (Delogu et al., 2001), and in Egypt, where the dominant species is *F. acuminatum* (Abdel-Kader et al., 1979).

Toxin formation and natural occurrence. Strains of *Fusarium* species commonly isolated from cereals in the Mediterranean area synthesise different mycotoxins, some at very high concentrations (Table 2). For example, Bakan et al. (2001b) analysed 60 strains of *F. culmorum* isolated from wheat grain collected from different wheat-growing areas in France. They found that 60/60 strains produced ZEA (up to 1700 mg kg^{-1}); 24/60 produced DON (up to 51.9 mg kg^{-1}); 35/60 produced NIV (up to 12 mg kg^{-1}); 12/60 produced FUS (up to 8.4 mg kg^{-1}); 5/60 produced 15-AcDON (up to 28 mg kg^{-1}); and 13/60 produced 3-AcDON (up to 21 mg kg^{-1}).

The mycotoxins most frequently encountered in field surveys of FHB of wheat across of the Mediterranean countries are DON and its derivatives, produced by

Table 2. Profile of mycotoxin production by strains of *Fusarium* from cereal grains in Mediterranean countries[a]

Fusarium species	Strain (ITEM)	Origin	Mycotoxins (mg kg^{-1})					
			ZEA	ZOH	DON	15-AcDON	3-AcDON	MON
F. culmorum	349	Italy	30	ND	20	ND	100	ND
	328	Italy	100	20	5	ND	140	ND
	345	France	270	ND	50	ND	31	ND
	682	Portugal	20	0.2	15	ND	9.5	ND
	627	Yugoslavia	40	0.8	1.9	ND	5.3	ND
	628	Yugoslavia	350	1.4	12	ND	37	ND
F. graminearum	623	Portugal	6	ND	7.7	21.6	ND	ND
	625	Yugoslavia	50	ND	38	90	ND	ND
	626	Yugoslavia	5	ND	14	32	ND	ND
	655	Yugoslavia	22	ND	14	38	ND	ND
	656	Yugoslavia	60	ND	22	71	ND	ND
	657	Yugoslavia	40	ND	21	51	ND	ND
F. cerealis[b]	619	Yugoslavia	60	4.1	ND	ND	ND	ND
F. equiseti	365	Italy	7.4	1.6	ND	ND	ND	ND
	367	Italy	ND	ND	ND	ND	ND	ND
F. avenaceum	314	Italy	ND	ND	ND	ND	ND	2000
	321	Italy	ND	ND	ND	ND	ND	67
	683	Portugal	ND	ND	ND	ND	ND	1200
	620	Yugoslavia	ND	ND	ND	ND	ND	1330
	621	Yugoslavia	ND	ND	ND	ND	ND	40

[a]Strains were grown on autoclaved maize kernels at 25 °C for 4 weeks. ND = not detected.
[b]This strain produced also fusarenon-X (29.2 mg kg^{-1}) and nivalenol (<1 mg kg^{-1}).

F. graminearum and *F. culmorum*. Freshly harvested grain in Italy contained up to 1000 ng g^{-1} of DON in samples of durum and up to 330 ng g^{-1} of DON in soft wheat (Pascale et al., 2001). Similar data were obtained from French wheat, where DON was present at levels up to 650 ng g^{-1} in 90% of freshly harvested grain samples (Bakan et al., 2001a).

NIV and FUS, which usually co-occur with DON, are also common in ears of small-grain cereals affected by FHB in Mediterranean localities and are usually attributed to the activity of *F. graminearum* and *F. culmorum* NIV-chemotypes, and to *F. poae* and *F. cerealis*. In particular, NIV, at levels up to 232 ng g^{-1}, was reported in samples of freshly harvested French wheat (Bakan et al., 2001a).

ZEA, which is produced mainly by *F. graminearum* and *F. culmorum* and commonly co-occurs with DON and its derivatives, is among one of the most frequently encountered mycotoxins in grain from FHB-diseased small-grain cereals throughout the Mediterranean countries. In particular, ZEA (up to 16 ng g^{-1}) was found, together with DON, in surveys of freshly harvested French wheat (Bakan et al., 2001a).

Several strains of *F. avenaceum* isolated from infected samples produced MON, BEA, and other hexadepsipeptides (enniatins B, B_1 and A_1) *in vitro* at high levels (up to 3703 mg kg^{-1}). Recently, these toxins also were found as natural contaminants in wheat kernels that were dominated by *F. avenaceum* (Logrieco et al., 2002).

Ear rot of maize

Fusarium species are responsible for at least two types of maize ear rot, roughly a 'red ear rot' or 'red fusariosis', caused mainly by species of the *Discolor* section, and 'pink-ear rot' or 'pink fusariosis' caused mainly by representatives of the *Liseola* section (Bottalico, 1998). The dominant species causing maize 'red ear rot' are *F. graminearum*, *F. culmorum*, *F. cerealis*, and *F. avenaceum*. The species frequently isolated from maize 'pink-ear rot' are essentially the widespread anamorphs of *F. verticillioides*, *F. proliferatum*, and *F. subglutinans*. Other toxigenic *Fusarium* species less frequently isolated from both types of maize ear rot include *F. equiseti*, *F. poae*, *F. sporotrichioides*, *F. acuminatum*, *F. semitectum*, *F. solani*, and *F. oxysporum*. Finally, there are some species isolated sporadically only from maize, although they may occasionally be reported as a regional problem, such as *F. anthophilum*, *F. chlamydosporum* (syn. *F. fusarioides*), *F. compactum*, *F. heterosporum* (syn.: *F. reticulatum*, *F. graminum*), *F. lateritium*, *F. sambucinum*, *F. torulosum*, and *F. venenatum*.

Fusarium *red ear rot of maize*

Causal agents and symptoms. The dominant species causing maize 'red ear rot' are *F. graminearum*, *F. culmorum*, *F. cerealis*, *F. avenaceum*, and *F. subglutinans*, which represent 90–95% of all isolated *Fusarium* strains from plants with this disease. Others species isolated include: *F. sporotrichioides*, *F. poae*, *F. equiseti*, and *F. acuminatum*, and to a lesser extent *F. verticillioides* and *F. proliferatum.* *F. graminearum* is becoming more widely distributed in continental parts of Europe (e.g. northern Italy, France, and Yugoslavia) and is often associated with many other *Fusarium* species. In maize 'red ear rot', symptoms usually appear at the tip of the ear, with a reddish mould eventually covering the ear extensively. The blue–black perithecia of the teleomorph, *G. zeae*, are commonly observed on infected husks and ear shanks. The severity of the disease and the occurrence and prevalence of the causal species varies from region to region and year to year, depending on the climatic parameters (temperature, relative humidity, rain, and location), and agronomic practices (farming systems, tillage, crop rotation, fertilization, planting area, irrigation, and measures taken for disease and pest control). In this context, the host genotype (e.g. constituents, maturity class, *Fusarium*-susceptibility) can be very important. The *Fusarium* profile, and consequently mycotoxin accumulation, can change drastically if there is insect damage. In particular, on maize ears damaged by the European corn-borer, which is an important vector of *Fusarium* species of the *Liseola* section, e.g. *F. verticillioides* and *F. subglutinans* are isolated more frequently than those of the *Discolor* Section, e.g. *F. graminearum*, *F. culmorum*, and *F. cerealis* (Lew et al., 1991). Control of the European corn borer can thus reduce of the levels of FB_1 and MON in diseased ears, but has little effect on the occurrence of ZEA, DON or NIV, mycotoxins which are produced by *Fusarium* species in the *Discolor* section.

Toxin formation and natural occurrence. In a study of ZEA production by 70 *Fusarium* strains from grain and vegetative parts of cereals, collected from several hosts all over Italy, all the 15 assayed strains

of *F. graminearum* and 15/16 strains of *F. culmorum* produced ZEA (up to 1500 and 6300 mg kg^{-1}), whereas ZEA was not produced by representatives of *F. avenaceum* (19 strains), *F. equiseti* (2), *F. sporotrichioides* (3), *F. tricinctum* (5), *F. solani* (1), *F. oxysporum* (1), *Microdochium nivale* (5) or an unidentified *Fusarium* spp. (3) (Bottalico and Logrieco, 2001). T2 was produced by the *F. sporotrichioides* strains in this study (Bottalico and Logrieco, 2001). Additional data on the potential toxigenic capability of *Fusarium* strains from cereals collected from some Mediterranean localities are illustrated in Table 2 (Logrieco et al., 1990c). The occurrence of toxigenic *Fusarium* strains, including *F. semitectum* and *F. acuminatum*, capable of producing ZEA and DAS was reported in freshly harvested maize kernels from several locations in Egypt (Abdel-Mallek et al., 1994).

Epidemics of maize red ear fusariosis, induced by *F. graminearum* and *F. culmorum*, usually result in contamination of the maize ears by DON, NIV, ZEA, and rarely T2 derivatives. The levels of toxin present are not always proportional to diseases severity. High concentrations of DON (up to 2.8 mg kg^{-1}), NIV (up to 1.3 mg kg^{-1}) and ZEA (up to 1.75 mg kg^{-1}) were found in freshly harvested infected maize ear samples in France (Bakan et al., 2001a). In addition, 107/156 samples of dried maize collected at 12 sites in France were contaminated by ZEA (up to 1.45 mg kg^{-1}) (Bakan, 2001). Occurrence of severe maize red-ear rot, caused primarily by *F. graminearum* and associated DON contamination (up to 67 mg kg^{-1}), has also been reported in Italy in years with cool rainy autumns, and especially on late maturing hybrids (FAO maturity class 500–700) (Bottalico and Logrieco, 2001). Furthermore, ZEA often is associated with DON. ZEA was found in Italy (up to 42 mg kg^{-1}) (Bottalico and Logrieco, 2001), Yugoslavia (up to 10 mg kg^{-1}) (Bočarov-Stančić et al., 1997), and in Egyptian commercial cereals (maize, rice, and wheat), although at relatively low incidence (24 out of 135 samples) and concentrations (5–50 ng g^{-1}) (Abd Alla, 1997).

NIV and FUS have often been reported from Mediterranean maize-growing areas associated with red-ear rot. The formation of these compounds may be due to the NIV chemotype of *F. graminearum*, when all three toxins occur together. NIV and FUS can be found with ZEA when the plant is infected with *F. cerealis*. In particular FUS (up to 0.9 mg kg^{-1}) and NIV (up to 0.7 mg kg^{-1}) were associated with ZEA (1300 mg kg^{-1}) in samples of *Fusarium*-infected maize ears collected in Italy and Austria (Bottalico ad Logrieco, 2001).

In infected maize ears, the main *Fusarium* species capable of producing MON are *F. subglutinans*, *F. proliferatum*, and *F. avenaceum*. While *F. subglutinans* is more widespread in northern Mediterranean localities, *F. proliferatum* is increasingly reported from southern locations. In infected maize ears, *F. subglutinans* appears to be a better producer of MON (up to 400 mg kg^{-1}) than *F. avenaceum* (up to 38 mg kg^{-1}), whereas the toxigenicity of *F. proliferatum* (up to 200) is similar to that of *F. subglutinans* (Logrieco et al., 1995). MON is potentially one of the more common mycotoxins in maize ear rot in Mediterranean basin, because it can be produced in both southern and northern areas. Its production is not limited to the predominant ear-rot agents *F. proliferatum*, *F. subglutinans*, and *F. avenaceum*, but is also produced by other less widespread or opportunistic species, including *F. acuminatum*, *F. tricinctum*, *F. chlamydosporum*, and *F. oxysporum* (Schütt et al., 1998).

Fusarium *pink-ear rot of maize*

Causal agents and symptoms. The species frequently isolated from maize 'pink-ear rot' is usually *G. fujikuroi* and its anamorphs *F. verticillioides*, *F. proliferatum*, and *F. subglutinans*. In maize 'pink-ear rot', the kernels may be infected through the silk but the pathogen may also be introduced into the ear from the tip by other types of external infections or be present as a systemic endophyte. Thus, ear colonisation is a complex process. In association with a more generalised colonisation originating from airborne inoculum colonising the ears from the tip, a 'random kernel rot' phase of the disease also may occur, in which randomly scattered kernels, either individuals or in groups, usually tan to brown, develop pink mycelium under wet conditions. In such cases, the mycotoxin (fumonisins) accumulation in infected kernels may begin early in the maize ear and continue as the kernels reach physiological maturity (Warfield and Gilchrist, 1999).

Maize 'pink-ear rot' is commonly observed in several Mediterranean areas, including Italy (Bottalico et al., 1995), France (Le Bars and Le Bars, 1995), Spain (Rapior et al., 1993), and Egypt (Fadl-Allah, 1997). The dominant species, *F. verticillioides*, is usually associated with *F. proliferatum* and, to a lesser extent, with

F. subglutinans. In particular, *F. proliferatum* usually occurs together with *F. verticillioides* in more southern areas, e.g. Italy (Logrieco et al., 1995), France (Melcion et al., 1998), and Egypt (Fadl-Allah et al., 1997), but is displaced by *F. subglutinans* in northern areas, e.g. Croatia (Jurjević et al., 1997). Logrieco et al. (1995) examined 42 samples of preharvest maize ear rot collected in 1992–1993 from different maize fields throughout Italy and found the following incidence of *Fusarium* species. *F. proliferatum* (34%) and *F. moniliforme* (54%) were the dominant species, followed by *F. equiseti* (8%) and *F. graminearum* (2%), and to a much lesser extent by *F. chlamydosporum*, *F. culmorum*, *F. oxysporum*, *F. semitectum*, *F. solani*, *F. sporotrichioides*, and *F. subglutinans*. This was the first report of a high incidence of toxigenic *F. proliferatum* strains as causal agents of maize ear rot, and strongly suggests a more significant role for *F. proliferatum* in maize ear rot and in associated fusariotoxicoses, than has previously been thought, especially since *F. proliferatum* is relatively easy to confuse with other closely related species in the *Liseola* section.

Toxin formation and natural occurrence. *Gibberella fujikuroi* has been divided into eight distinct mating populations (biological species), designated as A–H, including several *Fusarium* anamorphs that differ in their host preference and toxigenic capability. Of these, the three most commonly recovered from maize are *F. verticillioides* (mating population A), *F. proliferatum* (MP D), and *F. subglutinans* (MP E). Most strains of *F. verticillioides* from Italy produced FB_1 (up to 3800 mg kg^{-1}) and traces of BEA. Strains of *F. proliferatum* produced FB_1 (up to 2500 mg kg^{-1}), MON (5300 mg kg^{-1}), BEA (200 mg kg^{-1}) and FUP (300 mg kg^{-1}). Strains of *F. subglutinans* produced high amounts of MON (3300 mg kg^{-1}), BEA (180 mg kg^{-1}) and FUP (1500 mg kg^{-1}) (Moretti et al., 1997; Bottalico and Logrieco, 2001).

A survey of fumonisin production by 28 strains of *F. verticillioides* and 1 strain of *F. proliferatum* from Italy, Spain, and France showed that all of the assayed strains produced FB_1 (up to 4100 mg kg^{-1}) and FB_2, with the highest average production by strains from maize (average 1300 mg kg^{-1}), followed by those from wheat (average 770 mg kg^{-1}), barley (average 320 mg kg^{-1}), and sorghum (10–15 mg kg^{-1}); whereas only traces of fumonisins were produced by 11 strains of *F. subglutinans* (Visconti and Doko, 1994). *Fusarium* species from Spanish maize-based animal feeds (Sanchis et al., 2001) were dominated by *F. verticillioides*, which produced FB_1 (up to 4200 mg kg^{-1}) and FB_2 (up to 1100 mg kg^{-1}), followed by *F. proliferatum* which also produced FB_1 (up to 3900 mg kg^{-1}) and FB_2 (up to 1700 mg kg^{-1}); whereas none of the very few strains of *F. subglutinans*, *F. graminearum*, *F. avenaceum*, and *F. semitectum* produced any fumonisins. Mycotoxin production by 26 strains from Italian preharvest maize ear rot samples was as follows: all the 26 assayed strains (100%) produced FB_1 (up to 2250 mg kg^{-1}); 22 strains (85%) also produced BEA (up to 200 mg kg^{-1}); and 12 strains (46%) produced together FB_1 and BEA and also MON (up to 5300 mg kg^{-1}) (Logrieco et al., 1995).

High levels of FB_1 are frequently reported in freshly harvested maize ears in Spain (Rapior et al., 1993), Italy (up to 250 mg kg^{-1}) (Pietri et al., 1995; Visconti et al., 1996), France (up to 3.3 mg kg^{-1}) (Bakan et al., 2001a), the Adriatic Zadarska region of Croatia (up to 5753 mg kg^{-1}) (Jurjević et al., 1999) and Egypt (up to 780 ng g^{-1} in maize and maize-based products) (El-Sayed et al., 2001). In contrast, fumonisin levels are often lower in the central and northeastern Mediterranean areas (Bottalico, 1998). In the central Mediterranean areas, due to the co-occurrence of both the principal fungal producers of FB_1, i.e. *F. verticillioides* and *F. proliferatum*, the probability of finding fumonisin toxins in maize is higher than that in northern Mediterranean regions. Thus, Marín et al. (1995) found *F. proliferatum* strains that could produce fumonisins under grain conditions drier than normally suitable for *F. verticillioides*. These results suggest that the species recovered might vary with the weather conditions and appear in succession in the ripening maize ears. Given the widespread formation of fumonisins in preharvest *Fusarium*-infected maize ears, the occurrence of fumonisins in commercial grains and in maize-based foodstuffs is to be expected. In dried maize collected at 12 locations of France in 1997, FB_1 occurred in 107/156 samples (up to 4 mg kg^{-1}) (Bakan, 2001). In Spanish maize-based foods for human consumption purchased from different retail markets during two distinct surveys (Sanchis et al., 2001), 8/50 and 17/36 samples were contaminated with up to 200 ng g^{-1} or 940 ng g^{-1} fumonisins, respectively. Furthermore, analyses of moistened or moulded maize samples involved in disease out breaks in farm animals in Spain, from 1994 to 1999, revealed the presence of fumonisins in 18/48 samples (up to 1800 ng g^{-1}), associated with little if any presence of DON and ZEA (Jiménez and Mateo, 2001). Fumonisin

B_1 was also found (up to 86 ng mg^{-1}) in 14/96 sample of Spanish beer (Torres et al., 1998).

In maize pink-ear rot, associated with large amounts of FB_1 produced by *F. verticillioides* and *F. proliferatum*, the co-occurrence of MON, BEA or FUP produced by *F. proliferatum* and/or *F. subglutinans* has also been reported. MON is expected throughout the Mediterranean maize growing areas, because MON can be produced not only by the predominant ear rot agents, e.g., *F. proliferatum*, *F. subglutinans*, and *F. avenaceum*, but also by other less widespread or opportunistic species, e.g. *F. acuminatum*, *F. tricinctum*, *F. chlamydosporum*, and *F. oxysporum* (Schütt et al., 1998). In Italy, high levels of MON (up to 8200 mg kg^{-1}) were found in preharvest selected maize ears heavily infested with *F. proliferatum* in association with *F. verticillioides* (Bottalico and Logrieco, 2001).

The co-occurrence of BEA, with FB_1 and/or MON in maize ears infected by *F. proliferatum* was first reported in preharvest *F. proliferatum*-infected samples in Sardinia, and then in samples from other Italian localities (up to 520 mg kg^{-1}) (Bottalico and Logrieco, 2001). It is probably safe to assume that BEA is more widespread in Mediterranean areas than has been reported, because this toxin is also produced by several other *Fusarium* species found on maize ears, e.g. *F. subglutinans*, *F. avenaceum*, *F. acuminatum*, *F. equiseti*, *F. poae*, and *F. oxysporum.*

An important emerging toxicological problem appears to be associated with FUP. This fusariotoxin is commonly produced, often together with BEA, by strains of *F. proliferatum* and *F. subglutinans.* Since the first recovery of FUP from infected maize ears in Italy (up to 500 mg kg^{-1}), this toxin has been increasingly found in maize pink-ear rot associated with *F. proliferatum* (Bottalico and Logrieco, 2001).

Stalk rot of maize

Causal agents and symptoms. Several *Fusarium* species are commonly isolated from stalk rot of maize in Mediterranean regions, but the most common are *F. verticillioides, F. graminearum*, and *F. culmorum*. *F. verticillioides* is usually the most prominent and may spread within plants through systemic infections originating from seedborne inoculum. It also penetrates roots and stalks directly and may enter plants through wounds. The most important pathway for *F. verticillioides* to infect maize seed is through infections of the silks (Munkwold et al., 1997). *F. verticillioides* is commonly isolated from maize plants even in the absence of visible symptoms. This behaviour resembles that of some true endophytes and in this symptomless state this fungus may contribute to mycotoxin accumulation in both diseased and 'healthy' tissues and kernels (Bacon and Hinton, 1996). However, when plants are stressed by biotic or abiotic factors, they can be massively colonised by endophytic strains of *F. verticillioides* and develop typical stalk rot symptoms. *F. verticillioides* and *F. graminearum* are usually isolated from visibly infected plants. *F. culmorum* is common in cooler areas (Bottalico et al., 1989). Many other species can be isolated from maize, including *F. equiseti*, *F. semitectum*, *F. oxysporum*, and *F. acuminatum* in southern areas and *F. subglutinans*, *F. avenaceum*, *F cerealis*, and *F. poae* in central to northern areas (Bottalico, 1998). With respect to pathogenicity, maize seedling assays confirmed the aggressiveness of *F. verticillioides* and the strong pathogenicity of *F. graminearum* and *F. culmorum*, while indicating moderate to weak activity for *F. subglutinans*, *F. crookwellense*, *F. avenaceum*, *F. equiseti*, *F. oxysporum*, and *F. poae* (Pronczuk et al., 1991).

Toxin formation and natural occurrence. Samples of rotted stalk of maize collected in Italy were contaminated by ZEA (up to 7.4 mg kg^{-1}), DON (up to 0.67 mg kg^{-1}) and ZON (up to 0.086 mg kg^{-1}) which were produced by strains of *F. culmorum* and *F. graminearum* (ZEA, ZON, DON) and *F. equiseti* (ZEA and ZON) (Bottalico et al., 1985).

Fusarium *crown and root rot of Asparagus*

Symptoms and causal agents. The main symptom of *Fusarium* crown and root rot of Asparagus (*A. officinalis*) is the premature yellowing of the fronds in midsummer, consisting of chlorosis and wilting of individual stalks, progressive discoloration of plants and destruction of roots and crown. The *Fusarium* species most frequently reported as the casual agents of the disease are *F. oxysporum* f. sp. *asparagi* and *F. proliferatum*. The relative importance of these species depends on geographic location (Elmer, 1995). In Italy, diseased asparagus plants were colonized by *F. proliferatum* (72%) and *F. oxysporum* (28%) as the dominant species, with less frequently recovered species (23%), including *F. equiseti*, *F. solani*,

F. acuminatum subsp. *acuminatum*, *F. compactum*, *F. scirpi*, and *F. graminearum*. *F. proliferatum* was isolated more frequently from crowns than roots and *F. oxysporum* was isolated more frequently from roots than crowns (Logrieco et al., 1998).

Toxin formation and natural occurrence. On autoclaved maize kernels, nine of the *F. proliferatum* strains produced high levels of FB_1 (up to 2500 mg kg^{-1}), FB_2 (950 mg kg^{-1}), BEA (90 mg kg^{-1}) and FUP (300 mg kg^{-1}). In addition, FB_1 (up to 0.5 mg kg^{-1}), and FB_2 (0.1 mg kg^{-1}) were detected in samples of crowns and stems of asparagus plants, respectively (Logrieco et al., 1998). The natural occurrence of fumonisins in asparagus tissues colonized by *F. proliferatum* indicates a possible risk for human health (Nigh et al., 1999; Seefelder et al., 2002).

Fusarium *fig endosepsis*

Causal agents and symptoms. Fig endosepsis, also called pink rot or soft rot, is a serious disease of fruits of the Common Fig (*Ficus carica*) associated with several *Fusarium* species, particularly *F. verticillioides*, *F. solani* and *F. dimerum*, but also *F. proliferatum*, *F. subglutinans*, *F. lactis*, and *F. ramigenum* (Moretti et al., 2000).

Toxin formation and natural occurrence. All strains of *Fusarium* species isolated from rotted figs in Italy produced fusaric acid on autoclaved maize kernels at very low levels, with the exception of one strain of *F. subglutinans* which produced high levels of this toxin. In addition, strains of *F. subglutinans* and *F. proliferatum* produced BEA and FUP. Fumonisins were detected in cultures of all strains of *F. proliferatum*. Low levels of fumonisins were found in some samples of rotted fig fruits (Moretti et al., 2000).

Fusarium *on other crops*

Samples of dry pea (*Pisum sativum*) seeds were contaminated with several strains of *Fusarium* and contained T2 toxin (Saber et al., 1998), presumably produced by strains of *F. acuminatum*. Toxigenic *Fusarium* strains (presumably *F. semitectum* and *F. acuminatum*), which can produce ZEA and DAS, were recorded in sunflower (*Helianthus annuus)* seeds from several locations in Egypt (Abdel-Mallek et al., 1994). Also in Egypt, toxigenic strains of *F. verticillioides*, producing FB_1 (up to 73 ng ml^{-1}), were reported in the predominant fungal flora associated with decayed sugarcane (*Saccharum officinarum*) (Ziedan and Hegazy, 2002). *Fusarium* toxins have also been found at very low levels in beans, peanuts, chilli, curry, ginger, garlic, coriander, black tea, and medicinal plants. Although these food categories are usually consumed in small amounts and do not appear to represent a real risk, they could contribute to an intake of *Fusarium* toxins.

In general, exposure of plants to *Fusarium* toxins and their relative toxicological risk have not yet been well established. For example, toxigenic *Fusarium* species are important pathogens and root colonisers of various fruits (e.g. banana, mango, and pineapple) and vegetables (e.g. red clover and potato), but so far, there are no reports of the natural occurrence of *Fusarium* mycotoxins in products obtained from these plants.

ALTERNARIA DISEASES

The genus *Alternaria* is ubiquitous and abundant in the atmosphere as well as in soil, seeds, and agricultural commodities. It includes plant pathogenic and saprophytic species that may affect crops in the field or can cause harvest and postharvest decay of plant products. Of the many species belonging to *Alternaria*, the most common is *A. alternata* (*A. tenuis*), which is widespread across plants, seasons and geographic regions, and contains host-specific pathogenic strains, as well as opportunistic and saprophytic forms causing spoilage of freshly harvested crops. Species of *Alternaria* produce many secondary metabolites, mostly phytotoxins, which play an important role in the pathogenesis of plants. Some species, in particular *A. alternata*, can produce mycotoxins in infected plants and/or in agricultural commodities. The major *Alternaria* mycotoxins belong to three structural classes: the tetramic acid derivative, tenuazonic acid; the dibenzopyrone derivatives, alternariol, alternariol mono-methyl ether and altenuene; and the perylene derivatives, the altertoxins (Bottalico and Logrieco, 1998).

Tenuazonic acid. Tenuazonic acid (TA) is a well-known mycotoxin and phytotoxin, produced primarily by *A. alternata* (*A. tenuis*), but also by other phytopathogenic *Alternaria* species, including

A. capsici-annui, *A. citri*, *A. kikuchiana*, *A. japonica*, *A. longipes*, *A. porri*, *A. radicina*, *A. tenuissima*, and *A. tomato*. Tenuazonic acid is also produced by other species of fungi including, *Pyricularia oryzae* and *Phoma sorghina*. TA is toxic to a wide spectrum of viruses, bacteria, fungi and plants. Tenuazonic acid is toxic to mice, chick embryos and chickens. In particular, increasing TA in chicken feed from sublethal to lethal levels progressively reduced feed efficiency, suppressed weight gain and increased internal haemorrhaging. Sorghum grain colonised by *P. sorghina* that contained TA were associated with the human haematological disorder known as Onyalay (Bottalico and Logrieco, 1998).

Alternariols. Alternariol (AOH) and alternariol monomethyl ether (AME), are produced primarily by *A. alternata*, but also by other *Alternaria* species, including *A. brassicae*, *A. capsici-annui*, *A. citri*, *A. cucumerina*, *A. dauci*, *A. kikuchiana*, *A. tenuissima*, *A. tomato*, and *A. solani.* AME is also produced by *A. longipes* and *A. porri.* Toxic activity of the alternariols is greater towards bacteria than forwards fungi. AOH and AME are usually found in combination and have some teratogenic and fetotoxic effects in pregnant mice (Bottalico and Logrieco, 1998).

Altertoxins. Altertoxins I, II and III (ATX-I, ATX-II, and ATX-III) are produced primarily by *A. alternata*, but also by other *Alternaria* species, including *A. mali*, *A. radicina*, *A. tenuissima*, and *A. tomato*. The great interest in these toxins is related to their mutagenic activity, in particular that of ATX-III, whose mutagenicity is approximately ten times lower than that of aflatoxin B_1 (Bottalico and Logrieco, 1998).

Black mould of tomato

Causal agents and symptoms. *Alternaria* tomato fruit rot occurs on green and ripe fruit affected by physiological alterations, e.g. nutritional deficiency, skin sunburn, etc. The disease is favoured by warm rainy weather or dew formation on the fruit surface and is more severe if infection occurs when the fruit is ripe rather than green. The disease may cause substantial losses, especially of tomatoes for canning. Fruit rot lesions are circular to irregular in shape, slightly sunken and dark green to almost black from the abundant sporulation of the fungus. *Alternaria* rot sometimes develops on the fruit beneath the sepals and can colonise the internal tissues without an obvious external infection. In southern Italy, *A. alternata* is the dominant fungal species although *A. tenuissima* has also been recovered.

Toxin formation and natural occurrence. Sixteen of seventeen strains of *A. alternata* from black mould tomatoes collected in Italy, produced *in vitro* high amounts of TA (up to 4200 mg kg^{-1}), AOH (600 mg kg^{-1}), AME (100 mg kg^{-1}), ALT (30 mg kg^{-1}), and ATX-I (13 mg kg^{-1}), while three strains of *A. tenuissima* produced only very low levels of TA. In naturally infected tomato fruits collected in southern Italy, *Alternaria*-mycotoxins were present as TA (up to 7200 ng g^{-1}), mainly associated with AME (up to 270 ng g^{-1}) and AOH (up to 1300 ng g^{-1}) (Bottalico and Logrieco, 1998).

Black rot of olive

Causal agents and symptoms. Olives are often affected by *Alternaria* species, particularly if the fruits remain on the soil for a long time after ripening. Surface physical damage of the olive fruit due to unfavourable conditions, e.g. low temperature and insects, etc., is a major precondition for fungal penetration into the fruit pulp and for subsequent mycelial proliferation.

Toxin formation and natural occurrence. A study of mycotoxin production by strains of *A. alternata* isolated from olives showed that the toxigenic potential of *A. alternata* is much higher on rice than that it is on olives. In particular, the production of TA, AME and AOH on rice was 1000, 150, and 60 times higher than on olives. Moreover, ALT and ATX-I, which were produced on rice at average levels of 5.9 and 5 mg kg^{-1}, respectively, were not detected in olive cultures. The amount of TA produced on rice by these isolates of *A. alternata* is very high, up to 9800 mg kg^{-1} with an average over 6000 mg kg^{-1} (Visconti et al., 1986).

In moulded or damaged olive samples collected in Apulia (Italy), 4/13 samples were contaminated with two to four *Alternaria* mycotoxins, i.e. AOH, AME, ALT, ATX I or TA. The highest levels of contamination were found in a badly damaged sample containing 2900, 2300, 1400, and 260 ng g^{-1} of AME, AOH, ALT and TA, respectively. No mycotoxins were detected in olive oil for human consumption (six samples) or olive husks (three samples) collected from oil mills after the first pressing (Visconti et al., 1986).

Black and grey rot of citrus fruits, with reference to mandarin

Causal agents and symptoms. Mandarin fruits with *Alternaria* black rot have been observed in several locations in southern Italy at harvest time. In the first stage of the disease, the fruit have no visible symptoms, but later the surface turns dark, beginning at the peduncle. In advanced stages of the disease, the fruit usually falls to the ground. Two kinds of *Alternaria* heart rot are distinguished, based on the colour of the diseased tissues (grey and black). The causal agents produce grey and black colonies when cultured on potato-sucrose-agar under light. The black colour is associated with sporulation, whereas the grey colour is associated with felty grey mycelium with conidiophores growing on the aerial hyphae. Both strains colonise mandarin, but the black strain also colonises oranges and lemons (Logrieco et al., 1990a).

Toxin production and natural occurrence. Grey strains of *A. alternata* from mandarin fruits in southern Italy produced TA (up to 85 mg kg^{-1}), AOH (0.92 mg kg^{-1}), AME (0.17 mg kg^{-1}), and ATX-I (0.2 mg kg^{-1}). A black strain produced TA (up to 93 mg kg^{-1}), AOH (8.7 mg kg^{-1}), and AME (6.3 mg kg^{-1}), but did not produce ATX-I or ALT. Toxin formation occurred more readily on a rice substrate than in whole mandarin fruits. On autoclaved rice the grey strain of *A. alternata* produced TA (up to 6800 mg kg^{-1}), AOH (12 mg kg^{-1}), AME (7.5 mg kg^{-1}), ATX-I (83 mg kg^{-1}), and ALT (25 mg kg^{-1}), while the black strain produced TA (up to 170 mg kg^{-1}), AOH (20 mg kg^{-1}), AME (20 mg kg^{-1}), ATX-I (7.5 mg kg^{-1}), and ALT (50 mg kg^{-1}) (Logrieco et al., 1990). Oranges and lemons can also be contaminated with *Alternaria* and some toxins were detected when artificially infected with *A. citri*. Investigations carried out on the natural occurrence of mycotoxins in infected fruits showed that samples of the two kinds of mandarin heart rot contained different mycotoxin profiles. In black rot samples, TA, AME, and AOH (up to 87, 1.4, and 5.2 mg kg^{-1}) were found, whereas TA (up to 174 mg kg^{-1}) was the only detectable mycotoxin in grey rot samples. None of the natural *Alternaria* rot samples contained ALT or ATX-I (Logrieco et al., 1990a).

Black point of small-grain cereals

Causal agents and symptoms. Cereal grains are frequently infected by species of *Alternaria*, in particular *A. alternata*, which can cause a disease called 'black point', which consists of a discoloration of the germ and the seed due to mycelial and conidial masses. This

Table 3. Incidence of *Alternaria* species in cereal grains from Mediterranean countries

Cereal	Origin	Samples examined No.	Mean % of infected kernels[a]	Mean % of *Alternaria* infected grain			
				Total	*A. alternata*	*A. triticina*	Other[b]
Barley	Greece	1	100	86	53	33	0
	Lebanon	2	100	59	33	26	5
	Turkey	5	70	0	0	0	0
	Yugoslavia	2	100	17	15	2	0
Maize	France	1	95	0	0	0	0
Oat	Greece	1	100	49	45	4	0
Rice	Egypt	10	92	<1	0	<1	0
Rye	Greece	1	100	10	5	5	0
	Greece	1	70	0	0	0	0
Wheat	France	6	93	45	31	14	0
	Greece	5	93	58	42	9	0
	Italy	13	97	50	42	9	3
	Lebanon	5	72	0	0	0	0
	Turkey	10	98	48	37	11	2
	Yugoslavia	23	100	7	5	2	0

[a]Percentages are based on 50 kernels per sample.
[b]*A. tenuissima* and *Alternaria* anamorph of *Pleospora infectoria*.

disease is frequent and serious when persistent rainfall, heavy dews or irrigation occur during kernel development, although a high incidence has also been observed in relatively dry weather.

In a survey carried out on grain samples (wheat, barley, rice, oat, rye, and maize) from Egypt, France, Greece, Italy, Lebanon, Portugal, Turkey, and Yugoslavia, the highest percentage of *Alternaria* infection was found in kernels of barley (up to 86%), wheat and oats, whereas rice and maize were for the most part uninfected. Two predominant species of *Alternaria* were isolated, *A. triticina* and *A. alternata*. Occasionally, *A. tenuissima* and the *Alternaria* state of *Pleospora infectoria* were also recorded (Table 3) (Logrieco et al., 1990b).

Toxin production and natural occurrence. Investigations of mycotoxin production on autoclaved rice by strains of *A. alternata* from wheat, collected in Italy, Yugoslavia, Greece, Lebanon, Egypt, and Turkey, showed that 14/14 produced TA (up to 6000 mg kg^{-1}); 13/14 produced AOH (120 mg kg^{-1}), and AME (59 mg kg^{-1}); 13/14 produced ALT (37 mg kg^{-1}); 14/14 produced ATX-I (32 mg kg^{-1}), and 13/14 produced ATX-II (100 mg kg^{-1}) (Logrieco et al., 1990b). Additional investigations carried out with some of these isolates grown on wheat and rice showed that TA production was greater on wheat (up to 8700 mg kg^{-1}) than on rice, while the opposite was observed for the benzopyrenes and perylene derivatives (Table 4) (Bottalico and Logrieco, 1992). A high incidence of toxigenic strains was found among 176 isolates of *A. alternata* from barley collected in Spain, i.e., 88.6% produced TA, 15.3% produced AOH and 9% produced AME (Sanchis et al., 1993).

Alternaria *blackmould of other crops*

Sunflower. The sunflower head rot disease, caused by *A. alternata*, usually starts from the external basal part of the head and then, favoured by humid atmosphere, the mycelium colonises the seeds through the internal tissues. The superficial lesions become covered with dark spores. Sunflower seeds from infected heads collected in south Italy were contaminated with AOH and AME, up to 1.8 and 0.13 mg kg^{-1}, respectively (Bottalico and Logrieco, 2001).

*Oilseed rape (*Brassica napus*).* All of the 11 isolates of *A. alternata* from samples of siliquae black spot or from oilseed rape (nine from seeds and four from siliquae) collected all over Italy, when cultured on autoclaved rice produced TA (up to 12000 mg kg^{-1}), AOH (200 mg kg^{-1}), AME (200 mg kg^{-1}), ATX-I (250 mg kg^{-1}), and ATX-II (70 mg kg^{-1}) (Visconti et al., 1992). However, *Alternaria* mycotoxins, i.e. TA, AOH, AME, ANE, ATX-I, ATX-II, and ATX-III were not found in 16 samples of oilseed rape collected in oil processing plants, although the samples were contaminated with strains of *A. alternata* capable of producing high amounts of these mycotoxins on rice (Visconti et al., 1992). Strains of *A. alternata* were predominant

Table 4. Profile of mycotoxin production by strains of *A. alternata* from cereal grains in Mediterranean countries[a]

Alternaria strain	Origin	Mycotoxins (mg kg^{-1})[b]					
		TA	AME	AOH	ALT	ATX-I	ATX-II
ITEM-752	Greece	2500	19	62	3	4	6
ITEM-753	Greece	3500	52	120	37	7	5
ITEM-754	Greece	2800	7	48	10	3	2
ITEM-755	Greece	1400	5	5	1	2	3
ITEM-756	Yugoslavia	6000	21	69	14	5	ND
ITEM-757	Lebanon	3100	ND	ND	3	2	5
ITEM-758	Lebanon	4700	20	47	2	4	3
ITEM-761	Lebanon	1900	26	17	ND	26	100
ITEM-759	Egypt	2200	16	56	3	2	16
ITEM-760	Turkey	3200	13	9	2	5	13
ITEM-762	Italy	3200	32	80	4	32	3
ITEM-763	Italy	2800	59	100	7	15	11

[a]Strains grown on autoclaved rice kernels at 25 °C for 4 weeks. ND = not detected.
[b]Abbreviations: TA = tenuazonic acid; AME = alternariol monomlethyl ether; AOH = alternariol; ALT = altenuene; ATX-I = altertoxin I; ATX-II = altertoxin II.

among the mycoflora isolated from 20 oilseed rape samples collected in Catalonia (Spain), but none of the examined samples was contaminated with *Alternaria* mycotoxins (Vinas et al., 1994).

*Pepper (*Capsicum annuum*).* Black rot infection of *A. alternata* occurs on pepper fruits when they are over ripened or injured by sunlight. The colonised spots on the fruit surface reveal a dense, velvety-olive green or black spore mass. Moreover, the mycelium develops in the internal tissues invading either the pericarp or the placenta. Furthermore, the fungus can continue the colonisation during low-temperature refrigerated transport and storage. Samples of peppers with black spot, collected in southern Italy, were contaminated with low concentrations of TA (up to 54 ng g^{-1}) AME (49 ng g^{-1}), and AOH (640 ng g^{-1}) (Bottalico and Logrieco, 2001).

*Melon (*Cucumis melo*).* The black mould disease of melon is caused by *A. alternata*, but strains of *A. cucumerina* have also been found. Fruit which has suffered sun injury is especially prone to infection. Mycelium, especially in a humid atmosphere, can colonise large areas and develop in the internal tissues, while the superficial lesions become covered with dark spores. Samples of melon showing superficial black spot of *A. alternata*, collected in southern Italy, were contaminated with traces of TA (8 ng g^{-1}) and AME (5.1 ng g^{-1}) (Bottalico and Logrieco, 2001).

ASPERGILLUS DISEASES

Aspergillus is among the most ubiquitous fungal genera, mostly including saprophytic species which colonise plant debris or deteriorated agricultural commodities, but also a few strains which are able to colonise living plants. Besides their economic significance as spoilage agents of plant products, particularly grains and legumes, some species are capable of producing mycotoxins which are able to elicit tremendous effects on human and animal health. The mycotoxins of great interest produced by *Aspergillus* include aflatoxins and ochratoxins.

Aflatoxins. Aflatoxins are produced by some strains of *A. flavus*, which form essentially aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2), and by most strains of *A. parasiticus*, which also form aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2). Of these four main aflatoxins, AFB_1 and AFG_1, occur most frequently and in the largest amounts in plant products. Aflatoxin-producing strains of *A. flavus* and *A. parasiticus* are distributed worldwide in soil and air and are classifiable as storage fungi and field fungi, since some strains are phytopathogenic. These can infect plants in the field and then colonise harvested or stored plant products. Aflatoxins can thus accumulate in many important agricultural commodities. Plant products which become particularly contaminated are those from tropical and sub-tropical areas and include maize and other cereal grains, groundnuts and other legumes, cottonseed and other oilseeds, cassava and other roots and tubers, most nut crops, most fresh and dried fruits, spices and herbs, and most vegetables. Aflatoxins may also contaminate many processed foods and feeds. Aflatoxins exhibit many adverse biological effects on living organisms, such as a broad range of toxic activities, which are related to their reaction with nucleic acid and cell nucleoproteins, and the ultimate effect of these reactions on protein synthesis and cellular integrity. Aflatoxins are primarily potent hepatotoxins, causing aflatoxicoses in man and animals. They occur in farm animals, both as chronic diseases characterised by an impairment of resistance and immune responsiveness, which result in a reduction in growth rate and feed efficiency; and as acute poisoning characterised by severe clinical disease, liver tumours, and death. Because of their mutagenic, teratogenic, and carcinogenic potency, aflatoxins are classified within Group 1, as compound carcinogenic to humans (IARC, 1993).

Ochratoxins. Ochratoxins are produced by a few strains of *A. ochraceus* and allied species belonging to section *Circumdati* (particularly *A. alliaceus*), by almost all strains of *A. carbonarius* and other strains of the closely related *A. niger*, and by some *Penicillium* species, primarily *P. verrucosum*. These three groups of species show different behaviours in respect to ecological niches, the plant products affected and their geographical occurrence. *A. ochraceus* and its allied species grow at warm temperature and down to a_w 0.80, and can sporadically affect cereals, other plant products and stored food, as well as coffee and cocoa beans during sun-drying. *P. verrucosum* grows well below 30 °C and down to a_w 0.80, therefore it is usually found on cereals in cool temperate regions of northern Europe and Canada, though occasionally in Mediterranean sea localities. *A. carbonarius* grows at moderate to high

temperatures and is associated with maturing fruits, especially grapes.

Of the nine described ochratoxins, only ochratoxin A (OTA) plays a role as an environmental toxin. Field cases of ochratoxicosis in farm animals, mainly pigs and poultry, have been reported in many areas of the world. Its primary manifestation is a chronic nephropathy, characterised by kidney lesions and secondarily by carcinogenicity to the upper urinary system. However, the doses at which the renal tumours were observed in rodents were higher than those that caused nephrotoxicity. OTA has also been found in human blood samples, most notably in people living in the Balkans where a fatal human kidney disease (Balkan Endemic Nephropathy) occurs. This syndrome is usually associated with an increased incidence of tumours of the upper urinary tract. However, the clinical and epidemiological data available were deemed inadequate for calculating the carcinogenicity potency of OTA in humans and led to the inclusion of this toxins in Group 2A (IARC, 1993).

Black rot of grapes

Causal agents and symptoms. The disease is particularly severe in the warmer grape-producing southern localities of Spain, France, Italy, Greece, Egypt, and Morocco. The fungus survives on plant debris in the soil and the conidia are disseminated in vineyards by warm (25–30 °C) air currents. The fungus causes infection via injuries, essentially in mature berries, and then under warm conditions it spreads throughout the bunch. Infection and colonisation of the bunch may also occur through injuries caused by careless handling. The disease is caused by species of *Aspergillus* section *Nigri*, and in particular by *A. carbonarius*/*A. niger*. Because of its black spores, this fungus is highly resistant to sunlight, and survives sun-drying. It is the source of OTA in grapes, dried vine fruits, and wine, especially red wine. OTA is formed prior to alcoholic fermentation (Zimmerli and Dick, 1996) and it is more commonly detected in red wines (54%), than in rosé (40%) and white wines (25%), with concentrations higher in red wines. (Otteneder and Majerus, 2000). In addition, contamination of wine from northern areas is lower than that of wine from southern or warmer areas (for red wine 12% and 95%, respectively) (Otteneder and Majerus, 2000).

Of the 692 strains of *Aspergillus* isolated in Italy from black moulded grapes, 87% belonged to section *Nigri*, represented by *A. carbonarius* (26.6%) and the closely related species *A. niger* (28.8%), and by *A. japonicus* (44.6%) (Bottalico et al., 2002). Investigations carried out by Sage et al. (2002), showed that 6/11 grape samples collected from southern France were contaminated by potentially ochratoxigenic strains and all 14 strains of *A. carbonarius* tested were able to produce OTA (up to 87 mg kg^{-1}).

Toxin formation and natural occurrence. In Italy, 56 samples of red (38), rosé (8), white (9) and dessert (1) wine were contaminated with high levels of OTA ranging from <10 to 7600 ng l^{-1} with red wines more contaminated than rosé and white wines (Visconti et al., 1999). Additional data reported by Castellari et al. (2000), indicated the presence of OTA in samples of red wine from Italy (7/7, up to 500 ng l^{-1}) and Spain (1/1, 74 ng l^{-1}), and in samples of white wine from Italy (1/1, 12 ng l^{-1}) and France (1/1, 11 ng l^{-1}). In Italy, OTA was found in 14/96 red wines (up to 3200 ng l^{-1}) and in 6 out of 15 special dessert white wines (up to 3800 ng l^{-1}), with the distribution of OTA higher in wines produced in southern regions (Pietri et al., 2001).

In Spain, OTA was found in 267 samples of wines of different types and 18 samples of grape products. Contamination was evident in 92%, 91%, and 65% of red, rosé, and white wines (mean OTA concentration of 54, 31, and 20 ng l^{-1} (Burdaspal and Legarda, 2000). In addition, OTA was detected in 74 % of 47 samples of sherry wines (mean 40 ng l^{-1}), in 83% of 12 samples of sparkling wines (mean 12 ng l^{-1}), and in 94% of 16 samples of dessert wines (mean 1000 ng l^{-1}). In a preliminary study by Filali et al. (2001) all of the 30 Moroccan samples of wine were contaminated with OTA (up to 3200 ng l^{-1}).

Immunoaffinity HPLC on 31 samples of red wine originating from Mediterranean sea countries and 15 samples of vinegar, revealed that 72% of the wine samples and 100% of the vinegar samples were contaminated with OTA (up to 3.4 and 0.25 ng ml^{-1}) (Markaki et al., 2001). In southern France, 8/11 musts from grape samples were contaminated with OTA (up to 460 ng l^{-1}), and there was a strong correlation between the presence of *A. carbonarius* on grapes and OTA in musts (Sage et al., 2002). The average levels of OTA in locally produced red and white wines in Cyprus in 1999 year were 0.12 and 0.2 $\mu g\,kg^{-1}$ (Ioannou-Kakouri et al., 2001). Sanchis et al. (2001) also found OTA in samples of beer from Europe. Thirty-seven out of 38 Spanish samples were contaminated with OTA

(up to 75 ng l^{-1}) and it was present in all 41 samples from other EU countries (up to 121 ng l^{-1}).

Aspergillus *moulding of* cereals

Causal agents and symptoms. Among the many fungal species causing moulding of cereals in Mediterranean areas there are, in particular, some aflatoxigenic strains of *A. flavus* and *A. parasiticus*, and some ochratoxigenic strains of *A. ochraceus* and *P. verrucosum*. These toxigenic strains, essentially with an opportunistic or saprophytic behaviour, may colonise cereals before harvest under wet favourable climatic conditions, but particularly after harvest and during storage and processing when the water content of the product is favourable (>15–17%).

Aflatoxigenic fungi belonging to the *A. flavus* group, namely *A. flavus* and *A. parasiticus*, were reported in Spain to occur widely in cereal grain even at high contamination levels, i.e. maize (100%), wheat (100%), sorghum (94%), barley (74%), and rice (34%), but no aflatoxins were found in 165 samples collected from markets (Jiménez and Mateo, 2001). However, analysis of moistened or moulded maize samples associated with problems in farm animals in Spain, during 1994–1999, revealed aflatoxins in 13 of 38 samples (up to 3300 ng g^{-1}), and OTA in 3 of 18 samples (up to 150 ng g^{-1}). Other species of *Aspergillus*, occurring in commercial cereal samples from Spanish markets, with proven toxigenicity, were *A. niger* (OTA), *A. glaucus*, (OTA), *A. ochraceus* (OTA), *A. candidus* (OTA) and *A. fumigatus* (OTA), and in lower proportions *A. chevalieri* (sterygmatocystin (STG)), *A. clavatus* (PAT), *A. terreus* (patulin (PAT)), *A. versicolor* (STG) and *A. amstelodami* (STG) (Jiménez and Mateo, 2001).

Investigations carried out on the toxigenic mycoflora associated with freshly harvested wheat grains collected from different localities in Egypt led to the isolation, together with other fungal genera, of some representatives of *Aspergillus* associated with the occurrence of aflatoxins and ochratoxins (El-Kady and El-Maraghy, 1990). Additional surveys carried out by Atalla et al. (1999) on the mycoflora associated with diseased wheat grains collected from different regions, led to the isolation of some representatives of *Aspergillus* associated with the presence of AFB_1, AFB_2, AFG_1, AFG_2, OTA, OTB, and STG. It can be presumed that wheat grains may be colonised to some extent, from harvest to storage, by *A. flavus, A. parasiticus, A. ochraceus*, and *A. versicolor*. Furthermore, Abdel-Mallek et al. (1994) reported the occurrence of toxigenic *Aspergillus* strains in maize kernels. These were capable of producing aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2) (presumably from *A. flavus* and *A. parasiticus*), STG (presumably from *A. versicolor*), and OTA (presumably from *A. ochraceus*).

Toxin formation and natural occurrence of aflatoxins and ochratoxins. Aflatoxins (traces to 7 ng g^{-1}) were reported in 36 of 58 wheat samples collected from experimental field plots in northern Italy (Lombardy). This was probably due to bad climatic conditions at harvest time and/or in appropriate postharvest drying procedures (Perenzin et al., 2001). Such data confirmed the widespread occurrence of aflatoxigenic strains of *Aspergillus* in Italy and stressed the importance of the postharvest care of grains. In Croatia, OTA was present in 76% of 94 wheat samples (up to 160 ng g^{-1}) and in 33% of 51 maize samples (up to 40 ng g^{-1}) (Puntaric et al., 2001). In Cyprus, in 1997–1999, the average of positive samples (>0.2 ng mg^{-1} OTA) of locally grown cereals (maize and barley) and cereal products (wheatflour and biscuits) was 23%. The range of toxin levels (0.3–1 ng mg^{-1}) was lower than the maximum tolerable limits (5 ng mg^{-1}) adopted by EU countries for wheat (Ioannou-Kakouri et al., 2001). Analysis of 100 maize-based Spanish product revealed that only one feed sample was contaminated with AFB_1 (0.15 mg kg^{-1}) and AFB_2 (0.08 mg kg^{-1}) (Sanchis et al., 1995).

Aspergillus *on other crops*

The aflatoxigenic capability of 91 strains of *Aspergillus* belonging to 11 species groups of Raper and Fennell's nomenclature and 22 strains of *Penicillium*, isolated from plant products collected in Italy, confirmed the production of aflatoxins (AFB_1 and AFB_2) by strains of *A. flavus* (11 out of 33) and *A. parasiticus* (two out of two), capable of producing AFB_1, AFB_2, AFG_1, and AFG_2. Aflatoxins were not produced by representatives of the other *Aspergillus* groups, including *A. glaucus* (17 strains), *A. cervinus* (1), *A. fumigatus* (1), *A. ochraceus* (6), *A. niger* (12), *A. candidus* (2), *A. flavus* (33), *A. wentii* (9), *A. cremeus* (1), *A. flaviceps* (1), *A. terreus* (1), and unclassified *Aspergillus* spp. (7); or by 22 strains of *Penicillium*.

The ochratoxigenic capability of 195 strains of *Aspergillus* isolated from Spanish animal mixed feeds and raw plant products (maize, soybeans and peas) was determined. They included 1 strain of *A. alliaceus*, 12 of *A. candidus*, 45 of *A. flavus*, 15 of *A. fumigatus*, 12 of *A. ochraceus*, 2 of *A. tamarii* Kita, 10 of *A. terreus*, 10 of *A. versicolor*, 23 of *Eurotium amstelodami*, 31 of *E. chevalieri*, 15 of *E. rubrum*, and 19 of *A. niger*. OTA was found in cultures of 2 strains of *A. ochraceus*, 1 of *A. versicolor*, 1 of *A. fumigatus*, the only assayed strain of *A. alliaceus* and 2 of *A. niger* (Sanchis et al., 2001).

Peanuts (Arachys hypogea)
Determinable levels of aflatoxins were found in 14 of 41 samples of raw shelled peanuts imported into the United States from Egypt in 1981, with three samples exceeding the maximum tolerable concentration of 26 ng g^{-1} (Jelinek et al., 1989). Surveys carried out in Cyprus on 1580 unsorted samples of field peanuts indicated, for 1997 an incidence of 6.7% and a level of total aflatoxins, (up to 780 μ g kg^{-1}) confirming that peanuts are very susceptible to aflatoxin contamination (Ioannou-Kakouri et al., 2001). Aflatoxigenic strains belonging to the *A. flavus* group, namely *A. flavus and A. parasiticus* occur widely in Spanish peanuts Jiménez and Mateo (2001). AFB_1 (10 ng kg^{-1}) was found in one sample. In samples of Spanish peanuts, other species of *Aspergillus,* with proven toxigenicity were *A. niger, A. glaucus* and *A. ochraceus* (OTA), and in lower proportion *A. candidus, A. fumigatus*, and *A. chevalieri* (STG), *A. clavatus* (PAT), *A. terreus* (PAT) *A. versicolor*, and *A. amstelodami* (STG) (Jiménez and Mateo, 2001).

Almond (Prunus amygdalus)*, Hazelnut* (Corylus avellana)*, and Pistachio* (Pistacia vera)
The results of a preliminary survey on the occurrence of aflatoxigenic *Aspergillus* strains and aflatoxins in freshly harvested and processed (peeled, sliced, diced, and grounds) almonds, showed that a negligible aflatoxin risk, if any, was associated with Italian processed products (principally ground almond), whereas traces of aflatoxins were usually associated with whole almonds from Morocco (Bottalico and Logrieco, 2001). *A. flavus* and *A. parasiticus*, were reported by Jiménez and Mateo (2001) to occur in Spanish nut samples, i.e. pistachios (84%), almonds (76%), and hazelnuts (76%). AFB_1 (95 ng kg^{-1}) and AFB_2 (15 ng kg^{-1}) were found in one sample of almonds. Other species of *Aspergillus* with proven toxigenicity, occurring in nuts samples, were *A. niger* (OTA), and in lower proportions *A. glaucus* and *A. ochraceus* (OTA), *A. candidus, A. fumigatus, A. chevalieri, A. versicolor*, and *A. amstelodami* (STG), *A. clavatus* and *A. terreus* (PAT) (Jiménez and Mateo, 2001). Aflatoxins were also found in 4 of 11 samples of pistachios from Sicily (up to 45.07 ng g^{-1} of AFB_2 and AFG_2), in three out of seven samples from Greece (up to 87 ng g^{-1} of AFB_1, AFB_2, AFG_1, and AFG_2) and in three out of six samples from Turkey (up to 102 ng g^{-1} of AFB_1, AFB_2, AFG_1, and AFG_2) (Barbagallo and Russo, 1999).

Common Fig Tree (Ficus carica)
Dried fig samples (110) imported from Turkey were surveyed by Gelosa (1990). Sixty-one percent were contaminated with strains of *Aspergillus* and *Penicillium* and contained aflatoxins (14% up to 340 ng g^{-1}). In Cyprus, some samples of local figs from the 1998 crop were contaminated with aflatoxin, but the levels (up to 5 μg kg^{-1}) were within the maximum permitted levels (10 μg kg^{-1} total aflatoxins, of which AFB_1 should be not more than 5 μg kg^{-1}) (Ioannou-Kakouri et al., 2001).

Pea
Samples of dry pea seeds were contaminated by several strains of *Aspergillus* and aflatoxins (Saber et al., 1998).

Oilseed rape
Three of 40 strains of *A. flavus* isolated from 20 oilseed rape samples collected from Catalonia (Spain) were aflatoxigenic. One sample of oilseed rape was contaminated with AFB_1 (0.25 ng g^{-1}) (Vinas et al., 1994).

Sunflower seeds
Aspergillus strains, capable of producing the four aflatoxins and STG, and strains of *A. ochraceus*, capable of producing OTA, were recorded in sunflower seeds collected from several Egyptian localities (Abdel-Mallek et al., 1994). High levels of aflatoxins (up to 230 ng g^{-1}) were found in 7% of 135 samples of sunflower seeds in Tunisia (Jelinek et al., 1989). *A. flavus* and *A. parasiticus* were reported in samples of sunflower seeds from Spain, together with other toxigenic *Aspergillus* species, including *A. niger,* but in lower proportions also *A. glaucus, A. ochraceus, A. candidus*, *A. fumigatus, A. chevalieri, A. versicolor*, and *A. amstelodami* (STG), and *A. clavatus* and *A. terreus* (PAT) (Jiménez and Mateo, 2001).

Fruit juices of apple (Pyrus malus) *and guava* (Psidium guajava)
Investigations carried out by Abdel-Sater et al. (2001), on the natural contamination of Egyptian canned fruit juices and beverages, showed that the most prevalent fungi were members of *Aspergillus* and *Penicillium.* In addition, TLC analyses revealed that all five pear beverages examined contained AFB_1 and AFG_1 (both up to 30 ng ml^{-1}), and two of five guava juices contained AFB_1 (up to 12 ng ml^{-1}).

Dried raisins
Aspergillus flavus, A. niger, A. fumigatus, and *Eurotium chevalieri* were among the most frequently encountered fungi in 100 samples of dried raisins collected from retail markets throughout Egypt. Selected strains produced AFB_1, AFB_2, AFG_1, AFG_2, OTA, fumagillin, and STG. In addition, TLC analyses revealed the presence of AFB_1 (up to 300 ng g^{-1}) in two samples, and OTA (250 ng g^{-1}) in a third sample (Youssef et al., 2000).

Sesame (Sesamum indicum) *seeds and tahini (sesame butter)*
Tahini, an ingredient of many sweet foods widely consumed in Turkey and the Middle East, is produced by milling dehulled and roasted sesame seeds. Aflatoxigenic strains of *A. flavus* and *A. parasiticus* were reported to grow on sesame seed and the first surveys carried out in Turkey have shown the potential risk of contamination for commercial sesame seeds and tahini (Nilüfer and Boyacioglu, 2002).

Sugarcane
Aflatoxigenic strains of *A. flavus* and ochratoxigenic strains of *A. niger* were the predominant fungal flora associated with decayed sugarcane in Egypt (Ziedan and Hegazy, 2002).

PENICILLIUM DISEASES

Penicillium is certainly the most complex genus in terms of numbers of species and their range of habitats. They are of great economic importance primarily as a cause of food and feed spoilage. However, a few species are among the most common and destructive agents of postharvest diseases, affecting most kinds of fruits and vegetables. Several species also cause decay of grains and legumes during harvest, storage or transit, especially at temperatures slightly below normal and at moisture contents slightly above normal. Moreover, certain species decay hay and others colonise processed foods and feeds, including meats, cheeses, and spices. In addition to the losses caused by the rotting of fruits and vegetables, as well as by the deterioration of seeds, grains and processed foods, and feeds, the fungus may produce several mycotoxins that can accumulate in infected products. Notable is the occurrence of OTA in small-grain cereals colonised by *P. verrucosum*, which contaminates cereal-based foods and feeds, as well as malt and beer.

Patulin. PAT is among the most widespread of fungal metabolites, produced by many species of *Penicillium*, as well as by species of *Aspergillus* and *Byssochlamys*. Of great interest is the production of PAT by strains of *P. expansum* colonising apple and other fruits and forming toxins in rotted fruits, which is then transmitted to juices and jams. However, PAT is also formed in cereal grains and cereal-based foods and feeds by certain strains of *P. expansum, P. griseofulvum*, and *P. roquefiortii*.

Citrinin. Citrinin (CIT) is produced by at least by 14 species of *Penicillium* and three species of *Aspergillus*. Of particular importance is the formation of CIT by strains of *P. citrinum* causing yellowing of rice, and by strains of *P. verrucosum* colonising cereal grains and legumes. Citrinin is commonly associated with OTA in barley and other cereal grains naturally colonized by *P. verrucosum*. CIT is a nephrotoxin associated with OTA in certain mycotoxicoses of pigs and chickens and suspected also to be involved in human diseases such as yellowed-rice toxicosis and Balcanic endemic nephropathy.

Penicillic acid. Penicillic acid (PAC) is produced by many species of *Penicillium*, and in particular by strains of *P. aurantiogriseum* which cause 'blue-eye' of maize. Penicillic acid was found in samples of maize and other cereal grains, as well as in samples of dried bean. Penicillic acid is cytotoxic to plant and animal cell lines and is moderately genotoxic to microorganisms.

Penicillium *moulding of cereals*

Causal agents and symptoms. Favourable weather conditions, such as high rainfall and mild temperature

before harvest, can be conducive to colonisation of cereal ears by saprophytic or opportunistic fungi, including toxigenic *Penicillium* strains. The activity of such toxigenic strains may continue during harvest and storage under favourable conditions. Jurjević et al. (1999) reported a high occurrence (up to 93.6%) of species of *Penicillium* in freshly harvested maize samples collected from 14 regions of Croatia, whilst Jiménez and Mateo (2001) reported a wide occurrence of toxigenic *Penicillium* species, particularly *P. griseofulvum* (PAT), *P. variabile, P. islandicum, P. purpurogenum*, *P. chrysogenum* (PAC), and *P. capsulatum* in samples of cereal grains collected all over Spain markets

Toxin formation and natural occurrence. Samples (two out of four) of freshly harvested maize from Croatia, collected in 1996, which were characterised by high amount of rainfall and mild temperature at harvest, were found colonised by *Penicillium* species (up to 82%), and contaminated by OTA (up to 224 ng g^{-1}) (Jurjević et al., 1999). Sample of cereals from Cyprus were contaminated (23%) with low concentrations of OTA (up to 0.9 ng g^{-1}), with the highest contamination in barley, followed by wheat, while the contamination was quite negligible for maize (Ioannou-Kakouri et al., 2001). Investigations carried out on the toxigenic mycoflora associated with freshly harvested wheat grains collected from different localities in Egypt led to the isolation, together with other fungal genera, of some representatives of *Penicillium* associated with the occurrence of CIT (El-Kady and El-Maraghy, 1990). In addition, the occurrence of toxigenic *Penicillium* strains capable of producing CIT and rubratoxins was also recorded in maize kernels (Abdel-Mallek et al., 1994). Moistened or moulded wheat samples associated with problems in farm animal in Spain, revealed PAT in three of five samples (up to 38 ng g^{-1}) (Jiménez and Mateo, 2001).

Blue mould rot of apple and pear

Causal agents and symptoms. The disease is caused by strains of *P. expansum,* a pathogen which is generally considered to be a wound parasite. Mould growth normally occurs only where the surface tissue of fruit has been damaged. Infection commonly follows insect or storm damage during preharvest, rough gathering at harvest or strong washing and sorting procedures after harvest. During storage, infection can occur even at 0 °C, but decay proceeds slowly during cold storage, and usually only develops rapidly when fruits are returned to warm temperatures. The species includes strains with different pathogenicities, as determined by lesion diameter on fruits, and with different capacities to produce PAT (from 2 to 100 mg kg^{-1}).

Toxin formation and natural occurrence. Under laboratory conditions, PAT can be produced by a variety of different moulds on apples, grape juice and grains, but in natural conditions PAT is essentially known as a metabolite of *P. expansum* contaminating apples and apple juice. Patulin is stable in apple juice, grape juice, and dried maize, but not in oranges, orange juice, flour, bread, cheese, or wet maize. However, occasionally PAT has been detected in other naturally brown rot fruits like bananas, pineapples, grapes, peaches, apricots, and tomatoes. Fruit juices converted to cider or wine do not contain PAT because it is destroyed by alcoholic fermentation. High levels of PAT (up to 250 mg kg^{-1}) have been reported in 52% of the 104 Spanish apple samples (Jelinek et al., 1989), but the level of contamination in the juices is usually lower, probably due to the disposal of apples affected by rot. To this regard, the occurrence of PAT was reported in 43 out of 100 samples of Spanish apple juices in concentrations of 10–170 ng ml^{-1} (Prieta, 1994). Investigation carried out on Italian products, showed PAT in all five samples of apple juices examined (up to 60 ng ml^{-1}), in five out of six pear juices (up to 25 ng ml^{-1}), in two out of six peach juices (up to 3 ng ml^{-1}), in one out of three apricot juices (12 ng ml^{-1}) and in more than 50% of 20 samples of jam (up to 75 ng g^{-1}) (Valletrisco et al., 1983).

A very high incidence (100%) of PAT even though at low levels (5–75 ng ml^{-1}) was found in 44 samples of Turkish apple juices (Karadenizm and Eksi, 1997). But a similar survey carried by Gökmen and Acar (1998) showed higher PAT contamination of 215 apple juices from Turkey with 100% incidence and toxin range of 7–376 ng ml^{-1}. In France, PAT was found in 100% (up to 610 ng g^{-1}) and 69% (up to 300 ng g^{-1}) of 27 samples of concentrated apple juices, and 13 samples of apple cider (Jelinek et al., 1989). Laidou et al. (2001) reported the formation of PAT in pear inoculated with *P. expansum* and its diffusion in the apparently sound flesh, in concentration surpassing the accepted maximum Greek limits (50 ng ml^{-1}). Investigation carried out by Martins et al. (2002) on the presence of

mycotoxins in 351 samples of seven different varieties of apples with small rotten areas, collected throughout Portugal, revealed the occurrence of PAT (up to 80.5 mg kg^{-1}) and CIT (up to 5 mg kg^{-1}) found either together (20%) or alone (69% and 4% samples).

Penicillium *on other crops*

None of the strains of *Penicillium* isolated from 20 oilseed rape samples collected all over Catalonia (Spain) were mycotoxigenic (Vinas et al., 1994). However, strains of *Penicillium* species, including strains of *P. citrinum*, capable of producing, CIT were recorded in sunflower seeds collected from several Egyptian localities (Abdel-Mallek et al., 1994). In addition, Jiménez and Mateo (2001) reported the wide occurrence of *Penicillium* species, particularly *P. griseofulvum*, *P. variabile* and *P. chrysogenum* in 35 samples of sunflower seeds collected from Spain markets. *P. chrysogenum* and *P. oxalicum* were reported by Youssef et al. (2000) to be among the most frequently encountered fungal species in 100 dried raisins fruits collected from retail markets in Egypt. Selected strains were able to produce PAC and CIT. Jiménez and Mateo (2001) reported the wide occurrence of *Penicillium* species, particularly *P. griseofulvum, P. variabile*, and *P. chrysogenum* in 38 samples of peanuts collected from Spanish markets. Toxigenic strains of *Penicillium* spp. producing PAC (up to 48 ng ml^{-1}) were reported to be among the predominant fungal flora associated with decayed sugarcane in Egypt (Ziedan and Hegazy, 2002).

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Modelling plant disease epidemics

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Key words: epidemiology, model, differential equations, spatial pattern, disease forecasting, aggregation

Abstract

An epidemic is the progress of disease in time and space. Each epidemic has a structure whose temporal dynamics and spatial patterns are jointly determined by the pathosystem characteristics and environmental conditions. One of the important objectives in epidemiology is to understand such spatio-temporal dynamics via mathematical and statistical modelling. In this paper, we outline common methodologies that are used to quantify and model spatio-temporal dynamics of plant diseases, with emphasis on developing temporal forecast models and on quantifying spatial patterns. Several examples of epidemiological models in cereal crops are described, including one for Fusarium head blight.

Introduction

Mathematical modelling of crop disease is a rapidly expanding discipline within plant pathology. The first models of the temporal development of epidemics were developed by Van der Plank (1960; 1963), and have since formed the basis for disease modelling (Campbell and Madden, 1990; McCartney, 1997). In 1969, Waggoner and Horsfall published their model of potato early blight epidemics caused by *Alternaria solani*. Subsequently, various models have been developed (Jeger and Tamsett, 1983). A model is a simplification of reality and attempts to summarise the main processes, to put forward hypotheses and to verify their coherence and consequences. It also represents a trial to determine the minimal hypotheses which would allow minimal mathematical representation of real processes. In epidemiology, modelling aims to understand the main determinants of epidemic development in order to develop sustainable strategies for strategic and tactical management of diseases.

Epidemiological models can be classified in several ways. For convenience, Kranz and Royle (1978) classified them into three types – descriptive, predictive and conceptual – according to their main objective. Descriptive models provide hypotheses or generalise experimental results, but they do not usually reveal the mechanisms underlying the processes. Predictive models, which are also descriptive, allow the prediction of the occurrence and the severity of epidemics. Both descriptive and predictive models use mathematical tools, such as simple or complex functions, regression and differential equations, or simple decision models. The conceptual models, also known as explanatory or analytical models, allow the identification of problems by distinguishing cause from effect and quantify the effects of specific events on epidemic development. They are constructed as representations of underlying biological and ecological processes. These models may eventually lead to the development of complex simulation models. It should be pointed out that models can be disease-specific, but can also be very general. A descriptive model is often concerned with understanding and predicting development of specific diseases, and thus is generally used for assisting growers in making tactical decisions in managing diseases. A conceptual model is often concerned with the theoretical understanding of generic features of epidemic development and thus is used more for making policy and strategic decisions.

Recent advances in computer tools have made mathematical/statistical modelling more accessible and

have led to the development of more complex models for many diseases. In this paper, we attempt to provide a brief introduction to the framework for developing epidemiological models. For this purpose, various modelling processes, such as problem specification, are reviewed. Finally, several models for cereal diseases are briefly presented. We have focused on two areas: forecasting temporal disease dynamics and quantifying spatial patterns. We intentionally do not include any discussion of the more theoretical aspects of epidemiological modelling, nor recent approaches which attempt to combine temporal models with spatial models.

Problem specification

Since models are most useful when directed at a clearly specified and well-defined problem, the crucial first step in modelling is to precisely define the question(s) to be addressed. The nature of the problem is likely to determine what are the most appropriate modelling techniques to use (Kranz and Royle, 1978; Sutherst, 1993). In fungal epidemiology, possible objectives for modelling include (Norton et al., 1993):

1. predicting the timing of an event, such as when disease infection is likely to occur;
2. predicting the scale of an event, such as the severity of disease infection or crop loss;
3. estimating the frequency or the probability of an event, such as monocyclic or polycyclic epidemics;
4. assessing and comparing the performance of different management strategies.

Biological characteristics of a fungal pathosystem

Once the objective of a model is clearly established, the second step is usually to determine the variables that are needed for developing the model. These variables generally represent key features in the development of epidemics, including initial inoculum, progeny/parent ratio and the length of latent period (Van der Plank, 1982) as well as major external factors. Recently, de Vallavieille-Pope et al. (2000) assessed the use of various epidemiological parameters in modelling. They identified spore production, latent period, the number of infection cycles, host resistance and external factors as key components of disease development. The importance of the interactions between pathogen and host population dynamics has long been underestimated (Gilligan, 1985) and is now receiving due attention (Webb et al., 2000).

Fungal pathogens

Inoculum

Fungal inoculum is of prime concern; its source, density and type will greatly influence the design of the forecasting scheme. Without inoculum, there is no epidemic. A fungal population consists of individuals at various stages of their life-cycle. The population can be described by the proportion or absolute quantity of individuals at each stage, i.e. age structure of the population (Shaw, 1998). For example, for modelling purposes, *Blumeria graminis* on cereals may consist of three components: (1) spores, each potentially capable of infection, (2) mycelia, and (3) cleistothecia (Shaw, 1998). Passage from one stage to the next can be very fast, usually depending on environmental conditions. Inoculum may also be simply divided into primary and secondary; such division has been found to be very useful in modelling cereal soil-borne pathogens (Gilligan, 1985). van Maanen and Gourbiere (2000) showed the importance of the life-cycle stages in the control of fungal dynamics, by adapting a patch-occupancy model previously developed by Gourbiere et al. (1999).

Fungal populations are difficult to study under field conditions because individual mycelia and spores cannot generally be easily quantified (Siefert, 1981). The assessment of the amount of the pathogen on and in the leaves is notoriously difficult. For example, sporulating mycelia of *Stagonospora nodorum* or *Mycosphaerella graminicola* of wheat, causal agents of leaf blotching and spotting infection, are hidden within the leaves. Visual assessment of the percentage leaf area covered by lesions can be rapid, but is quite subjective, and hence may not be a reliable assessment of inoculum (de Vallavieille-Pope et al., 2000). Numbers of colonies or pustules can be counted accurately on a small scale, but this is not practical on a large scale. To simplify the matter, a pathogen population is often measured indirectly as disease incidence or severity. Other methods used to quantify pathogen populations include determination of the fungal biomass using enzyme-linked immunosorbent assay (ELISA) (Newton and McGurk, 1991), ergosterol and/or chitin content measured using high performance liquid chromatography (HPLC) (Johnson and McGill, 1990; Barajas-Aceves

et al., 2002) and competitive PCR methods (Nicholson et al., 2002). Quantifying inoculum of soil-borne pathogens is generally more problematic (Jeger, 2000).

Inoculum dispersal

Inoculum dispersal fulfils essentially three functions: (1) population survival, (2) colonisation of new habitats and (3) reproduction (Ingold, 1971). Dispersal can occur either by mycelium or spores. Spore dispersal comprises three phases; liberation, which can be passive or active (Dix and Webster, 1995), transport, and deposition (Ingold, 1978). The dispersal scale depends on inoculum properties as well as the transport vector, and may range from a few metres through rainsplash (Madden, 1992), to 100–10 000 m for airborne spores, such as those of powdery mildew of cereals (Andrivon and Limpert, 1992). Often, spore dispersal can be described by either an exponential or a power function (Fitt et al., 1987).

Disease models differ in the method by which the various steps in the disease-cycle are formulated, but each model includes the equivalent of a primary deposition function (McCartney and Fitt, 1998). The primary deposition function plays an important role in both the shape of the disease gradients and the expansion rate of the disease focus. Ferrandino (1993) derived a function to account for loss of spores both by escape from the canopy and by deposition. The model generated disease gradients that became shallower as the epidemics progressed. Such a pattern has been observed in diseased crops, e.g. potato late blight (Minogue and Fry, 1983b) and *A. linicola* on linseed (Vloutoglou et al., 1995). The efficiency of spore dispersal affects the density of new infections (de Vallavieille-Pope et al., 2000). Gourbiere et al. (1999) considered dispersal as the main parameter which determines the number of newly colonised units in their model, which has been further extended to simulate the distribution and frequency of new infections along weather gradients (van Maanen et al., 2000).

Latent and infectious periods

In epidemiology, the latent period is the interval between the onset of spore germination and the appearance of the next spore generation. The rate of epidemic development is largely influenced by the length of latent period, which determines the number of potential infection cycles that can be completed during a growing season (de Vallavieille-Pope et al., 2000). The shorter the latent period, the more reproduction cycles the fungus can have per season. In contrast to polycyclic diseases, monocyclic diseases have only one reproductive cycle throughout a single season.

The importance of the latent period and spore deposition frequency in modelling has been emphasised by Gumpert et al. (1987). Latent periods have been reported to depend on inoculum and lesion density (de Vallavieille-Pope et al., 2000), but are mainly influenced by temperature (e.g. Beresford and Royle, 1988; Xu, 1999; Xu and Robinson, 2000; 2001). They may also vary with the level of host susceptibility and with host growth stages, features that emphasise the importance of studying both pathogen and host dynamics. Another key factor influencing the development of an epidemic is the length of the infectious period (i.e. the length of time during which a single colony continues to produce spores), as this determines the quantity of spores that a single colony is likely to produce during its lifetime.

Pathogen dynamics regulation

Fleming (1980) argued that the role of predation and parasitism in pathogen regulation might be greater than previously recognised. Burgess and Hepworth (1996) recognised various hyperparasites attacking sclerotia of *Sclerotinia* spp. Brasier (1990) showed that the transfer of virus pathogens of fungi would occur more efficiently when the fungal population is at a high density. Another natural regulation comes from pathogen population competition, e.g. the competition between the eyespot pathogens *Tapesia yallundae*, *T. acuformis* and the sharp eyespot pathogen *Rhizoctonia cerealis* (Bateman et al., 1995). This aspect of microbial community interaction (symbiosis or competition) is, in general, poorly understood for fungal pathogens; it is now becoming gradually more important with the present moves to more integrated disease management strategies.

Host dynamics

For a long time, epidemic modelling has emphasised pathogen activity, ignoring effects of the host on pathogen development. Particular interests are changes in susceptibility, and the contribution of resistance, e.g., to the length of the latent period (Kranz and

Royle, 1978). Another reason why host dynamics should be included in epidemiological models arises from the fact that pathogen population dynamics are linked to host dynamics, and pathogens may affect growth and reproduction of their hosts (Anderson and May, 1979). Simple models can be developed to capture the essential features of host–pathogen interactions, though more complex models are usually necessary.

Host susceptibility and resistance

Among the host factors which need to be taken into account are the levels of intrinsic host resistance and age-related resistance associated with specific host tissues (Shtienberg, 2000). Some cultivars display increased tolerance or partial resistance (Walters and Hardwick, 2000). The nature of host resistance will affect the rate of disease development and must therefore be taken into consideration in modelling (Shtienberg, 2000). Theoretical models have been developed on the effects of cultivar mixtures or crop heterogeneity on epidemic development based on the gene-for-gene relationship (Barrett, 1978; Jeger et al., 1981a,b; Xu and Ridout, 2000). Host resistance and/or pathogen infectivity/aggressiveness may also depend on the age of host tissues. For example, studies have shown that rose tissue become resistant to infection by *Sphaerotheca pannosa* as leaves age (Rogers, 1959; Mence and Hildebrandt, 1966).

Multiple hosts and crop rotation

Paramount among farm practices is crop rotation, which has conventionally been adopted to reduce the carry-over of pathogens from one crop to another (Walters and Hardwick, 2000). However, crop rotations are also likely to favour certain pathogens when multiple hosts are available. *Sclerotinia sclerotiorum*, causal agent of stem rot in spring-sown oilseed rape, has a wide host range of about 400 species, including many weeds (Bolland and Hall, 1994). Maize rotating with wheat is known to be one of main reasons for recent severe epidemics of Fusarium head blight (Obst et al., 1997). The importance of crop rotation has been included in a forecasting system for Sclerotinia stem rot (Twengstrom et al., 1998). Crop rotation emphases the importance of the infection time-length, and as consequence, the survival of inoculum. *Polymyxa betae* produces resting spores which survive in the soil between successive crops. Survival of sclerotia for up to 5 or 10 years has been reported (Ben-Yephed et al., 1993).

Environmental factors

It is generally agreed that the environment is the driving force in the development of epidemics (Rabbinge and Bastiaans, 1989; Hardwick, 1998). This includes major climatic variables, such as rain, temperature and humidity. Wind and rain are essential for pathogen dispersal; rain provides free water on host surfaces for most pathogens to infect and sporulate and sun provides favourable temperatures for disease development (Ingold, 1971; Lacey, 1996; Francl and Panigrahi, 1997; de Vallavieille-Pope et al., 2000; Walters and Hardwick, 2000). The duration of each event as well as its timing is also important. For example, for apple scab, night rain results only in a much smaller proportion of mature ascospore being discharged compared to daytime rain (MacHardy, 1996).

Moisture, particularly the duration of wetness, is the dominant factor for most pathogens (Huber and Gillespie, 1992). Free water or near saturation moisture on the host surface is essential for germination and penetration of the host for many pathogens. Thus, not surprisingly, a single parameter indicating water availability is used in several forecasting systems. Prediction of actual wetness duration is preferable to prediction of occurrence because many pathogens cause more damage as the duration of wetness increases (Hosford et al., 1987; Francl and Panigrahi, 1997). Artificial neural network models have been developed to predict wetness on wheat flag leaves from both dew and rain (Francl et al., 1995; Francl and Panigrahi, 1997).

The role of temperature has been studied for many pathosystems, mostly for its influence on initial germination, infection, and the length of incubation, latent and infectious periods. For example, Newton (1989) showed that the infection efficiency of barley powdery mildew was reduced by over 50% at 7 °C compared to 20 °C. The different stages of the disease-cycle of *P. betae*, the fungal vector of beet necrotic yellow vein virus, are sensitive to soil temperature, which appeared to be a principal factor in influencing the occurrence and severity of the sugar beet disease rhizomania (Blunt et al., 1992).

The relationships between disease development and environmental factors are the key component and often

the only component of disease forecasting systems. Both past and future weather forecasts can be used in these systems for predicting epidemic development. Forecasting systems provide an indication or quantification of disease development, especially when the disease is likely to exceed an economic-injury threshold and thus warrants a treatment.

Mathematical representation of epidemic development

Overall, modelling can be divided into three steps: model development, model analysis and hypothesis testing. When developing a model, biological characteristics of the pathosystem are expressed as mathematical relationships. In model analysis, epidemic dynamics are investigated in relation to the parameters of interest (or variables, which may be formulated as functions of external factors such as rain and temperature). Finally in hypothesis testing, the results from model analysis are used to test or verify whether or under what conditions the hypothesis (i.e. the specified problem/question) is valid. Mathematical expression of biological features of the system is critical, since it will, more or less, determine the techniques to be used in model analysis and hypothesis testing. Here, we briefly introduce several common mathematical tools used to analyse plant disease epidemics.

Disease progress curves

Van der Plank (1960) used exponential, monomolecular and logistic models to describe the development of epidemics. He illustrated how polycyclic diseases could be described by logistic models, and monocyclic diseases by monomolecular models. Growth models (monomolecular, Gompertz and logistic models) provide a range of curves that are often similar to disease progress curves. These non-linear curves can be easily fitted to experimental data by any standard statistical package. Use of such growth curves has been described by Campbell and Madden (1990). The important parameters in these models are the initial amount of disease, the apparent rate of disease increase and the level of maximum disease. These parameters can be estimated separately for each individual treatment and their relationships with treatment or environmental factors can then be investigated. Alternatively, the mathematical relationships between the model parameters and treatment/environmental factors can be incorporated into the growth curve models and fitted to the observed data directly. More complex model fitting procedures are required for the latter approach.

Most analyses of disease progress data rely on time as the independent factor. This may not be appropriate when data are collected in different years, seasons, locations, etc. A measure of heat-sum or degree-days provides an alternative method. This assumes that temperature is the most important factor driving growth rate of the host, the pathogen and the disease. This method was used to model various epidemics, such as the cotton-Verticillium wilt system (Gutierrez et al., 1983), powdery mildew on tomato (Correll et al., 1988), and more recently, take-all on wheat (Brassett and Gilligan, 1989; Cohlbach et al., 1997). Many other modifications to standard growth models are possible. For example, to take into account the temporal variability of host susceptibility to pathogens, Lalancette and Hickey (1986) modelled disease progress as a function of host growth, which was used to represent biological time. Van der Plank (1963) introduced a correction factor for the rate of disease increase based on the exponential change in mass of susceptible host tissue. This correction factor was studied by Kranz (1975), and Waggoner (1986), and reviewed by Campbell (1998).

Area under disease progress curve

Not all disease progress curves are well or easily described by a growth curve model. Alternative methods to quantify epidemic development include the area under the disease progress curve (AUDPC). Van der Plank (1963) related area under the stem rust progress curve to the yield loss in wheat. Jeger and Viljanen-Rollinson (2001) listed a range of publications using AUDPC to assess quantitative resistance to disease. The resulting AUDPC values can be used as a measure of epidemic development and used in further analysis and hypothesis testing, such as regression and in variance analyses.

Linked differential equations

One of most commonly used mathematical techniques in modelling epidemics is the linked differential

equation (LDE), which is usually used to investigate theoretical questions concerning the dynamics of plant disease in relation to host, environment and human interventions. Van der Plank (1963) demonstrated how analytical models written as differential equations could be integrated and used to quantify the various parameters associated with disease progress. The LDE models are of the susceptible, exposed, infectious and removed (SEIR) type, which is the standard modelling approach in human disease epidemiology, and is also widely used in plant disease epidemiology (Jeger et al., 1998; Jeger, 2000).

In this approach, the host population is usually divided into several non-overlapping categories, such as healthy susceptible, latently infected, infectious and removed (post-infectious). When an individual plant becomes infected, the pathogen moves through the latent stage to become infectious at a rate which is the inverse of mean latent period. Infected plants lose infectiousness and proceed into the removed or post-infectious stage at a rate which is the inverse of mean infectious period (Segarra et al., 2001). Plant populations may be constant, but may also assume increase or decrease to model host growth or senescence processes. Depending on the hypothesis to be tested, the number of these categories used varies greatly. For example, when modelling the effect of induced resistance, an extra category, healthy resistant, may also be required.

Linked differential equation models are specified for each defined plant category, written generically as

$$\frac{\mathrm{d}P}{\mathrm{d}t} = B(P) - D(P),$$

where $B(P)$ and $D(P)$ are functions describing the increase and decrease of the host population of category P. $B(P)$ and $D(P)$ are jointly determined by host growth functions, pathogen attributes, pathogen transmission/dispersal characteristics and disease management. Some density-dependent population regulation can also be included.

Linked differential equation models are usually evaluated analytically to determine the key dynamic features of the system, and then numerically to explore the dynamics in the important conditions identified. Typical questions to be asked include (1) what are the values and stability of equilibria? (2) how are the values and stability of equilibria affected by model parameters/variables? (3) how does the epidemic approach the equilibria? (4) what are the conditions necessary for the epidemic to persist? and (5) how sensitive are the system dynamics to model parameters/variables?

Computer simulation

Many computer simulation models have been developed in the past decades. Computer simulation is in general a natural extension of LDE modelling. In computer simulation, model parameters in LDE are often assumed to be functions of external factors such as temperature and humidity. These functions can either be of simple linear type or complex non-linear type. Computer simulation can be used to study both theoretical and applied problems. Using a stochastic simulation model, the relationships of spatio-temporal statistics with underlying biological, physical and biological factors have been successfully studied (Xu and Ridout, 1998; 2000; 2001). One of the earliest spatio-temporal simulation models was EPIMUL (Kampmeijer and Zadoks, 1974), which laid the foundations for further developments (Minogue and Fry, 1983a,b; Van den Bosch et al., 1988; Zawolek and Zadoks, 1992; Ferrandino, 1993; Maddison et al., 1996). Jeger (1986) discussed the advantages and disadvantages of the simulation approach in comparison to the analytical approach.

Examples of forecasting models

Rust development of epidemics

Rust development of epidemics (RustDEp) is a dynamic simulator of the daily progress of brown rust severity on wheat (Rossi et al., 1997). It takes into account (1) the proportion of spores able to establish new infections influenced by temperature and leaf wetness (de Vallavieille-Pope et al., 1995), (2) the fact that the latent period depends on temperature (Johnson, 1980), and (3) the fact that the infectious period depends on temperature and host growth stage (Tomerlin et al., 1983). In the RustDEp model, the inputs of meteorological data are recorded by a weather station, allowing more accurate simulation of the disease progress (Rossi et al., 1997).

Sclerotinia stem rot forecasting systems

Several approaches have been used to develop forecasting methods for *Sclerotinia* stem rot, caused by

S. sclerotiorum, including checklists (Thomas, 1984), risk point tables (Ahlers, 1989), serological tests (Jamaux and Spire, 1994; Lefol and Morrall, 1996) and petal infestation assessments (Turkinson and Morrall, 1993). Nevertheless, their practical use has not been adequately evaluated and their accuracy was sometimes not satisfactory. Twengstrom et al. (1998) improved an existing risk point system using logistic regressions. The factors that affect *Sclerotinia* infection were given points with regard to the risk of heavy infestations. The choice of risk factors and risk points in the improved model was made on the basis of statistical and biological considerations. New threshold values for spraying recommendations were determined. Mild *Sclerotinia* epidemics were predicted for <40 points and spraying was not recommended. If the risk point was ≥ 50, spraying was recommended. This system is a type of decision model, which has several advantages, including ease of use and distribution.

Disease forecasting system for Fusarium head blight in the USA

A series of severe Fusarium head blight epidemics experienced in the USA (McMullen et al., 1997), led to a collaborative project between the States of Ohio, Dakota, Minnesota and Manitoba to create a forecasting model. De Wolf et al. (2000) used weather data (temperature, RH, rainfall) and mean disease levels collected in Ohio from 1982 to 1999 to identify critical environmental periods. Correlation analysis was used to identify weather variables that were potentially associated with epidemic development and then to use these variables to develop a logistic regression. The resulting risk model had an 84% level of accuracy in predicting epidemics. The model is currently under evaluation.

Another model, which incorporates rainfall, temperature and wheat heading date has been developed to produce a risk map for the production of the *Fusarium*-associated mycotoxin deoxynivalenol (DON). These maps forecast the amount of DON (in ppm) that would accumulate if the wheat was at Zadoks growth stage 59 (75% of the heads completely emerged from the flag leaf). The model was improved using data from 399 farm fields across Ontario from 1996 to 2000, using both forecast and actual weather data. DON predictions are meant to serve as a guide; a high DON level may warrant a timely application of fungicide. The DON level maps are available on the Ontario Weather Network website (www.ownweb.ca).

Quantifying spatial patterns

Recently, there has been increased interest in the statistical description and theoretical modelling of the spatio-temporal dynamics of epidemics, especially for disease incidence data, where individual plants or plant-parts are classified as diseased or healthy (Hughes and Madden, 1992; 1993; Madden and Hughes, 1995; Yang, 1995; Hughes et al., 1996; 1997; Xu and Ridout, 1998). The individuals often occur in groups that may arise naturally (e.g. leaves on a shoot) or artificially (e.g. plants in a quadrat). Many different methods have been used to characterise spatial aggregation for disease incidence data, including fitting of the beta-binomial distribution (Hughes and Madden, 1993; Madden and Hughes, 1995), variance–mean relationships (Hughes and Madden, 1992; Madden and Hughes, 1995), spatial autocorrelation (Campbell and Madden, 1990; Gottwald, 1995; Madden and Hughes, 1995), geostatistical methods (Chellemi et al., 1988; Stein et al., 1994; Gottwald et al., 1996), and distance class methods (Gray et al., 1986; Nelson et al., 1992; Gottwald, 1995; Nelson, 1995; Ferrandino, 1996; 1998). In this paper, only the most common and recent statistical methods for quantifying spatial heterogeneity of disease incidence data are briefly introduced.

To assess spatial heterogeneity of plant disease, researchers usually collect data in two ways. First, disease incidence is assessed within a sampling unit comprising a group of plants at a particular spatial point (often called a quadrat); a number of such quadrats would be randomly taken over space or time. Second, disease is assessed on all individual plants or on a subset of plants; in this case, spatial location is recorded as well as disease incidence. Sometimes, a hybrid of these two methods can also be used, i.e. disease is assessed within a quadrat but a number of such quadrats is selected on a set of predefined spatial locations. In the former method, the heterogeneity can be investigated within the quadrat. In the latter case, the spatial dependence of disease can be studied between sampling units. Usually, the information extracted from the data is positively related to the amount of effort put into the disease assessment.

When the spatial dynamics of plant diseases are studied, the following questions can be considered: (1) is

the disease aggregated? (2) how does the degree of aggregation vary with time? (3) is there any spatial dependence in disease development? (4) can such spatial dependence be quantified? (5) what is the rate of disease spread, disease gradient or spore dispersal gradient? (6) what is the impact of disease aggregation on disease management? and (7) what factors have caused the observed spatial pattern. To understand many spatial analytical tools, there is a need to first understand the expected variance of a number of infected plants within a sampling quadrat.

Disease incidence and its variance

Disease incidence is an example of a *binary* variable, one that can take only two possible values, i.e. diseased or not. Thus if X denotes the disease assessment on a particular plant (i.e., $X = 0$ for a healthy plant; $X = 1$ for a diseased plant) and π denotes the probability that the plant is diseased, then $\Pr(X = 0) = 1 - \pi$ and the value of π determines the distribution of X completely. In particular, the mean and variance are $E(X) = \pi$ and $\text{var}(X) = \pi(1 - \pi)$, respectively. This distribution, known as the Bernoulli distribution, is the only possible probability distribution for a binary random variable.

Suppose that a rectangular quadrat is sampled, with n number of plants in the quadrat, and a binary random variable X_i indicates the disease status of the ith plant in the quadrat. The total number of diseased plants (Y) in the quadrat is therefore $Y = \sum_i X_i$. If π denotes the probability that a randomly selected plant from the population is infected, then $E(X_i) = \pi$ and hence $E(Y) = n\pi$. Using the standard formula for the variance of a sum of random variables, the variance of Y is then calculated as:

$$\text{var}(Y) = n\pi(1 - \pi)[1 + (n - 1)\rho] \quad (1)$$

where

$$\rho = \frac{1}{n(n-1)/2} \sum_{i<j} \text{corr}(X_i, X_j) \quad (2)$$

Thus, the variance has two components: the variance of a binomial distribution, $\{n\pi(1 - \pi)\}$, and an additional component due to pairwise correlation in disease status between plants within a quadrat, $\{n(n - 1)\pi(1 - \pi)\rho\}$. The parameter ρ is often called the intracluster correlation (Ridout et al., 1999). In terms of spatial pattern, positive values of ρ imply spatial aggregation or clustering, whereas $\rho = 0$ implies a random pattern of disease incidence. A negative value of ρ implies some sort of regularity in the pattern, whereby the presence of a diseased plant reduces the likelihood of neighbouring plants being diseased. Thus, the degree of spatial aggregation can be assessed by determining the magnitude and significance of ρ, either directly or indirectly.

In assessing spatial heterogeneity, either incidence (i.e. proportion or percentage infected plants within a sampling unit) or counts data (number of infected plants within a quadrat) can be used. Madden and Hughes (1995) discussed the relative merits of both approaches. Here, if not specified otherwise, incidence is assumed.

Beta-binomial distribution

It follows from equation (1) above that if the incidence data can be satisfactorily fitted by a binomial distribution then this implies that intracluster correlation is expected not to be significantly different from zero. Therefore, there is no spatial aggregation of diseased plants. Typically, for an aggregated data set, there are more observed units in either the upper or lower disease incidences and correspondingly less in the mid-level of incidence than that predicted by the binomial distribution at a given level of overall incidence.

If the binomial distribution fails to describe the observed data satisfactorily, other types of distributions may be fitted to the data. One commonly used distribution is beta-binomial (Hughes and Madden, 1993; Madden and Hughes, 1995). This distribution can be derived by assuming that the incidence of disease varies from quadrat to quadrat in a random way, for example due to spatial variability in environmental factors, which gives rise to a *mixture* distribution for Y. The binomial parameter π is replaced by a random variable P, with $\text{E}(P) = \pi$ and $\text{var}(P) = \rho\pi(1 - \pi)$. Unconditionally the variance of Y is given by equation (1) above. Commonly, P is assumed to follow a beta distribution as this gives a flexible range of distributions for P, and hence for Y (Hughes and Madden, 1993; Madden and Hughes, 1995). The resulting mixture distribution is thus called beta-binomial distribution with two parameters: p and θ. Parameter p is the expected or average value of π, i.e. overall disease incidence; θ is an index of aggregation, ranging from 0 to ∞, although typically less than 1. This distribution has been successfully fitted to many observed data sets (Hughes and Madden, 1993;

Madden and Hughes, 1995; Xu et al., 2001). If the beta-binomial distribution fits observed incidence data satisfactorily, θ can then be used to indicate the degree of aggregation. In general, beta-binomial distribution may not be sensitive enough to detect aggregation for small sample sizes.

Corresponding to the incidence, Poisson distribution is equivalent to binomial distribution for counts data in assessing the randomness of the observed data. Negative-binomial distribution is often used to describe aggregated counts data; one of its parameters, k, is also used to indicate the degree of aggregation. This parameter has been incorporated into temporal epidemic models to investigate the effects of spatial aggregations on temporal disease development. Other distributions for describing aggregated counts data are also possible.

Index of dispersion

Poisson distribution fits counts data if the counts data are randomly distributed. For counts data, the variance-to-mean (VM) ratio is usually used to measure aggregation. This is essentially a variance-to-variance (observed-to-expected) ratio since the mean is the expected variance for a Poisson distribution. Thus, for a random distribution of observed data VM is expected to be 1. This VM ratio can be generalised to give the definition of index of dispersion (D) as the ratio of the observed to theoretical variance. $D = 1$ for random data, $D > 1$ for over-dispersed data and $D < 1$ for under-dispersed data. For binary data such as disease incidence:

$$D = \frac{S^2}{np(1-p)} \tag{3}$$

where $np(1-p)$ is the expected variance of incidence data assuming that the binomial distribution fits the data, and S^2 is the observed variance. A chi-square test has been used to determine whether to reject or accept the null hypothesis (randomly distributed) (Pielou, 1977). This is based on the fact that $(n-1)D$ has a chi-square distribution with $n-1$ degree of freedom for a random distribution and a constant p.

Intracluster correlation

Intraclass correlation (ρ) measures the tendency of the plants within a sampling unit (quadrat) to have a similar disease status. Positive values of ρ indicate aggregation of disease. ρ can be calculated directly from the quadrat data as (Fleiss, 1981):

$$\rho = 1 - \frac{1}{p(1-p)NT(T-1)} \sum_{i=1}^{N} Y_i(T - Y_i) \tag{4}$$

where Y_i is the number of infected plants in the ith quadrat, N is the total number of quadrats and T is the number of plants in a quadrat and is the same for all quadrats. ρ can also be calculated from equation 1 directly as $\rho = (D-1)/(n-1)$, where D is the index of dispersion assuming n is a constant. In addition, if the beta-binomial distribution fits the data, ρ is related to θ as $\rho = \theta/(1+\theta)$.

Power–law relationship

It has been shown that for counts data there is usually a linear relationship between logarithm of the observed variance and the observed mean (Taylor, 1961), which can also be interpreted as variance–variance relationship as pointed out above. To estimate this linear relationship, many pairs of variance and mean are required. This empirical power–law has successfully fitted numerous experimental as well as simulated data, though there is much debate about the biological and mathematical basis for this relationship.

Hughes and Madden (1992) have modified this power law for a binary variable, called binary power law, relating the variance of disease incidence to mean incidence, with the general form:

$$\text{var}(Y) = A\pi^b(1-\pi)^c \tag{5}$$

This might be used, e.g., to model the variance–mean relationship at different times during the development of an epidemic or to compare data from different experimental treatments. It has provided a good fit to many data sets (Madden and Hughes, 1995). Frequently the simpler two-parameter model (i.e., the symmetric form) with $c = b$ is adequate. This simpler model implies that the variance is greatest when $\pi = 1/2$. More generally, the maximum variance occurs when $\pi = b/(b+c)$, provided b and c are positive. The θ parameter of the beta-binomial distribution, hence ρ and D, is also mathematically related to the binary power law parameters (Madden and Hughes, 1995).

The interpretation of binary power law parameters can be found in several published papers (Hughes and Madden, 1992; Madden and Hughes, 1995; Ridout and Xu, 2000).

Spatial autocorrelation

Spatial autocorrelation determines the spatial dependence of disease incidence between sampling units over various lags, thus measuring inter-cluster correlation in contrast to ρ. Details can be found in Campbell and Madden (1990). This technique has been successfully used to characterise the spatial dependence of disease. Typically, spatial autocorrelation declines exponentially over distance; the rate of this decline indicates the degree of disease gradient. Spatial autocorrelation is closely related to the semi-variogram in geostatistical methods (Burrough, 1987), which has been used to analyse spatial disease patterns (Chellemi et al., 1988; Stein et al., 1994; Gottwald et al., 1996).

Xu and Ridout (1998) found the following model to be useful in describing the relationship of spatial autocorrelation with mean disease incidence and spatial lag for data generated from computer-simulated epidemics:

$$\gamma(d, \pi) = S\pi^{u}(1-\pi)^{v} \exp[-td(1-\pi)] \qquad (6)$$

where S is a scaling parameter, u and v are power law parameters, d is the distance, and t determines the rate of decrease of correlation with distance at fixed incidence. In this model, the relationship between spatial autocorrelation and incidence at any spatial lag was of a binary power–law form; at any level of disease incidence spatial autocorrelation declined exponentially over distance. Ridout and Xu (2000) further showed that quadrat-based statistics such as index of dispersion, intra-cluster correlation and binary power law can all be derived from a given model that describes the spatial autocorrelation at the level of individual plants.

Concluding remarks

There are other methods that have been developed to detect and quantify spatial patterns of plant diseases, such as distance class analysis (Nelson et al., 1992; Nelson, 1995; Ferrandino, 1998), which requires data that are more intensive than those methods outlined above. This approach is based on the thinking that the joint disease status of two plants may depend not only on the distance between them, but also on their orientation relative to one another, due to the directional effects in pathogen dispersal under field conditions. Spatio-temporal stochastic models have also been fitted directly to experimental data using Markov Chain Monte Carlo Methods (Gibson, 1997). A new method was recently developed, called spatial analysis by distance indices (SADIE) (Perry, 1995; 1998). The key concept behind SADIE is the distance to regularity, i.e. the total effort in terms of distance moved that individuals in the observed sample must expend to move such that the individuals in the samples are spaced as uniformly as possible. The degree of non-randomness within a data set is quantified by comparing the observed spatial pattern with rearrangements in which the sampled counts are randomly redistributed among the sampling units.

The spatio-temporal statistics and models are very useful for describing and summarising observed epidemics. However, to compare them between studies, there is a need to understand their relationships to the underlying biological and physical processes. In a series of papers based on simulation studies, Xu and Ridout (1998; 2000; 2001) demonstrated the importance of initial epidemic conditions, especially the spatial pattern of initially infected plants, and the size and shape of sampling quadrat in relation to prevailing wind in influencing some spatial statistics, as shown in a few experimental studies, e.g. for rice sheath blight. (Savary et al., 1997), as well as the effect of quadrat size on spatio-temporal statistics (Gottwald et al., 1995; Madden et al., 1995).

It is not expected that a single method could capture everything and answer all the questions concerned with a complex spatial pattern that may be a hierarchical and multi-scaled phenomenon (Kotliar and Wiens, 1990). It is thus important for researchers to use appropriate statistical methods either individually or in combinations that are most appropriate to their research objectives. Biological processes are in a constant state of flux and it is unlikely that all eventualities can be considered by even the most complex model. This is an important constraint, for if the model is too complex it may be impractical. Neither should a model cover all these possibilities, since, if it does, it is no longer a 'model'. A model should be sufficiently complex but no more than necessary to answer the posed question(s). Although several modelling activities have been undertaken in plant disease epidemiology, only one type of model is

likely to be most used by the agriculture industry, that is a disease forecasting system. Often, these systems have been developed, but not properly followed through or supported in their use. One of the challenges facing epidemiologists is to demonstrate convincingly the benefit of using disease forecasting systems, not only in experimental plots but also on a commercial scale, and to ensure the systems are used widely and correctly.

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Effects of environmental conditions on the development of *Fusarium* ear blight

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Abstract

Recent research on the epidemiology of *Fusarium* ear (or head) blight (FEB or FHB) of small-grain cereals is reviewed, focusing on inoculum, infection and disease forecasting. Both conidia and ascospores have been shown to be important for causing FEB. For *Fusarium graminearum*, propagules from crop debris are the main source of initial inoculum. Inoculum production is critically dependent on rainfall although the precise relationship is not clear. Recent work on understanding the effects of climatic variables on FEB development has been based on field observations. These field-based studies confirmed that warm and moist conditions during anthesis are the key factors for FEB development. Several empirical models were derived from the field data and proposed for use in disease forecasting. However, these models may not be applicable to a broader range of areas because of the limited nature of the field data. Several areas are proposed for future research, focusing on the development of more generally applicable forecasting models and on understanding the relationships between disease severity, fungal biomass and the production of associated mycotoxins.

Introduction

Fusarium ear (or head) blight (FEB or FHB) of small grains has recently become an important issue for two reasons. First, the incidence and severity of FEB has increased worldwide and these have resulted in significant yield losses. The International Maize and Wheat Improvement Centre (CIMMYT) has identified FHB as a major factor limiting wheat production in many parts of the world. In addition to causing yield losses, FEB is of greater significance under certain circumstances because of mycotoxin accumulation which can occur in *Fusarium*-infected grain. Grain contaminated with *Fusarium* mycotoxins is unsuitable for both human and animal consumption because of adverse health effects of such toxins.

The main causal agents of FEB are *Fusarium culmorum*, *F. graminearum*, *Microdochium nivale* var *nivale* and var *majus* (formerly *F. nivale*), *F. avenaceum* and *F. poae*. The distribution and predominance of these pathogens is, to a large extent, determined by climatic parameters, particularly temperature and moisture. Hence, in hotter regions, *F. graminearum* predominates, whereas in cooler maritime areas *M. nivale* is favoured. In addition to small-grain crops, *Fusarium* has a wide host range among other grass hosts. Combinations of these pathogens can often occur on wheat ears. The impact of the environment on such disease complexes is poorly understood. The sporadic nature of FEB is largely attributed to the observation that wheat plants are most susceptible to the disease during anthesis, which is a relatively short phase of the growth. For successful infection during anthesis, inoculum must be available and optimum weather conditions must prevail. There is a general consensus, which has been arrived at largely through anecdotal evidence, that FEB is most severe in cereals where warm and wet conditions occur. However, there are still insufficient quantitative data on the impact of environmental parameters on FEB development caused by individual species, and combinations of species, to draw any firm conclusions.

Because of its economic importance, there is an immense literature on FEB worldwide. Central to the understanding of FEB development is the nature of the initial inoculum of *Fusarium* from the soil, which survives either as saprophytic mycelium or as thick-walled resting chlamydospores, depending on the species. This initial inoculum may result in the infection of seedlings, resulting in the development of seedling blight and foot rot. Later, during anthesis and the early seed development period, airborne conidia or ascospores may infect the ears of cereal plants and consequently this results in development of FEB. Research on FEB has been reviewed by Parry et al. (1995) and Dubin et al. (1997). The FEB situation in North America has also been reviewed (Bai and Shaner, 1994; McMullen et al., 1997). Consequently, this paper concentrates on reviewing research on FHB conducted over the last 7–8 years, focusing on three aspects of the epidemiology of the disease: inoculum, infection and forecasting.

Inoculum

Most species of *Fusarium* are spread by the dispersal of conidia that are blown or splashed to new infection courts. Among the species causing FEB, *F. graminearum* has an additional epidemiological advantage because it regularly forms abundant perithecia (*Gibberella zeae*), resulting in production of ascospores. Ascospores are forcibly discharged into the air, which greatly increases the dispersal distance from the colonised residue where perithecia form. Ascospores and conidia cause significant infections (Fernado et al., 1997; Scholz and Steffenson, 2001). A genetically modified strain of *G. zeae* was obtained by deleting the entire mating type locus (MAT) that controls sexual reproduction from a wild type (Brown et al., 2001). This MAT-deleted strain appears similar to the wild type in morphology and in its ability to produce macroconidia. Field inoculations showed that the MAT-deleted strain resulted in less disease and less trichothecenes than the wild strain, indicating that ascospores play an important role in the development of FEB epidemics and the production of FEB associated mycotoxins.

Production of conidia is critically influenced by temperature as well as moisture. Rossi et al. (pers. comm.) studied the effect of constant temperature regimes on sporulation of *F. avenaceum*, *F. culmorum*, *F. graminearium* and *M. nivale* on potato dextrose agar media. Different isolates within each species showed similar relationships of sporulation with temperature. *F. avenaceum* produced the greatest amount of spores, followed by *M. nivale* and then by *F. graminearium* and *F. culmorum*. The optimum temperature for production of macroconidia was 32 °C for *F. culmorum* and *F. graminearium*, 28 °C for *F. avenaceum* and 26 °C for *M. nivale*. Mathematical models were developed to describe these relationships for each species and validated against data obtained on artificially inoculated wheat stems.

Conidia are generally splash dispersed and thus the dispersal distance is generally limited. Laboratory research showed that the maximum dispersal height and distance of *F. culmorum* and *F. poae* are 60 cm and 70 cm, respectively (Horberg, 2002). Furthermore, the splash dispersal patterns are indistinguishable for the two species. Thus only a very small proportion of conidia on the crop debris at soil level can reach the ears by rain splash. It has been speculated that symptomless infections on green leaves, such as the flag leaf, may provide an important bridge between conidia on crop debris and ears. Isolation of apparently healthy and diseased leaves (with necrotic leaf spots) indicated that *Fusarium* species associated with FEB survive parasitically and saprophytically on leaves throughout the season (Ali and Francl, 2001).

One of the main sources of inoculum is colonised crop debris. This provides a site for abundant sporulation during the next growing season. The mixture of maize, wheat and barley production, especially when combined with widespread use of reduced tillage, has increased residue retention and set the stage for major epidemics of FHB to occur in small-grain cereals whenever weather is favourable for disease during flowering and heading. Andries et al. (2000) found that in Michigan the peak of perithecial production occurred just prior to flowering and that most (83%) of these perithecia were on maize debris. The proportion of mature perithecia was highly correlated with temperature during the 14 days prior to sampling, but not with relative humidity (RH). Temperatures below 3 °C appeared to inhibit perithecium formation. These findings closely parallel the 2-week cycle of development and maturation of perithecia demonstrated in the laboratory (Trail and Common, 2000). In China, where rice is rotated with wheat, the incidence of rice debris bearing ascospores could be as high as 80% (Lu et al., 2001). Ascospore production appears to be

critically dependent on soil moisture. When the soil moisture content is below 30%, ascospore production is not possible. When it is greater than 80%, ascospore production is at its maximum. Minimum and optimum temperatures for ascospore production are about 7–10 and 15–20 °C, respectively. Burning crop residues reduces the inoculum potential of *F. graminearium* present in residues and hence the potential inoculum for FEB (Dill-Macky and Salas, 2001). However, there are many other hosts, such as grasses and some broad-leaved weeds, which may provide an important alternative inoculum source.

Several researchers have observed a clear diurnal pattern of ascospore discharge (Paulitz, 1996; Fernado et al., 2000; Francl et al., 2000), beginning from 1600–1800 h, which peaked around midnight and then gradually declined. Peak ascospore release occurred 2–4 days after rainfall (>5 mm). Similarly, in China, ascospore release only occurred at near-saturation humidity (Lu et al., 2001). Paradoxically, Maldonado-Ramirez and Bergstrom (2000) found that most ascospores were discharged during daylight hours when atmospheric turbulence was highest. This pattern may provide the maximum opportunities for ascospores to be moved into the planetary layer, where vertical mixing occurs up to cloud level and the potential for long distance dispersal is greatest.

In general, ascospore dispersal is associated with rainfall events, although Thomas et al. (1999) and Francl et al. (1999) did not find any consistent relationships between the numbers of ascospores caught in trapping experiments and the actual amount of rainfall. The numbers of spores caught during anthesis were generally low but increased substantially thereafter (Thomas et al., 1999). Spores were not detected or only occurred sporadically during dry periods (Francl et al., 1999). Inoculum increased during rainy periods but the timing of this increase was variable. These results suggest that, whilst rainfall may be needed for perithecial and ascospore formation and maturation, it may not actually trigger the release of ascospores.

Infection

As outlined above, anthesis appears to be a period of increased susceptibility of ears to *Fusarium* infection. Much research has been directed towards understanding the exact pathways through which *Fusarium* spores infect and colonise ears. To facilitate such research, Bushnell et al. (1999) produced a transformed isolate of *F. graminearum* with a constitutively expressed gene for green fluorescent protein (GFP). Preliminary investigations using this GFP-transformed isolate showed that hyphae within host tissues were subcuticular and intercellular and that hyphae also appeared to grow into and out of leaf stomata. Furthermore, fungal development was limited in paleas, lemmas and coleoptiles. Infection and colonisation pathways on ears are currently being further investigated using this fungal isolate. Pritsch et al. (2000) found that spores germinated and penetrated the glume stomata and spread throughout the wheat head resulting in chlorosis of infected kernels. There were no differences in the infection structures between a resistant and a susceptible cultivar investigated. Under warm, mist-irrigated field conditions, colonies that formed on the abaxial (exterior) surface of the palea (near the kernel) and on the adaxial (interior) surface of the palea and lemma facing the floret mouth served as starting points for floret invasion (Lewandowski and Bushnell, 2001).

Infection of seeds by *F. graminearium* was investigated in plots inoculated with colonised corn debris and irrigated prior to and during anthesis (Argyris et al., 2001). The incidence of infection increased from c. 20% at 10 days after anthesis (DAA) to a maximum level >95%v at 37–40 DAA (harvest was at c. 50 DAA; physiological maturity, i.e. maximum dry seed weight, occurred between 30 and 32 DAA). There was a significant relationship between visual estimates of spikelet infection and seed infection in the four cultivars studied. Germination of seeds from inoculated plots declined progressively from 10 DAA to harvest (50 DAA); there was a significant negative correlation between germination and seed infection.

Knowledge of environmental factors that influence infection and subsequent FEB development is essential for assessing the potential disease risks and for developing efficient disease management strategies. There seems little doubt that the most important environmental factor that limits FEB development is moisture. Given a wet environment for an extended time, even a low initial inoculum level or a sub-optimal temperature does not prevent FEB from developing. Most studies which investigated the relationship between environmental factors and FEB development have been based on intensive field monitoring of inoculum strength, weather variables and disease development.

Development of FEB has been monitored in states of the USA over several years (De Wolf et al., 2000; 2001).

Differences in observed disease incidence between different field sites can be attributed partially to the differences in temperature and moisture. Lower temperatures and lack of surface wetness during anthesis are believed to be limiting factors for the low incidence of FEB. Modelling of these field data showed that environmental conditions prior to flowering were less important than those during anthesis. One of the important variables identified was the duration (hours) of RH >90% and temperature at 15–30 °C. Similar results were also obtained in Argentina indicating the importance of warm and moist conditions for FEB development (Moschini and Fortugno, 1996). Chinese researchers showed that ascospore germination is critically affected by temperature and humidity (Lu et al., 2001). At 25 °C and 100% RH, germination can be as high as 97.5% after 8 h and incidence of infection on stamens was about 58% after 20 h. Below 90% RH, germination was only about 0.4%.

The effects of temperature and humidity on the infection of wheat ears by *F. avenaceum*, *F. graminearum*, *F. culmorum* and *M. nivale* were studied recently using detached spikes in controlled environments (Rossi et al., 2001b). Infection of glumes was determined following incubation in wet conditions (4–72 h) at different temperatures. *F. avenaceum* and *F. graminearum* showed the highest incidence of infection, with optima at 28–29 °C. *M. nivale* and *F. culmorum* had lower incidences of infection, with optimum temperatures of 18 and 26.5 °C, respectively. Overall, the incidence of infection increased with increasing duration of wet periods, with *F. avenaceum* and *F. graminearum* increasing at a much faster rate than the other two species. For example, at the optimum temperature 30 °C, the incidence of infection by *F. avenaceum* increased from 0% after an 8 h wet period to 84% after a 72 h wet period, whereas with *M. nivale* at 15 °C, it increased from 0 to 22% under the same conditions. Empirical regression models were developed to relate the incidence of infection to the length of wet period and temperature and these models were used as a basis for developing forecasting models.

Recently, there has been a growing interest in understanding the spatial as well as the temporal dynamics of FEB epidemics. The spatial pattern of disease incidence appeared to be completely random in three out of four sampled fields (Shah et al., 2000; 2001), indicating that the primary inocula for infecting ears were distributed randomly and were thus more likely to be composed of external airborne ascospores. Only in one field was there some degree of clustering of diseased ears within the sampling quadrat. This may indicate that the inoculum was from clustered corn debris in soil. The field with an aggregated pattern had much a higher disease incidence than the other three fields. In another study, infected heads were found to be aggregated, in some cases highly so, within an individual wheat field, with the degree of aggregation increasing over time as disease incidence increased. The proportion of infected seeds varied greatly between seed lots, and this variability in seed infection was significantly greater than would be expected for a binomial (i.e. random) distribution in 72% of data sets (Shah et al., 2002).

Forecasting

A disease forecasting system is generally based on the combined effects of host susceptibility, inoculum strength and meteorological conditions on disease development. For FEB, the most susceptible stage appears to be anthesis, although considerable infection is still possible at the milky stage. Usually, inoculum strength, which is dependent on rainfall, RH, temperature and disease carry-over, etc. is very difficult to estimate. Many forecasting models have been developed for FEB and almost all are empirical regression models derived from long-term field observations. These models describe the overall effects of weather variables on epidemic development such as sporulation, spore dispersal, infection and subsequent disease development. These models may therefore not perform equally well in other regions because of the local nature of the field data. Details of these models are published and the models have been validated, although their usefulness in assisting practical disease management is often not known.

In China, there have been 19 documented forecasting models used in various regions (Lu et al., 2001). Most of these models use rainfall and temperature over various periods of time to estimate FEB risk. Some models also use measures of disease severity in the previous season and current spore catches to predict the disease risk. Similar models have been developed in other countries (Moschini and Fortugno, 1996; Lipps et al., 2001). A prediction model based on a single variable that combines both moisture and temperature correctly identified 83% of epidemic severity classes across several states in the USA (De Wolf et al., 2000; Lipps et al., 2001). In general, these models include the

effects of weather variables on two aspects of epidemic development:

1. Spore production. Sufficient rainfall about 8–10 days prior to and during anthesis facilitates production of both ascospores and conidia.
2. Spore dispersal and infection. Sufficient rainfall may be needed to disperse ascospores and/or conidia, followed by prolonged periods of warm humid conditions that are conducive for infection of ears.

The nature of these empirically derived models means that the exact weather variables and their weight in estimating the infection risk differed significantly between each model. This reflects differences in many of the factors in the data sets used to develop the models, such as the frequency/duration of field observations, the cultivars used, fungal pathogen species, inoculum strengths and climates. It remains a challenge for plant pathologists to develop a FEB forecasting model that can be used reliably over a wider range of conditions.

Recently, a system-based model development framework was adopted for developing a risk assessment model for FEB (Ross et al., 2001a). Such system-based models are usually developed by dividing the whole process into several sub-processes such that the individual sub-processes can be predicted accurately from variables that can be easily obtained. Risk indices are then derived by linking individual sub-models sequentially. This model included three sub-processes: sporulation, spore dispersal and infection. The model finally estimates daily infection risks. However, no validation results are yet available.

The future

There are several key areas in the epidemiology of FEB that need to be investigated before an effective management system can be developed for FEB in cereal production.

1. Most current research on the effects of weather conditions on FEB is based on intensive field observations. Such data are very useful for many purposes, but they cannot usually be used to develop a forecasting system applicable to a wide range of conditions. Infection studies need to be conducted in controlled environment conditions on whole plants instead of detached ears or spikelets.
2. The interrelationships between disease incidence/severity, fungal biomass and concentration of the associated mycotoxins need to be understood. Such knowledge should enable disease development to be forecast, and the potential production of mycotoxins to be predicted. Of course, the production of mycotoxins is likely to be influenced by post-infection and post-harvest conditions/events.
3. As FEB is a disease complex in most regions, the consequence of infection by multiple *Fusarium* species on disease development and the production of mycotoxins needs to be understood.
4. A better understanding of the relationship between the actual moisture level on the ear surface and atmospheric moisture, and how this relates to our laboratory measurement of 'wetness' is required.
5. There is a need to understand and characterise the spatial heterogeneity and patterns of FEB and their relationships to mycotoxins. Further research is also needed to understand the underlying physical and biological processes that are responsible for the resulting spatial heterogeneity and patterns. Such knowledge will enable an effective sampling scheme for disease assessment and quantification of mycotoxins to be designed.
6. Finally, in addition to the need to develop and validate forecasting models, it is necessary to integrate these models into practical production systems and to demonstrate their usefulness to a wider audience.

Currently, HRI-East Malling is co-ordinating an EU-funded project (RAMFIC) that attempts to obtain key knowledge in areas (1)–(3). The overall objective of this project is to obtain a large body of quantitative data on FEB (both visual disease severity and fungal biomass) and the production of associated mycotoxins in controlled environments and to develop quantitative risk assessment models, which will be refined using data collected from a large number of field sites in Europe with contrasting climatic conditions.

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Molecular tools to study epidemiology and toxicology of fusarium head blight of cereals

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Abstract

Fusarium head blight (FHB) of cereals is a disease complex. *Fusarium graminearum* is the major pathogen worldwide, while *F. culmorum*, *F. avenaceum* and *F. poae* are also associated with this disease. In addition to the true *Fusarium* species, *Microdochium nivale* may also cause head blight and is particularly prevalent where cooler, wetter conditions prevail. Other species such as *F. sporotrichioides, F. equiseti* and even *F. verticillioides* may also be of significance in particular situations. FHB is of particular concern because of the ability of the *Fusarium* species to produce mycotoxins in the grain that are harmful to human and animal consumers. The predominant mycotoxins within cereals are the trichothecenes, chiefly deoxynivalenol, nivalenol and their acetylated derivatives, along with T-2, HT-2, diacetoxyscirpenol and neosolaniol. This paper reviews the use of molecular techniques to identify the individual causal agents and to quantify their relative amounts within plant tissue. Diagnostic and quantitative polymerase chain reaction assays have been developed to detect and quantify individual fungal species within the disease complex and, where relevant, to differentiate between chemotypes within a single species. Assays to determine the type of toxin produced, or monitor the regulation of toxin production also provide valuable tools for understanding this disease. These techniques are being used to dissect the disease complex into its component parts in order to study interactions between the pathogens and their host and between the pathogens themselves as well as to determine the influence of environmental factors on the disease and the toxins produced by these fungi.

Introduction

Fusarium head blight (FHB) of wheat and other small-grain cereals constitutes a disease complex in which several fungal species may cause largely indistinguishable symptoms (Parry et al., 1995). Within the complex are fungi that produce mycotoxins (*Fusarium* species) and others that do not (*Microdochium nivale* varieties). To further complicate the situation, the different *Fusarium* species, and even different isolates, produce different toxins. Although a large number of *Fusarium* species have been isolated from blighted wheat, relatively few are considered to be of overall significance (Parry et al., 1995). *Fusarium graminearum* is the major pathogen worldwide, while *F. culmorum* tends to predominate in cooler, maritime regions where *F. cerealis* (syn. *F. crookwellense*) (Nirenberg, 1990) may also be found. *F. avenaceum* and *F. poae* are also frequently associated with FHB, with *F. poae* causing symptoms termed 'glume spot' (Kemp et al., 1996). In addition to the actual *Fusarium* species, *M. nivale* (formerly *F. nivale*) may also cause head blight and is particularly prevalent where cooler, wetter conditions prevail. Although these *Fusarium* species are of the greatest overall significance, it should be emphasised that other species such as *F. sporotrichioides, F. equiseti* and even *F. verticillioides* may be of significance in particular situations (Sugiura et al., 1993; Miller 1994; Tekauz et al., 2000). *F. verticillioides* is generally considered to be a pathogen of maize but has also been isolated from wheat (Chelkowski et al., 1995).

In addition to causing disease which leads to reduced yield and quality of grain, FHB is of particular concern because of the ability of the majority of the causal organisms to produce mycotoxins in the grain that are harmful to human and animal consumers (Joffe, 1986). Among these are trichothecenes, zearalenone, moniliformin, enniatins, beauvericin, and fumonisins. The predominant mycotoxins produced within cereals are the trichothecenes, a group of sesquiterpenoid

secondary metabolites. Four basic classes have been designated within the trichothecenes with types A and B being of most relevance to FHB. These two differ in the presence of a carbonyl group at position C-8 of type B trichothecenes. Type A trichothecenes such as T-2, HT-2, diacetoxyscirpenol (DAS) and neosolaniol (NEO) are significantly more toxic than type B trichothecenes such as deoxynivalenol (DON), acetylated derivatives including 3-acetyl (3-ADON) and 15-acetyl (15-ADON) and nivalenol (NIV) (Madhyastha et al., 1994; European Commission, 2002).

Fusarium graminearum and *F. culmorum* produce type B trichothecenes including DON, 3-ADON, 15-ADON and NIV. DON and NIV chemotypes (chemotypes I and II, respectively) are recognised within both species although the majority of isolates of *F. graminearum* and, possibly also *F. culmorum*, produce DON. With these species being the chief cause of FHB it is not surprising that DON and NIV are the most common trichothecenes detected in cereal grain worldwide (Placinta et al., 1999). It is unclear how or whether these compounds differ with respect to their phytotoxicity but chemotype and aggressiveness may be linked (see below). NIV is believed to be more toxic to humans and animals than DON or its acetylated derivatives, and hence is of importance with respect to food safety (Perkowski et al., 1997). NIV is particularly significant, as the monoclonal ELISA-based detection kits currently commercially available do not react to this compound.

Fusarium cerealis, F. poae and *F. equiseti* have also been reported to produce NIV (Sugiura et al., 1993; Liu et al., 1998; Langseth et al., 1999). In addition, *F. poae* and *F. equiseti* also produce type A trichothecenes including DAS (Langseth et al., 1999) and hence can produce both type A and type B trichothecenes. *F. sporotrichioides* produces type A trichothecenes including T-2 and HT-2 (Langseth et al., 1999). *F. avenaceum* is not known to produce trichothecenes but does produce other mycotoxins including moniliformin, enniatins and beauvericin (Golinski et al., 1996; Herrmann et al., 1996a; Logrieco et al., 1998). *F. verticillioides* also is not known to produce trichothecene mycotoxins but produces fumonisins (B1, B2 and B3) as well as beauvericin and moniliformin. Two groups, termed var. *nivale* and var. *majus* have been identified within *M. nivale* (Woolenweber and Reinking, 1935; Lees et al., 1995) but, in contrast to the *Fusarium* species, neither variety is known to produce mycotoxins (Logrieco et al., 1991).

Several parameters are used to quantify disease levels in epidemiological studies and in studies of host resistance and fungal pathogenicity. Visual symptoms of necrosis/bleaching of the glume tissues are assessed on a severity scale or as a percentage of infected spikelets either at a single time point or over several dates to produce an area under the disease progress curve (AUDPC). Harvested grain can be visually assessed for discolouration and shrivelling (tombstone kernels) and for yield loss (percentage yield or 1000 grain weight relative to controls). These disease parameters may then be related to the amount of mycotoxin present in order to establish the relationship between disease and toxin accumulation. Even when crops are inoculated with a single species or isolate the relationship between disease parameters and toxin content may not be closely related (Mesterhazy et al., 1999). Miedaner et al. (2001a) reported that no relationship could be observed between disease severity and DON content across environments with similar disease severities. Such results indicate the important influence of the environment although differences in the status of the inoculum may also be important. Under natural conditions, where pathogen populations are involved in producing disease, the relationship between disease and toxin content will inevitably be yet more complicated.

Dissection of the disease complex: The need for new tools

The presence of toxin-producing and non-toxin-producing species within the disease complex, along with isolates of differing chemotype greatly complicates attempts to understand the factors that influence disease development and toxin accumulation. The relative contribution of each of the species in causing disease in a particular situation will depend upon a range of variables, as will the amount of toxin produced by that species. Only by identifying and understanding the nature of the interactions between the environment and the causal organisms will it be possible to understand the consequences with respect to mycotoxin accumulation in grain and, ultimately, the subsequent threat posed to human and animal consumers.

Attempts to evaluate the relative role of each fungal species in a particular context by means of isolation into axenic culture may be confounded, because in the absence of selective media, the relative amounts of

each pathogen may not be accurately determined. For example, *F. culmorum* tends to out-compete *M. nivale* on most growth media and this may result in an underestimation of the prevalence of the latter in mixed infections (Pettitt et al., 1993). Whatever the method, the isolation of pathogens from plant tissues can only reveal what may be grown out of the plant rather than what is within the plant. It would be most desirable to be able to determine the identity, amount and, if possible, the location of each pathogen within the host tissues.

A number of molecular techniques are being utilised in order to understand the nature and diversity of the pathogens responsible for FHB. Molecular techniques are also being used to study the interactions between the pathogens and their hosts and between the pathogens themselves along with the influence of environmental factors such as fungicides on these interactions. The majority of techniques are based upon analysis of DNA or RNA although measurement of ergosterol using high pressure liquid chromatography (HPLC) is also used as a generic estimator of fungal biomass in many studies involving inoculation of plants with characterised isolates of known species.

An important aspect of these studies is the influence of the environment (in the broadest sense) on the production and accumulation of mycotoxins within grain and other plant tissues. With respect to the mycotoxins, most attention is paid to the trichothecenes but the potential of species to produce many different related compounds complicates attempts to study the factors affecting overall regulation of toxin biosynthesis. Assays to permit study of the regulation of trichothecene biosynthesis would aid investigations into the effect of host and environment on the toxigenic fungi involved in FHB. It is likely, however, that detailed chemical analysis will continue to be required to determine the amount of each trichothecene product within harvested grain.

Detection and identification of the causal agents by polymerase chain reaction (PCR)

The PCR offers a sensitive and potentially specific means to detect, identify and quantify the species present within plant tissues. A number of assays have been produced to permit detection of many of the major pathogens associated with FHB. The PCR assays are available for *F. graminearum* (Schilling et al., 1996; Niessen and Vogel, 1998, Nicholson et al., 1998), *F. culmorum* (Schilling et al., 1996; Nicholson et al., 1998), *M. nivale* var. *majus* and var. *nivale* (Nicholson et al., 1996), *F. poae* (Parry and Nicholson, 1996), *F. cerealis* (syn. *F. crookwellense*) (Yoder and Christianson, 1998), *F. avenaceum* (Schilling et al., 1996; Turner et al., 1998) and *F. verticillioides* (Möller et al., 1999). The development and use of these assays is complicated by the difficulty of correctly identifying isolates in axenic culture and our still limited understanding of phylogeny within the *Fusarium* genus. For example, several assays for *F. avenaceum* have subsequently been shown to cross react with *F. tricinctum* (Turner et al., 1998). Conventional taxonomy places these two species in separate sections (*Roseum* and *Sporotrichiella*, respectively). Molecular studies, however, have demonstrated that the *Sporotrichiella* section is a phenetically diverse group and that *F. tricinctum* and *F. avenaceum* are indeed closely related (Bateman et al., 1996; Turner et al., 1998). This demonstrates the importance of testing the specificity of assays against all species with the potential of being co-isolated and not just those believed to be closely related on the basis of our current understanding.

FHB pathogen diversity and populations

Molecular phylogeny is revealing the presence of cryptic variation that may have significance with respect to the epidemiology of FHB. Although *F. graminearum* has been isolated in all major agricultural regions of the world little was known, until recently, about the population structure of *F. graminearum* from different geographical areas and whether these differences were related to pathogenicity or any other trait. Genetic variation within *F. graminearum* has been detected by random amplified polymorphic DNA (RAPD) analysis. Although isolates from different continents have been examined (Schilling et al., 1994; Carter et al., 2002), most analyses have compared isolates from closer geographical sites, e.g. within Europe (Schilling et al., 1997), Canada (Dusabenyagasani et al., 1999), Nepal (Carter et al., 2000) and North Carolina, USA (Walker et al., 2001). While isolates from two regions (Ontario and Quebec) in Canada were considered to form a single population pool (Dusabenyagasani et al., 1999), two distinct groups were identified among Nepalese isolates (Carter et al., 2000). The latter study also characterised isolates using a sequence characterised amplified region (SCAR). This revealed

that all isolates of one group (termed B) possessed a single SCAR polymorphism while those of the second group (group A) had one of five different SCAR polymorphisms (Carter et al., 2000).

RAPDs have also been used to assess variability within *F. culmorum* (Miedaner et al., 2001b). These workers reported a relatively high level of genotypic variability within a population of *F. culmorum* from a field in Novgorod, Russia. The level of variability was even greater than that within a population of *F. graminearum* from a field in Sersheim, Germany. The reasons for the high level of variability within a fungus with no known teleomorph are unclear but these findings may reflect the consequences of a wide range of selective pressures upon fungi, such as *F. culmorum,* which may have both saprophytic and parasitic phases within their life-cycle.

Isolates of *F. graminearum* collected from wheat, maize and rice in Nepal separated into two groups (termed A and B) on the basis of RAPD analysis (Carter et al., 2000). The distinction might be related to host preference, with isolates of group B being less frequently isolated from wheat or rice than maize. Similarly, Satyaprasad and Bateman (1997) observed two groups within *F. avenaceum* on the basis of PCR-RFLP of the internal transcribed spacer (ITS) region of ribosomal DNA, with one being more frequently isolated from white lupin than the other. It was concluded that sub-groups related to host preference may exist within *F. avenaceum* (Satyaprasad and Bateman, 1997).

Isolates of *F. graminearum* from different geographic origins vary in their pathogenicity towards different hosts. The pathogenicity of Chinese and USA isolates has been tested on wheat, and in a comparison of six isolates, one Chinese isolate caused significantly greater disease symptoms than did any of four isolates from the USA (Bai and Shaner, 1996). In a study of pathogenicity towards rye, the genetic variation among isolates from three continents, America, Australia and Europe, was greater than that obtained for strains from populations of *F. graminearum* from individual sites in Europe (Miedaner and Schilling, 1996). The individual field populations, however, contained 60% of the total variation observed, indicating that a high degree of variation in pathogenicity was present at each site. Isolates of *F. graminearum* across Europe and the USA differ from those in the two groups (A and B) found in Nepal and form a distinct group (Carter et al., 2002). This group (group C) was found to be more aggressive towards wheat and maize seedlings than group A or B although the pathogenicity of individual isolates within each group also varied.

DNA sequence analysis has been used to differentiate *F. graminearum* into at least seven biogeographically structured lineages (O'Donnell et al., 2000). The relationship between the RAPD groups (Carter et al., 2000; 2002) and those of the sequence-derived lineages (O'Donnell et al., 2000) has yet to be fully determined but groups A, B and C appear to be largely congruent with lineages 6, 2 and 7, respectively. Evidence obtained to date indicates that these groupings are of biological significance with respect to host preference and aggressiveness (Carter et al., 2002) and hence it is important that epidemiological studies define the causal agent down to the level of group/lineage in order to place the results into context.

Serendipitously, the region amplified by the *F. graminearum* PCR assay developed by Nicholson et al. (1998), is polymorphic and amplicon size and/or sequence is generally associated with a RAPD group. This permits an immediate tentative designation of *F. graminearum* isolate/causal agents within plant tissues to different RAPD groups (Carter et al., 2000; 2002). The use of single strand conformational polymorphism (SSCP) enables differentiation between amplicons that differ only in sequence (Qu Bo, unpublished results), thus increasing the resolving power of this assay. These assays can be used to establish the distribution of FHB causal agents, down to the level of group/lineage, across regions and within crops and even within individual plants. At present the groups/lineages appear to be related to geographic regions, but such distinctions may disappear as a result of global trade in grain and plant products aiding distribution of these pathogens.

Diagnostic PCR assays for FHB pathogens

The annual Department for Environment, Food and Rural Affairs (DEFRA)-funded survey of winter wheat diseases revealed that the incidence of FHB was significantly greater in 1998 than in previous years (J.A. Turner and P. Jennings, Central Science Laboratory, personal communication). Harvested grain from severely affected sites was analysed for trichothecene mycotoxins and for the major FHB pathogens using competitive PCR. The predominant pathogen was *M. nivale* var. *majus*, which was present in 94% of samples. The preponderance of non-mycotoxin producing species in the UK epidemic of 1998, may

largely have accounted for the relatively low levels of mycotoxins present in the majority of samples. The detection of *F. graminearum* in over 40% of samples was of particular significance as *F. graminearum* had previously been found only very rarely in the UK. The cause of the apparent increase in this species is not known but it may be related to altered agronomic practices, such as the increased area of forage maize in the UK, or to environmental factors. DON and NIV were the major toxins detected in grain and there was a strong correlation (0.68) between the levels of these two toxins and the amount of *F. culmorum* and *F. graminearum* detected, indicating that these two species were probably largely responsible for the presence of the type B trichothecenes detected.

HT-2 toxin was detected in 38% of the samples but the causal agent could not be determined because, at that time, no PCR assays were available for species known to produce these toxins. Recently, Wilson et al. (unpublished) have developed assays for *F. sporotrichioides* and *F. langsethiae*, both producers of T-2 and HT-2. When comparisons were made between the incidence of *F. langsethiae* and *F. sporotrichioides*, and the incidence of toxin in the UK samples from 1998 (HGCA, 1999), it was found that up to 90% of type A trichothecene (T-2 and HT-2) production could be accounted for by the presence of these two species within the samples. Moreover, it was found that the incidence of *F. langsethiae* and *F. sporotrichioides* were independent of each other but the factors that determine the distribution of the two species are not known.

PCR has also been used to detect individual species in single field plot experiments and to determine their tissue localisation and relationship to visual disease symptoms (Doohan et al., 1998). Whereas *F. culmorum* was detected in grain, glume and rachis tissues, *M. nivale* varieties were found mainly in rachis tissue. *F. poae* was detected predominantly in glume tissues and was rarely found in the rachis indicating that this pathogen may be less able to colonise wheat heads than *F. culmorum.* Polley and Turner (1995) reported that *F. poae* was most commonly isolated from glumes, a finding in agreement with that of Doohan et al. (1998). PCR also detected *Fusarium* species in tissues from heads lacking disease symptoms. This is of significance because other workers have reported finding trichothecene mycotoxins in apparently healthy wheat heads (A. Mesterhazy, personal communication).

Quantification of FHB causal agents

Ergosterol has been used in a number of studies to estimate the degree of fungal colonisation of grain (Miedaner and Perkowski, 1996; Gang et al., 1998; Miedaner et al., 2000). Such studies are possible where known isolates are used to inoculate plants and where the influence of secondary infection is minimal. Host resistance was found to be the major factor affecting fungal colonisation and disease development on winter rye by *F. culmorum*. Mycotoxin content, however, was most influenced by environmental factors interacting with the host genotype (Miedaner and Perkowski, 1996). These results support those of others who reported the strong influence of environmental factors on the relationship between disease severity and DON content (Mesterhazy et al., 1999). In a separate study, Miedaner et al. (2001) reported that, while disease levels were similar in wheat, triticale and rye cultivars, the level of DON was generally higher in wheat than in rye indicating a potential role of the host species in determining the accumulation of DON. The ability to degrade DON has been proposed as a potential resistance mechanism (Miller et al., 1985) and naturally occurring plant compounds have been shown to inhibit trichothecene toxin biosynthesis by *F. sporotrichioides in vitro* (Desjardins et al., 1988). While the ratio of DON to disease differed among the cultivars, the ratio of DON to ergosterol was similar in wheat and rye indicating that similar levels of visual disease may not reflect similar levels of colonisation and, hence DON content, in different species.

Ergosterol measurement has also been used to assess aggressiveness among FHB pathogens. When isolates of *F. culmorum* and *F. graminearum* of differing aggressiveness were examined, increased levels of disease correlated with increased colonisation, as measured by ergosterol content, and with increased DON content of grain (Miedaner et al., 2000; Gang et al., 1998). Thus the greater the amount of fungus present in the grain, the higher the level of DON. The ratio of DON to ergosterol, however, did not correlate with disease in either study indicating that the differences in aggressiveness are probably not due to differences in the rate of production of DON. The use of ergosterol to estimate fungal colonisation is severely limited as the assay measures the total amount of all fungi present rather than the amount of a specific species.

Although conventional PCR is an extremely sensitive and potentially specific technique, due to the nature of the assay it is generally unsuitable for quantification.

Minor differences in amplification efficiencies, such as might occur where two samples differ in the level of inhibitory impurities present, would result in large differences in amplicon yield at the end of the reaction. Competitive PCR and, more recently, 'real-time' PCR assays have been developed to address this problem with respect to the study of FHB. These assays can be used to detect and quantify the individual fungal species in plant tissues even where they are present as part of mixed infections. Competitive PCR assays to detect and quantify the amount of each of the target species, based upon the amount of DNA of that species present, have been developed for *F. graminearum* and *F. culmorum* (Nicholson et al., 1998), *M. nivale* var. *majus* and var. *nivale* (Nicholson et al., 1996), *F. poae* (Parry and Nicholson, 1996) and *F. avenaceum* (Turner et al., 1998). Recently, Simpson et al. (unpublished) developed a 'real-time' PCR assay for *F. culmorum*. These assays provide the means to dissect the FHB complex into its component parts and thus determine the prevalence and role of each component in disease development and under various conditions.

Competitive PCR has been used in a number of studies of FHB, principally to study the effect of fungicides on disease and mycotoxin accumulation. Doohan et al. (1999) found that competitive PCR provided greater resolution than visual disease assessment for determination of fungicide efficacy and highlighted the difficulty of relating symptoms on glume tissues to colonisation of grain, even where only a single pathogen is present.

Attempts to determine fungicide efficacy against disease complexes such as FHB are complicated because of the potential for fungal components controlled by the treatment to be replaced by others less susceptible to the fungicide. It is conceivable for non-toxin producing species to be replaced by toxin producers. Control of FHB by fungicides has often proved to be erratic and it is possible that environmental factors and replacement of one pathogen by others may have contributed to this. Simpson et al. (2001) determined the levels of FHB pathogens in naturally infected and inoculated field experiments. The results demonstrated that fungicide application influenced the pathogen population through selective suppression of particular species by individual fungicides. Application of tebuconazole suppressed *F. culmorum* whereas azoxystrobin suppressed *M. nivale*. Suppression of either species tended to result in an increase in the amount of the other, indicating that an antagonistic/competitive interaction exists between these two species. The finding from other studies that *F. culmorum* and *M. nivale* were found together less often in single wheat heads than expected by chance supports the view that a competitive/antagonistic interaction exists between these species (Doohan et al., 1998).

The interaction between *Fusarium* species and *M. nivale* has important consequences for mycotoxin accumulation. Grain from plots treated with azoxystrobin had more DON than untreated plots or those treated with tebuconazole. The increase in DON may largely be due to increased colonisation by *Fusarium* species where the azoxystrobin fungicide has suppressed the competing *M. nivale* (Edwards et al., 2002; Nicholson et al., unpublished). However, in some instances the increase in DON is not associated with an increase in the amount of fungus detected in the grain suggesting that the fungicide may have a stimulatory effect on the production of toxin (Simpson et al., 2001; Nicholson et al., unpublished).

Quantitative PCR enables the detection and quantification of individual species within plant tissues or other matrices. Thus this technique is ideal for studying the interactions between FHB species. Simpson et al. (2000) co-inoculated seedlings of wheat, rye and oats with *M. nivale* vars. *majus* and *nivale*. At 15 °C var. *majus* colonised wheat and oat stems more than var. *nivale.* In contrast, on rye var. *nivale* had a significant competitive advantage over var. *majus*. Further studies have shown that the effect of the host species on competition between the two varieties also occurs at 10 and 20 °C (Simpson et al., unpublished).

More recently, competitive PCR has been used to investigate interactions between *F. culmorum* and *M. nivale* vars. *majus* and *nivale* on seedling cereal plants (Simpson et al., unpublished). When wheat seedlings were inoculated with both *F. culmorum* and *M. nivale* var. *majus*, colonisation by both species was reduced relative to when they were inoculated alone (Figure 1). Such studies confirm that these head blight pathogens are antagonistic to one another. When *F. culmorum* and *M. nivale* var. *nivale* were inoculated together, *F. culmorum* appeared to be unaffected whereas colonisation by *M. nivale* var. *nivale* was significantly reduced (Figure 1) which would suggest that *F. culmorum* interacts differently with the two varieties of *M. nivale*. At present, it is not known whether competition between *Fusarium* species is influenced by the host species. Such information would yield important information regarding the relative abilities of particular

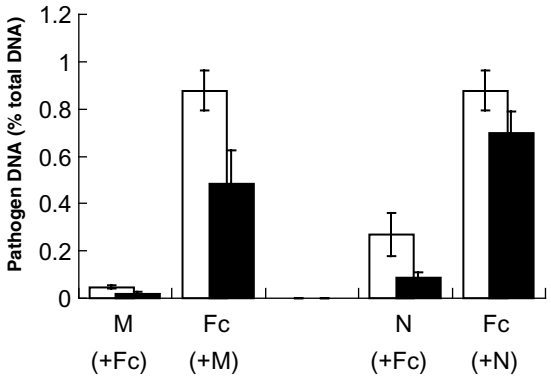

Figure 1. Amount of pathogen DNA detected (mean and one standard error) of *M. nivale* var. *majus* (M), *M. nivale* var. *nivale* (N) and *F. culmorum* (Fc) inoculated on wheat seedlings grown at 20 °C for 18 days. Open columns represent the DNA of each pathogen when inoculated alone. Hatched columns represent the amount present following mixed inoculation of *F. culmorum* and either variety of *M. nivale*. Error bars are one standard error.

fungal species to develop on particular cereal hosts and thus highlight the potential for particular mycotoxins to accumulate in grain of infected plants.

As well as interactions between the FHB pathogens themselves, it should be borne in mind that saprophytic elements of the microflora may also affect growth of pathogenic species (Liggitt et al., 1997) and also influence accumulation of mycotoxins (Ramakriskna et al., 1996). The use of similar assays designed for relevant species such as *Alternaria alternata*, and *Cladosporium herbarum* is highly desirable to increase our ability to identify all the players present in a particular niche and to partition effects accordingly. Competitive or 'real time' PCR assays such as those described above provide a means to detect and quantify each pathogen species and thus determine how they interact with one another and with other components of the microflora. These tools can also reveal how the interactions between pathogens and the host are influenced by environmental factors, including fungicide application.

The work described above highlights the role of host resistance and host species in influencing the development of FHB disease and hence mycotoxin accumulation. It is important to bear in mind that many of the FHB pathogens may also have a significant saprophytic element to their life-cycle, either through survival on colonised crop debris or as resting structures such as chlamydospores (Cook and Bruel, 1968; Nyvall, 1970). The ability to survive and multiply to become a significant source of inoculum for FHB may differ between species (Sitton and Cook, 1981) and contribute to determine the particular species that predominate in a given situation. There have been few attempts to examine this element of FHB epidemiology with molecular tools, but they should provide an insight into this component of a pathogen's life-cycle.

Detection and quantification by metabolite (toxin) profile

While determination of the relative proportions of individual FHB species is important, attention in many studies focuses on the amount of toxin accumulating in grain. In some instances, it is of more interest to determine whether toxigenic species are present than to identify exactly what species are present. The PCR-based tools have been developed to address diagnosis of pathogens by their toxigenic potential. For example, the first dedicated step in the trichothecene biosynthetic pathway is the isomerisation and cyclisation of farnesyl pyrophosphate to trichodiene by the enzyme trichodiene synthase (encoded by the gene *tri5*) (Proctor et al., 1995). The *tri5* gene is common to all known trichothecene-producing *Fusarium* species. PCR assays based upon sequences of this gene conserved among *Fusarium* species have been developed to enable the detection of the presence of fungi with the potential to produce trichothecenes. Doohan et al. (1999) and Edwards et al. (2001) developed competitive PCR assays based upon *tri5* to quantify the amount of potential trichothecene-producing *Fusarium* species present in plant tissues. Schnerr et al. (2001) have recently produced a 'real time' PCR assay for this gene. Similarly, a PCR assay has been developed to the *esyn1* gene that encodes enniatin synthetase to permit detection of *Fusarium* species with the potential to produce enniatins (Doohan et al., unpublished; Simpson et al., unpublished).

The importance of trichothecenes to the producing fungus and their role in causing disease has been much debated (Adams and Hart, 1989; Atanassov et al., 1994). Trichothecene-deficient mutants of *F. graminearum* have been produced through molecular gene disruption of *tri5* (Proctor et al., 1995) and a number of studies have provided information on the role of trichothecenes in pathogenicity of *Fusarium* species towards wheat and other species (Proctor et al., 1995; Desjardins et al., 1992; 1996) These studies have demonstrated that trichothecenes are not essential for

pathogenicity but may act as virulence/aggressiveness factors that result in toxin producers causing more disease than non-toxin producers (see below). Using competitive PCR strains of *F. graminearum* capable of producing trichothecenes have been shown to colonise wheat grain to a greater extent than non-producing strains (Nicholson et al., 1998; Smith unpublished). Similar studies of the infection of maize indicate that trichothecene-producing isolates of *F. graminearum* also colonise maize kernels to a greater extent than non-producing strains (Harris et al., 1999). The effect of trichothecenes on the aggressiveness of the pathogen appears, however, to be dependent upon the host species. Disruption of the *tri5* gene of *F. sambucinum* led to reduced virulence on parsnip root slices but not upon slices of potato tuber (Desjardins et al., 1992). Similarly, while disruption of the enniatin synthetase gene (*esyn1*) of *F. avenaceum* led to reduced levels of virulence against slices of potato tuber (Herrman et al., 1996b), it has not been possible to detect any effect on virulence against wheat and rye seedlings (Thomsett et al., unpublished). Trichothecenes and other toxins produced by *Fusarium* species may therefore aid infection and colonisation (aggressiveness) on some hosts while they have no such apparent role on others.

It is recognised that environmental factors play an important role in infection and development of FHB and mycotoxin accumulation (Miller, 1994; Schaafsma et al., 2001). The weather conditions during the latter stages of development are most important because wheat is most susceptible to infection at anthesis (Miller, 1994). The amount of mycotoxin accumulating also depends upon the resistance to infection of the host (Schaafsma et al., 2001), although significant genotype × environment interactions have also been observed in many studies (Miedaner et al., 2001). Trichothecene accumulation has been assessed in grain and chaff components of cultivars that differ in their susceptibility to FHB caused by *F. culmorum*. Ears of a susceptible wheat cultivar and a moderately resistant cultivar were spray-inoculated with conidia of *F. culmorum* at mid-anthesis (GS 65) (Zadoks et al., 1974). Inoculated ears were harvested at GS 90 and separated into chaff and grain components. Fungal colonisation was assessed using competitive PCR and levels of DON were determined using a commercial antibody test kit (Ridascreen – DON fast kit). Similar levels of fungal colonisation and toxin content were observed in the chaff of both cultivars (Gosman, 2001). As anticipated, the grain of the susceptible cultivar contained significantly greater amounts of DNA of *F. culmorum* than that of the resistant cultivar. The grain of the susceptible variety also contained higher levels of DON than the resistant variety. However, the ratio of DON to fungal DNA was significantly greater in the resistant than in the susceptible cultivar. A similar situation was observed among doubled haploid lines produced from a cross between a susceptible wheat cultivar (Riband) and a resistant cultivar (Arina) (Figure 2). Lines with higher resistance to FHB had much lower amounts of fungal DNA in the grain and reduced levels of DON, with the consequence that the ratio of DON to fungal DNA increased with greater resistance. Such findings suggests that, in the more hostile environment of the resistant cultivar, the fungus may produce more toxin than in the relatively benign environment of the susceptible host. Further studies are required to determine what temporal and/or spatial differences in the production of trichothecenes occur during infection of cultivars differing in their resistance to FHB. It is conceivable that toxin biosynthesis is enhanced when the fungus is experiencing stresses such as nutrient deprivation or exposure to antifungal substances produced by the resistant host cultivar. The form and level of stress will probably also vary between tissues.

Regulation of trichothecene biosynthesis

Because of the potential number of trichothecenes produced by a particular fungal species, or even isolate,

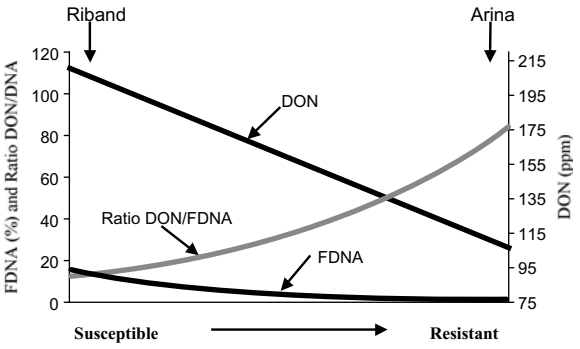

Figure 2. Trend for fungal colonisation, measured as fungal DNA (FDNA; fungal DNA as % of total DNA per sample) content determined using competitive PCR, DON (ppm) content and ratio of DON/FDNA in grain of a doubled haploid population (60 lines) derived from a cross between a susceptible (Riband) and a resistant (Arina) cultivar of wheat. Lines are ordered according to increasing resistance.

techniques to permit study of the overall regulation of the trichothecene biosynthetic pathway might be particularly useful for the identification of factors that prevent or enhance toxin production. The isomerisation and cyclisation of farnesyl pyrophosphate to trichodiene by the product of *tri5* occurs before the branching off to form the various trichothecenes and can act as a marker of overall activity in the pathway. Previous studies have shown that transcriptional control plays an important part in regulation of *tri5* expression, making this a suitable target for the study of factors affecting regulation of the biosynthesis of trichothecene mycotoxins (Hohn et al., 1993). A reverse transcription (RT)-PCR assay has been developed to enable study of *tri5* gene expression in trichothecene-producing strains of *Fusarium* species (Doohan et al., 1999). As noted above, stress may play a role in the regulation of trichothecene biosynthesis. As well as host-derived factors, environmental factors, including fungicides, may affect toxin production. There is growing evidence that sub-lethal concentrations of certain fungicides may increase mycotoxin production by *Fusarium* species (D'Mello et al., 1998). The possibility that this may occur under field conditions is supported by a limited number of reports in which the mycotoxin content of grain has increased following fungicide treatment (Simpson et al., 2001). *In vitro* studies using the *tri5* RT-PCR assay demonstrated that Folicur and Sportak fungicides induced a transient increase in expression of *tri5* of *F. culmorum* before levels fell to below those in the control cultures (Doohan et al., 1999).

Very little is known about the temporal and spatial regulation of trichothecene biosynthesis during infection and colonisation of wheat heads. Preliminary studies of the expression of *tri5* in grain and chaff of heads of a susceptible cultivar have been undertaken (Doohan et al., 1999). Expression of *tri5* was significantly greater in chaff (glume tissues) than in grain at GS 70 and GS 80. While expression levels in the chaff remained similar at GS 70 and GS 80, *tri5* expression was not detected in grain at this time (Doohan et al., 1999). These results indicate that, at least until GS 80, expression of *tri5* declines in grain while remaining high in glume tissues. It is of interest to determine which factors underlie the differential regulation of expression in the different tissues. Differential tissue-related patterns of *tri5* expression have since been observed in other wheat cultivars that differ in their susceptibility to FHB (Draeger et al., unpublished).

From the toxicological perspective, focusing upon *tri5* as a generic marker for trichothecenes is problematic since the toxicity of type A and type B trichothecenes differs markedly (Madhyastha et al., 1994; European Commission, 2002). Sequence analysis of 28S ribosomal DNA revealed that trichothecene producing *Fusarium* species can be resolved into two monophyletic groups (Mulè et al., 1997). The phylogenetic relatedness of species producing type A or type B trichothecenes has been utilised to develop PCR assays to detect species on the basis of trichothecene type (Simpson et al., unpublished). Such assays may provide sufficient resolution for many epidemiological studies.

Identification of chemotype and relevance to FHB

Even within a species, the potential for different isolates to produce different trichothecene toxins is significant when attempting to relate environmental factors to disease development and toxin accumulation. Although DON is the predominant toxin produced by most isolates of *F. graminearum* found across Europe (group C or lineage 7) some isolates produce NIV. The aggressiveness of DON-producers towards rye was found to be slightly greater than that for NIV-producers (Miedaner et al., 2000). To date, all isolates of *F. graminearum* group B/lineage 2 produce NIV, while both chemotypes are present within group A/lineage 6 isolates (Carter et al., 2002). While isolates of both DON and NIV chemotypes of group A were similarly aggressive towards wheat seedlings, NIV chemotypes were more aggressive towards maize than DON chemotypes (Carter et al., 2002). Thus, when evaluating the effect of particular factors upon disease development the chemotype of the isolate may be of significance. Both chemotypes have been observed within *F. culmorum* and both types appear common in Europe (Bakan et al., 2001). The aggressiveness of DON chemotypes of *F. culmorum* is generally greater than that of NIV types against both wheat and rye (Gang et al., 1998; Muthomi et al., 2000). Only very recently has the genetic basis for the difference in chemotype been determined (Lee et al., 2001; 2002). Two genes (*tri7* and *tri13*) appear non-functional among isolates of the DON chemotype, making them unable to produce NIV. Assays for both genes have been developed to allow the detection and differentiation of NIV and DON chemotypes among type B

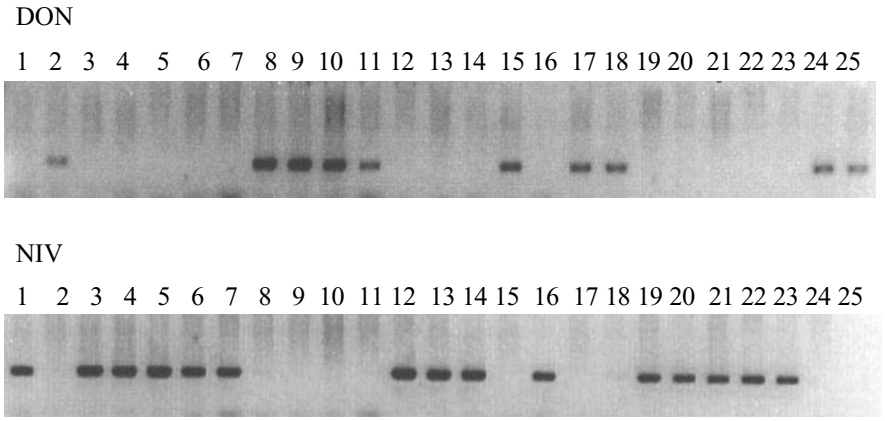

Figure 3. PCR assays of 25 isolates of *F. graminearum* with primers designed to specifically amplify from non-functional DON and functional NIV alleles of *tri7* in order to determine anticipated trichothecene production: DON or NIV.

trichothecene producing species (Chandler et al., unpublished) (Figure 3). These primers will permit detailed study of interactions between, and the competitive advantage of, NIV and DON chemotypes on different cereal hosts and against different saprophytic and pathogenic species associated with FHB.

Summary

The study and control of FHB is complicated because of the involvement of several fungal species and several mycotoxins in the disease. A number of molecular tools have been developed to permit dissection of this complex and enable the identification of the factors important in determining where the disease becomes established and which species might predominate in a particular situation. Diagnostic PCR assays have contributed to the detection of species new to the UK, such as *F. graminearum*, which was formerly rare in this country. Competitive and 'real time' PCR assays permit the relative amount of each species to be determined, even where they are present as part of a mixture. These assays are revealing how the different species interact with each other and with the cereal host. As the number of assays and ease of use increases, our ability to relate the presence and amount of particular causal agents to the amount and type of mycotoxin present in grain will also improve. This will contribute towards our understanding of the disease complex to reveal how host and environmental factors such as fungicide application affect the balance between species as well as the consequences for mycotoxin accumulation. Molecular tools such as the *tri5* RT-PCR assay provide the ability to study temporal and spatial regulation of the trichothecene biosynthetic pathway and to understand the effect of environmental factors and other components of the microflora of the wheat plant on mycotoxin production. Where isolates of the same species differ in chemotype, molecular assays related to the relevant genes provide a means to include the aspect of chemotype into efforts to understand the complex interactions between the FHB pathogens, the host, other microbes and the environment. These assays, along with those developed elsewhere, are useful tools with which to increase our understanding of the factors that influence FHB and, ultimately, our ability to control this disease and eliminate the risk of mycotoxin contamination of grain and foodstuffs.

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Epidemiology of *Fusarium* diseases and their mycotoxins in maize ears

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Abstract

Fusarium species cause two distinct diseases on ears of maize, Fusarium ear rot (or pink ear rot) and Gibberella ear rot (or red ear rot), both of which can result in mycotoxin contamination of maize grain. The primary causal agent for Fusarium ear rot is *Fusarium verticillioides*, but *F. subglutinans* and *F. proliferatum* are also important. Gibberella ear rot is caused primarily by *F. graminearum*, but *F. culmorum* can also be important, especially in Europe. Aspects of the epidemiology of both diseases have been studied for decades, but only recently have efforts been made to synthesize this information into comprehensive models of disease development. Much of the work on *F. graminearum* has focused on Fusarium head blight of small-grain crops, but some of the results obtained are also relevant to maize. The primary mycotoxins produced by these fungi, fumonisins and deoxynivalenol, have differing roles in the disease-cycle, and these roles are not completely understood, especially in the case of fumonisins. Progress is being made toward accurate models for risk assessment of both diseases, but key challenges remain in terms of integrating models of pre- and post-infection events, quantifying the roles of insects in these diseases, and characterizing interactions among competing fungi and the environment.

Introduction

Fungi in the genus *Fusarium* are the most common causes of ear and kernel rot diseases of maize. The importance of these diseases has been recognized for many decades, but they remain difficult problems because high levels of genetic resistance have not been discovered and incorporated into high-yielding, agronomically desirable hybrids. The complexity of developing resistant maize hybrids is related to the multi-faceted epidemiology of *Fusarium* diseases. With a more complete knowledge of *Fusarium* epidemiology with respect to maize comes a greater probability of identifying effective resistance mechanisms.

Fusarium infection of maize ears and kernels comprises two distinct diseases that differ, but also overlap, in their epidemiological characteristics. Gibberella ear rot or 'red ear rot' usually initiates from the tip of the ear and develops a red or pink mold covering a large proportion of the ear. Usually, it is caused by *Fusarium graminearum* (teleomorph *Gibberella zeae* (Koehler, 1959; Miller, 1994; Smith and White, 1988), although in Europe several other *Fusarium* species may be associated with this disease, especially *F. culmorum* (Logrieco et al., 2002; Miller, 1994). Gibberella ear rot predominates in cooler areas or those with higher precipitation during the growing season (Bottalico, 1998; Koehler, 1959; Logrieco et al., 1993; Smith and White, 1988). Fusarium ear rot typically occurs on random kernels, groups of kernels or on physically injured kernels (Koehler, 1959; Miller, 1994) and consists of a white or light pink mold. Identical symptoms are caused by *F. verticillioides* (syn. *F. moniliforme*), *F. proliferatum* or *F. subglutinans*, but occasionally other *Fusarium* species are associated with these symptoms (Miller 1994; Smith and White, 1988). Historically, *F. moniliforme* has been reported as the most common pathogen causing Fusarium ear rot; currently, its synonym *F. verticillioides* is considered the predominant species, and it seems clear that some previous reports of *F. moniliforme* included other species such as *F. subglutinans* and *F. proliferatum*.

In the United States, Fusarium ear rot is the most common disease associated with corn ears and it can be found at low severity levels in nearly all corn fields late in the season (Koehler, 1959; Kommedahl and Windels, 1981). Aside from causing ear rot symptoms, *F. verticillioides* frequently can be isolated from symptomless kernels (Foley, 1962; Smith and White, 1988).

The epidemiology of Gibberella ear rot has been the subject of at least two reviews (Miller, 1994; Sutton, 1982), and a third review (Parry et al., 1995), focusing on Fusarium head blight of wheat, also contains information relevant to maize. The general epidemiology of Fusarium ear rot has received relatively less thorough attention; some aspects were reviewed by Miller (1994; 2001) and by Nelson (1992). In this paper, the objective is to summarize key points from the previous reviews, discuss the results of recent research not covered in those reviews (with emphasis on the infection process), and explore the epidemiology of these diseases in relation to their primary associated mycotoxins (deoxynivalenol and fumonisins).

Survival and sources of inoculum

Currently, maize is typically grown in a short rotation with one or two other crops in crop production areas with little overall crop diversity. As a result, most fields where the crop is grown have some remnants of maize plant residue in or on the soil, or such residue is present in nearby fields. This residue is the primary source of inoculum for infections of maize kernels (Smith and White, 1988). Recent studies on Fusarium head blight of wheat also confirm that maize residue is a much more important source of *F. graminearum* inoculum than wheat residue (Dill-Macky and Jones, 2000; Schaafsma et al., 2001). *Fusarium* species survive well on maize crop residue (Nyvall and Kommedahl, 1970; Sutton, 1982) as mycelium or other survival structures. *F. graminearum* produces chlamydospores that can survive periods between host crops (Sutton, 1982), whereas *F. verticillioides* (reported as *F. moniliforme*) can produce thickened hyphae which also have survival capabilities (Nyvall and Kommedahl, 1968). Additionally, existing populations in the soil can colonize senescent maize tissues that are deposited there without prior infection (Cotten and Munkvold, 1998). *Fusarium* species can colonize senescent tissues of other crop and weed species that are not considered hosts for these pathogens (Parry et al., 1995). Several types of infectious propagules can be generated from these residues. *F. graminearum* forms perithecia on the residue, and these fruiting structures forcibly discharge ascospores into the air. A great deal of research has been conducted in order to characterize the temporal patterns of ascospore dispersal and the effects of environmental variables on their formation and release. Perithecial development occurs over a wide range of temperatures, but the optimal temperature is about 28 °C, whereas the optimal temperature for ascospore release is about 16 °C (Tschanz et al., 1976). Ascospore release requires a period of perithecial dehydration and follows a diurnal pattern, with release occurring primarily at night. Paulitz (1996) suggested that 'perithecial drying during the day followed by sharp increases in RH, may provide the stimulus for release of ascospores.' Rain events are not needed for ascospore release; in fact, heavy rainfall can inhibit release. *F. graminearum* also produces macroconidia in sporodochia on crop residue. In general, it appears that ascospores are the more important infectious type of propagule for Fusarium head blight of wheat, but the situation for maize has not been investigated as intensively. *F. verticillioides* and the other species causing Fusarium ear rot do not form perithecia as readily as *F. graminearum*. These species are heterothallic and while sexual reproduction probably plays an important role in genetic recombination, it is less important in the overall epidemiology of this disease, compared to its role in Gibberella ear rot. *F. verticillioides*, *F. subglutinans* and *F. proliferatum* produce large numbers of microconidia and macroconidia on crop residues, and these asexual spores comprise the most important inoculum for Fusarium ear rot and symptomless kernel infection.

The role of seed as an inoculum source for subsequent ear infection has been controversial. *Fusarium* species are commonly seedborne in maize (McGee, 1988), although current seed production practices such as gravity separation can reduce the incidence of seedborne *Fusarium* infection (Gillette, 1999). *F. graminearum*, *F. subglutinans* and *F. verticillioides* can all be transmitted from seeds to seedlings (Cotten, 1996; Kabeere et al., 1997). *F. verticillioides* can also systemically colonize the entire maize plant without causing symptoms, and in this way can be transmitted from seed to plant to kernels (Foley 1962; Munkvold and Carlton, 1997; Munkvold et al., 1997). A similar mechanism of kernel infection has recently been reported for *F. subglutinans* (Wilke et al., 2001). In general, seed appears to be a minor source of inoculum in relation to airborne spores infecting through the silks

(Munkvold et al., 1997), but this issue is still under investigation.

Kernels can also be infected by *F. verticillioides* strains that originate from the rhizosphere. The pathway for this type of infection is probably a symptomless systemic infection of the plants, initiated through the seedling roots. This has been demonstrated in experiments where toothpicks infested with marked strains of *F. verticillioides* were placed in the soil adjacent to the seed and the strains were later recovered from kernels (Desjardins et al., 1998).

Dispersal

Recent research focused on Fusarium head blight of wheat has also yielded valuable information about the dispersal of *F. graminearum* by air. Within-field spread of ascospores has been described using a two-dimensional Gaussian model (Paulitz et al., 1999). Gradients varied from steep to relatively shallow, depending on wind direction. However, ascospores and macroconidia that escape the crop canopy can be distributed long distances. Effective inoculum can be detected at kilometer distances from any known inoculum source (Bergstrom and Shields, 2002; Francl et al., 1999).

Macroconidia of *F. graminearum* are dispersed initially by water splashes, but they are a component of the atmospheric population of *F. graminearum* propagules (Bergstrom and Shields, 2002), indicating that once they are dispersed from the sporodochium, they can be dispersed relatively long distances by air.

Fusarium verticillioides and other species causing Fusarium ear rot are dispersed primarily as microconidia, although macroconidia also act as infectious propagules. Microconidia typically are more numerous and more easily wind-dispersed than macroconidia. Propagules of *F. verticillioides*, *F. subglutinans* and *F. proliferatum* are common in the air within fields of maize (Gillette, 1999), and also at locations distant from maize fields. Ooka and Kommedahl (1977) estimated that viable spores of *F. verticillioides* (reported as *F. moniliforme*) traveled as much as 300–400 km.

Insects play a key role in the dispersal of *F. verticillioides*, but less so for *F. graminearum*. The role of insects in dispersing other *Fusarium* spp. has not been reported, but it is likely that other microconidial species such as *F. proliferatum* and *F. subglutinans* have dispersal mechanisms very similar to *F. verticillioides*. A variety of insect species have been implicated in the dispersal of *F. verticillioides*, including European corn borers (*Ostrinia nubilalis*), sap beetles (*Carpophilus* spp. and *Glischrochilus quadrisignatus*), western flower thrips (*Frankliniella occidentalis*), and corn rootworm beetles (*Diabrotica* spp.) (Dowd, 1998; Gilbertson et al., 1986). Several lepidopteran and coleopteran species were associated with increased ear rot and *F. verticillioides* infection in Africa (Cardwell et al., 2000). European corn borer larvae can acquire spores of *F. verticillioides* from leaf surfaces and transport them to kernels (Sobek and Munkvold, 1999). Adult rootworm beetles and sap beetles commonly carry spores of *F. verticillioides* and *F. graminearum*. Rootworm beetles feed on maize silks, where spores of either *Fusarium* species may be deposited and cause kernel infection. Sap beetles are attracted to maize ears that have been damaged by other insects such as the European corn borer or corn earworm. In addition, they are attracted to volatile compounds produced by *F. verticillioides* (Bartelt and Wicklow, 1999). The beetles, therefore, may be well-situated for acquiring *Fusarium* spores from infested plant material and delivering them to wounded kernels, which are very susceptible to infection. Attraction of insects to *F. verticillioides*-infected plant material may not be limited to sap beetles. Schulthess et al. (2002) reported that several lepidopteran and coleopteran insect species were more numerous on *F. verticillioides*-inoculated plants than on non-inoculated control plants.

Deposition and infection pathways

The primary infection pathway for infection of maize kernels by *F. graminearum* is via the silks, which are highly susceptible during the first 6 days after silk emergence, but less so afterwards (Reid and Hamilton, 1996). Spores reach the silks by splashing, wind dispersal or insect vectors. Some infections by *F. graminearum* are clearly initiated by direct injury to the kernels by lepidopteran insects, but this appears to be a less important pathway than silk infection.

For *F. verticillioides*, several infection pathways have been identified including silk infection, insect injury and systemic transmission from seeds or roots to kernels (Munkvold et al., 1997; Sobek and Munkvold, 1999). The relative importance of different pathways may vary among geographic areas. In the central United States, severity of Fusarium ear rot and symptomless kernel infection are closely correlated with insect injury

Table 1. Linear correlation coefficients for insect injury (kernels/ear), Fusarium ear rot severity (kernels/ear), and fumonisin concentrations (mg/kg $FB_1 + FB_2 + FB_3$) for maize grain from field experiments in Iowa, USA, from 1996 to 2001. All correlations were highly significant ($P \leq 0.001$)

	Fusarium ear rot	Fumonisins
Insect injury – 1996	0.66	0.50
Insect injury – 1997	0.89	0.69
Insect injury – 1998	0.81	0.77
Insect injury – 1999	0.92	0.77
Insect injury – 2000	0.80	0.58
Insect injury – 2001	0.73	0.72
Fusarium ear rot – 1996	—	0.69
Fusarium ear rot – 1997	—	0.76
Fusarium ear rot – 1998	—	0.73
Fusarium ear rot – 1999	—	0.89
Fusarium ear rot – 2000	—	0.76
Fusarium ear rot – 2001	—	0.62

(Table 1), primarily due to *O. nubilalis*, and this appears to be the most important infection pathway in this area. The relative importance of lepidopteran insects in disease development for *F. graminearum versus F. verticillioides* can be illustrated by how insect control affects the two diseases. Transgenic Bt maize hybrids, which are highly resistant to European corn borer injury, have much lower levels of Fusarium ear rot and fumonisins (up to 90% reduction), compared to conventional hybrids (Munkvold et al., 1999). Bt hybrids have also been shown to have lower levels of DON (as a result of reduced *F. graminearum* infection), but the reductions have been more modest (up to 59% reduction) (Schaafsma et al., 2002). A similar situation exists in Europe. As early as 1991, Lew et al. predicted, 'measures to combat the European corn borer will mainly reduce moniliformin and fumonisin contamination, but will affect zearalenone, deoxynivalenol and nivalenol contents of the ears to a much lesser extent.' Recent results have confirmed the association of mycotoxin contamination with insect injury due to the insects *O. nubilalis* (Bakan et al., 2002) and *Sesamia nonagrioides* (Avantaggiato et al., 2003) in Europe. As predicted by Lew et al. (1991), reductions in deoxynivalenol, nivalenol and zearalenone were not as great as reductions in fumonisins when these insects were controlled with transgenic insect resistance in the field in France and Spain (Bakan et al., 2002).

Infection through silks is also a significant source of Fusarium ear rot and symptomless infection by *F. verticillioides* (Desjardins et al., 2002; Munkvold et al., 1997; Nelson, 1992). In the absence of, or in addition to, insect injury, infection through silks appears to be the most important infection pathway for *F. verticillioides* (and probably *F. proliferatum* and *F. subglutinans*). Most methods for screening maize hybrids for resistance to Fusarium ear rot have employed silk inoculation or a method that wounds the kernels (Clements et al., 2003). Resistance factors have thus been identified both in the silks (Headrick and Pataky, 1991) and in the pericarp of the kernels (Hoenisch and Davis, 1994; Scott and King, 1984), which may reflect relative ability to avoid kernel injury.

Systemic transmission of the fungus seems to be of lesser importance, but the frequency of systemic transmission has varied widely among reports (Desjardins et al., 1998; Munkvold and Carlton, 1997). There may be environmental conditions under which systemic transmission is more prevalent, but this has not yet been demonstrated. Systemic transmission also has been reported for *F. subglutinans* in maize (Wilke et al., 2001), but not for other *Fusarium* species.

Factors affecting disease development and mycotoxin production

One of the most important factors that determines the level of disease and mycotoxin accumulation is genetic resistance of maize hybrids. Hybrids differ significantly in this trait, for both Gibberella ear rot (Reid and Hamilton, 1996) and Fusarium ear rot (Clements et al., 2003). In addition, physical traits of hybrids, such as husk coverage (Warfield and Davis, 1996), are related to disease susceptibility. Genetic variability in the pathogen population is a relatively unexplored factor that also may contribute to variability in disease and mycotoxins (Carter et al., 2002; Melcion et al., 1997).

Fusarium ear rot and Gibberella ear rot are favored by distinctly different conditions. Fusarium ear rot is more common in warmer and drier areas, in comparison with Gibberella ear rot (Bottalico, 1998; Miller, 1994). This pattern is associated with different temperature optima for *F. graminearum versus F. verticillioides*. The optimum temperature for *F. graminearum* has been reported as 24–26 °C (Booth, 1971) or about 28 °C (Reid et al., 1999), whereas the optimum temperature for *F. verticillioides* is reported as about 30 °C (Marín et al., 1999; Reid et al., 1999). Gibberella ear rot is favored by high levels of moisture around silking, followed by moderate temperatures and high rainfall during the maturation period (Sutton, 1982). Fusarium ear

rot generally is favored by warm, dry weather during the grain-filling period (Marasas et al., 2000). Several lines of evidence indicate that drought stress is associated with elevated levels of *F. verticillioides* infection and fumonisin accumulation in kernels (Miller, 2001). Shelby et al. (1994) showed that, in a group of maize hybrids planted in different locations in the United States, fumonisin levels were inversely correlated with June rainfall. An earlier report from Illinois indicated that Fusarium ear rot was negatively correlated with rainfall during June and July but positively correlated with rainfall during the period from August to October (Koehler, 1959). These results are consistent, in that a dry period before or during grain filling favors more severe Fusarium ear rot and higher levels of fumonisins.

There may be an interaction between drought stress, insect populations, and Fusarium ear rot and fumonisins. During several years (e.g., 1989; 1997) when drought stress occurred in the central United States, fumonisin accumulation (Munkvold et al., 1999; Murphy et al., 1993) and *O. nubilalis* populations (Bullock and Nitsi, 2001) were higher than average. Although the hypothesis is difficult to test, annual fluctuations in insect populations are likely to be a significant factor in fumonisin content of maize grain.

Environmental influences on *F. graminearum* have been incorporated into risk assessment models for head blight of wheat in North America (DeWolf et al., 2003; Hooker et al., 2002). While this type of analysis could potentially be useful for risk assessment in maize, the efforts so far have not been sufficiently detailed to facilitate application to this crop. In both cases, environmental variables were empirically related to head blight severity or deoxynivalenol levels; environmental effects on components of the disease-cycle that apply to maize cannot be extracted from the existing risk assessment models for wheat.

Efforts to synthesize the effects of environment on *Fusarium* ear diseases of maize have been more limited. Sutton et al. (1980) correlated summer precipitation with incidence of zearalenone in maize. Vigier et al. (1997) developed a regression model for ear rot incidence (caused by *F. graminearum*, *F. subglutinans* and *F. verticillioides*) based on July rainfall and an examination of ear damage and incidence of fungal infection. This model did not have predictive capabilities, since it relied on assessment of the infected ears for physical injury and kernel infection. A mechanistic model of post-inoculation fungal growth was developed by Stewart et al. (2002). This model comprised differential equations relating growth rates of *F. graminearum* and *F. verticillioides* to temperature, relative humidity and precipitation, and to a non-linear silk function that described changes in silk susceptibility over time. The key determinants of fungal growth were temperature and ear wetness, which were estimated using precipitation and relative humidity data. This model effectively predicted ear rot severity following inoculation with a coefficient of determination of 0.89. However, its predictive capability is limited by the lack of assessment of pre-infection events.

Conditions for growth of *F. verticillioides* and production of fumonisins on solid corn kernels *in vitro* have been studied extensively, primarily in a series of papers by Marín and co-workers. Marín et al. (1999) developed a polynomial regression describing fumonisin production as a function of temperature and water activity. They reported optimal conditions of 30 °C and 0.97 a_w for fumonisin B_1 production by *F. verticillioides* and 15 °C at 0.97 a_w for *F. proliferatum*. Other studies confirmed 30 °C as the optimal temperature for *F. verticillioides* growth and fumonisin production, but indicated an optimal temperature for growth of *F. proliferatum* higher than 15 °C (Marín et al., 1995). The authors also pointed out that differences in optima occurred among isolates of the same species. There were significant interactions between the effects of temperature and water activity. Growth and fumonisin production for both fungi were greatly reduced at a_w values around 0.92. Germination of both fungi was optimal around 30 °C, but this was affected by water activity (Marín et al., 1996); the minimum a_w for germination was 0.88, and both species germinated rapidly at 0.94 and above. These results have relevance to predicting fumonisin development in field-drying grain, but have not been tested as such.

Fusarium species that infect maize kernels clearly encounter other fungi, including other *Fusarium* species, and competition among these fungi can have a distinct effect on the eventual levels of infection and mycotoxin contamination. In mixed field inoculations, *F. verticillioides* interfered with the growth of *F. graminearum* and markedly reduced the levels of deoxynivalenol in grain compared to *F. graminearum* alone (Reid et al., 1999). *F. verticillioides* growth and fumonisin levels were affected inconsistently by the presence of *F. graminearum*. The correlation between ear rot symptoms and deoxynivalenol is typically high in *F. graminearum*-inoculated ears, but this correlation was poor in ears receiving a mixed inoculation

(Stewart et al., 2002). Competition among *Fusarium* species and with other fungi has also been studied on irradiated grain (Marín et al., 2001; Velluti et al., 2000). In contrast to the field results, deoxynivalenol production by *F. graminearum* was stimulated in the presence of *F. verticillioides* (Velluti et al., 2001). *Aspergillus parasiticus* reduced growth of *F. verticillioides* and *F. proliferatum* but did not affect their fumonisin production, whereas the *Fusarium* species inhibited production of aflatoxin but not growth of *A. parasiticus* (Marín et al., 2001). Interactions among all these fungi were influenced by complex interactions between temperature and water activity.

Role of mycotoxins in the disease-cycle

The ecological role of mycotoxins has been a subject of much speculation, in particular their possible role in pathogenesis in the plant. In this respect there seems to be a contrast between Gibberella ear rot and Fusarium ear rot. Initially, some evidence pointed toward a role for fumonisins in the infection process for *F. verticillioides* and *F. proliferatum*. Fumonisins are phytotoxic to maize (Lamprecht et al., 1994), and virulence of *F. verticillioides* isolates co-segregated with fumonisin production (Desjardins et al., 1995). Thorough tests of the possible role of fumonisins in ear rot disease have now been conducted. In contrast to the initial hypothesis, strains of *F. verticillioides* that lack fumonisin production due to a natural mutation or having a biosynthesis gene disrupted had the same capability to infect maize kernels and cause ear rot as wild-type, fumonisin-producing strains (Desjardins and Plattner, 2000; Desjardins et al., 2002).

Similar tests with *F. graminearum* strains that lacked trichothecene production yielded different results. On both wheat and maize, strains that lacked DON production due to disruption of the biosynthesis gene were less able to cause disease than their wild-type counterparts (Desjardins et al., 1996; Harris et al., 1999).

While DON appears to aid plant infection, the ecological role of fumonisins remains unknown. One possibility is that fumonisins aid during competition with other fungi. Fumonisins are toxic to some fungi (Keyser et al., 1999). Studies assessing competition between *F. verticillioides* and other fungi have shown that fumonisin production is not inhibited by competing fungi (Marín et al., 2001), and that *F. verticillioides* is dominant against *Penicillium* and *Aspergillus* species in grain (Marín et al., 2001) and against *F. graminearum* in inoculated maize ears (Reid et al., 1999).

A key element in eventual efforts to predict the development of mycotoxins in maize is the relationship between fungal growth and mycotoxin production. Can predictions of fungal growth or visible disease be assumed to also predict mycotoxin contamination? In general, conditions favorable for fungal growth are indeed favorable for production of deoxynivalenol and fumonisins. This is evident from the generally close correlations among ear rot symptoms, fungal growth (measured by species-specific DNA assays or ergosterol accumulation) and mycotoxin content (Bakan et al., 2002; Munkvold et al., 1999; Reid et al., 1996; Stewart et al., 2002), and the finding that the large majority of fumonisins and deoxynivalenol resides in the symptomatic kernels (Desjardins et al., 1998; Reid et al., 1996). However, it has not been demonstrated that growth and mycotoxin production by these fungi respond to temperature and water activity in an identical manner. Furthermore, correlations between fungal growth and mycotoxins are sometimes quite variable and affected by competition with other fungi (Melcion et al., 1997; Stewart et al., 2002).

Future efforts toward understanding the epidemiology of these diseases must focus on more precise relationships between environmental variables and specific components of the disease-cycle, possibly facilitated by a stronger linkage to wheat head blight research efforts already underway. For Fusarium ear rot, the role of systemic infection by *F. verticillioides* and *F. subglutinans* has not been completely elucidated; conditions under which this is an important disease-cycle component have not been identified. Perhaps more importantly, a greater understanding is needed of the conditions under which insect activity is or is not crucial, along with a connection to prediction models for insect activity.

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Epidemiology of toxin-producing fungi and ochratoxin A occurrence in grape

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Key words: Aspergillus section *Nigri*, cropping system, meteorology

Abstract

Fungi responsible for ochratoxin A (OTA) production have been studied especially on cereals, where *Penicillium verrucosum* and *Aspergillus ochraceus* are to be considered the main producers. Until 1998, these fungi were also believed to be responsible for the production of the toxin in grape, but OTA-producing *A. carbonarius* and *A. niger* were identified in dried vine fruits in 1999. Further studies pointed out that mycoflora potentially responsible for the presence of OTA in grapes are present in the field. *Aspergilli* are dominant to *Penicillia*, and among these *Aspergilli* section *Nigri*. *A. carbonarius* probably plays an important role because of the high percentage of positive strains and the amount of OTA produced. *Aspergilli* section *Nigri* are present on grape bunches early in the season and their frequency increases during later growth stages. At early veraison and ripening, the incidence of colonised berries is more related to the year than to the growth stage, but not to visible symptoms, since it is normal to isolate fungi from intact berries. Differences in ochratoxin content of berries have been detected between years, when the same vineyards, managed in the same way, showed high levels (1999) or the absence (2000) of the toxin. The results suggest that meteorological differences between years and grape-growing areas are responsible for differences in OTA levels, but the data are at present insufficient to draw firm conclusions.

Introduction

Epidemiology is the study of changes in the intensity of disease in a population of hosts over time and considers how a plant disease, resulting from the interaction between a host and a pathogen, can be influenced by the environment. When the pathogen is a mycotoxigenic fungus, the disease is not necessarily a visible alteration of the host, but an invisible presence of its metabolites. As a consequence, information has to be acquired not only by monitoring host, pathogen, environment and disease (Campbell and Madden, 1990), but also the toxins which may accumulate. This paper summarises information obtained from published literature regarding ochratoxin A (OTA) in grape and presents further data collected in Italy in order to contribute to the understanding of the epidemiology of the fungi involved.

The host: grapes

Grape is a fruit appreciated by consumers as fresh (table grapes), dried (raisins), or as processed products, such as juice and wine. Grape production in Europe is estimated at 306 million tons per year, which is 54% of world production. Annual yields of table grape and raisin are 2.2 and 0.5 million tons, while 190 million hl of wine are produced (74% of world production). Furthermore, grape and its derivatives play a major role in import–export exchanges (Dutruc-Rosset, 1998). Grape was considered a safe product, free from mycotoxins, until 1996 when OTA was detected during a survey carried out in Switzerland on wine samples of different geographic origins (Zimmerli and Dick, 1996). Until now, OTA is the only mycotoxin to be detected in wine.

The toxin: Ochratoxin A

Ochratoxin A is carcinogenic to rodents and possesses teratogenic, immunotoxic and possibly neurotoxic and genotoxic properties. Furthermore, it has been implicated as a causal agent of Balkan Endemic Nephropathy and the development of urinary tract tumours in humans. In 1993, the International Agency for Research on Cancer (IARC) classified OTA as a possible human carcinogen (group 2B). The worldwide occurrence of OTA contamination of raw agricultural products has been amply documented. It occurs in a variety of plant products such as cereals, coffee beans, pulses, cocoa and spices. It has also been found in beer and in animal products such as pig meat, sausages and other meat products. Human exposure to OTA can be evaluated by blood analysis (FAO/WHO, 2001). During the last 5 years, several papers regarding the presence of OTA in grape and its processed products have been published. The information collected suggests that the intake of OTA related to grape is a real risk, especially for consumers of red wine, dessert wine and dried vine fruits. Red wine is more contaminated than white, especially if produced in Southern Europe. OTA has been detected in wine and in raisins at levels up to 7.6 $\mu g\, l^{-1}$ and 53.6 $\mu g\, kg^{-1}$, respectively (Battilani and Pietri, 2002).

The pathogens

Fungi responsible for OTA production have been studied especially on cereals, where *Penicillium verrucosum* and *Aspergillus ochraceus* (formerly known as *A. alutaceus*) are considered the main producers. Until 1998, they were also believed to be responsible for the production of the toxin in grape (Ospital et al., 1998), but OTA-producing *A. carbonarius* and *A. niger* were identified in dried vine fruits in 1999 (Codex Alimentarius Commission, 1999).

Few studies have been published regarding the occurrence of OTA-producing fungi in grapes. During the 1997–98 harvests, 50 grape samples were collected from Malbec and Chardonnay varieties in Argentina and Brazil. *Aspergilli* and *Penicillia* were isolated in both countries. *A. ochraceus* was found only in Brazil with a very low incidence and *P. verrucosum* was not identified. All the *Aspergilli* section *Nigri* that were collected (131 strains) were checked for OTA production and 25% were positive. *A. carbonarius* was isolated only in Brazil and 25% of isolates were ochratoxigenic (Da Rocha et al., 2002). Similar results were obtained in France, where 11 samples of grape and must used in red table wine making were investigated. Several *Aspergilli* and *Penicillia* were identified, but *A. ochraceus* and *P. verrucosum* were absent. Only *A. carbonarius* was tested for OTA production and all the isolates (14) were positive (Sage et al., 2002).

During 1999 and 2000, nine vineyards in Italy were sampled at different growth stages. Five hundred and eight fungal isolates were collected, 477 belonging to *Aspergillus* spp. and 31 to *Penicillium* spp. *P. verrucosum* was not found. Among the *Aspergilli*, species from sections *Fumigati*, *Circumdati* and *Nigri* were identified, with section *Nigri* (464 isolates) dominant. Examples of section *Circumdati*, which includes *A. ochraceus*, were isolated only occasionally. Eighty-six isolates of section *Nigri* were identified as *A. carbonarius* and they represented 19% of the black *Aspergilli* collected in both years. They proved to be the most toxigenic strains: about 60% of the isolates were positive for OTA production (Battilani et al., 2002).

These studies led to some preliminary conclusions. Mycoflora potentially responsible for OTA presence in grapes is present in the field and *Aspergilli* are dominant to *Penicillia*. The identified species did not include *P. verrucosum*. *A. ochraceus* was only found occasionally. As a consequence, they do not seem relevant to grape contamination. *Aspergillus* section *Nigri* was always present and included OTA-producing strains (Abarca et al., 2001). Among the species, *A. carbonarius* probably plays a relevant role, because the percentage of positive strains and the amount of OTA produced *in vitro* were generally higher than in the other black *Aspergilli* (Teren et al., 1996; Heenan et al., 1998; Battilani et al., 2002; Cabanes et al., 2001). The reported results identified *Aspergilli* section *Nigri* *A. carbonarius*, as being mainly responsible for OTA presence in grapes and wine, but no data are available on the dynamics of the fungus in the field or on the relationship between the fungi and OTA content in grapes.

In order to improve knowledge of the epidemiology of black *Aspergilli* in grapes, during a survey carried out in 1999 and 2000 (Battilani et al., 2002), data were collected with the aim of describing the dynamics of fungal populations on berries during grape development, possible interactions between fungal species, and cultural and meteorological factors. OTA content of berries were also investigated.

Materials and methods

Grape sampling

The survey carried out in 1999 and 2000 involved nine vineyards (designated V1–V9). Three were located in Emilia-Romagna (Northern Italy) and six in Puglia (Southern Italy). The vineyards chosen were representative of their grape-growing area as regards grape variety and farming methods (Table 1). Five plants were chosen along diagonal transects of each vineyard and two bunches were taken from each plant. When the training system consisted of two levels of bunches above ground, both were sampled, one bunch per level. In 1999, two growth stages were chosen for sampling: early veraison and ripening. In 2000, samples were also collected at two earlier growth stages, setting and berry enlargement (Table 2). Data on cropping systems were collected and meteorological data (mean daily air temperature, relative humidity and rainfall) were gathered from stations close to the vineyards.

Fungal isolation and characterisation

Samples of berries and rachis were incubated in moist chambers and the growing fungal colonies were transferred to Petri dishes containing Czapek Yeast Agar (CYA). After incubation, the fungal isolates were identified to genus level (Battilani et al., 2002). *Aspergilli* section *Nigri* isolates were identified as uniseriates, biseriates or *A. carbonarius.* Uniseriates are those with uniseriate conidial heads, biseriates are those with biseriate heads and among these *A. carbonarius* isolates were identified at species level. All *Aspergilli* and *Penicillia* isolated were tested for OTA production (Battilani et al., 2002).

Ochratoxin content in berries

All bunches collected, after selecting samples for fungal isolation, were manually crushed and OTA content was determined. The toxin was extracted with a mixture of acetonitrile : water (60:40) and, after dilution with water, an aliquot of the solution was purified using an immuno-affinity column and analysed by reverse-phase HPLC with fluorescence detection (Battilani et al., 2002).

Statistical analysis

Data on samples colonised by *Aspergilli* section *Nigri* were analysed using non-parametric Kruskal–Wallis

Table 2. Date of bunches sampling during the 2 years considered in the study

Year	Setting	Berries increase	Early veraison	Ripening
1999	—	—	2 August	10 September
2000	8 June	6 July	2 August	10 September

Table 1. Data on vineyards sampled in Italy in 1999 and 2000

Vineyard	Latitude	Longitude	Place	Grape variety	Training system	Height of bunches
V1 (South)	40°23′N	17°58′E	Salice salentino (LE)	Negroamaro	Bilateral spur pruned cordon	60
V2 (South)	40°23′N	17°58′E	Salice salentino (LE)	Malvasia nero	Bilateral cane pruned cordon	60
V3 (South)	40°23′N	17°58′E	Salice salentino (LE)	Negroamaro	Head-trained spur pruned	40–50 100–120
V4 (South)	40°24′N	17°57′E	Guagnano (LE)	Sangiovese	Overhead trellis, cane pruned (*Tendone*)	170–180
V5 (South)	40°24′N	17°38′E	Manduria (TA)	Primitivo	Cane pruned cordon (*Guyot*)	40–50
V6 (South)	40°44′N	17°25′E	Cisternino (BR)	Verdeca	Cane pruned cordon (*Guyot*)	60
V7 (North)	44°17′N	11°53′E	Faenza (RA)	Trebbiano	Overhead trellis, cane pruned (*Pergoletta*)	130–160
V8 (North)	44°17′N	11°53′E	Faenza (RA)	Trebbiano	Cane pruned cordon	40 170
V9 (North)	44°17′N	11°53′E	Faenza (RA)	Sangiovese	Spur pruned cordon	140–150

Italian provinces: LE: Lecce; TA: Taranto; BR: Brindisi; RA: Ravenna.

analysis of variance in order to study the significance of the considered factors (year, vineyard, growth stage and plant). Correlation analysis was used to check the relationship between the presence of fungi and the OTA content in berries.

Results

Fungal isolation and characterisation

Aspergillus and/or *Penicillium* strains were present on grapes, starting from setting in two vineyards, V3 and V9. One month later these fungi were detected in more vineyards: V1, V3, V4, V8 and V9. Vineyard V6 was free from fungi at early veraison in both years, as was V8 in both samplings in 1999 and V7 at ripening in 2000 (Figure 1). The number of colonised samples (berries or pieces of rachis) was low (4–10%) during early growth stages, but it increased up to 70% at ripening. Five hundred and twenty samples were colonised by *Aspergilli*, 506 of them by *Aspergilli* section *Nigri*. The height of bunches above ground level was not related to the fungi present. In fact, almost the same number of moulded samples was obtained in V3 and V8, independently of the distance of bunches from the ground. The percentage of colonised samples was higher in berries than in pieces of rachis. The percentage of colonised berries was 9% and 15% in 1999 and 2000, while the percentage of colonised rachis was 7% in both years.

The incidence of samples colonised by black *Aspergilli* was significantly influenced by the vineyard in both years and by the growth stage in 2000. Also, the year showed a significant effect when data were considered together (Table 3). The dynamic of samples colonised by black *Aspergilli* is shown in Figure 1 for early veraison and ripening, the most relevant growth stages. Fungal incidence was higher in 2000 than in 1999, especially at ripening. Most samples were colonised by *A. niger* aggregate in both years and at both growth stages. *A. carbonarius* was relevant at early veraison in 1999 and at ripening in 2000. In both

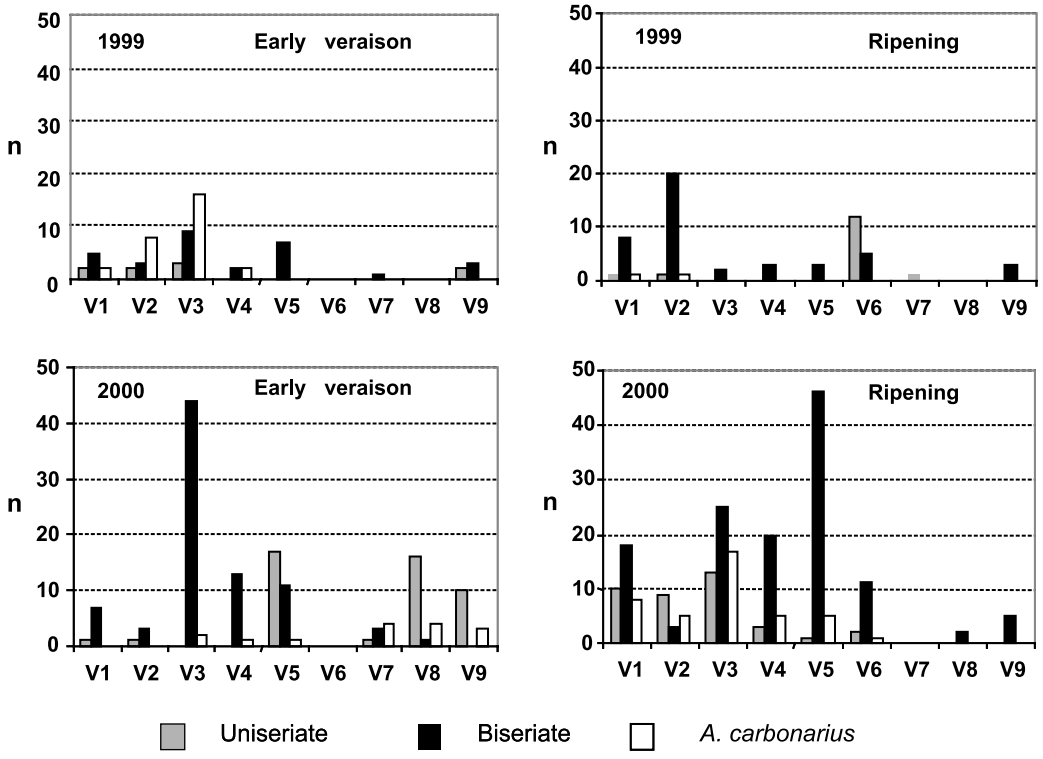

Figure 1. Dynamic of *Aspergilli* section *Nigri* identified as uniseriate, *A. niger* aggregate and *A. carbonarius* in nine Italian vineyards (V1–V9, see Table 1) at early veraison and ripening in 1999 and 2000.

cases, vineyards V1, V2 and especially V3 were the most colonised. The incidence of *A. carbonarius* was higher at early veraison in 1999 and at ripening in 2000, in both cases in Southern Italy. In Northern Italy, fungal contamination was always low, especially at harvesting.

The results on fungal dynamics are important, both because these genera are usually considered post-harvest moulds and because they were all isolated from berries without visible symptoms. Taking into account the dynamic of berries colonised by OTA-producing fungi, the higher incidence at early veraison in 1999 and at ripening in 2000 was confirmed. Several vineyards, including V7 and V8, were free of OTA-producing fungi at ripening in both years, and V6 which was free of toxins in both samplings in 2000 (Figure 2).

Table 3. Kruskal–Wallis analysis on incidence of berries colonised by *Aspergilli* or *Penicillia* in nine Italian vineyards in 1999 and 2000

Year	Vineyard	Plant	Growth stage	
1999				
χ^2	28.13	0.62	1.23	
Sign	0.00	0.43	0.27	
2000				
χ^2	19.70	0.54	233.22	
Sign	0.00	0.82	0.00	
1999–2000				
χ^2	39.62	0.31	107.20	49.42
Sign	0.00	0.58	0.00	0.00

Cropping system

Six grape varieties were included in the study. Varieties Negroamaro and Trebbiano were cultivated in two vineyards, the former in the South and the latter in the North, while Sangiovese was present in both grape-growing areas (Table 1). The training system was variable, as was the height of bunches from the soil. Four vineyards in the South (V1, V2, V5 and V6) had bunches close to the soil (40–60 cm) while in the North bunches were commonly at a greater height, around 150 cm above the soil level.

Chemicals were normally sprayed in all vineyards (Table 4), but the total number of sprays was greater in the North, with a mean of 15 applications in each cropping season, compared to 10 in the South. Active ingredients used were normally different between vineyards, especially if North and South are compared, but copper

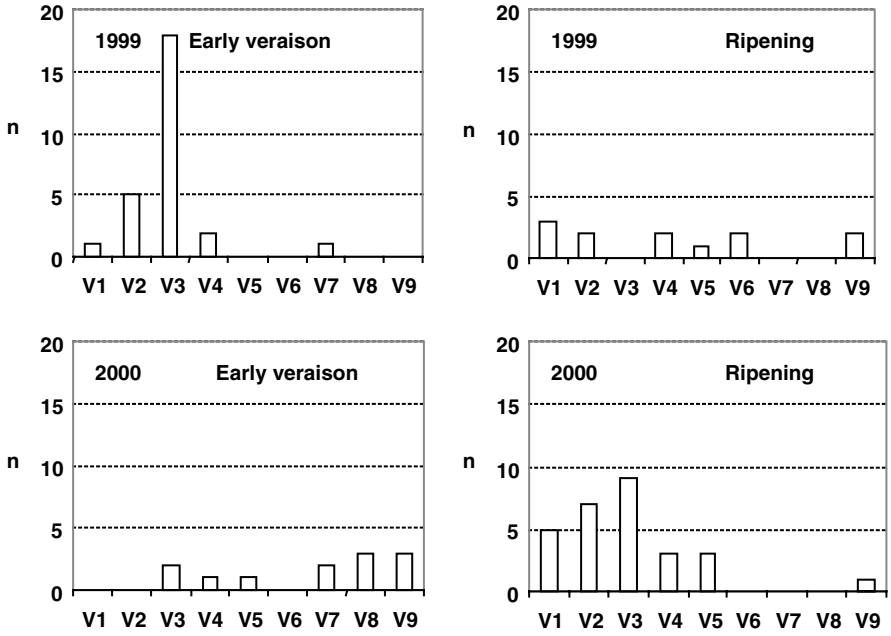

Figure 2. Dynamic of *Aspergilli* section *Nigri* positive for OTA production in nine Italian vineyards (V1–V9, see Table 1) at early veraison and ripening in 1999 and 2000.

Table 4. Active ingredients and total number of sprays distributed in the nine vineyards sampled in 1999 and 2000

	Vineyard																	
	V1		V2		V3		V4		V5		V6		V7		V8		V9	
Year	99	00	99	00	99	00	99	00	99	00	99	00	99	00	99	00	99	00
Azoxystrobin											■						▒	
Benalaxil											■	▒						
Cymoxanil	▒	▒	▒	▒	▒	▒	▒	▒		▒	■							
Dimethomorph									▒		■			▒		▒		
Dinocap	▒		▒		▒				▒		■							
Famoxadone											■							▒
Fenarimol	▒	▒	▒	▒	▒	▒			▒		■	▒	▒		▒			
Mancozeb	▒		▒		▒		▒		▒	▒	■						▒	▒
Metalaxyl	▒	▒	▒	▒	▒	▒				▒	■							
Metiram											■			▒		▒		
Penconazole											■							▒
Procymidone											■							▒
Propiconazole											■			▒		▒		
Quinoxifen											■							▒
Tetraconazole									▒	▒	■							
Thiophanate methyl	▒		▒		▒		▒	▒			■							
Copper	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒	■	▒	▒	▒	▒	▒	▒	▒
Sulphur	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒	■	▒	▒	▒	▒	▒	▒	▒
No. sprays	10	10	10	10	10	10	15	11	10	9	■	8	17	16	17	16	11	15

■: No data available; ▒: Sprays; □: No sprays.

and sulphur were always applied. Vineyards V1–V3 were managed in the same way, being included in the same farm, as were V7 and V8. None of the active ingredients sprayed is known to be active against *A. niger* (Khatri and Shekhawat, 1989; Suryawanshi and Deokar, 2001).

Meteorological data

Meteorological data show differences both between the places and years considered in the study (Figure 3). In Faenza (Northern Italy), the mean daily temperature ranged between 13 and 23 °C in both years and the summation in degree-days from 1 April to 30 September was 3650. In Brindisi (Southern Italy), the temperature was higher, with mean daily values varying between 15 and 30 °C in 1999 and between 19 and 30 °C in 2000, with a summation in degree-days of 4500 and 4800, respectively. The two grape-growing areas were also quite different regarding rainfall, especially in 2000. The total April–September rainfall in Faenza and Brindisi was 330 and 305 mm in 1999, and 182 and 62 mm in 2000. August and September were rainy months in 1999, with more than 70 mm/month in both areas, whereas they were dry in 2000, with 0 mm in August in the South.

Ochratoxin content in berries

The 2 years were quite different for OTA content in berries. In 1999 (Table 5), OTA content at early veraison was below $1\,\mu g\,kg^{-1}$ in all vineyards except V1 and V4. The situation remained more or less the same at ripening, except for V3, where OTA concentration was $13\,\mu g\,kg^{-1}$. In 2000, only traces of OTA (maximum $1\,ng\,kg^{-1}$) were occasionally detected in bunch samples. According to correlation analysis, the number of samples colonised by black *Aspergilli* and the OTA content in berries were not significantly related ($r = 0.46$), while the correlation was significant when only samples colonised by OTA-producing fungi were considered ($r = 0.94$).

Discussion

Aspergilli section *Nigri* are present on bunches early in the season and their frequency increases in later grape growth stages. At early veraison and ripening, the incidence of colonised berries is similar and the differences seem to be more related to the year than to the growth stage. It was normal to isolate fungi from intact berries. Independently of the growth stage considered, OTA was not always detected in berries, even

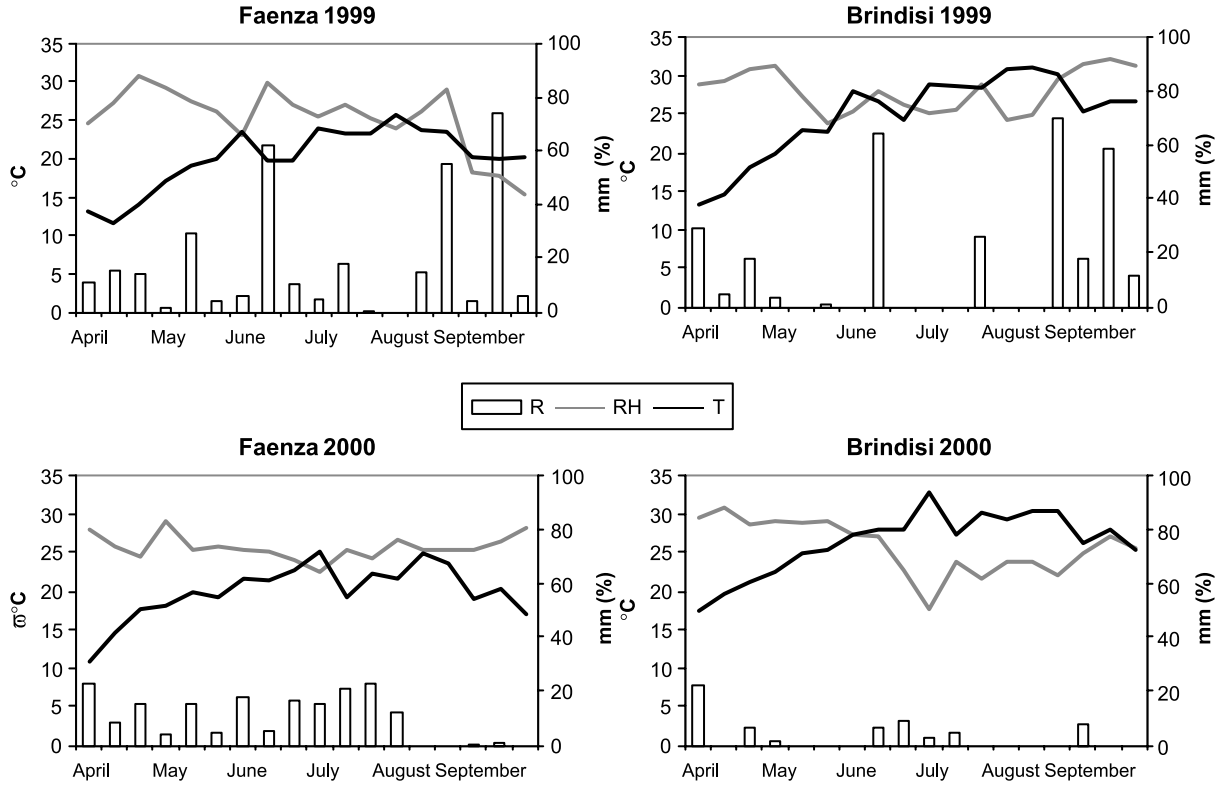

Figure 3. Temperature (T), relative humidity (RH) and rain (R), computed on 10 day periods, from April to September in Faenza and Brindisi in the 2 years of sampling.

Table 5. Ochratoxin A content ($\mu g\,kg^{-1}$) in bunches collected in nine Italian vineyards in 1999

Vineyard	Early veraison	Ripening
V1	1.29	1.51
V2	0.01	0.15
V3	0.03	13.08
V4	2.30	1.78
V5	0	N.A.
V6	0.12	0.03
V7	0.03	0.02
V8	0.00	0.01
V9	0.01	0.00

N.A. = Not available.

in those colonised by ochratoxigenic fungi. When OTA was present, it seemed to be correlated to the nature of the fungal isolates which were present. The presence of a fungus does not mean OTA synthesis. There are other factors that clearly play a role. Vineyards V1–V3, located in the same farm and managed following the same annual practices, showed differences in OTA contamination. OTA was practically absent in V2, but was detected at concentrations of 1.51 and 13.08 $\mu g\,kg^{-1}$ in V1 and V3, vineyards at the same site but trained with a different system. In addition, the Sangiovese grape variety was present both in V4 and V9. However, while the former vineyard contained OTA ($1.78\,\mu g\,kg^{-1}$), the latter was practically free from OTA.

The most remarkable difference was detected between years, where the same vineyards, managed in the same way, showed high levels of OTA in 1999 but OTA was absent in 2000. It appears that meteorological differences between years and at different grape-growing areas may be responsible for differences in OTA levels. Unfortunately, the existing data are not sufficient to draw final conclusions. Knowledge of the epidemiology of OTA-producing fungi in grape is actually very poor. A milestone has been gained: the pathogen is defined, but the role of the host, in particular of different grape varieties, as well as environment is still not clear.

Because of the importance of the problem and of the wide geographic area interested in it, a project supported by EU, Quality of Life, Key Action 1, started in 2001. In this project several European Countries in the Mediterranean Basin, relevant for grape growing, are involved. The overall objective of the project is the risk assessment of OTA presence in grapes and wine in Europe and protection of the consumer's health by decreasing the amount of toxin with the aid of integrated management of production and processing. The objectives will be followed by defining the critical control points for OTA synthesis during grape production and processing, modelling the effect of ecological conditions on fungal growth and OTA synthesis, and assessing possible preventive and corrective actions. All the information available about the pathosystem will be put together in a Decision Support System that will identify the actions likely to reduce the risk of OTA accumulating in grapes and its processed products.

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Post-harvest fungal ecology: Impact of fungal growth and mycotoxin accumulation in stored grain

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Key words: ecology, biotic, abiotic factors, fungal interactions, niche occupation, mycotoxins, insects

Abstract

Grain quality after harvest is influenced by a wide variety of abiotic and biotic factors and has been studied as a stored grain ecosystem. Important factors include grain and contaminant mould respiration, insects and mites, and the key environmental factors of water availability and temperature. Interactions between these factors influence the dominance of fungi, particularly mycotoxigenic species. Studies have shown that growth, mycotoxin production, competitiveness and niche occupation by mycotoxigenic species are influenced by the presence of other contaminant moulds and environmental factors. This has been demonstrated for both *Fusarium culmorum* and deoxynivalenol production, *Aspergillus ochraceus/Penicillium verruscosum* and ochratoxin production and *Fusarium* section Liseola and fumonisin production. Interactions between mycotoxigenic spoilage fungi and insects do occur but have not been studied thoroughly. Some insects disseminate mycotoxigenic species, others are known to use spoilage moulds as a food source, while others avoid certain fungal species. Thus, a more holistic ecological view is needed when considering management approaches to long-term-safe storage of cereal grains after harvest.

Stored grain as an ecosystem

Grain entering store carries a wide range of microorganisms including bacteria, yeasts and filamentous fungi, the population structure being dependent on field climatic conditions and harvesting processes (Magan and Lacey, 1986; Lacey and Magan, 1991). Poor post-harvest management can lead to rapid deterioration in grain quality, severely decreasing germinability and nutritional value of stored grain. Fungal activity can cause undesirable effects in grain including discolouration, contribute to heating and losses in nutritional value, produce off-odours, losses in germination, deterioration in baking and milling quality, and can result in contamination with mycotoxins.

Wallace and Sinha (1981) were the first to consider stored grain as a man-made ecosystem and use multivariate statistics to examine the complex interactions between abiotic and biotic factors to identify key parameters for safe storage. They believed that unless this more holistic and ecological approach was adopted it was not possible to understand the processes occurring and thus improve post-harvest management of stored grain. Factors such as grain type and quality, fungal population and community structure, mycotoxin production and pest infestation were all interlinked (Figure 1). The key environmental factors of temperature, water availability and gas composition influence both the rate of fungal spoilage and the production of mycotoxins.

Generally, provided grain is stored at a moisture content equivalent to $\leq$0.70 water activity (a_w) then no spoilage will occur. However, since grain is often traded on a wet weight basis, inefficient drying systems can lead to fungal activity and concomitant mycotoxin production which renders grain useless for food or feed. During initial storage, fungal inoculum can become redistributed in grain. Mechanical damage is also conducive to entry of spoilage fungi in insufficiently dried grain. It must also be remembered that

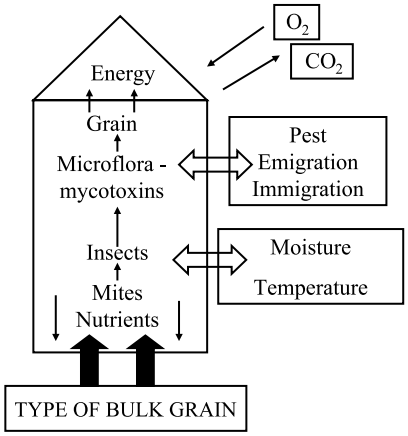

Figure 1. Diagrammatic representation of interactions between biotic and abiotic factors in stored grain ecosystems (adapted from Sinha, 1995).

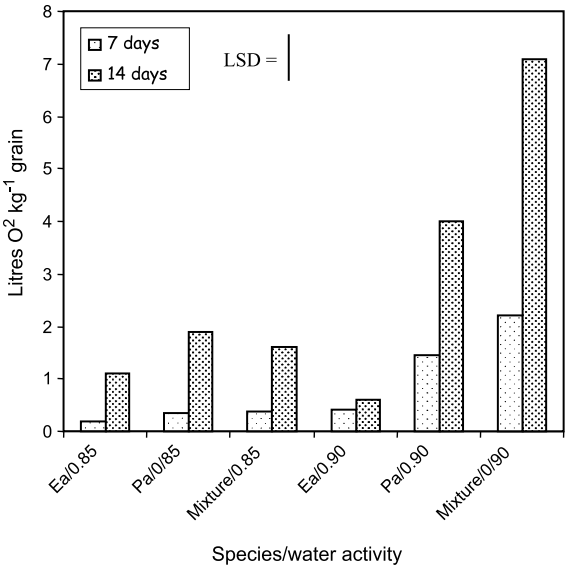

Figure 2. Measurement of the total respiratory activity of *E. amstelodami* (Ea), *P. auratiogriseum* (Pa) or a mixture of the two (mixture) at 0.85 and 0.90 water activity after 7 and 14 days incubation (Magan, unpublished data). Bar indicates least significant difference ($P = 0.05$) between treatments.

stored grain ecosystems offer an excellent but finite nutritional substrate for spoilage fungi.

Ecological considerations of interactions between spoilage fungi post-harvest

Fungi seldom occur on grains in isolation, but usually as a mixed consortium of bacteria, yeasts and filamentous fungi. It is thus inevitable that interspecific and intraspecific interactions will occur depending on the nutritional status of the grain and the prevailing environmental conditions. Indeed, environmental factors may exert a selective pressure influencing community structure and dominance of individual species, especially mycotoxigenic species. Figure 2 shows the effect of a_w on respiration of single spoilage fungi (e.g. *Eurotium amstelodami* and *Penicillium aurantiogriseum*) when grown individually or co-inoculated on wheat grain using an automated electrolytic respirometer system (Willcock and Magan, 2001). Respiration of co-inoculated species was less than additive especially at intermediate a_w conditions after 7 days. After 14 days, patterns changed again when total O_2 utilisation was considered (Hamer and Magan, unpublished data). This is indicative of competition between species. The situation becomes even more complex when a mixture of species colonising cereal substrates is considered.

From an ecophysiological point of view, it has to be remembered that spoilage fungi colonising grain use different primary and secondary strategies to occupy the niche. They may have combative (C-selected), stress (S-selected) or ruderal (R-selected) strategies or merged secondary strategies (C–R, S–R, C–S, C–S–R; Cooke and Whipps, 1993). Primary resource capture of grain is influenced by the germination rate, growth rate, enzyme production and the capacity for sporulation. Subsequent interactions between spoilage fungi result in combat, antagonism and niche overlap which all influence secondary resource capture.

We will consider two approaches which have been used to understand the type of interactions which occur between fungi under different environmental regimes in grain to enable better prediction of not just dominance by key spoilage fungi, but also the potential for production of mycotoxins. Magan and Lacey (1984, 1985) used categories of mutual intermingling (1/1), antagonism (2/2; 3/3) on contact or at a distance respectively, and dominance on contact or at a distance (4/0; 5/0). By giving a higher numerical score to fungi able to dominate *in vitro* rather than antagonism and adding the scores for each species, an Index of Dominance (I_D) was developed to assist with interpreting patterns of colonisation and dominance in grain ecosystems. The I_D was found to significantly change with a_w and temperature, and with grain substrate. Of 15 species, the most competitive species in wheat grain in the United Kingdom were *P. brevicompactum*,

P. hordei, *P. roqueforti*, *Aspergillus fumigatus* and *A. nidulans*. Decreasing the a_w led to conditions increased competitiveness of *P. brevicompactum*. Only *Fusarium culmorum* could compete with storage moulds, at >0.93–0.95 a_w. Interestingly, the rate of growth was not related to dominance. Previously, studies by Ayerst (1969) had suggested that speed of germination and growth were key determinants of colonisation of nutrient-rich matrices, such as grain.

Table 1 shows the effect of interactions between a mycotoxigenic strain of *F. culmorum* and other species in relation to water availability on wheat grain using the I_D scoring system. It is interesting to note that *F. graminearum* is more competitive than *F. culmorum*, regardless of temperature or water availability. *F. culmorum* is, however, dominant against other grain fungi including *Microdochium nivale*. This is indicative of why *F. culmorum* has become such an important pre- and post-harvest pathogen of temperate cereals and also indicates that *F. graminearum* is more competitive when both colonise grain. Thus interactions can change with different abiotic factors and with interacting species. Table 2 gives an example for an ochratoxin (OTA)-producing strain of *A. ochraceus* and other species, both *in vitro* and on maize grain at two different temperatures. *A. ochraceus* is dominant against *A. candidus* and *A. flavus* at 18 °C, but not against the latter at 30 °C *in situ*.

Table 1. F. culmorum interaction and I_D determined after 30 day incubation at 15 or 25 °C on irradiated wheat grain adjusted to different a_w levels (Hope and Magan, unpublished data)

a_w	Species[a]	Temperature		ID
		15 °C	25 °C	
0.995	F.c : F.g	0[b]/4[c]	0/4	0/8
	F.c : F.p	2/2	1/1	3/3
	F.c : A.t	4/0	4/0	8/0
	F.c : C.h	4/0	4/0	8/0
	F.c : M.n	2/2	4/0	6/2
	F.c : M.m	2/2	4/0	6/2
	F.c : P.v	4/0	2/2	6/2
I_D		16/10	19/7	35/17
0.955	F.c : F.g	0/4	0/4	0/8
	F.c : F.p	0/4	2/2	2/6
	F.c : A.t	4/0	4/0	8/0
	F.c : C.h	4/0	4/0	8/0
	F.c : M.n	4/0	4/0	8/0
	F.c : M.m	0/4	4/0	4/4
	F.c : P.v	2/2	4/0	6/2
I_D		14/14	20/6	34/20

[a]F.c., *Fusarium culmorum*; F.g., *Fusarium graminearum*; F.p., *Fusarium paoe*; A.t., *Alternaria tenuissima*; C.h., *Cladosporium herbarum*; M.n., *Microdochium nivale*; M.m; *M. nivale* var. *majus*; P.v., *Penicillium verrucosum*.

[b]Refers to interaction score for first species only.

[c]Refers to interaction score for second species only.

I_D refers to total addition of scores for an individual species competing with a range of other species based on the interaction scores for each species of 1 : 1 (mutual intermingling), 2 : 2 (mutual antagonism on contact), 3 : 3 (mutual antagonism at a distance), 4 : 0 (first species dominant over the other on contact), 5 : 0 (first species dominant at a distance over the other; from Magan and Lacey, 1984).

More recently, alternative approaches were utilised to understand the relative competitiveness of different species within fungal communities colonising grain. Wilson and Lindow (1994a,b), working with biocontrol systems, suggested that the co-existence of microorganisms particularly on plant surfaces may be mediated by nutritional resource partitioning. Thus *in vitro* carbon utilisation patterns (Niche size) could be used to determine Niche overlap indices (NOI) and thus the level of ecological similarity. Based on the ratio of the number of similar C-sources utilised and those unique to an individual isolate or species, a value between 0 and 1 was obtained. NOI of >0.9 were indicative of co-existence between species in an ecological niche, while scores of <0.9 represented occupation of separate niches. This approach was modified by Marin et al. (1998a) and Lee and Magan (1999) for a multifactorial approach by including water availability and temperature into the system. This demonstrated that based on utilisation of maize C-sources, the NOIs for fumonisin-producing strains of *F. verticillioides* and *F. proliferatum* were >0.90 at >0.96 a_w at 25 and 30 °C, indicative of co-existence with other fungi such as *Penicillium* species, *A. flavus* and *A. ochraceus*. However, for some species, pairing with *F. verticillioides* resulted in NOI values <0.80 indicating occupation of different niches. Figure 3 shows a diagrammatic example of the impact that environmental factors and interaction have on Niche size and NOI for *A. ochraceus* against *Alternaria alternata*. This shows how interactions may change with environment. Table 3 shows results for interactions between an OTA-producing *P. verrucosum* strain and a *Eurotioum* species at 15 and 25 °C. These results suggest that Niche overlap is in a state of flux and significantly influenced by both temperature and water availability. Nutrient status is very important. Lee and Magan (1999) demonstrated that comparison of C-sources in a standard BIOLOG test plate (95 carbon sources) with

Table 2. *In vitro* and *in situ* interaction scores and I_D between *A. ochraceus* and other species in relation to environmental factors (adapted from Lee and Magan, 2000a,b)

a_w	Species[a]	*In vitro*		*In situ*	
		18 °C	30 °C	18 °C	30 °C
0.95	*A. ochraceus* : *A. candidus*	4[b]/0[c]	5/0	4/0	4/0
	A. ochraceus : *A. flavus*	4/0	4/0	4/0	0/4
	A. ochraceus : *A. niger*	4/0	0/4	0/4	0/4
	A. ochraceus : *E. amstelodami*	2/2	2/2	0/4	0/4
Index of Dominance[d]		14/2	11/6	8/8	4/12

[a]Interacting fungal species.
[b]Refers to interaction score for *A. ochraceus* only.
[c]Refers to interaction score for interacting species only.
[d]Refers to total addition of scores for an individual species competing with a range of other species based on the interaction scores for each species of 1 : 1 (mutual intermingling), 2 : 2 (mutual antagonism on contact), 3 : 3 (mutual antagonism at a distance), 4 : 0 (first species dominant over the other on contact), 5 : 0 (first species dominant at a distance over the other; from Magan and Lacey, 1984).

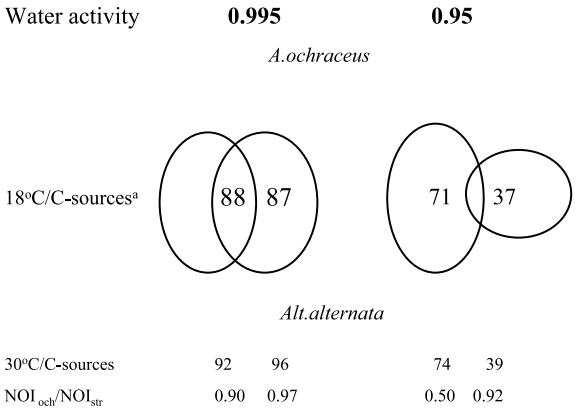

Figure 3. Diagrammatic representation of the Niche size and NOI for *A. ochraceus* when interacting with *A. alternata* at different temperatures and water activity levels (adapted from Lee and Magan, 1999). [a]Number of C-sources utilised out of 95; NOI_{och}, number of C-sources in common divided by total number utilised by *A. ochraceus*; NOI_{str}, number of C-sources utilised in common divided by the number utilised by *A. alternata*.

those only relevant to maize grain (18 carbon sources) gave very different results in terms of Niche size and NOI under different environmental conditions. This approach confirms that interactions and dominance are dynamic, not static, and emphasises the importance of taking account of such fluxes in any integrated approach to understanding and controlling the activity of mycotoxigenic spoilage moulds in the stored grain ecosystem.

Table 3. Example of impact of environmental factors on Niche size and NOI between *P. verrucosum* and other spoilage fungi (Cairns and Magan, unpublished data)

Water activity	0.995		0.93	
	Niche size[a]	NOI_{Pv}/ NOI_{Sp}	Niche size	NOI_{Pv}/ NOI_{Sp}
15 °C				
P. verrucosum	92		74	
A. ochraceus	71	0.71/0.92	52	0.50/0.74
F. culmorum	90	0.88/0.92	63	0.58/0.74
P. aurantiogriseum	90	0.78/0.92	75	0.69/0.74
25 °C				
P. verrucosum	85		69	
A. ochraceus	88	0.84/0.85	63	0.55/0.69
F. culmorum	89	0.82/0.85	51	0.45/0.69
P. aurantiogriseum	74	0.84/0.85	64	0.59/0.60

[a]Niche size, the total number of carbon sources utilised by a species based on Biolog GN plate of 95 Carbon sources.
NOI_{pv}, total number of carbon sources utilised in common divided by the total number utilised by *P. verrucosum* only, under each set of conditions.
NOI_{sp}, total number of carbon sources utilised in common, divided by the total utilised by the competitor, under each set of conditions.

Effect of interactions on growth and mycotoxin production

Figure 4 shows effects of fungal interactions and a_w on growth of *F. culmorum* on layers of irradiated wheat grain at two different a_w levels. The results show that when interacting with some species, e.g. *M. nivale* or

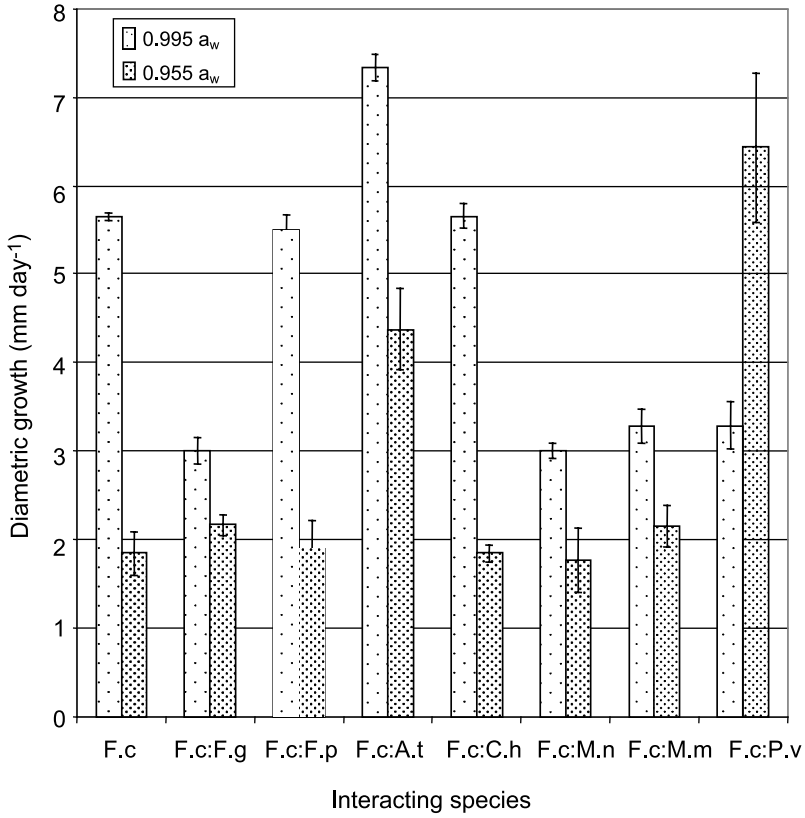

Figure 4. Effect of environmental factors on relative growth (diametric extension ± SE) of *F. culmorum* when paired with other interacting fungi on wheat grain at 0.995 and 0.955 water activities and 25 °C. Growth rates are means of five replicates per treatment. Key to fungi: F.c., *Fusarium culmorum*; F.g., *Fusarium graminearum*; F.p., *Fusarium paoe*; A.t., *Alternaria tenuissima*; C.h., *Cladosporium herbarum*; M.n., *Microdochium nivale*; M.m.; *M. nivale* var. *majus*; P.v., *Penicillium verrucosum* (Hope and Magan, unpublished data).

P. verrucosum, growth of *F. culmorum* is significantly faster than when growing alone on grain. Similar effects on growth and on OTA production were observed for *A. ochraceus* on maize grain (Lee and Magan, 2000b).

The question arises whether such effects on growth also influence mycotoxin production in poorly stored grain. *In vitro* and *in situ* studies have previously suggested that interaction between some species can result in a significant accumulation of mycotoxins, while in other cases an inhibition of mycotoxin production is observed. For example, interactions between section Liseola *Fusarium* species with *A. niger* resulted in a tenfold increase in fumonisin production especially at 0.98 a_w, although under drier conditions no increase in fumonsin occurred on maize grain (Marin et al., 1998b). Also in maize, *A. flavus*, *A. niger* and *E. amsteladami* all significantly inhibited OTA production by *A. ochraceus* (Figure 5; Lee and Magan, 2000b). Recent studies with *F. culmorum* show that interaction with *M. nivale* stimulated DON production on wheat grain with freely available water (= 0.995 a_w), while under drier conditions (0.955 a_w) interaction with *A. tenuissima*, *Cladosporium herbarum* and *P. verrucosum* reduced DON production (Table 4, Hope and Magan, unpublished data). OTA production by *P. verrucosum* was also influenced by competition with other spoilage fungi on wheat grain (Table 5).

Relationship between insects and mycotoxin producing fungi in stored grain

It is important to remember that insect pests are a common problem in stored grain ecosystems. They grow and multiply at water availabilities much drier than those allowing fungal growth. Insects can produce

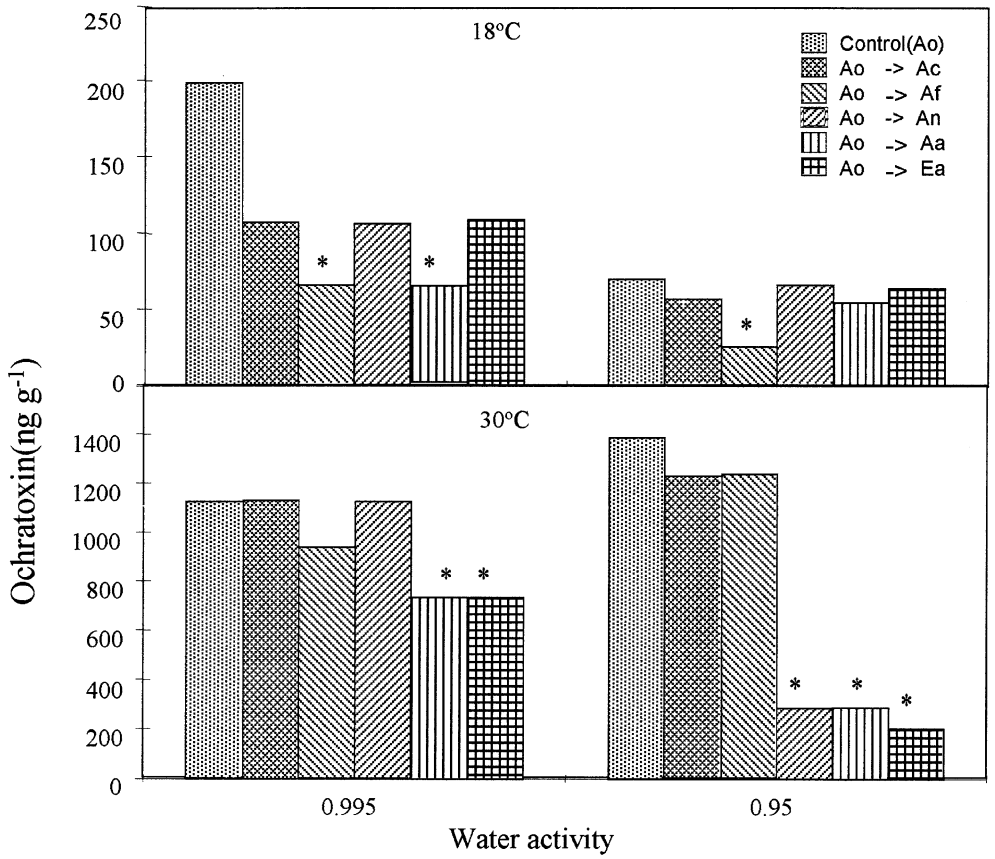

Figure 5. Effect of temperature and water activity on relative ochratoxin production by *A. ochraceus* when interacting with other fungi on maize grain at 18 and 30 °C when compared to *A. ochraceus* colonisation alone. Key to fungi: Ao, *Aspergillus ochraceus*; Ac, *Aspergillus candidus*; An, *Aspergillus niger*; Aa, *Alternaria alternata*; Ea, *Eurotium amstelodami* (adapted from Lee and Magan, 2000b). *Indicates significant differences from the control ($P = 0.05$).

Table 4. Effect of interactions between *F. culmorum* and other species on deoxynivalenol and nivalenol (ng g^{-1} grain) production on irradiated wheat grain at two water activity levels at 25 °C (Hope and Magan, unpublished data)

	Mycotoxin			
	Deoxynivalenol		Nivalenol	
Water activity	0.995	0.955	0.995	0.955
F. culmorum	7669	447	289	298
F. culmorum + *C. herbarum*	634	0	316	412
F. culmorum + *A. tenuissima*	459	444	0	288
F. culmorum + *M. nivale*	451	600	868	0
F. culmorum + *M. majus*	0	440	292	0
F. culmorum + *P. verrucosum*	3264	450	0	0

LSD ($P = 0.05$): DON = 180.5: NIV = 123.2.
Key to fungi: F, *Fusarium;* A, *Alternaria*; M, *Microdochium; M. majus*, *M. nivale* var. *majus*; P, *Penicillium verrucosum*.

metabolic heat which generates water via condensation on surfaces due to temperature differentials and develop classic hot spots which can quickly result in heating and complete spoilage. Pre-harvest insect infection can lead to increased post-harvest production of aflatoxin in maize (Sauer et al., 1984).

Some storage insects are disseminators of storage fungi, while others are exterminators (Sinha, 1971). Some storage fungi attract insects as food sources and promote population increases. Some fungi produce metabolites which repel insects. Indeed, loss in calorific value is due to the combined effects of spoilage fungi and insects. These interactions have often been neglected, although they are important. Physiological and biochemical similarities between fungi and early developmental stages of insects mean that potential exists for combined insecticidal/fungicidal control.

Table 5. Effect of interactions between *P. verrucosum* and two other mycotoxigenic *Fusarium* species (*F. culmorum*, *F. poae*) on ochratoxin production (ng $g^{-1} \pm$SE) on wheat-based medium under different water activity and temperature conditions after 56 days incubation

Water activity	Temperature (°C)			
	15		25	
	0.99	0.95	0.99	0.95
P. verrucosum alone	3000 ± 327	1800 ± 645	150 ± 36	3600 ± 409
P. verrucosum + *F. culmorum*	0	10 ± 7	0	0
P. verrucosum + *F. poae*	60	200 ± 18	0	0

There are no recent studies of interactions between insects and mycotoxigenic fungi. Dix (1984) found that *Penicillium* spp. and *A. flavus* were associated with *Sitophilus zeamais*. As adults they carried a high density of spores without succumbing to aflatoxicoses. Earlier Eugenio et al. (1970) showed that the lesser mealworm and the confused flour beetle retained zearelenone (ZEA) through metamorphoses from larvae to adult. Wright (1973) found that neither ZEA or T-2 toxin produced by *F. graminearum* and *F. tricinctum*, respectively, caused any mortality in the life-cycle of *Tribolium confusum*. Since this insect also feeds on these fungi, it may be a non-propagative vector or disperser of these fungal metabolites.

Dunkel (1988) carried out elegant studies to examine the efficacy of different concentrations of OTA, citrinin, rubratoxin B and patulin on larval weights and development time of three different insect species at between 0 and 1000 ppm concentrations. Larval weight of *T. confusum* was only significantly affected by citrinin, rubratoxin B and patulin at 1000 ppm concentration, with little effect on adult emergence. Of the three insect pests examined, only *Attagenus megatoma* was significantly affected at 100–1000 ppm of the mycotoxins. Surprisingly, no studies have been conducted with regard to fumonsins, DON or nivalenol. Such studies are necessary to evaluate the interactions which might occur between insect pests and spoilage moulds in stored grain ecosystems.

Conclusions

The activity of mycotoxigenic fungi in stored grain must be examined in the context of the ecosystem as a whole in order to understand the dominance of certain species under certain environmental conditions. Interactions between these fungi and other contaminants are complex and are significantly affected by the prevailing and changing environmental factors. Niche overlap and dominance of mycotoxigenic species have been shown to fluctuate with environmental factors. Studies *in vitro* and *in situ* suggest that interactions between toxigenic species and other spoilage fungi markedly influence mycotoxin production, with some species stimulating and others inhibiting production. The role of insect pests should not be neglected as they may be integrally involved in the dominance of mycotoxigenic species by helping in dispersal and acting as vectors and carriers of the toxin through grain. Overall, conditions in stored grain are not in a steady state and thus the dynamics of the system will vary over time. This needs to be taken into account in determining safe storage times for cereals without risks of spoilage and mycotoxin contamination. Any decision support system must take all these factors into account for the effective development of good management systems post-harvest.

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Strategies for the control of *Fusarium* head blight in cereals

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Key words: blight, control, *Fusarium*, mycotoxin, wheat

Abstract

Fusarium head blight (FHB) is a widespread and destructive disease of small grained cereals caused by a number of *Fusarium* species and *Microdochium nivale.* In addition to causing significant reductions in grain yield, FHB can result in the reduction of grain quality, either by affecting grain processing qualities or by producing a range of toxic metabolites that have adverse effects on humans and livestock. Control of FHB can be achieved by a number of cultural, biological and chemical strategies along with the exploitation of host plant resistance. In recent years, much of the research undertaken for the control of FHB has been concentrated on understanding and exploiting the genetic resistance of cereal plants to FHB-causing pathogens. Although, a brief overview of genetic resistance is presented, this review seeks to summarise the significance of FHB and review the effectiveness of cultural, biological and chemical control strategies that have been investigated for the control the disease.

Introduction

Fusarium head blight (FHB), also known as scab, is a significant disease of small grain cereals and has been reported throughout the world. The disease is caused by five major species, *Fusarium avenaceum* (teleomorph, *Gibberella avenacea*), *F. culmorum*, *F. graminearum* (teleomorph, *G. zeae*), *F. poae* and *Microdochium nivale* (teleomorph, *Monographella nivalis*) (Parry et al., 1995). Initial symptoms of FHB appear as slightly brown water-soaked spots present on the glumes. The lesions increase in size until the whole spikelet is covered and, depending on weather conditions, spreads to the neighbouring spikelets. Infected plant tissues senesce, taking on the typical colour of ripe heads in contrast with green uninfected heads. In some cases, infection of the rachis causes blighting or death to those spikelets situated above the point of infection. The production of sporodochia at the base of infected glumes gives rise to a pinkish colour on severely infected ears (Atanasoff, 1920). Grain harvested from FHB-affected ears is often shrivelled and may have a red discolouration due to the presence of fungal growth. In North America, *Fusarium* damaged kernels (FDK) are assessed and categorised according to their colour: 'white tombstone' (shrivelled, white and chalky) and 'pink tombstone' (shrivelled, with pinkish appearance) (Sinha and Savard, 1997).

Significance of *Fusarium* head blight

Effect on grain yield

According to McKay (1957), a severe head blight outbreak in Ireland in 1942, decreased yield in wheat by between 21% and 55%. A second outbreak during 1954 was responsible for yield reductions in wheat and oat crops by up to 50%. An extensive field survey of wheat crops in the Atlantic Provinces of Canada during 1980 (Martin and Johnston, 1982) revealed that FHB was responsible for between 30% and 70% yield loss. Scab epidemics in wheat and barley occurred in southern Idaho in 1982 and 1984 and resulted in estimated yield losses as high as 50% (Michuta-Grimm and Foster, 1989). In China, the largest area

affected by FHB is in the mid and lower regions of the Yangtze river valley. Surveys carried out between 1951 and 1985 recorded 19 FHB outbreaks with grain yields of wheat reduced by 5–15% in years when moderate epidemics of FHB were recorded and up to 40% in years when disease epidemics were severe (Zhuping, 1994). According to Sayler (1998), in nine US states between 1991 and 1996, wheat producers lost 501 million bushels of grain, equivalent to $2.6 billion. Hard red spring wheat crops were worst affected with ca. 52% production losses, whilst soft red wheat and durum wheat experienced 38% and 10% production losses. During head blight epidemics in the Northern Argentinean Pampas areas, yield losses between 10% and 50% were recorded (Moschini et al., 2001).

These surveys provide an indication of the potential yield loss that may be associated with FHB, but they provide no indication as to how the disease reduces grain yield. More precise data on the effect of FHB on grain yield have been obtained from artificially inoculated field trial studies. Arseniuk et al. (1993) for example reported that under experimental conditions, 1000-grain weight, the number of grains per head and the head weights in four triticale cultivars were reduced by 15%, 18% and 22%, respectively. Following the artificial inoculation of double haploid barley genotypes with *F. culmorum,* Surma et al. (2000) observed significant reductions in grain number per ear (4–31%) and 1000-grain weight (14–31%) when compared to uninoculated plots of the same barley genotypes.

Effect on grain quality

The presence of *Fusarium* spp. in wheat can cause deleterious effects on grain processing qualities. Bechtel et al. (1985), for example, found that *F. graminearum* was capable of destroying starch granules, storage proteins and cell walls during invasion of wheat grains. Dexter et al. (1997) showed that Canadian hard red spring wheat grain samples that contained *Fusarium* damaged grains exhibited weak dough properties and unsatisfactory baking quality. Following a study of the effects of fungal proteases on wheat storage proteins, Nightingale et al. (1999) suggested that *F. graminearum* and *F. avenaceum* produced proteolytic enzymes. These enzymes hydrolyse endosperm proteins during dough mixing and fermentation and result in weaker dough and decreased loaf volume. In barley, infection of grains with *Fusarium* spp. reduces malt yield and quality, as well as causing uncontrolled foaming of beer (gushing) during the malting process. (Narziss et al., 1990; Schwarz et al., 2001; 2002).

Seed quality

Fusarium head blight can result in grain becoming infected by the causal pathogen. Such infections can result in seedling blight if seed is subsequently sown (Winson et al., 2001). The drilling of *Fusarium*-infected cereal seed has been linked with the subsequent development of seedling blight and foot rot in growing crops (Nelson, 1929), as well as a reduction in plant establishment, number of ears m^{-2} and grain yield (Wong et al., 1992; Humphreys et al., 1995; 1998).

Mycotoxins

Apart from the effects on seed and grain processing qualities, *Fusarium* species produce a range of toxic metabolites. These include a number of mycotoxins belonging to the trichothecene group. The different trichothecenes produced by members of the *Fusarium* are classified as type A or type B according to the structural components (Krska et al., 2001). Type A trichothecenes includes T-2 and HT-2 toxins whilst type B trichothecenes are represented by deoxynivalenol (DON), nivalenol (NIV), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON) and fusarenon-X (FUS-X). *F. graminearum*, *F. culmorum* and *F. crookwellense* also produce an oestrogenic mycotoxin zearalenone (ZEN), also known as F-2 toxin which does not belong to the trichothecene group of toxins (Hussein and Brasel, 2001).

If grain contaminated with *Fusarium* toxins is used as feed for animal or human consumption, a range of adverse toxicosis as well as other health disorders are observed. In the middle of the last century, in Russia, the consumption of food prepared from over-wintered cereals, contaminated with *F. poae* and *F. sporotrichioides*, caused human poisoning known as Alimentary Toxic Aleukia (ATA). Symptoms of ATA include fever, necrotic angina, leukopenia, haemorrhaging and exhaustion of bone narrow. In some cases there are fatalities (Joffe, 1978). In 1987, an outbreak of a gastrointestinal disorder in the Kashmir Valley, India was associated with the ingestion of *Fusarium* mycotoxins (Bhat et al., 1989). Similarly, in China, 53 outbreaks of human food poisoning was associated with scabby and mouldy cereals occurred between 1960 and

1991 (Luo, 1992). In the Anhui Province of China in 1991, approximately 130,000 people were affected by gastrointestinal disorders, accompanied with abdominal pain, nausea, vomiting, fatigue and fever (Huang, 1992). Analysis of eight wheat and two barley samples revealed that DON was present in all samples at concentrations ranging from 0.016 to 51.45 mg kg^{-1}. NIV was also detected in all these eight wheat samples and one of the barley samples (0.001–6.93 mg kg^{-1}). Furthermore, both barley samples and six wheat samples contained ZEN at concentrations of between 0.046 and 0.3 mg kg^{-1} (Li et al., 1999) In 1998 and 1999, Li et al. (2002) analysed wheat samples taken from crops in the Henan Province of China, where cases of human toxicosis had been reported (Luo et al., 1987). Thirty samples out of the 31 tested (97%) from the Puyang area of this province contained DON, and 21 of them (70%) exceeded the Chinese advisory limit of 1 mg DON kg^{-1} grain.

The effect of DON-contaminated feed grain on domestic livestock is dependent on the animal species involved and on the severity and duration of exposure to contaminated grain (Rotter et al., 1995; Rotter et al., 1996). Among farm animals, pigs show greatest sensitivity to DON, while poultry and ruminants appear to show higher tolerance to the toxin (Trenholm et al., 1984). The oestrogenic compound ZEN, causes a range of reproductive disorders in young pigs ranging from vulva vaginitis and vaginal prolapses to enlargement of the uterus and atrophy of the ovaries (Mirocha et al., 1971). Consumption of grain contaminated with ZEN by pregnant sows resulted in an increase in stillborn pigs and small litters (Miller et al.,1973). T-2 toxin reduces feed consumption and weight gain in chickens due to severe oral lesions (Kubena et al., 1994). The toxin has also been associated with coagulopathy (Doerr et al., 1981) and altered feathering (Wyatt et al., 1975).

As a result of the adverse effects induced by *Fusarium* toxins, several countries have adopted advisory limits to ensure minimum levels of DON in finished products intended for human consumption and for animal feeds (Van Egmond, 1989). For example, the Food and Drug Administration (FDA) in the United States recommends that DON levels should not exceed 1000 $\mu g\,kg^{-1}$ in finished wheat products and should not exceed 5000 or 10,000 $\mu g\,kg^{-1}$ for feed intended for swine and cattle, respectively. The proposed advisory limits for trichothecene mycotoxins to be adopted within the European Union are 500 $\mu g\,kg^{-1}$ for retail products such as breakfast cereals, bread and pasta and 750 $\mu g\,kg^{-1}$ for flour and grain (Pricket et al., 2000). Although the development of trichothecene mycotoxins occurs primarily under field conditions, inappropriate grain storage can result in further increases in mycotoxin content (Birzele et al., 2000; Homdork et al., 2000).

Control of *Fusarium* head blight

Cultural control

In order to reduce the risk of FHB epidemics, several cultural control techniques can be employed, including suitable crop rotation, appropriate use of fertilisers and weed control. Crop rotation is one of the most effective cultural control measures that can be adopted. A survey involving 28 wheat crops grown in Illinois and Indiana (Holbert et al., 1919) showed that when wheat was sown following maize, 15% became infected by FHB. However, when wheat was grown following either alfalfa or oats, only 4% of crops became infected. Similar observations were later recorded by Koehler et al. (1924). Where wheat was grown after maize, up to 43% ears were observed to be affected by FHB. In wheat crops grown following rye or oats, 27% and 23% of ears showed symptoms, whilst following clover or timothy grass, only 11% and 8% of infected ears were observed. The average incidence of wheat crops affected by FHB on 72 farms in southwestern Ontario was six times greater when wheat followed maize than when wheat crops followed soybeans or cereals (Teich and Nelson, 1984). Recent studies on the effect of previous crop residues on FHB development also demonstrated that the incidence and severity of FHB was greater when wheat followed corn and lower when wheat followed soybeans (Dill-Macky and Jones, 2000).

Removing or burying crop residues leads to a reduced source of FHB inoculum. When wheat was planted after maize plots were ploughed, only 99 scabbed heads 10^{-5} were observed, whilst in disc-cultivated plots 209 wheat heads 10^{-5} showed symptoms. Such observations appeared to be due to the greater quantity of maize residue left on the soil surface (Teich, 1989). Other workers have also demonstrated that removal or ploughing in of crop debris reduces the incidence of FHB in cereals. For example, in Germany it has been observed wheat following grain maize had 0.5 mg kg^{-1} DON in comparison to that found in wheat following forage maize (0.3 mg kg^{-1})

(Obst et al., 1997). This effect was probably due to less crop debris remaining on the field after forage maize. In Canada, during a 3 year field study on the effect of tillage practices on FHB in wheat, Miller et al. (1998) isolated *F. graminearum* from 79%, 55% and 46% of kernels in year one, two and three, respectively, in no-till plots, whilst the incidence of infected kernels was 20%, 40% and 13% in the 3 years when plots were tilled.

The incidence of FHB can also be affected by fertiliser applications. During field trials investigating the effect of nitrogen inputs on the development of FHB, Martin et al. (1991) observed that increasing nitrogen applications from 70 to 170 kg N ha^{-1} resulted in increases in the incidence of *Fusarium*-infected grain in wheat, barley and triticale. In 1985 in Lambton County, Ontario, Canada, a comparative study showed that applications of ammonium nitrate resulted in 79 FHB-infected heads 10^{-5} whilst applications of urea resulted in 59 infected heads 10^{-5} (Teich, 1987). Applications of nitrolime to wheat plots reduced the incidence of FHB by 59% when compared to plots treated with calcium ammonium nitrate (Yi et al., 2001). However, there was no significant effect on DON concentration in harvested grain. It is not clear how nitrogen affects FHB development, however, several hypotheses can be postulated, including nitrogen influencing the water potential of the plant which in turn could influence the susceptibility of ears to infection by *Fusarium* species.

Field surveys in southwestern Ontario showed that fields with high weed densities had twice as many heads with FHB symptoms compared to weed-free fields (Teich and Nelson, 1984). The potential significance of weeds in the development of FHB epidemics has also been demonstrated by Jenkinson and Parry (1994), who isolated *Fusarium* species, which proved to be pathogenic to wheat, from 14 species of common broad-leaved weeds. These workers suggested that weeds provide an alternative source of inoculum for FHB epidemics and that weed control may reduce inoculum availability.

Cultivar resistance

The importance of wheat cultivars, which are resistant to FHB has been recognised since the early part of the last century (Dickson and Mains, 1929; Christensen et al., 1929). As a result, screening for resistance to FHB is conducted in most major wheat-growing countries. A simplified model for FHB resistance was proposed by Schroeder and Christensen (1963) who suggested that resistance was of two types; resistance to initial infection (Type I) and resistance to colonisation within the ear (Type II). Two further types of resistance based on the degradation of the mycotoxin DON (Type III) (Miller and Arnison, 1986) and tolerance to high DON concentrations (Type IV) (Wang and Miller, 1988) have also been proposed.

Types I and II resistances have been associated with certain morphological characters of wheat cultivars. Hilton et al. (1999) observed a significant negative relationship between plant height and resistance to FHB following artificial inoculation of ears of 17 cultivars of winter wheat with *F. culmorum*. Furthermore, while assessing plant height and disease severity on segregating populations from tall × short-strawed cultivars, a clear tendency was observed for tall-strawed lines to show less severe symptoms of FHB than shorter strawed lines. Such observations are supported by genetic mapping of quantitative trait loci (QTL), where QTLs for plant morphological traits such as plant height and heading date coincide with QTLs for lower FHB severity and DON concentration (Zhu et al., 1999; Ma et al., 2000).

It is beyond the scope of this paper to review the considerable amount of literature that has been published on host resistance to FHB and DON accumulation. The authors, therefore, refer the reader to Kolb et al. (2001) who have reviewed information on molecular markers associated with QTL for resistance to FHB in wheat and barley and the use of those markers for marker-assisted selection, and Ruckenbauer et al. (2001) who recently reviewed breeding strategies in resistance breeding against FHB.

Biological control

There is limited information on the control of FHB by biocontrol agents, although recent reports demonstrate that biocontrol of FHB pathogens has potential. For example, field antagonists of *G. zeae* affected the production of perithecia and ascospores of this pathogen (Bujold et al., 2001). *In vitro* studies on wheat and maize residues (straw/stalk and grain) showed that inoculating residues with a *Microsphaerosis* species (isolate P130A) significantly reduced *G. zeae* ascospore production by 73% (Bujold et al., 2001). When applied to crop residues in the field, the *Microsphaerosis* species had no effect on the pattern of perithecial formation, but significantly reduced

perithecial production. Under glasshouse conditions, the inoculation of wheat ears with *Phoma betae* at anthesis, reduced the severity of FHB symptoms caused by *F. culmorum* by 60% (Diamond and Cooke, 2003). In addition to these observations, it was also recorded that inoculating wheat ears with either *Pythium ultimum* or cell-free germination fluids obtained from a number of FHB-causing pathogens, significantly increased the latent period of *M. nivale*.

Bacterial biocontrol agents have also been investigated. Applications of the bacterial strain AS 43.4 (*Bacillus* spp.) isolated from wheat anthers decreased disease severity of FHB under glasshouse conditions by 67–95% and DON concentration in grain by 89–97% (Khan et al., 1999). In another glasshouse investigation, three (*Bacillus* strains 43.3; 43.4 and *Cryptococcus* strain OH 182.9) out of seven FHB antagonists reduced disease severity by 48–95% and decreased DON quantity in grain by 83–98% (Schisler et al., 2002). Unfortunately, under field conditions, the same antagonistic strains gave variable results. The *Bacillus* strains had no effect on either FHB severity or DON concentration in grain, while strain OH 182.9 reduced FHB and DON by 50%. Field studies undertaken by McMullen et al. (2002) showed that whilst the fungicide tebuconazole provided significant control of FHB, strain OH 182.9 had no effect on disease development.

Unfortunately, the discrepancy between the performance of biocontrol agents under environmentally controlled and field conditions is an issue that is commonly observed, and which provides a major obstacle to the development of commercial biocontrol products.

Chemical control

In vitro work determining the efficacy of fungicides to inhibit the growth and mycotoxin production of *Fusarium* spp. has been undertaken. Moss and Frank (1985) studied the effect of various concentrations of tridemorph on T-2 toxin and diacetoxyscirpenol (DAS) production by *F. sporotrichioides in vitro*. At low concentrations (6–8 mg kg^{-1}) tridemorph caused slight enhancement of fungal growth, whilst significantly reducing the production of T-2 toxin and DAS. However, at higher concentrations of tridemorph (30–50 mg kg^{-1}), fungal growth was inhibited by ca. 50%, but T-2 toxin production was stimulated fivefold. Dichloran, iprodione and vinclozolin were also effective against *F. graminearum* (Hasan, 1993). All three fungicides, when added to potato-dextrose broth at 10, 100, 150 and 500 $\mu g\,ml^{-1}$, produced a significant reduction of mycelial mass, and in production of DAS and ZEN. Dichloran eliminated DAS production at 500 $\mu g\,ml^{-1}$ and eliminated ZEN at 250 $\mu g\,ml^{-1}$. Iprodione reduced DAS production at 100 $\mu g\,ml^{-1}$ and prevented it at 250 $\mu g\,ml^{-1}$. Vinclozolin prevented DAS production at 250 $\mu g\,ml^{-1}$ and significantly reduced the production of ZEN at 500 $\mu g\,ml^{-1}$. In contrast, *F. graminearum* reacted in various ways when incubated in the presence of sub-lethal concentrations of some fungicides such as prochloraz, tebuconazole, benomyl, carbendazim and thiabendazole (Matthies et al., 1999). These workers showed that prochloraz inhibited mycelial growth of *F. graminearum* and reduced 3-ADON production. Tebuconazole inhibited fungal growth at 0.1, 0.5 and 1.0 $\mu g\,ml^{-1}$, however, at 0.5 $\mu g\,ml^{-1}$, 3-ADON production was increased fourfold when compared to control treatments. At 0.1 $\mu g\,ml^{-1}$, benomyl increased mycelial growth of *F. graminearum* by 22% and reduced 3-ADON production by 22% compared to the untreated control. Dose-related inhibition of mycelial growth and mycotoxin production was observed when carbendazim was added to the media at 0.5, 0.7, 1, 1.5 and 2 $\mu g\,ml^{-1}$. Guazatine and iminoctadine significantly reduced mycelial growth of *F. graminearum in vitro*, but increased 3-ADON production by up to 200%. The influence of fungicides on fungal growth and toxin production by *F. sporotrichioides* is affected by temperature (Placinta et al., 1996). In a laboratory study, the fungus was placed on carbendazim-amended potato dextrose agar at concentrations ranging from 1.0 to 10 $\mu g\,ml^{-1}$ and incubated at 25 °C. After 5 days of incubation, half the plates were incubated at 11 °C. The results indicated that at 5 $\mu g\,ml^{-1}$ carbendazim and 25 °C, production of T2-toxin increased, however, no effect on ZEN or neosolaniol (NEO) production was observed. Conversely, following the 25/11 °C temperature regime, dose-related inhibition of ZEN and T-2 toxin was observed. One possible explanation for these variable reactions of *F. sporotrichioides* is the existence of fungicide-resistant strains within the species. D'Mello et al. (2000) demonstrated that when two strains of *F. sporotrichioides,* a control strain (CS) and a strain resistant to carbendazim (RS), were grown at 25 °C in a peptone broth containing 1, 2, or 4 $\mu g\,ml^{-1}$ carbendazim, the CS showed dose-related effects on inhibition of fungal growth and T-2 toxin production, whilst 2 $\mu g\,ml^{-1}$ carbendazim enhanced T-toxin production by the RS, with no effect on mycelial mass.

Effective chemical control of FHB under field conditions has generally been inconsistent. Glasshouse and field trials conducted to assess the efficacy of fungicides against FHB have yielded conflicting results. Jacobsen (1977) studied the effect of benomyl (0.55 kg a.i ha^{-1}), mancozeb (1.76 kg a.i ha^{-1}), mancozeb + benomyl (1.1 kg a.i ha^{-1} + 0.27 kg a.i ha^{-1}) and benomyl + carbendazim (0.55 kg a.i ha^{-1} + 1.1 kg a.i ha^{-1}) on septoria leaf and glume blotch, and FHB caused by *F. graminearum,* on grain yield and test weight of wheat. Fungicide applications resulted in an increase in test weights by up to 2% over unsprayed control plots for benomyl, by 1.2% for the mixture benomyl + mancozeb and by 1.7% for mancozeb. All treatments reduced the percentage of scab-infected grain by 50%. Similarly an application of benomyl + thiabendazole applied 5 days before expected flowering date reduced the incidence of wheat grain infected with *F. graminearum* by 88%, and increased grain germination by 65% and 1000-grain weight by 48% (Carranza, 1988). In contrast, Michail (1989), who observed the incidence of *Fusarium*-infected seed in 32 samples of 16 wheat cultivars from crops sprayed with a range of fungicides (triadimefon, captfol + triadimefon, fenpropimorph, carbendazim, propiconazole, captafol + pyrazofos and prochloraz) at growth stages between GS 32 and GS 50 (Zadoks et al., 1974) failed to find any significant effect of the fungicide treatments on grain infection.

Carbendazim, prochloraz and propiconazole were effective against *F. avenaceum*, *F. culmorum* and *F. graminearum* by reducing them by 70% in the wheat spikelets over the control treatment (Hutcheon and Jordan, 1990). In naturally infected trials in the Atlantic Provinces in Canada, propiconazole, at a rate of 250 g a.i ha^{-1} applied at GS 51 and GS 73 (Zadoks et al., 1974) provided good control of FHB and increased yield by 34% compared to the control (Martin and Johnston, 1982). No effect of the fungicide treatments was observed on the concentration of DON in grain. Milus and Parsons (1994) studied the effects of benomyl, chlorothalonil, fenbuconazole, flusilazole, myclobutanil, potassium bicarbonate, propiconazole, tebuconazole, thiabendazole and triadimefon + mancozeb. These fungicides had no effect on FHB, mycotoxin levels or yield of harvested grain. Fungicides have, however, been shown to affect mycotoxin concentration in harvested wheat grain following FHB infection in the field. A 16-fold increase in NIV concentration occurred in harvested grain when the fungicide mixture (tebuconazole + thiabendazole) was applied 3 h post-inoculation and a sixfold increase when applied 24 h post-inoculation (Gareis and Ceynova, 1994). The severity of FHB symptoms was reduced by 54%. It is difficult to draw firm conclusions from just one trial, however, such observations raise the question of how each of the trichothecenes produced by *Fusarium* spp. are affected by different fungicides.

During fungicide efficacy field trials in Hungary, triadimefon, carbendazim, bromuconazole, cyproconazole + carbendazim, propiconazole, tebuconazole + triadimenol and tebuconazole were applied to plots of the FHB susceptible wheat cultivars Zombor and Csaba 1 day after artificial inoculation of ears with *F. graminearum* and *F. culmorum* (Mesterhazy and Bartok, 1996). Results showed that fungicide treatments that included the triazole tebuconazole, reduced grain infection by between 97% and 99% and DON contamination by 100%. Conversely, those treatments, which did not include tebuconazole, reduced, grain infection by 43–87% and DON contamination by 50%. The efficacy of 'azole' fungicides against FHB was confirmed by Ellner (1997). The fungicides tebuconazole, tebuconazole + triadimenol and prochloraz, significantly reduced FHB severity by 50% and DON concentration by 85%. One possible reason for the inconsistent control of FHB achieved by fungicides under field conditions is the complex interaction, which may occur between fungicide *Fusarium* species and other ear colonising fungi such as *Alternaria, Septoria, Cladosporium* and *Botrytis cinerea*. For example, Bateman (1979) studied the relationship between saprophytic ear colonising species and *M. nivale* on wheat ears and wheat seed. Grain collected from wheat ears which were artificially inoculated with either *Alternaria* spp., *Cladosporium* spp. or *Sporobolodomyces* spp. at anthesis prior to the inoculation of ears with *M. nivale,* yielded significantly less *M. nivale* in comparison with saprophyte-free ears. Similar results were obtained by Liggitt et al. (1997) during glasshouse studies where wheat plants were inoculated with either *A. alternata*, *B. cinerea* or *C. herbarum* at GS 59 prior to inoculation with *F. culmorum* at GS 65. The presence of any one of the three saprophytic species reduced FHB severity by between 46% and 78% compared to plants inoculated only with *F. culmorum*. However, when each of the saprophytic species were introduced to ears after their inoculation with *F. culmorum*, neither *B. cinerea* nor *C. herbarum* had any effect on FHB severity, although *A. alternata* significantly increased disease symptoms by 34%. Liggitt et al. (1997) also

demonstrated that fungicides had different effects. For example, pyrimethanil reduced mycelial growth of *A. alternata* by up to 92%, but failed to reduce growth of *F. culmorum*, *B. cinerea* or *C. herbarum* by more than 27%. Conversely, flusilazole reduced mycelial growth of *F. culmorum* by up to 90%, but failed to reduce mycelial growth of *B. cinerea* or *C. herbarum* by more than 59%. These workers suggested that the application of fungicides which have limited activity against *F. culmorum*, but which have significant activity against saprophytic species, may lead to greater colonisation of wheat ears by the pathogen, due to the removal of antagonistic saprophytes.

Interactions between fungicides and FHB pathogens have also been observed. Applications of the strobilurin fungicide, azoxystrobin, may have a significant effect on the interaction between *F. culmorum* and *M. nivale* colonising wheat ears. During 2 years of field studies, applications of tebuconazole, metconazole or carbendazim caused a significant reduction in both DON concentration and the extent of grain colonisation by *Fusarium* spp. quantified by a competitive PCR assay (Jennings et al., 2000). Conversely, applications of the same fungicides resulted in an increase in the extent of grain colonised by *M. nivale.* In the first year of the study, effective reduction of *M. nivale* on wheat ears, achieved following applications of azoxystrobin, alleviated competition between *M. nivale* and *Fusarium* spp. As a result, greater colonisation of ears by *Fusarium* spp. was observed and DON contamination was increased by 41%. In the second year, *M. nivale* was not present and no significant increase of DON concentration in grain was detected after treatment with azoxystrobin. More recently, Simpson et al. (2001) also associated applications of azoxystrobin with increased DON concentrations in harvested wheat grain. During a field trial where wheat ears were artificially inoculated with a mixture of *F. avenaceum*, *F. culmorum* and *M. nivale* at GS 65 and then sprayed with an application of azoxystrobin three days later, a 40% increase in DON concentration was observed in harvested grain, compared to grain harvested from unsprayed plots. The quantification of *F. culmorum* DNA did not indicate an increase of this species in grain, although *M. nivale* DNA was significantly reduced by azoxystrobin.

It can thus be postulated that applications of azoxystrobin might have a direct effect on DON production, through imparting a stress factor on *F. culmorum*, inducing the pathogen to produce more mycotoxin. However, by quantifying the amount of the *Tri*5 gene present in grain following fungicide efficacy trials under both glasshouse and field trials, azoxystrobin appeared to have no direct effect on DON production (Edwards et al., 2001; Pirgozliev et al., 2002). Indeed, when the concentration of *Tri*5 DNA was related to DON content, a significant and strong positive linear relationship was observed between the two variables. Plotting *Tri*5 DNA and DON concentrations for each of a range of fungicides and dose rates revealed that those fungicides which were ineffective at controlling *F. culmorum*, such as azoxystrobin, allowed greater colonisation of grain by the fungus and hence more *Tri*5 DNA: this was reflected in a greater DON content. None of the fungicide treatments resulted in an elevated production of DON per copy of *Tri*5 DNA, an observation that would be expected if a particular fungicide directly influenced DON production. It would appear, therefore, that fungicides can influence DON content in harvested grain, and that this influence is indirect by affecting the extent of grain colonisation by toxin-producing *Fusarium* species.

In contrast to the observations of Jennings et al. (2000) and Simpson et al. (2001), Jones (2000) observed a decrease in FHB severity of 12% compared to unsprayed controls and a reduction of DON by 25% when azoxystrobin was applied during field trials inoculated with *F. graminearum* between 1995 and 1997. Siranidou and Buchenauer (2001) also showed that applications of azoxystrobin reduced FHB severity, although DON concentration was similar to that in unsprayed controls. Similarly, azoxystrobin provided some control of FHB, without any detrimental effects on the mycotoxin production (Cromey et al., 2001). Azoxystrobin, tebuconazole and carbendazim were applied at GS 59 or 65 on winter wheat plots naturally infected with *Fusarium* spp. (predominantly *F. graminearum*). Tebuconazole reduced disease severity by 41%, whilst azoxystrobin and carbendazim reduced FHB by 29% compared to the control treatment. Tebuconazole and carbendazim significantly reduced DON and NIV in grain, whilst azoxystrobin did not have any effect on these mycotoxins.

Another possible reason for the inconsistent control of FHB achieved by fungicides under field conditions could be the timing of application. Studies where fungicides were applied between GS 32 and GS 50 failed to reveal any significant reduction of FHB (Michail, 1989; Hutcheon and Jordan, 1992). However, when fungicides were applied between GS 59 and 70, significant reductions were achieved in both the severity of FHB and concentration of mycotoxin in harvested

grain. For example, during a field trial where wheat plots were artificially inoculated with *F. graminearum* at anthesis, Boyacioglu et al. (1992) observed that triadimefon reduced grain infection and DON concentration when applied either 2 days pre-inoculation, at the time of inoculation or 2 days post-inoculation of ears. Propiconazole also reduced *F. graminearum* infection by 39–56% and DON concentration by 62–79% when applied at the time of inoculation and 2 days post-inoculation of ears. Thiabendazole was most effective, reducing DON concentration by 83% when applied 2 days pre-inoculation despite the fungicide having no effect on the incidence of grain infection. In Germany, Matthies and Buchenauer (2000) reported that in wheat inoculated with *F. culmorum,* applications of either tebuconazole or prochloraz, 2 days post-inoculation (GS 65), reduced disease severity by 56% and 41%, whilst applications 8 days pre-inoculation or 9 days post-inoculation were less effective. Tebuconazole and prochloraz applied 2 days post-inoculation reduced DON content in grain by 43% and 22%. However, an application of tebuconazole against FHB (*F. culmorum*) 3 days pre-inoculation reduced disease severity by 92% and DON concentration in grain by 69% in comparison with control treatment, whilst the same fungicide applied 5 days post-inoculation reduced FHB by 57% and DON in grain by 54% (Homdork et al., 2000). The greatest reduction of DON concentration in grain (80%) and FHB severity (90%) was achieved when tebuconazole was applied twice (pre- and post-inoculation). Suty et al. (1996), also reported that double treatment of tebuconazole at GS 55 and 69 was more effective than a single treatment at either GS 55 or 69. However, this approach may be uneconomical in commercial situations as it would result in increased fungicide input.

More recently, Siranidou and Buchenauer (2001) showed that applications of tebuconazole 2 days before and 2 days post-inoculation of wheat plots with *F. culmorum* reduced severity of FHB and DON content in wheat grain by 61–89% and 50–70%, respectively. Metconazole was applied at only 2 days pre-inoculation and reduced DON and FHB severity by 69% and 71% respectively. Chlorothalonil, prochloraz and benomyl failed to effectively control FHB.

Future developments

Due to the lack of consistently effective control measures, FHB continues to pose a significant threat to the yield and quality of small grained cereals. Although cultural control strategies such as rotation, land preparation and weed control can have an effect on inoculum load, the ubiquitous nature of the causal pathogens means that such control measures will always be limited. A range of fungicides have been identified with good activity against FHB pathogens. Unfortunately, the efficacy of these fungicides is significantly influenced by the dose rate used, time of application and perhaps even the method of application. Effective chemical control of FHB is further confounded by the fact that the disease is caused by a complex of pathogens which interact with one another and with saprophytic species such as *Alternaria* spp. and *Cladosporium* spp. (Liggitt et al., 1997; Pirgozliev, 2002). For example, glasshouse studies have shown that introducing either *Alternaria tenuissima, C. herbarum* or *M. nivale* to wheat ears before inoculation with *F. culmorum*, significantly increased FHB symptoms and DON content (Pirgozliev, 2002). Furthermore, applying azoxystrobin, a fungicide know to be effective against *M. nivale*, after the inoculation of wheat ears with this pathogen (Simpson et al., 2001) at GS 57 resulted in a 56% increase on DON content caused by *F. culmorum*. In order to optimise the control of FHB and mycotoxin contamination of grain, therefore, detailed studies on the effect of fungicide treatments on the cereal ear disease complex needs to be undertaken. The quantification of FHB-causing pathogens using molecular PCR assays following fungicide treatments will prove invaluable in advancing our understanding of the disease complex and in optimising disease and mycotoxin control.

The most reliable and consistent strategy for controlling FHB and mycotoxins is the exploitation of cultivars with good resistance. Large quantitative variation for FHB resistance in wheat has been described (Buerstmayr et al., 1996). Molecular mapping of QTL associated with FHB resistance, such as that carried out by Buerstmayr et al. (2002) for spring wheat, will prove invaluable in marker-assisted selection and will significantly speed up the development of cultivars with good resistance to FHB and mycotoxin contamination.

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Major changes in *Fusarium* spp. in wheat in the Netherlands

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Key words: fusarium head blight, multiplex PCR, population diversity, molecular identification, mycotoxin

Abstract

The re-emergence of fusarium head blight throughout the world and especially in Western Europe prompted a survey of the situation in the Netherlands. To allow for a high throughput screening of large numbers of samples, a diagnostic PCR method was developed to detect the most common species of *Fusarium* occurring on wheat. Seven primer pairs were tested for their ability to identify isolates of *Fusarium avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. proliferatum* and *Microdochium nivale* var. *majus* and *M. nivale* var. *nivale*. Each primer pair only generated a PCR product with the corresponding *Fusarium* species and all PCR fragments had different molecular sizes. This allowed the generation of these amplicons using a mixture of all seven primer pairs. The robustness of this multiplex PCR encouraged us to screen a large series of isolates collected in 2000 and 2001. In both years 40 fields were sampled leading to a collection of 209 isolates from 2000 and 145 isolates from 2001. The results of the multiplex PCR demonstrated that *F. graminearum* was the most abundant species in the *Fusarium* complex on wheat in both years. This is in sharp contrast to reports from the 1980s and early 1990s, which found *F. culmorum* as the predominant species. Primers derived from the *tri7* and *tri13* genes, which are implicated in the acetylation and oxygenation of the C-4 atom of the backbone of the trichothecene molecule, were used to discriminate between deoxynivalenol and nivalenol (NIV) producers. The populations of *F. culmorum* and *F. graminearum* both showed a slight increase in NIV-producers in 2001.

Introduction

Fusarium head blight (FHB) or fusarium scab of small-grain cereals is caused by a complex of species. Although up to 17 species have been associated with the disease (Parry et al., 1995), the predominant species are *Fusarium avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *Microdochium nivale*. FHB can reduce yield by 30–70% (Bai and Shaner, 1994) but currently the major concern regarding FHB arises from the ability of the majority of species to produce mycotoxins. It is well documented that *F. culmorum*, *F. graminearum* and *F. poae* can produce different trichothecenes. Moreover, *F. avenaceum* produces moniliformin (Schütt et al., 1998). The most predominant mycotoxins found in small-grain cereals are deoxynivalenol (DON) (also known as vomitoxin) and nivalenol (NIV) and their derivatives.

Reports from all continents demonstrate the re-emergence of this devastating disease and its high economic impact. In the mid 1990s, *Fusarium* epidemics caused large losses in wheat production in the US and Canada. Economic damage was estimated at US $2500 million in wheat and US $400 million in barley in the US alone and US $220 million and US $300 million in Canada for wheat and barley respectively (Windels, 2000). The epidemics in the US were primarily caused by *F. graminearum*, which has been shown to be a complex in its own right, with seven or even eight lineages with different geographic and phytopathological characteristics (O'Donnell et al., 2000). In China and New Zealand, *F. graminearum* also seems prominent,

but large variations were observed between years (Carter et al., 2000; Cromey et al., 2002). Studies in many countries in Europe, on the other hand showed that other species of the complex are more dominant. Surveys performed in Scandinavia, Hungary and other mid-European countries identified *F. avenaceum* and *F. poae* as the most important species (Lukanowski and Sadowski, 2002). In Germany, particularly in the Bavarian region, *F. graminearum* was frequently encountered. This might be a special situation since *F. graminearum* is also found on maize. Crop rotations of wheat and maize generate conditions that are very conducive for *F. graminearum*. This may be aggravated by the fact that ascospores can contribute substantially to the inoculum pressure, with two or even more cycles per growing season (Obst et al., 2002). Studies performed in the UK, on the other hand, identified *F. culmorum* as the most important cause of head blight (reviewed by Parry et al., 1995). In more recent years *M. nivale* and *F. poae* were also frequently encountered (http://www.csl.gov.uk/resdev/AH/PDCP/epid/fusarium/inc2.cfm).

In the Netherlands, studies in the 1980s by Daamen et al. (1991) and in the early 1990s by De Nijs et al. (1996) identified *F. culmorum* as the predominant agent for FHB. The majority of the older studies were based on the morphological characterization of the isolated individuals, but more recent work relied on the application of DNA fingerprinting methodologies. Using RAPD analyses, De Nijs et al. (1997) confirmed that the predominant species in the Netherlands was indeed *F. culmorum*. The isolates used had been sampled in the early 1990s (1991 and 1993). Reports from neighbouring countries, where an increased importance of *F. graminearum* was observed, stimulated a new survey in 2000 (Waalwijk et al., 2001). The results of this survey, presented in this paper, demonstrate a drift in the *Fusarium* populations from *F. culmorum* in the early 1990s to *F. graminearum* in 2000 and 2001.

The aim was to develop a molecular screen for field isolates, that in principle belong to any of the 17 species within the Fusarium head scab complex (Parry et al., 1995). To enable screening of large numbers of samples, a multiplex PCR was developed that detects the major species within the complex.

The major mycotoxins that contaminate small-grain cereals are the type A trichothecenes T-2 and HT-2, primarily produced by *F. poae* and *F. sporotrichioides*, and the type B trichothecenes DON (or vomitoxin) and NIV produced mainly by *F. graminearum* and *F. culmorum*. Although the synthesis and regulation of expression of these mycotoxins are largely unknown, some major steps in the biosynthesis have been elucidated and the genes involved appear to be clustered. Comparative analyses of these gene clusters in DON-producers and NIV-producers have identified the genes *tri13* and *tri7* as vital in the synthesis of either NIV or DON. Both genes appear intact in NIV-producing isolates but in DON-producers they contain multiple mutations. Alignment of the *tri13* gene from a NIV-producer with its homologue in a DON-producer reveals many alterations including several deletions (Lee et al., 2002; Brown et al., 2002). Disruption of the *tri13* gene in a NIV producer converts it into a DON producer. Moreover, heterologous expression of an intact *tri13* gene isolate in a DON producer conveys the ability to produce NIV. (Lee et al., 2002; Brown et al., 2002). The *tri7* gene, in turn, appears to be involved in the acetylation of NIV leading to 4-acetylnivalenol (4-ANIV). The most striking feature of the *tri7* gene in DON-producers is the presence of a multiple repetition of 11 bp that occurs only once in NIV-producers. Moreover, this gene apparently misses a functional starting codon (Lee et al., 2001). Gene sequences from both genes were used to develop primers that were utilized to screen our 2000 and 2001 populations for frequencies of DON- and NIV-producers. Part of this work has been presented at the 7th European Fusarium Seminar (Waalwijk, 2002).

Materials and methods

Fungal isolates and sampling strategy

Tester isolates from the collection at Plant Research International B.V. (PRI) were used to obtain DNA samples from *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *M. nivale* (see Table 1). Species-specific primers from *F. proliferatum*, developed in our laboratory, were also included. Isolates representing the seven lineages of *F. graminearum* (O'Donnell et al., 2000) were included for comparison.

In 2000 and 2001, wheat ears with visual symptoms of FHB were sampled from 40 different fields throughout the Netherlands. Isolates were obtained by hierarchical sampling: on five different places in each field a visible diseased ear was collected and from each ear two kernels were taken for isolation of *Fusarium* spp. These kernels were surface disinfected by washing them sequentially with 70% ethanol and 2% sodium hypochlorite. Subsequently, the kernels

Table 1. Isolates from the PRI collection used to develop multiplex PCR, primer designations, anticipated sizes of the PCR fragments and literature sources

Species	Isolate	Primer name	Sequence	Sizes (bp)	Ref[b]
F. avenaceum	IPO 92-3	FaF	CAAGCATTGTCGCCACTCTC	920	A
		FaR	GTTTGGCTCTACCGGGACTG		
F. culmorum	PD90-283	Fc01F	ATGGTGAACTCGTCGTGGC	570	B
		Fc01R	CCCTTCTTACGCCAATCTCG		
F. graminearum	PD88-790	Fg11F	CTCCGGATATGTTGCGTCAA	450	B
		Fg11R	GGTAGGTATCCGACATGGCAA		
F. poae	PD93-1780	Fp8F	ACGACGAAGGTGGTTATG	1600	C
		Fp8R	GGTGAAGAGCCTGTTTGCTTG		
F. proliferatum	ITEM2287	TH5-F	GATAACGTCCAAGGCTACG	330	D
		TH6-R	GGGGTCGTTCAGCTCAAGG		
M. nivale var. *nivale*	—	Y13NF	ACCAGCCGATTTGTGGTTATG	310	A
		Y13NR	GGTCACGAGGCAGAGTTCG		
M. nivale var. *majus*[a]	IPO 1.21	Mnm2F	TGCAACGTGCCAGAAGCT	750	A
		Mnm2R	AATCGGCGCTGTCTACTAAAAGC		
		GzTri7/F	GGCTTTACGACTCCTCAACAATGG	$162 + [11]_n^c$	E
		GzTri7/R	AGAGCCCTGCGAAAGYACTGGTGC		
		Tri13F	TACGTGAAACATTGTTGGC	234 or 415	F
		Tri13R	GGTGTCCCAGGATCTGCG		

[a]The original isolate in the PRI collection was not determined to the variety level, but an amplified product of 750 bp with primer pair Mnm2F/Mnm2R and absence of a product with primer pair Y13MF/Y13MR identified it as *M. nivale* var. *majus*.

[b]References used: (A) Doohan et al. (1998); (B) Nicholson et al. (1998); (C) Parry and Nicholson, (1996); (D) unpublished; (E) Lee et al. (2001) and (F) this study.

[c]$162 + [11]_n$, PCR fragments were obtained of 162 bp in the case of NIV-producers or 162 bp plus a multitude of 11 bp, e.g. 173, 184, 195 etc. in DON-producers (Lee et al., 2001).

were washed extensively with sterile distilled water and placed on PDA plates containing 10 mg l^{-1} tetracycline and 100 mg l^{-1} streptomycin. Plates were incubated for 5 days at 20 °C and *Fusarium*-like mycelium was transferred to new PDA plates containing the antibiotics. In this way, 209 and 145 isolates were obtained in 2000 and 2001, respectively.

DNA manipulations

Isolates were grown on PDA for 3–5 days and mycelium was harvested by filtration though cheesecloth. After lyophilization and grinding, DNA was obtained using the Puregene extraction protocol (Kema et al., 2002). High throughput DNA isolations from field isolates were done as follows: isolates were grown at 20 °C for 4–5 days in 96 well format blocks with individual tubes containing 0.7 ml of potato dextrose medium per well, with daily inversions to mix the content of the tubes. After addition of a tungsten bead and lyophilization, mycelium was ground by vigorous shaking of the blocks in a MM300 mixer mill (Retch, Ochten, the Netherlands), followed by the DNA extraction using the Puregene kit. Gel electrophoresis was used to check the quality of the DNA as well as to estimate the concentration of each sample.

Standard PCRs with a single primer set were performed for 40 cycles (1 min denaturation at 94 °C, 30 s annealing at 60 °C and 1 min extension at 72 °C) followed by a final extension of 5 min at 72 °C and storage at 4 °C until harvest of the samples. Template DNA was used at 20 ng, species-specific primers (Table 1) were added at 6 μM and amplicons were separated on 1.0% agarose. *Tri7* PCRs, using the primers Gztri7F and Gztri7R (Lee et al., 2001) and *tri13* PCRs with the primers tri13F and tri13R (Table 1) were also performed under these conditions.

Multiplex PCRs were performed under identical conditions, except that all seven species-specific primer pairs, i.e. 14 primers, were combined in the same reaction at 6 μM each. PCRs with the ITS primers

ITS1 and ITS4 were done for 30 cycles at 57 °C annealing temperature (Waalwijk et al., 1996). Mating type specific primers for the *Mat1-1* and *Mat1-2* loci in *Fusarium* spp. were used to confirm the homothallic nature of the isolates identified as *F. graminearum*. These reactions were performed as described before (Moretti et al., 2002).

Sequencing of fragments was done using the Big Dye technology of Applied Biosystems and sequence reactions were run on ABI 3700 equipment of the Greenomics® facility at PRI. DNA sequence analyses were performed using the multiple sequence alignment programme MegAlign (DNASTAR).

Results

To test whether a multiplex PCR approach would be feasible for the molecular identification of the field isolates obtained in 2000 and 2001, primers documented in literature were tested on laboratory isolates under regular PCR conditions. DNA from isolates of the major pathogens from the FHB complex, i.e. *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. proliferatum* and *M. nivale* was subjected to PCRs with their corresponding species-specific primers (Table 1). As the isolate from *M. nivale* in our collection, IPO 1.21, was not identified to the variety level, it was unclear which PCR fragment to expect. The fragment obtained was 750 bp in size thereby identifying IPO 1.21 as *M. nivale* var. *majus* (Doohan et al., 1998). At a temperature of 60 °C, each of these DNAs generated amplicons with sizes that were expected (Table 1). Since these reactions were performed under the same conditions and each species-specific fragment has its unique size, the number of species-specific primers within the same PCR reaction was increased to a combination of all 14 primers for five *Fusarium* and two *Microdochium* species. As with the singular PCR, each of the isolates generated only a single PCR fragment and the sizes of these fragments were in accordance with those expected for the respective species (Figure 1A).

Subsequently, 209 isolates from 2000 and 145 isolates from 2001 were analysed using this multiplex PCR. The majority of isolates (>90%) from these fields generated PCR fragments that allowed classification into the major pathogens of the FHB complex (e.g. Figure 1B). Only a small subset of isolates from each year could not be classified, because (i) no PCR product was obtained (Figure 1B) (ii) a combination

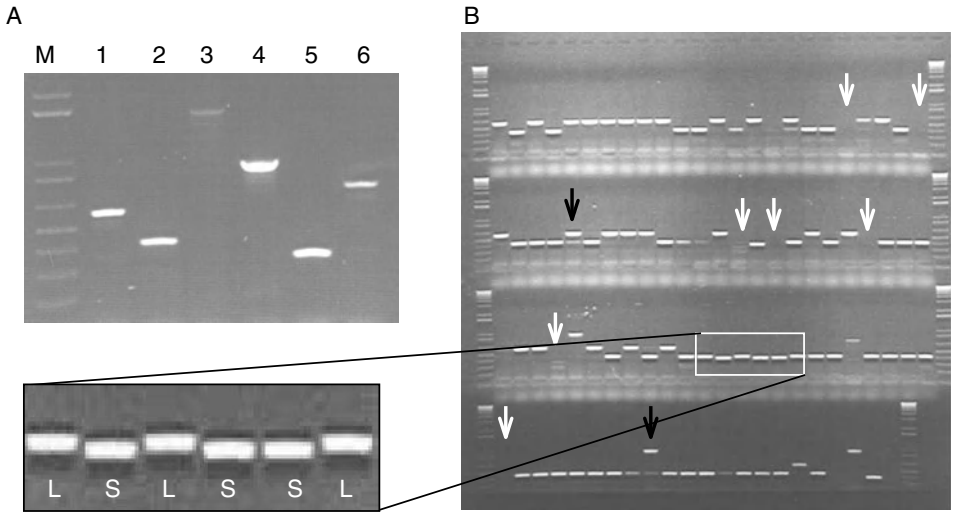

Figure 1. (A) Multiplex PCR with mixture of 14 primers on DNA purified from tester isolates. M, 1 kb molecular markers; 1, *F. culmorum* isolate PD90-283; 2, *F. graminearum* PD88-790; 3, *F. poae* PD93-1780; 4, *F. avenaceum* IPO 92-3; 5, *F. proliferatum* ITEM 2287 and 6, *M. nivale* var. *majus* IPO1.21. (B) Multiplex PCR on a series of field isolates ($n = 88$). On the bottom row the tester isolates were also run for comparison. White arrows indicate lanes without clear fragments and black arrows indicate lanes with dual fragments. The boxed area was zoomed in to illustrate the presence of size variation within the *F. graminearum* isolates (designated L and S).

of two PCR fragments was observed, each corresponding to a different species of the FHB complex (Figure 1B), or (iii) fragments of aberrant size were obtained (Figure 1B). Individuals that did not produce a PCR product, were tested with the generic primers ITS1 and ITS4, which amplify the ITS1 and ITS2 region of the ribosomal RNA genes. Some isolates from this category again failed to generate a PCR product, suggesting that the quality and/or the quantity of DNA was insufficient for proper amplification. However, the majority of these strains generated a clear ITS fragment, indicating that the quality of the DNAs was good. To resolve the inconsistency of a robust ITS-PCR but no species-specific fragment, the amplified ITS regions from these isolates were sequenced. The sequences obtained were very distinct from the *Fusarium* ITS sequences deposited in the publicly accessible databases. Six out of nine ITS sequences obtained were identical to *Epicoccum* spp. and three showed substantial deviation from any ITS sequence in the databases. Further morphological identification at the Fungal Biodiversity Center, CBS, Utrecht, the Netherlands identified isolates 1H2 and 6H1 as *Ascochyta* spp. and isolate 6G1 as *Ascochyta skagwayensis*. The sequences of these isolates have been deposited at Genbank under AF520640–AF520642.

To clarify the category containing dual fragments, monospore cultures were produced which generated only a single fragment indicating that the sample was a mixture of two different species for the FHB complex. Fragments of aberrant size were occasionally obtained, as can be seen in the boxed area in Figure 1B. In PCR reactions with mating type primers these isolates gave both *Mat 1-1* and *Mat 1-2* fragments indicating that these isolates were homothallic, i.e. *F. graminearum*. This was confirmed by sequencing the fragments from the multiplex reactions which identified them as being generated by the *F. graminearum* specific primers Fg11F/R. The sequences from long and short fragments were identical except for 30 nucleotides that were absent in the aberrant fragments (Figure 2A). When isolates representing the seven lineages of *F. graminearum* (O'Donnell et al., 2000) were tested, only Fg11F/R products were obtained in lineages 1, 2, 6 and 7 and no products were found in lineages 3–5. Comparison of the sequences of the Fg11R/F amplicons from the Dutch isolates with those from lineages 1, 2, 6 and 7 identified the presence of one, two or three copies of this 30 bp fragment (Figure 2A). Interestingly all isolates from lineage 6 contained an additional insertion of 86 bp in the Fg11F/R amplicon. The presence of this latter insertion is causing the size increase between isolate PD88-790 and isolate NRRL6101 (Figure 2B). PD88-790 was isolated in 1988 in the Netherlands and therefore likely to belong to lineage 7. Isolate NRRL6101 was identified as a lineage 6 isolate (O'Donnell et al., 2000). Multiple sequence alignment including isolates from the lineages 1, 2, 6 and 7 clearly showed that the Dutch isolates cluster with isolates of lineage 7 (Figure 2C).

The sampled fields were located in different parts of the country. For comparative analyses they were grouped according to the regional structure used by De Nijs et al. (1996). Irrespective of the region of isolation the most common species of the FHB complex observed in 2000 was *F. graminearum*. The percentage of isolates identified as *F. graminearum* ranged from 47.9% in the south-west to 80.0% in the south-east, with an average of 58.4% (Table 2). *Fusarium culmorum* ranged from 8.2% in the south-east to 36.7% in the south-west and *M. nivale* var. *majus* from 0.0% in the south-east to 27.4% in the polders in the centre of the Netherlands (Figure 3). In 2001, the same regions, but different fields, were sampled and again *F. graminearum* was the dominant species, with an average frequency of 58.6%, with *F. culmorum* at 29.7% and *M. nivale* var. *majus* at 6.9% (Table 3). Interestingly, only *M. nivale* var. *majus* was observed and *M. nivale* var. *nivale* was never encountered. The identification of other species in the complex, i.e. *F. avenaceum* and *F. poae*, was very rare in both years and *F. proliferatum* was not found (Table 2). The frequencies of isolation of the different species from various cultivars did not show any interaction with the sole exception of *cv*. Renan in 2000 where 7 out of 10 isolates were *M. nivale var. majus* (Table 4). In total, 81 pairs of isolates from the same ear were obtained, that contained two *F. graminearum* ($n = 26$), two *F. culmorum* ($n = 9$) or two *M. nivale* var. *majus* ($n = 2$) isolates. The remainder ($n = 44$) contained two different species.

The genes *tri13* and *tri7*, involved in oxygenation and acetylation of the C-4 residue of the trichothecene backbone respectively, were used to identify the putative chemotype of each of the isolates. With the primer pair Gztri7F/R, NIV-producers generated a 162 bp fragment and DON-producers generated a fragment of 162 bp plus a multiple of 11 bp, i.e., 173, 184, 195 bp, in size (Lee et al., 2001). Several of these differently sized amplicons were obtained

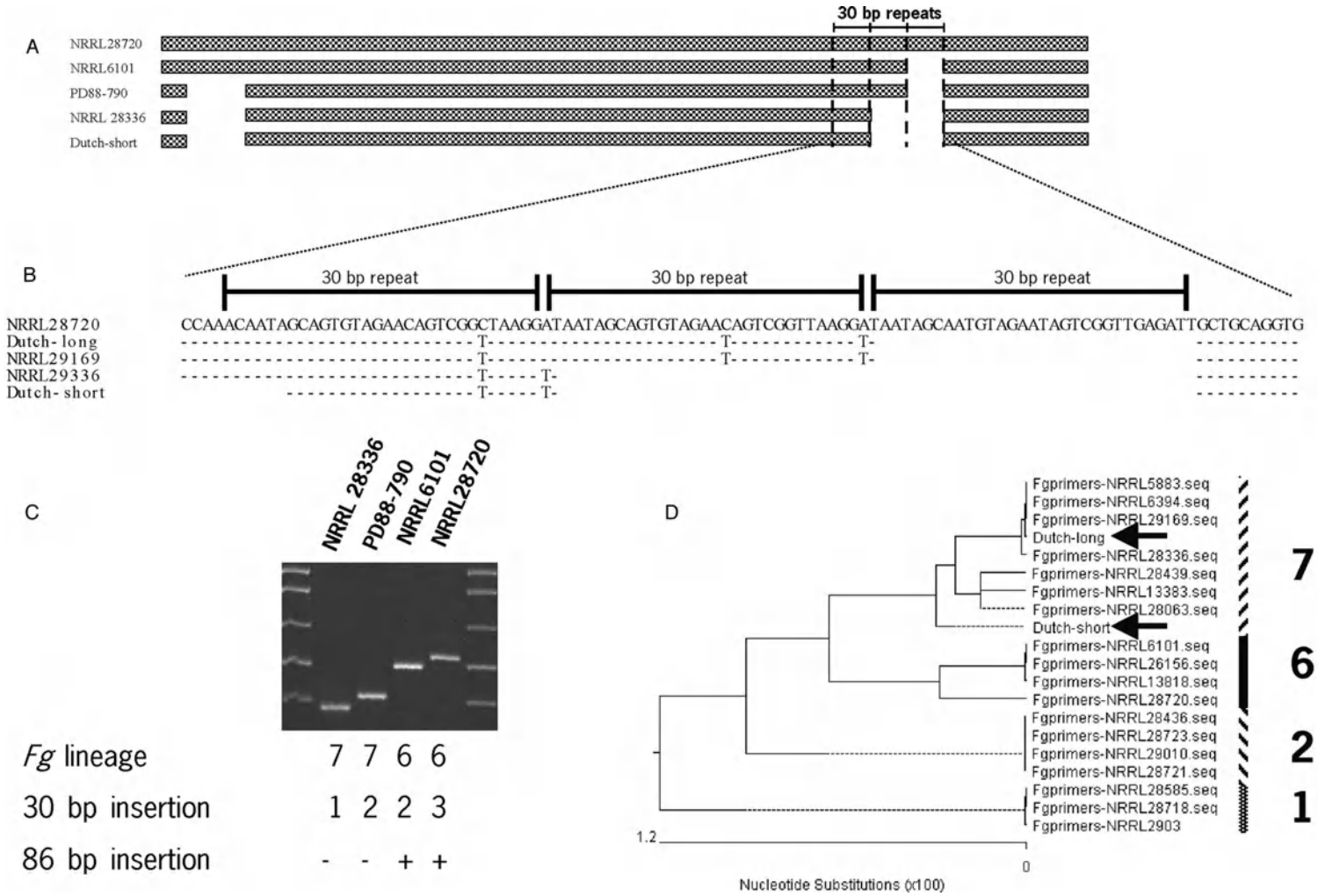

Figure 2. (A) Schematic representation of the alignment of the sequences obtained from *F. graminearum* isolates generating size variation with the primers Fg11F and Fg11R. NRRL28720, isolate of *F. graminearum* from lineage 6 containing three 30 bp repeats and the 86 bp insertion; NRRL6101, isolate of *F. graminearum* from lineage 6 containing two 30 bp repeats and the 86 bp insertion; PD88-790, *F. graminearum* tester isolate from The Netherlands, with two 30 bp repeats but missing the 86 bp insertion; NRRL28336, isolate of *F. graminearum* from lineage 7 containing one 30 bp repeat and missing the 86 bp insertion; Dutch-short, isolate 47E1, from the 2001 survey containing one 30 bp repeat and missing the 86 bp insertion. (B) Multiple sequence alignment of part of the Fg11F/R fragments from different isolates of *F. graminearum*, illustrating the presence of a (several) copies of a 30 bp direct repeat. NRRL28720, isolate of *F. graminearum* from lineage 6 containing three 30 bp repeats: Dutch-long, sequence of isolates from this survey (designated L in Figure 1B) and NRRL29169, isolate of *F. graminearum* from lineage 7 both containing two repeats; NRRL28336, isolate of *F. graminearum* from lineage 7 and Dutch-short (designated S in Figure 1B) containing one repeat. (C) Size variation in the Fg11 F/R amplicon among isolates from different lineages. (D) Multiple alignment using MegAlign (DNASTAR) of the FG11F/R fragments generated from representatives of the lineages of *F. graminearum*. No amplicon was obtained with lineages 3–5. Clustering of the remaining lineages was according to that observed by O'Donnell et al. (2000).

Table 2. Numbers of *Fusarium* spp. in different regions (2000/2001)

Region	*F. avenaceum*	*F. culmorum*	*F. graminearum*	*M. nivale* var. *majus*	*F. poae*	Others	Total
South-west	0/0	18/9	25/10	5/7	0/0	1/1	49/27
East	1/0	4/1	14/6	3/0	0/1	1/0	23/8
Central	1/0	14/15	35/27	20/3	0/1	3/1	73/47
North-east	1/1	4/12	36/29	6/0	0/0	2/1	49/43
South-east	0/0	2/6	12/13	0/0	0/1	1/0	15/20
Total	3/1	42/43	122/85	34/10	0/3	8/3	209/145

when the populations of 2000 and 2001 were screened with these primers (Figure 4A). The primer pair *tri13F/R* was designed by comparing the published sequences for this gene from known NIV- and DON-producers (Accession# AF336365, AF366366 and AY057841–AY057844). They generated a 415 bp fragment in NIV-producers and a fragment of 234 bp in DON-producers (Figure 4B). In general, the *tri13* primers were more robust with 78.8% of the *F. culmorum* and 87.9% of the *F. graminearum* isolates giving a fragment. With the *tri7* primers these values were 74.1% and 86.0%, respectively. A comparison between both primer sets showed a correlation (DON or NIV with both the *tri7* and *tri13* primers) of 76.5% for *F. culmorum* and 91.8% for *F. graminearum*. Table 5 shows the distribution of NIV- and DON-producers for both *F. culmorum* and *F. graminearum*. In general, *F. culmorum* comprised primarily NIV-producers and *F. graminearum* isolates were mainly DON-producers. Nevertheless, both populations showed a small shift towards a higher percentage of NIV producers in 2001 (Table 5).

Discussion

The re-emergence of FHB on many continents has caused much concern related to food and feed production and safety. This prompted a survey of the situation in the Netherlands. To allow for high throughput screening of large numbers of samples, a multiplex PCR was developed to simultaneously detect the most frequently encountered species of the FHB complex. This diagnostic PCR appeared to be very robust, since only a small percentage of isolates were not readily identified. Most of these isolates appeared to belong to other fungal genera, like *Epicoccum* and *Ascochyta* and were apparently erroneously considered to be *Fusarium* spp. during isolation from the surface-sterilized kernels. Although these remote genera can be easily recognized using morphological criteria, the regular emergence of new *Fusarium* spp. in the disease complex is a taxonomic challenge that is appropriate only to experts. The multiplex PCR, contained a combination of primers that allowed simultaneous detection of seven species, but we anticipate that this number can be increased further. The only prerequisite is that the species-specific amplicons can be identified unambiguously.

The results clearly underline the reports from other European surveys that point to an increase in the importance of *F. graminearum* as a major pathogen of wheat in temperate climates. In the Netherlands, *F. culmorum* was the major component of the FHB complex in the 1980s and 1990s (Daamen et al., 1991; De Nijs et al., 1996). However, *F. graminearum* was the primary species in our surveys for two consecutive years which suggests a dramatic shift in the composition of the FHB complex. Although there is a gap of 7 years between the 1990s samplings and the present surveys, this trend seems to have occurred in the mid-1990s, since *F. graminearum* was also predominant in 1997 (S. Edwards, pers. commun.). The causal factors for this shift have not been elucidated, but an increase in maize production has been suggested to play an important role. *F. graminearum*, in contrast to *F. culmorum,* is well recognized as a major pathogen on maize and, more importantly, has the capacity to survive on maize stubble. Nevertheless, other factors can be put forward to explain the increased occurrence of *F. graminearum*. Climatic changes might favour the propagation of *F. graminearum* over *F. culmorum,* as the former species has a higher temperature optimum. The homothallic nature of *F. graminearum* allows the production of large masses of ascospores that can play a role in the epidemiology. In a recent study in Germany the important contribution of ascospores to inoculum

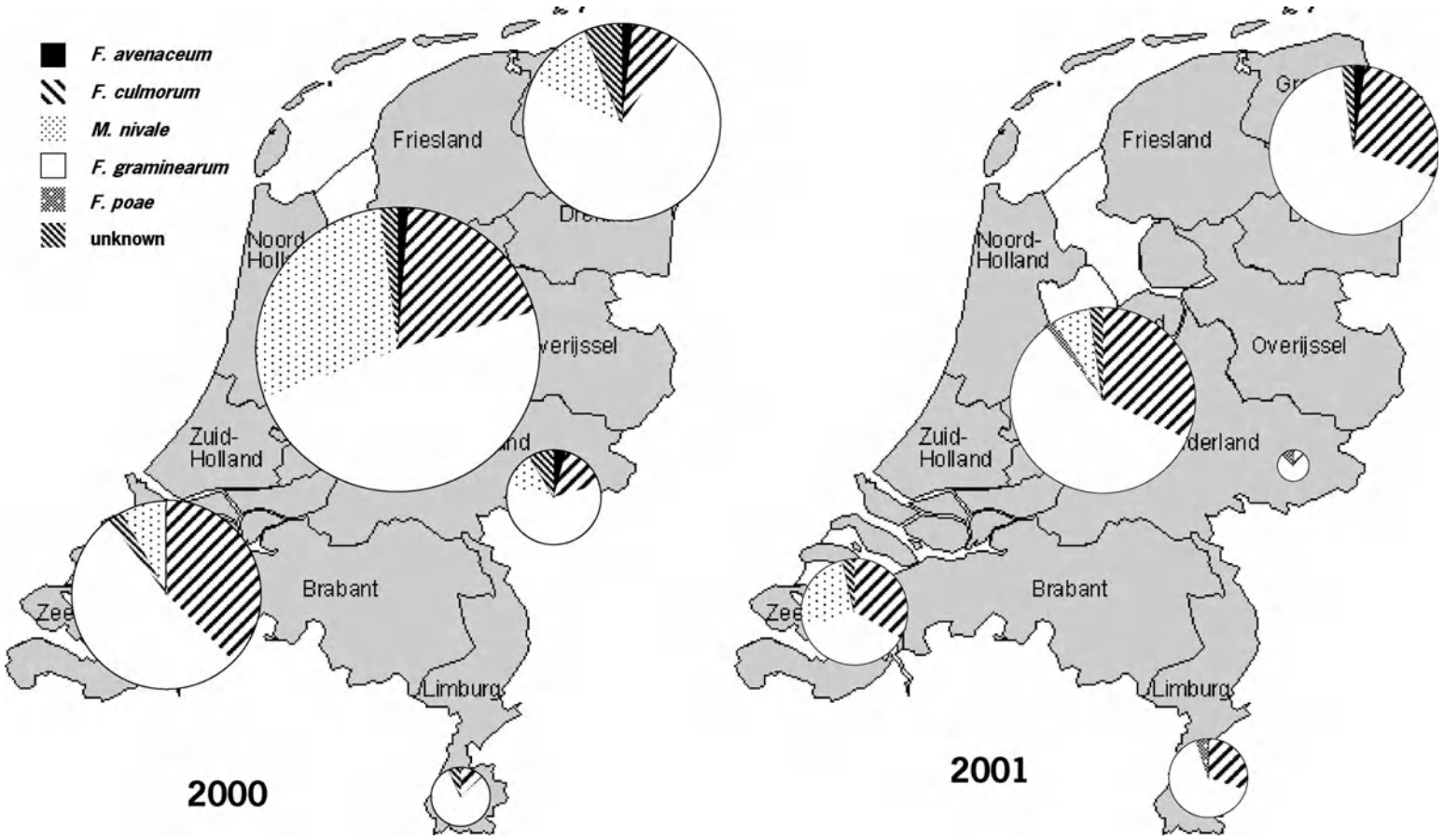

Figure 3. Numbers of *Fusarium* spp. in the various regions of the Netherlands in 2000 (a) and 2001 (b). The numbers of isolates obtained from the five regions are indicated. The sizes of the circles correspond to these numbers.

pressure was emphasized (Obst et al., 2002). Finally, the resistance of cultivars may influence the composition of the FHB complex. It has generally been accepted that resistance to *F. culmorum* and to *F. graminearum* are indistinguishable, but since selection is usually performed under natural disease pressure, selection efficiency towards the individual species in the complex is hardly possible. The distribution of the *Fusarium* species over the different regions and over the different cultivars did not reveal any clear interactions. The sole exception to this was *M. nivale* var. *majus* on Renan (7 out of 10) in 2000. Due to the absence of this cultivar in 2001, we could not substantiate this finding. The frequency of *F. culmorum* on cv. Kampa in 2001 (80% of the isolates) and on a mixture of cultivars in 2000 (80% of the isolates) also showed a strong bias, but samples sizes were very small ($n = 5$). Moreover, in both cases the isolates originated from a single field and were indistinguishable with PCR, suggesting potential clonality. Only a minority of the isolate pairs originating from the same ear were identified as the same species (37 out of 81 pairs) and several of these pairs could be resolved on the basis of the *tri7*, *tri13* and/or mating type PCRs. Only accurate fingerprinting, e.g. with AFLP, will identify clones within ears, field or regions, but when populations were considered at field level, both NIV-producers and DON-producers were frequently encountered in the same field. The mating type PCR was not capable of differentiating *F. graminearum* genotypes because its homothallic nature leads to both *Mat1-1* and *Mat1-2* specific fragments. The *F. culmorum* populations, on the other hand, belonged to either *Mat1-1* or *Mat1-2* and within a single field both mating types were recovered. This suggests that at least some of the *F. culmorum* populations were not clonal, as suggested by De Nijs et al. (1997) and Schilling et al. (1996), but contained at least two different genotypes. Within the scope of the two surveys described here, isolates of *M. nivale* var. *nivale* were not encountered. This is in agreement with results from surveys in the UK (P. Nicholson, pers. comm.) and could be attributed to the postulated difference in pathogenicity between the two fungal varieties (Diamond and Cooke, 1997).

Careful examination of the size variation obtained with the Fg11F and Fg11R primers led to the identification of two groups of isolates having either one or two copies of the 30 bp repeat. When these data were compared with the isolates from the seven lineages described by O'Donnell et al. (2000) isolate NRRL 28720 was found to carry three copies of the repeat. Carter et al. (2000; 2002) encountered an isolate

Table 3. Occurrence of *Fusarium* spp. in the Netherlands (percentages are given in parentheses)

Species	2000	2001
F. avenaceum	3 (1.4)	1 (0.7)
F. culmorum	42 (20.1)	43 (29.7)
F. graminearum	122 (58.4)	85 (58.6)
F. poae	0	3 (2.1)
F. proliferatum	0	0
M. nivale var. *majus*	34 (16.3)	10 (6.9)
Others[a]	8 (3.4)	3 (2.1)
Total	209	145

[a]Others: these isolates did not react with any combination of primers in the multiplex PCR primer mix. A subset did not generate a PCR fragments with the generic primers ITS1 and ITS4, suggesting insufficient extraction from these isolates. The remainder was subjected to morphological and molecular identification that confirmed that these isolates had been falsely identified as for *Fusarium* spp. Morphological characterization and molecular identification through sequencing of the ITS region identified these strains as *Epicoccum* spp. or *Ascochyta* spp.

Table 4. Numbers of *Fusarium* spp. on different cultivars (2000/2001)

Cultivar	*F. avenaceum*	*F. culmorum*	*F. graminearum*	*M. nivale* var. *majus*	*F. poae*	Others	Total
Drifter	0/0	7/15	18/28	7/4	0/1	0/1	32/49
Kampa	0/0	0/4	0/1	0/0	0/0	0/0	0/5
Renan	0/0	1/1	2/0	7/0	0/0	0/0	10/1
Residence	0/0	9/5	32/13	10/1	0/0	1/0	52/19
Ritmo	1/0	7/4	16/6	0/0	0/0	2/0	26/10
Tower	0/0	1/1	6/0	0/0	0/1	2/0	9/2
Vivant	2/1	13/13	47/37	9/5	0/1	2/2	73/59
Mixture	0/0	4/0	1/0	1/0	0/0	1/0	7/0
Total	3/1	42/43	122/85	34/10	0/3	8/3	209/145

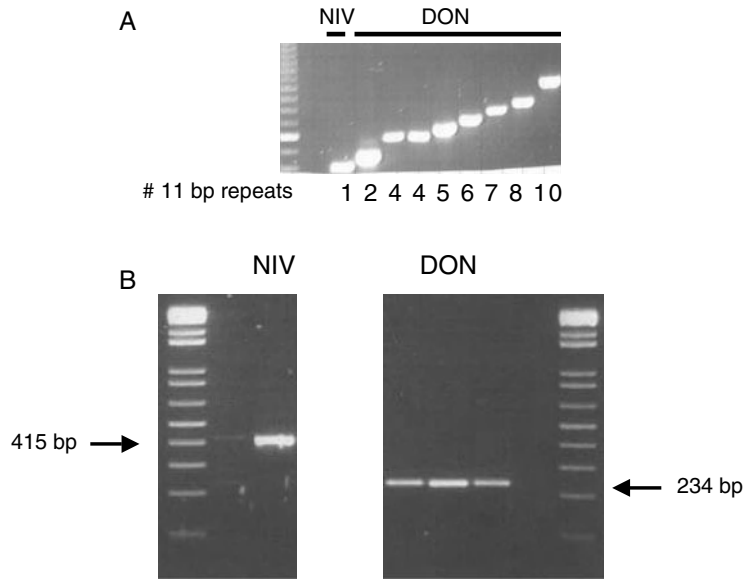

Figure 4. Amplification products generated by primer set Gztri7F/R(A) or tri13F/R (B). (A) Amplification products generated by primer set Gztri7F/R from field isolates of *F. graminearum* from 2000 and 2001 sorted by amplicon size. From left to right: water control, NIV-producing isolate 43B2, DON-producing isolates 45A1, 64C2, 42B1, 44A1, 38D1, 42C1 and 26G1. All isolates originated from 2001 except isolates 38D1 and 26 G1. At the far right lane isolate NRRL 28063 was included which contains 10 copies of the 11 bp repeat. (B) Amplification products of NIV- or DON-producing isolates amplified with primer set tri13F/R. From left to right: 1 kb markers, water control, NIV-producer NRRL28439, DON-producers NRRL5883, NRRL28063 and NRRL6394, water control and 1 kb markers.

Table 5. Frequency of NIV- and DON-producers of *F. culmorum* and *F. graminearum* in 2000 and 2000

	2000		2001	
	DON (%)	NIV (%)	DON (%)	NIV (%)
F. culmorum	11 (26.2)	23 (54.8)	8 (18.6)	31 (72.1)
F. graminearum	93 (76.2)	16 (13.1)	58 (68.2)	18 (21.2)

carrying four copies of the repeat in their studies on Nepalese isolates of *F. graminearum*. Comparison of the sequences from isolates of all lineages revealed that within Fg11F/R amplicons an additional insertion/deletion occurred involving an 86 bp fragment. This fragment was present in isolates from lineages 2 and 6, but was missing in lineages 1 and 7. Interestingly, when a series of Chinese *F. graminearum* isolates from wheat was analysed all ($n = 137$) generated an amplicon that contained the 86 bp insertion (Waalwijk, unpublished). This suggests that this insertion is specific for lineage 6 (and lineage 2) and may provide a tool for the discrimination of these groups of lineages. This could be valuable in Asia where lineages 2, 6 and 7 occur (Carter et al., 2000; O'Donnell et al., 2000). For the European and American situation, such a tool could be used to monitor the introduction of new lineages, since in these regions only lineage 7 has so far been reported (O'Donnell et al., 2000; Ward et al., 2002).

The recent publication of sequences from the *tri7* and *tri13* genes from the trichothecene cluster has allowed the screening of the populations for the production of NIV or DON. Lee et al. (2001; 2002) and Brown et al. (2001; 2002) demonstrated the role of these genes in the biosynthesis of trichothecenes in general and the coordination of either NIV or DON production in particular. Using primers derived from these genes it was possible to discriminate NIV-producers from DON-producers in both *F. graminearum* and *F. culmorum*. Amplification using the *tri7* primers in DON-producing isolates from *F. culmorum* was found to be unsuccessful, since the entire gene appeared to be missing (P. Nicholson, pers. commun.). In general, the *F. culmorum* populations consisted primarily of NIV-producers whereas the *F. graminearum* populations contained mainly DON producers. When the surveys of both years where compared, a slight but consistent increase of NIV-producers in 2001 was observed. Sample sizes were not very

large and the shift was not dramatic, but when populations were separated over their geographic origin the same tendency was observed. As NIV-producers were reported to be more pathogenic on maize than DON-producers (Carter et al., 2002), such a shift might be caused by the inclusion of maize in the crop rotation. Clearly, additional studies are required to confirm this preliminary observation. Studies on the toxicological characteristics of NIV and DON towards animals have led to more stringent recommendations for NIV. If the shifts observed in our studies prove to be consistent, monitoring the chemotypes will form an essential part of future surveys of FHB in wheat and other cereals.

Acknowledgements

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Influence of climatic factors on *Fusarium* species pathogenic to cereals

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Abstract

Fusarium head blight of small-grain cereals, ear rot of maize, seedling blight and foot rot of cereals are important diseases throughout the world. *Fusarium graminearum, F. culmorum*, *F. poae*, *F. avenaceum* and *Microdochium nivale* (formerly known as *F. nivale*) predominantly cause *Fusarium* diseases of small-grain cereals. Maize is predominantly attacked by *F. graminearum*, *F. moniliforme*, *F. proliferatum* and *F. subglutinans*. These species differ in their climatic distribution and in the optimum climatic conditions required for their persistence. This review deals with the influence of climate on the production and dispersal of inocula, growth, competition, mycotoxin production and pathogenicity. Most species produce inocula, grow best, and are most pathogenic to cereal heads at warm temperatures and under humid conditions. However, the optimal conditions for *F. moniliforme* and *F. proliferatum* maize ear rot tend to be hot and dry and *M. nivale* head blight, seedling blight and foot rot of small-grain cereals tend to occur under cooler conditions. Seedling blight and foot rot caused by other species are favoured by warm dry weather. Between them, these fungi produce four important classes of mycotoxins: trichothecenes, zearalenone, fumonisins and moniliformin. Conditions favourable for *in vitro* growth are also generally the most favourable for mycotoxin production on cereal grains. These fungi rarely exist in isolation, but occur as a complex with each other and with other *Fusaria* and other fungal genera. Climatic conditions will influence competition between, and the predominance of, different fungi within this complex.

Introduction

The genus *Fusarium* comprises a diverse array of fungi, members of which are phytopathogenic to a wide range of plants under diverse environmental conditions. Phytopathogenic *Fusarium* fungi cause several diseases of small-grain cereals, including seedling blight and foot rot, Fusarium head blight (FHB) (also known as 'scab' or ear blight) and ear rot of maize (Sutton, 1982; Parry et al., 1995). The *Fusarium* species *Fusarium graminearum* (teleomorph *Gibberella zeae*), *F. culmorum, F. poae*, *F. avenaceum* (teleomorph *G. avenacea*) and *Microdochium nivale* (formerly known as *Fusarium nivale*, teleomorph *Monographella nivalis*) are common pathogens of wheat and barley (Sutton, 1982; Parry et al., 1995; Miedaner, 1997; Tekauz et al., 2000; Brennan et al., 2003). Three species of *Fusarium* are frequently isolated from infected maize: *F. graminearum, F. moniliforme* (syn. *F. verticillioides*, teleomorph *G. fujikuroi* mating population A) and *F. subglutinans* (teleomorph *G. fujikuroi* mating population E). Other species responsible for ear rot of maize include *F. culmorum*, *F. proliferatum* (teleomorph *G. fujikuroi* mating population D) and *F. equisti* (Sutton, 1982; Leslie et al., 1986; Pomeranz et al., 1990; Odiemah and Manninger, 1994; Vigier et al., 1997; Velluti et al., 2000; Torres et al., 2001).

Fusarium diseases of wheat, barley and maize cause significant yield losses world-wide and are therefore of great economic importance (Sutton, 1982; Parry et al., 1995; Miedaner, 1997; Mesterhazy et al., 1999). In addition, many of these *Fusarium* species have the potential to produce a range of toxic secondary metabolites known as mycotoxins that cause a potential health risk when contaminated grain is consumed in human and animal food products (D'Mello and Macdonald, 1997; D'Mello et al., 1999; Placinta et al., 1999).

Host and climatic factors influence the growth, survival, dissemination and hence the incidence of *Fusarium* fungi and the disease severity. The influence

of host cultivars on the pathogenicity and toxicity of *Fusarium* fungi has been extensively reviewed (Miedaner, 1997; Miedaner et al., 2001; Mesterhazy et al., 1999; Magg et al., 2002). The influence of climatic factors on *Fusarium* diseases is complicated by the fact that *Fusarium* fungi can cause disease individually or in complex infections (Doohan et al., 1998), and there are numerous reports on how species differentially respond to different environmental variations, particularly temperature and humidity. Also, host susceptibility to fungal disease is directly influenced by temperature and osmotic stress (Conrath et al., 2002).

This review focuses on the influence of climatic variables, particularly temperature, humidity and rainfall, on the *in vitro* and *in vivo* growth, reproduction, survival, competitive ability, mycotoxicity and pathogenicity of *Fusarium* fungi commonly isolated from wheat, barley and maize.

Climatic distribution of *Fusaria*

The incidence of the causal organisms of FHB of wheat, barley and ear rot of maize is often correlated to different climatic conditions (temperature and rainfall) in different geographic locations. *F. culmorum*, *F. poae, F. avenaceum* and *M. nivale* are common pathogens of wheat and barley in the cooler maritime regions of the world such as the UK, while *F. graminearum* tends to be the predominant *Fusarium* species pathogenic to these cereals in hotter regions of the world such as the USA (Parry et al., 1995; Brennan et al., 2003). *F. graminearum*, *F. moniliforme* and *F. subglutinans* are the *Fusarium* species most frequently isolated from infected maize, but depending on geographical location, other causal species of ear rot include *F. culmorum*, *F. proliferatum* and *F. equisti* (Leslie et al., 1986; Vigier et al., 1997; Pomeranz et al., 1990; Odiemah and Manninger, 1994; Velluti et al, 2000; Torres et al., 2001).

Varying the temperature in a simple model ecosystem produces changes in the community structure of *Fusarium* species that mimic those found along climatic temperature and rainfall gradients (Saremi et al., 1999). The influence of climatic conditions on the incidence of *Fusarium* species is probably both direct (e.g. an effect on mode of reproduction) and indirect (e.g. an effect of soil and vegetation type). More research is required to determine the indirect effect of climate on the incidence of *Fusarium* fungi and how this affects species-specific factors.

Production and dispersal of *Fusaria* inocula

Temperature, humidity, light intensity and wind are the critical climatic factors affecting the production and dispersal of asexual conidia and sexual ascospores of *Fusarium*. Hence these factors critically influence the propagation and survival of *Fusarium* fungi. The optimal environmental conditions for the production and dispersal of inoculum vary depending on whether the fungus reproduces sexually and/or asexually. Of the common causal agents of FHB and ear rot, *F. graminearum, F. avenaceum, M. nivale, F. moniliforme, F. proliferatum* and *F. subglutinans* reproduce both sexually and asexually, while only asexual conidial reproduction has been observed for *F. culmorum* and *F. poae* (Parry et al., 1995; Leslie, 1996).

Production of inoculum

Temperature, water availability (a_w), aeration and light are key climatic factors influencing the production of *Fusarium inoculum*. Sutton (1982) showed that *F. graminearum* inoculum is generally formed under warm rather than cool conditions. In the case of sexual reproduction, the optimal temperatures for *F. graminearum* perithecial and ascospore production were 29 and 25–28 °C, respectively. The discharge of inoculum is triggered by a drop in air temperature accompanied by a rise in relative humidity (Paulitz and Seaman, 1994; Paulitz, 1996). Ascospore release occurs over a range of temperatures (10–30 °C) and this explains why the optimal temperature observed for ascospore dispersal was 16 °C (Sutton, 1982). However, ascospore release is inhibited by rain or continuously high relative humidity (>80%) and Gilbert and Tekauz (2000) postulated that there is a threshold humidity beyond which release slows or stops.

Macroconidia of *F. graminearum* are produced at an optimal temperature of 28–32 °C and their production is severely inhibited below 16 and above 36 °C (Tschanz et al., 1976). On wheat spikelets, Andersen (1948) showed that millions of conidia of *F. graminearum* were produced on moist wheat heads at 20–30 °C on, and lesser numbers at 15 °C. Macroconidia appeared within 5 days at 20 °C and within 3 days at 25–30 °C. Exposure of spikelets to moisture reduced conidial formation time to 1–2 days, with conidial numbers increasing with increasing humidity. Using osmotically adjusted agar plates, maximal ascospore and/or conidial production for *F. culmorum*, *F. graminearum*

Group I and *F. avenaceum* occurred at approximately −10 to −20 bars (Cook and Christen, 1976; Sung and Cook 1981). However, Sung and Cook (1981) found that for *F. graminearum* Group II, conidial production was maximal at −1.4 to 3.0 bars. Although macroconidia are more readily formed in older *in vitro* cultures (Winder, 1999), which have decreased water potential, such cultures may also be depleted in nutrients and perhaps contain increased levels of toxic metabolites.

Chlamydospores are produced by some species of *Fusarium* fungi in response to adverse conditions (Griffiths, 1974). High temperatures and anaerobic conditions favoured the conversion of macroconidia of *F. sulphureum* to chlamydospores (Barran et al., 1977). The switch between the production of ascospores, macroconidia, mesoconidia, microconidia and chlamydospore formation may well be related to both nutritional and environmental factors, such as temperature and water potential. Higher daytime temperatures (30 vs. 20 °C) caused a greater proportion of macroconidia to form, and lowered the abundance of mesoconidia in *in vitro* cultures of *F. avenaceum* (Winder, 1999).

Light levels influence the production of inoculum by *Fusarium* fungi. Low intensity ultraviolet light (<390 nm) is required for perithecial initiation: the most effective wavelength range being 300–320 nm (Tschanz et al., 1976). Continuous darkness, continuous illumination, or a transition from light to darkness failed to induce conidiation in cultures of *F. solani* (Das and Busse, 1990). Conidia were only produced from hyphae that grew in darkness for less than 20 h and were subsequently illuminated.

Dispersal of inoculum

Parry et al. (1995) have extensively reviewed the role of wind and rain-splash in the dispersal of Fusaria inocula. Splash dispersal of *Fusarium* conidia has previously been demonstrated (Jenkinson and Parry, 1994; Hörberg, 2002; Rossi et al., 2002) and Parry et al. (1995) suggested that macroconidia are more suited for splash rather than wind dispersal. Rossi et al. (2002) studied the dynamics of airborne Fusarium macroconidia in wheat fields naturally infected by head blight. They detected no or very few conidia before rainfall, but their numbers progressively increasing during rainfall. With subsequent humid conditions, conidia continued to be produced for some hours after rainfall and usually reached their peak under these conditions. Fernando et al. (1997) showed that *F. graminearum* inoculum (macroconidia and ascospores) was usually displaced downwind from inoculated wheat heads and that the incidence of disease was higher and more diffuse in irrigated as opposed to non-irrigated plots. High humidity is required for the initial release of ascospores, although dry periods may be required for their forceful discharge into the air from perithecia (Parry et al., 1995). Also, the *F. graminearum* disease development gradients in wheat plots were more diffuse from ascospores (from infected maize grain) than from macroconidia (inoculated wheat heads) applied at anthesis (Fernando et al. 1997). Presumably air-borne dispersal of facilitates more widespread displacement of ascospores than does splash/rain dispersal of conidia.

While large differences in spore morphology (e.g. ascospore vs. macroconidum) influence inoculum dispersal, it appears that smaller inter-species differences in macroconidial shape do not. Hörberg (2002) compared the splash dispersal of *F. culmorum* and *F. poae* macroconidia and found essentially identical patterns, although *F. culmorum* produced fewer colonies than *F. poae*. However, it would be interesting to investigate whether there are differences in the splash dispersal patterns of macro and microconidia of *F. poae*.

Germination, growth and competition between *Fusaria*

Germination, growth and competition between *Fusaria* are dependent upon the availability of nutrients and environmental factors such as temperature, pH, humidity, aeration and light. The influence of nutritional availability is outside the scope of this review. It is generally not a limiting factor during infection and colonisation of host tissue, but may be limiting or growth-inhibiting during saprophytic survival (e.g. humic acids in soil) (Moliszewska and Pisarek, 1996).

Germination

Germination is influenced by a_w and temperature: warm humid conditions favour this developmental stage. Marín et al. (1996) found that the a_w minima for the microconidial germination of Spanish isolates of *F. moniliforme* and *F. proliferatum* were 0.88 on maize meal extract medium. Microconidia of *F. moniliforme* germinated optimally at 25–37 °C and 0.96–0.98 a_w, but at 30 °C when the a_w was 0.90–0.94, with intra-isolate variation. The germination of microconidia of *F. proliferatum* was optimal at 30 °C, regardless of a_w,

and with significant intra-isolate variation. However, Etcheverry et al. (2002) found that Argentinean isolates of *F. moniliforme* and *F. proliferatum* grew very slowly, if at all, at a_w 0.93 and 25 °C. At marginal temperatures and a_w levels, the germination lag time increases (Marín et al., 1996; Etcheverry et al., 2002). Earlier, Francis and Burgess (1977) found that the percentage germination of conidia, ascospores and chlamydospores of *F. graminearum* Group II isolates was reduced as water potential was lowered from −1 to −20 bars.

Growth

Temperature and a_w differentially affect the growth of *Fusarium* species (Table 1). *Fusarium* species differed in their temperature requirements for optimal growth on potato dextrose agar (Cook and Christen, 1976; Pettitt et al., 1996; Brennan et al., 2003). Irrespective of the European origin of isolates, *in vitro* culture experiments showed that optimal growth occurred at 25 °C for *F. graminearum*, at 20–25 °C for *F. culmorum and F. poae* and at 20 °C for *F. avenaceum* and *M. nivale*. *M. nivale* and *F. culmorum* were the fastest growing of all five species over the range 20–30 °C. Figure 1 depicts the typical growth of *F. graminearum*, *F. poae* and *M. nivale* and *F. poae* following 5 days incubation at 10 and 30 °C on potato dextrose agar. The fastest growing species at 10 °C was *M. nivale*, while at 30 °C they were *F. graminearum* and *F. culmorum* (Figure 1) (Brennan et al., submitted for publication). In general, *F. culmorum* had the fastest growth rate of all five species over the range 10–30 °C. Species accounted for 51–63% and country of origin accounted for 23–52% of growth rate variation. At the low temperature of 5 °C, Pettitt et al. (1996) found that of *F. culmorum*, *F. avenaceum* and *M. nivale*, the latter species was significantly the fastest growing. At the higher temperature of 35 °C, Cook and Christen (1976) found that *F. graminearum* did not grow, even after 30 days. Marín et al. (1998a) found that the maize pathogens *F. moniliforme* and *F. proliferatum* had a faster growth rate than *Eurotium* and *Penicillium* species and on sterile layers of maize grew best at 30 °C (Table 1).

The temperature optima for growth of *Fusaria* appears to be dependent on a_w. Cook and Christen (1976) found that the optimal growth temperature for European isolates of *F. graminearum* (24–28 °C) increased slightly when lower water potentials prevailed. *Fusarium graminearum* grew optimally at −10 to −20 bars and *F. culmorum* at −8 to −14 bars. Increasing a_w (>0.925) favoured growth of *F. moniliforme* and *F. proliferatum* on sterile layers of maize at 30 °C (Marín et al., 1995). More research is required to better understand the influence of a_w on the growth of *F. culmorum*, *F. poae* and *M. nivale.*

It must be noted that drawing comparisons between growth rate studies is difficult, as the rates are very dependent on the growth substrates used. For example, on maize culture media *F. subglutinans* grew optimally

Table 1. Optimum temperature and water potential/availability for the *in vitro* growth of *Fusarium* species

Species	Substrate[a]	Optimum growth conditions		References
		Temperature (°C)	Water potential/availability[b]	
F. graminearum	BM, PDA	24–28	−10 to −20 bars	Cook and Christen (1976), Brennan et al. (2003)
F. culmorum	BM, CMA, PDA	20–25	−8 to −14 bars	Cook and Christen (1976), Parry et al. (1994), Brennan et al. (2003)
F. avenaceum	PDA	20–25	ND	Parry et al. (1994), Brennan et al. (2003)
F. poae	PDA	20–25	ND	Brennan et al. (2003)
M. nivale	PDA	15–20	ND	Parry et al. (1994), Brennan et al. (2003)
F. moniliforme	Sterile maize layers	30	$a_w > 0.925$	Marin et al. (1995)
F. proliferatum	Sterile maize layers	30	$a_w > 0.925$	Marin et al. (1995)
F. subglutinans	MCM, RCM	15–25	ND	Castellá et al. (1999)

[a]BM = basal medium, PDA = potato dextrose agar, CMA = corn meal agar, MCM = maize culture media, RCM = rice culture media. [b]ND = no data.

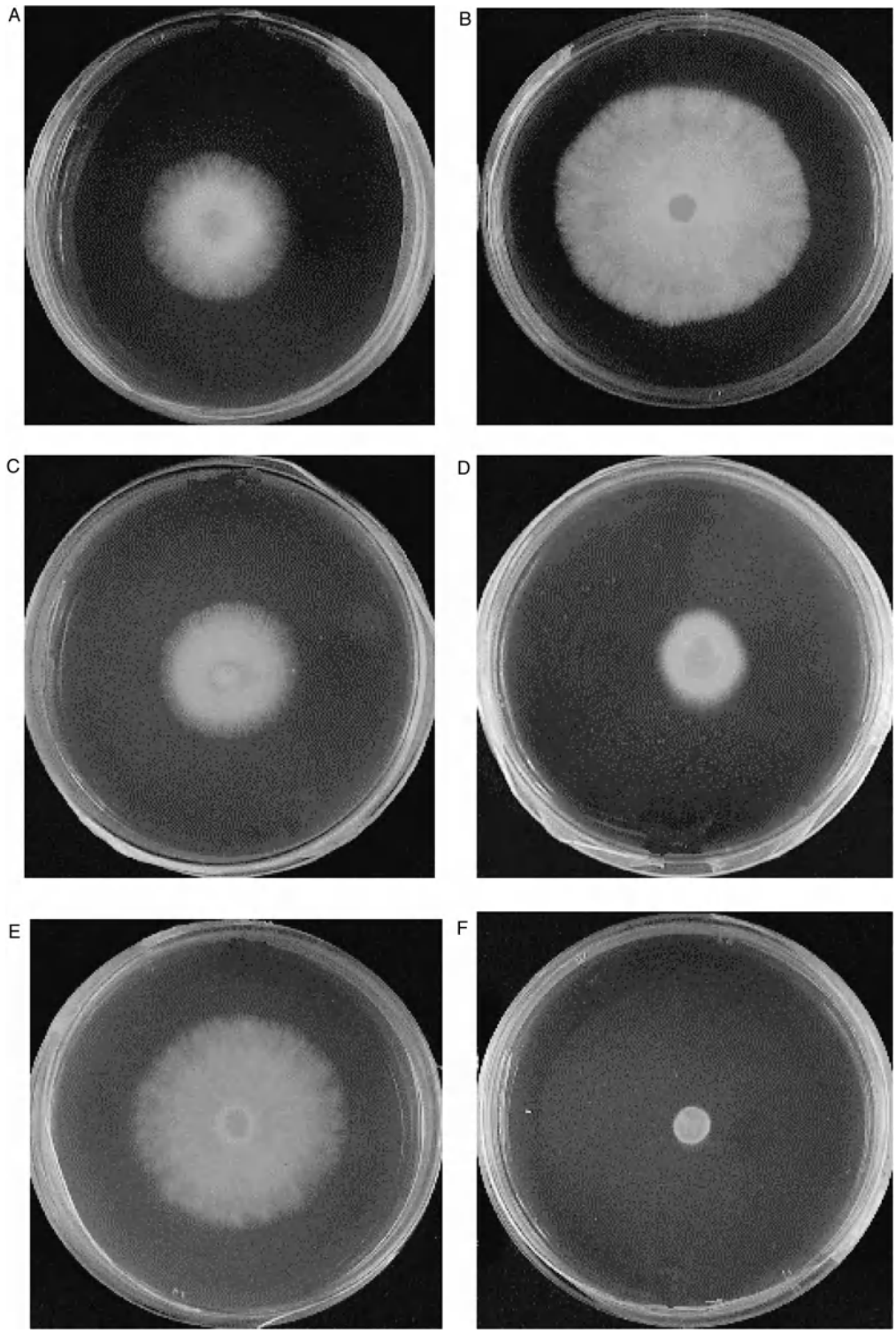

Figure 1. Growth of *F. graminearum* (strain HugR8) (A and B), *F. poae* (strain I105) (C and D) and *M. nivale* (strain 12/1/N) (E and F) on potato dextrose agar, following 5 days incubation at 10 and 30 °C, respectively (Brennan et al., 2003).

at 20–25 °C, but faster on rice culture media at 15 °C (Castellá et al., 1999).

Competition: Temperature and a_w

Fusarium fungi do not exist in isolation, either in the soil, on debris, or on the host, but are continually competing with other organisms, particularly microorganisms. Microbial interactions and the balance between microbial communities are influenced by the prevailing environmental conditions. It has previously been shown that temperature and a_w significantly influence the growth and interaction between *F. moniliforme* and *F. proliferatum*, and between *F. graminearum*, *F. subglutinans*, *F. proliferatum, Aspergillus, Penicillium*, *Eurotium* and *Trichoderma* species (Marín et al., 1998a,b). In a study of the competing abilities of *Fusarium*, *Aspergillus*, *Penicillium*, *Eurotium* and *Trichoderma* species, Marín et al. (1998a) found that *Fusarium* species were only dominant at high a_w (0.995). Magan and Lacey (1984) found that of a range of field fungi, *F. culmorum* was the only one able to compete with and dominate other fungi, particularly at $a_w > 0.95$.

Within the *Fusarium* genus, *F. graminearum* appears to have a competitive advantage over other species under cooler conditions (Marín et al., 1998b; Velluti et al., 2000). Marín et al. (1998b) suggested that *F. graminearum* has a competitive advantage over *F. moniliforme* and *F. proliferatum* at 15 °C, while at 25–30 °C, these species coexisted in the same niche. Similar results were found by Velluti et al. (2000), regardless of a_w (0.93, 0.95 and 0.98). Later in this review, the occurrence of *Fusarium* complexes and their impact on mycotoxin production will be discussed.

Mycotoxin production

One of the most serious consequences of FHB and ear rot of cereals is the contamination of grain with mycotoxins (D'Mello and Macdonald, 1997; D'Mello et al., 1999; Placinta et al., 1999). The most important classes of*Fusarium* mycotoxins, based on their harmful effects on human and animal health, are the trichothecenes, fumonisins, moniliformin and zearalenone (ZEA) (D'Mello et al., 1999). Trichothecene mycotoxins are tricyclic sesquiterpenes and two classes; types A and B, are commonly found in cereals along with the oestrogenic mycotoxin ZEA (D'Mello and MacDonald, 1997; D'Mello et al., 1999). The fumonisin class of mycotoxins comprises a group of structurally related metabolites of which fumonisin B_1 (FB_1) and B_2 (FB_2) are commonly found in maize grain with moniliformin (D'Mello and Macdonald, 1997; D'Mello et al., 1999).

Mycotoxin production in grain can begin in the field and continue throughout storage. Mycotoxin production is dependent mainly on both well-defined ranges of temperature and a_w. But in turn, the optimum climatic conditions for mycotoxin production in infected grains depends on the substrate, *Fusarium* species and isolate. The influence of temperature and a_w on mycotoxin production by *Fusarium* fungi is probably not entirely direct but rather a function of the influence of these parameters on fungal growth.

Trichothecenes and ZEA

Many *Fusarium* species, including *F. graminearum*, *F. culmorum*, *F. poae*, *F. oxysporum* and *F. sporotrichioides* are producers of trichothecenes and ZEA (D'Mello and Macdonald, 1997; D'Mello et al., 1999) (Table 2). *F. sporotrichioides* and perhaps *F. poae* predominately produce type A trichothecenes, which includes T-2 toxin, HT-2 toxin, neosolaniol and diacetooxyscirpenol (DAS). *F. culmorum* and *F. graminearum* predominately produce type B trichothecenes, including deoxynivalenol (DON, also known as vomitoxin), its 3-acetyl and 15-acetyl derivatives (3-ACDON and 15-ACDON, respectively) and nivalenol (NIV). Most studies indicate that high moisture favours the production of both classes of mycotoxins, but the optimum temperatures for trichothecene and ZEA production in *Fusarium*-infected grain appears to be specific to the substrate, species and individual metabolites (Table 2).

Moderate rather than warm temperatures favour the production of type A trichothecenes by *F. sporotrichioides* (Miller, 1994; Mateo et al., 2002) (Table 2). While the optimum production conditions varied depending on the substrate and toxic metabolite, in general *F. sporotrichioides*-infected maize, wheat and rice grains contained more type A trichothecenes when moistened with 35% water ($a_w = 0.990$) and incubated at 20 °C for 3 weeks than when incubated at higher temperatures or a_w. However, Rabie et al. (1986) detected relatively large amounts of T-2 toxin in *F. acuminatum*-infected oats

Table 2. The major classes of *Fusarium* mycotoxin, their principal producers and optimal production conditions on cereal grains

Toxin	Species	Substrates	Optimum production conditions[a]	References
Type A trichothecenes [T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpenol (DAS)]	*F. sporotrichioides* *F. poae*	Barley, oats, rice, wheat, maize	Moderately warm and humid (20–25 °C, $a_w = 0.990$)	Mateo et al. (2002), Miller (1994), Rabie et al. (1986)
Type B trichothecenes [deoxynivalenol (DON), 3-acetyl DON, 15-acetyl DON, nivalenol (NIV)]	*F. graminearum* *F.culmorum*	Barley, wheat, rice, maize	Warm and humid (25–28 °C, $a_w = 0.97$)	Greenhalgh et al. (1983), Lori et al. (1990), Beattie et al. (1998), Homdork et al. (2000)
ZEA	*F. graminearum* *F. culmorum*	Wheat, rice, maize	Warm (17–28 °C), or temperature cycles (e.g. 25–28 °C for 14–15 days; 12–15 °C for 20–28 days) and humid ($a_w = 0.97$ or 90% RH)	Jiménez et al. (1996), Lori et al. (1990), Ryu and Bullerman (1999), Homdork et al. (2000), Martins and Martins (2002)
Fumonisins	*F. moniliforme* *F. proliferatum* *F. subglutinans*	Maize	Cool to warm conditions and humid (15–30 °C, $a_w = 0.98$)	Cahagnier et al. (1995), Marin et al. (1999a,b)
Moniliformin	*F. subglutinans* *F. moniliforme* *F. avenaceum*	Wheat, rye, barley, oats, maize	Warm temperatures (25–30 °C)	Kostecki et al. (1999), Schütt (2001)

[a]Optimum temperature and humidity vary depending on substrate, species and isolate; typical conditions are given in parentheses. Time of production varies from 3 to 8 weeks.

stored at 25 °C, although a comparison was not drawn between different incubation conditions.

In the case of type B trichothecenes, warm humid conditions favour their production during storage of *F. culmorum* and *F. graminearum*-infected grain (Greenhlagh et al., 1983; Lori et al., 1990; Beattie et al., 1998; Homdork et al., 2000; Martin and Martins, 2002) (Table 2). Martins and Martins (2002) found that on *F. graminearum*-infected cracked corn ($a_w = 0.97$), more of the type B trichothecene DON was produced following incubation at 28 °C for 35 days, rather than at 22 or 28 °C for 15 days followed by 12 °C for 20 days; their results agreed with those of Greenhlagh et al. (1983). Also, maximal DON was produced by *F. graminearum* on infected wheat and polished rice following incubation in the dark at 27 °C, but in hulled rice, DON production was maximised when incubated at 27 °C in the light (Lori et al., 1990).

The effect of initial infection level may outweigh the effect of environmental conditions on mycotoxin contamination of grain, depending on the toxic metabolite in question. Following 7 months storage of barley grain with high initial *Fusarium* infection levels (85%), DON contents did not change significantly, irrespective of conditions (−4, 20 or 24 °C, quiescent or forced aeration), although it was lowest in malt produced from the grain stored at 24 °C (Beattie et al., 1998). Initial infection levels would not normally be so high. In wheat stored for 6–8 weeks under warm humid conditions (25 °C, 90% RH), Homdork et al. (2000) found that, while the DON content significantly increased in grain with a low to moderate (4–15%) initial *F. culmorum* infection level, it did not increase in samples with high (>50%) initial infection levels. However, the influence of initial infection levels on mycotoxin production may be toxin-specific, as while these conditions were optimal for the production of NIV, unlike DON, it was not present at harvest and levels increased irrespective of initial infection level.

As for trichothecenes, the conditions for optimal ZEA production appear to be species, isolate and substrate specific, and may vary from those for DON production. Several studies have found that maximum ZEA was produced in *F. graminearum* and *F. oxysporum*-infected maize at a_w 0.97 and by cycling the incubation temperatures from 25 to 28 °C for 14–15 days, followed by 12–15 °C for 20–28 days (Jiménez et al., 1996; Ryu and Bullerman, 1999;

Martins and Martins, 2002) (Table 2). However, the optimum temperature for ZEA production may vary with isolate and substrate. Jiménez et al. (1996) found that, while the aforementioned conditions were optimal for ZEA production in maize grain infected by two isolates each of *F. graminearum* and *F. oxysporum*, another *F. graminearum* and two *F. culmorum* isolates produced maximal ZEA after 30 days incubation at room temperature (16–25 °C) rather than at 28 or 37 °C ($a_w = 0.97$). In wheat grain with moderate to high levels (4–15%) of *F. culmorum* infection, ZEA production was favoured by warm and humid (25 °C, 90% RH) rather than cool and dry storage conditions. Most ZEA was produced towards the end of the storage period (6–8 weeks) (Homdork et al., 2000). Lori et al. (1990) reported a lower optimal substrate-dependent temperature for ZEA production by a *F. graminearum* isolate. ZEA production was maximised by incubation of *F. graminearum*-infected wheat and polished rice in the dark at 17 and 21 °C, respectively, while production was maximised in hulled rice incubated at 27 °C in the light (Lori et al., 1990).

Fumonisins and moniliformin

Fumonisins and moniliformin are commonly produced in maize infected by *F. moniliforme* and *F. proliferatum*, species which tend to grow better at higher temperatures (Keller et al., 1997; Kostechi et al., 1999; Miller, 2001; Marín et al., 1999a,b). Moniliformin has also been detected in cereals infected with *F. avenaceum* and *F. subglutinans* (Kostechi et al., 1999; Torres et al., 2001; Kiecana et al., 2002). While the temperature optima for the production of fumonisins by these pathogens vary, they all prefer $a_w \sim 0.98$ and fumonisin production generally decreases with temperature and higher a_w (Cahagnier et al., 1995; Marin et al., 1999a,b). Marín et al. (1999a,b) found that a_w had a more significant effect than temperature on total fumonisin production in maize grain and ground maize by *F. moniliforme* and *F. proliferatum*. In general, fumonisin production and fungal biomass decreased with temperature and a_w and was optimal at 15–30 °C and 0.98 a_w, depending on the isolate. At marginal temperatures (especially 15 °C), there was an increase in fumonisin production at lower a_w levels (0.92 and 0.95) when compared to the concentrations produced at higher temperatures and higher a_w levels. But even at 37 °C, Marín et al. (1999b) found that an isolate of *F. moniliforme* could produce significant amounts of fumonisin. Ono et al. (1999) attributed the higher fumonisin content of maize in the Northern region of the State of Párana, Brazil compared to the Central-South to higher rainfall in the former during the month preceding harvest (202 and 92.8 mm, respectively). Oxygen limitation retards the growth of *F. moniliforme* and *F. proliferatum* and under such conditions it was found that no FB_1 was produced (Keller et al., 1997).

Higher temperatures favour moniliformin production in cereal grains infected by *F. avenaceum* or *F. subglutinans* (Kostecki et al., 1999; Schütt, 2001). Moniliformin production by a *F. subglutinans* isolate from maize was higher at 30 than 20 or 25 °C and on rice rather than on wheat, rye, barley, oat or maize grains (Kostecki et al., 1999). Temperature greatly influenced moniliformin production by *F. avenaceum* on wheat, with more being produced under mediterranean rather than temperate conditions (Schütt, 2001).

Microbial interactions and mycotoxin production

Fusaria and other microorganisms often occur as a complex on cereal crops, but limited information is available regarding the effects of fungal interactions on mycotoxin production by *Fusarium* complexes under different environmental conditions. The two fumonisin-producing species *F. moniliforme* and *F. proliferatum* are dominant over other fungal genera that contaminate maize over a wide range of temperature and a_w conditions, but little is known about the effect of such interactions on mycotoxin production. Orsi et al. (2000) found that in a mixed mycoflora-infected maize, FB_2 correlated positively with maize grain moisture content; significant negative correlations existed between relative FB_1 and FB_2 contents of maize and relative humidity, and between FB_2 and mean temperature. In terms of inter-species interactions, it is known that growth and FB_1 production by these pathogens on irradiated maize grain was significantly reduced at 15 °C in the presence of *F. graminearum* (Marín et al., 1998b; Velluti et al., 2000). More interestingly, at 25 °C, growth was not significantly affected, but there was an increase in FB_1 production by *F. moniliforme* in the presence of *F. graminearum*, regardless of a_w (0.92–0.98) (Velluti et al., 2000). ZEA production by *F. graminearum* was not significantly higher in the presence of *F. moniliforme* or *F. proliferatum*, regardless of temperature or a_w.

Pathogenicity of *Fusaria*

Ultimately, all of the above factors, i.e. production and dispersal of inoculum, germination, growth, competition and mycotoxin production might facilitate the pathogenesis of plants by *Fusarium* fungi. Hence, even prior to infection of the host, climate influences *Fusarium* potential for disease incidence and severity. Once *Fusarium* inoculum has been dispersed to the host, temperature and humidity play a significant role in the infection and colonisation of cereals by *Fusarium* fungi. The optimum conditions for disease development vary depending on the species, inoculum type and virulence and the affected tissue, i.e. seed, stem-base or head (Sutton, 1982; Miller, 1994; Parry et al., 1994; 1995; Miller et al., 1995; Tekauz et al., 2000; Vigier et al., 1997; Velluti et al., 2000; Bateman and Murray, 2001). Osmotic or temperature stress may indirectly affect disease development by inducing or 'priming' the host antifungal defence mechanisms prior to pathogen attack (Conrath et al., 2002). While *in vitro* growth tests suggested that temperature ecotypes existed amongst *Fusarium* on the basis of both species and country of origin, *in vitro* pathogenicity tests indicated that species is a much more important factor than climatic origin in determining the pathogenicity of *Fusarium*, irrespective of temperature (Brennan et al., 2003).

As discussed earlier, climate can influence the quantity and type of inoculum produced, but the production conditions may also influence the subsequent pathogenicity of inoculum. Winder (1999) showed that incubation temperature affected the pathogenicity of *F. avenaceum* inoculum (as did substrate type). Inoculum produced at 20 °C caused significantly more leaf damage on marsh reed grass plants that did that produced at 30 °C. More research is required to determine the effect of climatic conditions on the pathogenicity of FHB and maize ear rot inoculum.

FHB and ear rot

The climatic conditions optimal for the development of maize ear rot differ from those for small-grain cereals. This is more a function of the causal species rather than the host (Parry et al., 1995; Vigier et al., 1997) (Table 3). Precipitation plays a key role in the development of epidemics of FHB of small-grain cereals, which are usually associated with warm wet weather at the time of anthesis (Parry et al., 1995; Tekauz et al., 2000). Risk assessment and disease forecasting models for FHB are usually based on climatic conditions around the flowering to the early milky-ripe stage. For wheat, wetness periods of at least 24 h and temperatures above 15 °C are required for significant infection of heads by *F. graminearum*, *F. culmorum*, *F. poae*, *F. avenaceum* and *M. nivale*. The temperature optima for infection were 25, 25, 25, 25 and 15 °C, respectively (Parry et al., 1994; 1995).

In the first half of the twentieth century, it was recognised that temperature and humidity influence the severity of FHB (Atanasoff, 1920; Dickson et al., 1921; Pugh et al., 1933; Andersen, 1948). Under damp

Table 3. Optimal climatic conditions for the development of *Fusarium* cereal diseases

Species	Optimal conditions for disease development		References
	FHB and ear rot	Seedling blight and foot rot	
F. graminearum, *F. culmorum*, *F. poae*, *F. avenaceum*	Warm wet weather at anthesis (25 °C and >20 h rainfall)	Warm dry weather (>16 °C)	Atansoff (1920), Dickson et al. (1921), Pugh et al. (1933), Andersen (1948), Sutton (1982), Parry et al. (1994; 1995) Reid et al. (1995), McMullen et al. (1997), Hall and Sutton (1998), Tekauz et al. (2000), Brennan et al. (2003)
M. nivale	Moderately warm wet weather at anthesis (20 °C and >20 h rainfall)	Cool dry weather (10–15 °C)	Parry et al. (1994; 1995), Brennan et al. (2003)
F. moniliforme, *F. proliferatum*	Hot and dry conditions, especially at maize silking	—	Miller et al. (1995), Vigier et al. (1997), Reid et al. (2002)
F. subglutinans	Moderately warm humid climates	—	Vigier et al. (1997), Reid et al. (2002)

conditions, FHB symptoms on wheat developed 6 days post-*F. graminearum* inoculation, while under dry conditions symptoms were not observed until the onset of rain or heavy dew (Atansoff, 1920). Pugh et al. (1933) and Andersen (1948) found that the period of continuous wetness required for the development of moderate *F. graminearum* FHB disease scores was dependent on temperature (20–48 h at 25 °C and 60–72 h at 20 °C), with negligible symptoms developing at 15 °C. However, severe FHB infections can occur below 20 °C if precipitation is abundant (McMullen et al., 1997; Hall and Sutton, 1998). Tekauz et al. (2000) postulated that the increase in the incidence of *F. graminearum* in barley and wheat grown in the cooler and moist Black Soil Zone of the Canadian prairies supports the view that moisture is the overriding determinant of FHB severity.

Fusarium spp. infect maize heads either by growing down the silks, or through wounds created by birds or insects. Optimal conditions for the development of maize ear rot vary from warm to hot dry weather, depending on the causal species (Sutton, 1982; Vigier et al., 1997) (Table 3). *F. graminearum* and *F. subglutinans* tend to be the causal organisms in cooler to moderately warm and humid climates, while *F. moniliforme* and *F. proliferatum* are common causal organisms under hot and dry conditions (Sutton, 1982; Miller et al., 1995; Vigier et al., 1997). A survey of maize in Ontario during 1991–1993 showed that *F. subglutinans* was the predominant species and its incidence was negatively correlated with rainfall. Incidence of *F. graminearum* increased with precipitation and the results were consistent with a previous finding that lower *F. graminearum* infection levels were observed in Canada during dry years (Reid et al., 1995). In a 2-year study (1994–1995), Reid et al. (2002) observed lower disease severity in heads inoculated with *F. moniliforme* than in those inoculated with *F. graminearum* or *F. subglutinans.* This may have been due to the fact that the temperature never exceeded 25 °C or to the fact that the pathogenicity of *F. moniliforme* is considered moderate in comparison to that of *F. graminearum* and *F. subglutinans* (Miller, 1994).

Seedling blight and foot rot

Fusarium seedling blight arises mainly as a result of seed-borne inoculum, although inoculum may be soil-borne. Data on the influence of climatic factors on *Fusarium* seedling blight and foot rot is limited, but it is known that periods of dry weather predispose cereals to seedling blight and foot rot disease, the optimum temperature for the development of both diseases being dependent upon the causal species (Table 3) (Parry et al., 1994; Bateman and Murray, 2001). While cooler temperatures favour *M. nivale* seedling blight (10–15 °C), warmer conditions favour disease development for the other causal species (>16 °C) (Brennan et al., submitted for publication, Parry et al., 1994). Similar results were obtained in *in vitro* seedling blight experiments (Brennan et al., 2003). The effect of temperature (10–30 °C) on coleoptile growth retardation caused by *Fusarium* infection was examined. Greatest retardation was caused by *F. graminearum*, *F. poae* and *F. avenaceum* at 20–25 °C (>83% reduction), and by *F. poae* and *M. nivale* at 10–15 °C (>45.6% reduction). Figure 2 depicts the differences in the *in vitro* pathogenicity of *F.culmorum* and *M. nivale* at 25 °C (Brennan et al., 2003).

Conclusions

Temperature and humidity/wetness are the main climatic factors influencing the development of *Fusarium* diseases of cereals, although the influence of these climatic factors is not independent of other environmental and host factors. Many gaps exist in our knowledge of the influence of environmental parameters on *Fusarium* diseases of cereals. A risk assessment model for the forecasting of FHB epidemics in Ireland, based on environmental conditions is currently being developed (van Maanen, Cook and Doohan, unpubl. data). These data will also form part of an EU risk assessment model (EU RAMFIC project QLRT-1999-31517).

Particularly interesting questions for future research are: the influence of humidity on *Fusarium* diseases of small-grain cereals and the influence of environmental parameters on both the mycotoxin profiles (rather than individual metabolites) and biological control of *Fusaria*. Knowledge of the influence of climatic conditions on *Fusarium* diseases may prove useful towards developing novel disease control methods. More research is required to understand whether osmotic or temperature stress affect *Fusarium* disease development by inducing or 'priming' the host antifungal defence mechanisms prior to pathogen attack (Conrath et al., 2002). In terms of manipulating environmental conditions to control *Fusarium* diseases, adjustment of soil temperature and moisture through soil solarisation has successfully been applied in

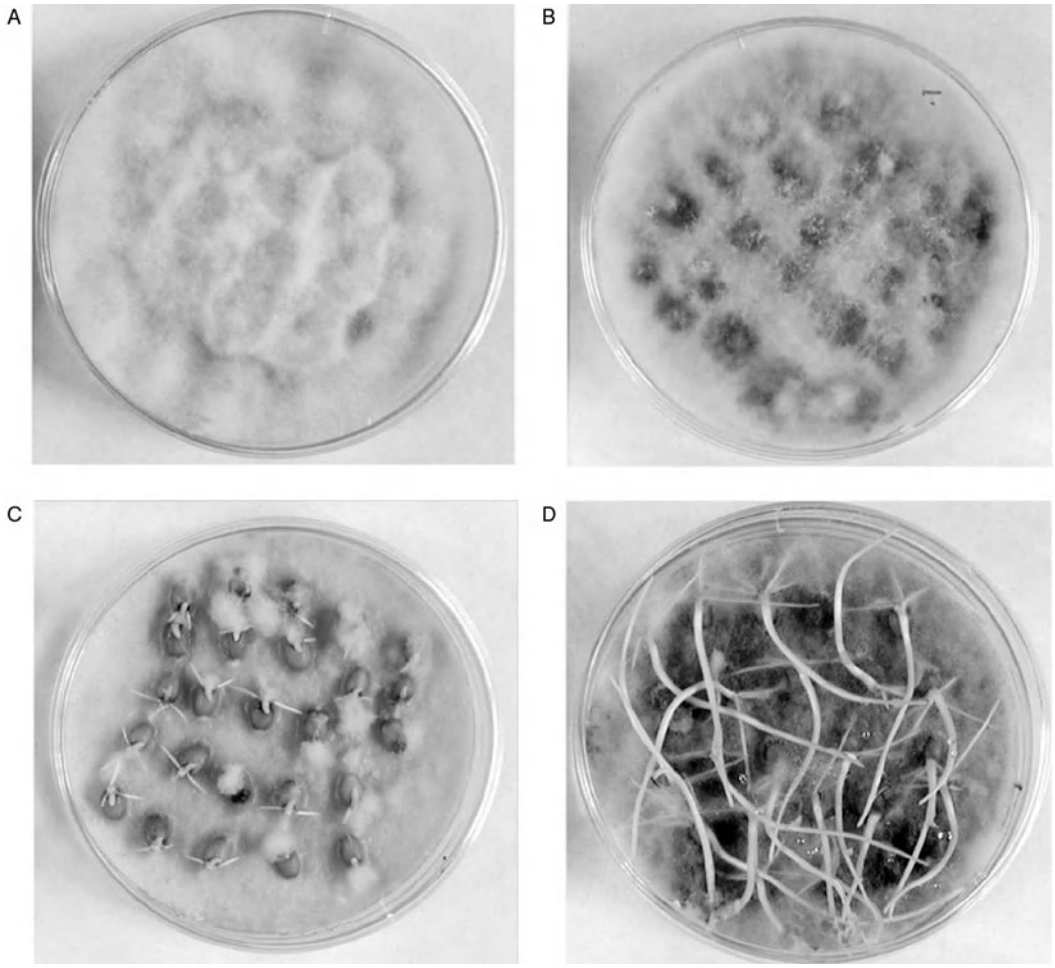

Figure 2. Effect of *F. graminearum* (strain 074) (A and B) and *F. poae* (strain 2742) (C and D) on coleoptile growth rate of wheat seedlings (cv. Falstaff) at 10 and 25 °C, respectively (Brennan et al., 2003).

many countries for the control of soil-borne pathogens (Katan, 1981). Soil solarisation is a method of heating moist soil by covering it with plastic sheets to trap solar radiation. Al-Karraghouli and Al-Kayssi (2001) showed that the efficiency of soil solarisation in killing *F. oxysporum* generally decreased with increasing soil moisture content, as the soil heat capacity increased and maximum soil temperatures decreased with increasing volumetric moisture content. The percentage eradication of the fungus was faster and higher in the soil surface layer (0.05 m) and decreased gradually with depth (as did temperature). While soil solarisation may be effective in eradicating *Fusarium* pathogens of cereals in drier climates, its applicability in maritime climates requires further analyses.

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Association of *Fusarium* species in the wheat stem rot complex

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Abstract

Data from a national survey were analysed to investigate whether there was interdependence among the *Fusarium* species, which cause the stem rot complex of wheat. About 25 wheat stems were sampled from each of 260 sites over the main wheat growing areas in the UK. Occurrence of each *Fusarium* species on individual stems was determined. *Fusarium culmorum*, *F. avenaceum* and *Microdochium nivale* were the three dominant species, detected in 248, 185 and 239 out of the 260 sites. There were no interactions among species in the distribution of the three species over the 260 sites. Several statistical tests were used to determine whether there was interdependence among the three species on the same stem within each site. Of the three species, there was only limited evidence of competition between *F. culmorum* and *F. avenaceum*.

Introduction

Fusarium foot rot of wheat is caused by a number of species which can either infect stem bases individually or as part of a disease complex (Parry et al., 1994). The predominant species present in UK wheat crops are *Fusarium culmorum*, *Microdochium nivale* and, to a lesser extent, *F. avenaceum* (Parry, 1990; Pettitt et al., 1993). Colonisation of wheat stem bases by these pathogens appears to follow a seasonal succession (Duben and Fehrman, 1979; Parry, 1990), which may in part be mediated by temperature (Pettitt and Parry, 2001). The predominant species in any one year is important in determining the severity of foot rot symptoms seen and appears to change from year to year (Pettitt et al., 1996). However, the importance of competition between these species for infection sites in determining predominance has not been explored in detail. In this paper the interdependence of the three main members of the UK *Fusarium* foot rot complex of wheat is considered by various statistical methods and by using isolation data collected from over 260 fields distributed across eastern England.

Materials and methods

Field samples

As part of an annual cereal disease survey of England and Wales carried out by Central Science Laboratory in Harpenden in collaboration with the Agricultural Development and Advisory Service, winter wheat stem-bases were collected in 1993 from between 350 and 400 crops, at growth stage 73–75 ('milky ripe', Zadoks et al., 1974), following the procedure described by Polley and Thomas (1991). From each site sampled, 50 fertile tillers were taken at random from along a diagonal traverse of the field. Detailed isolations were carried out on samples from 260 of these sites, mostly from the eastern counties of England (Figure 1). Subsamples of 25 tillers were taken for isolations once information on disease incidence and severity in each tiller sample had been recorded. At each site, monthly average temperatures were recorded.

Isolation and identification of pathogens

Isolations were made following the procedures described previously (Pettitt et al., 1993; 1996). Stem

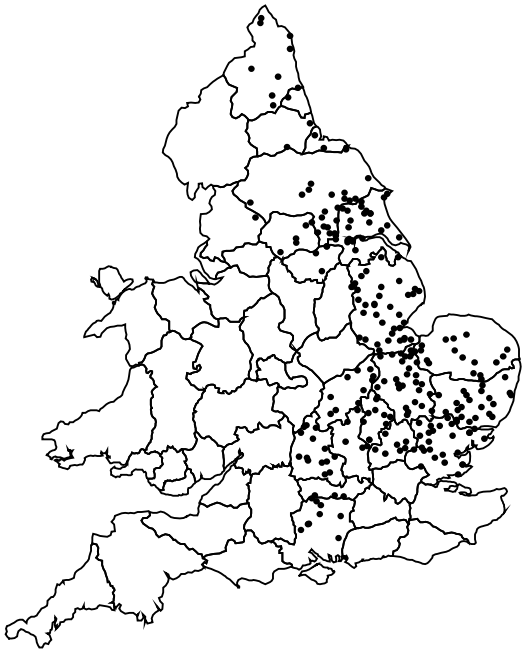

Figure 1. Map of England and Wales showing the distribution of the field sites (•) sampled in this study.

bases and first nodes were excised from each sampled tiller, surface sterilised in sodium hypochlorite solution (5% available chlorine) and washed three times in sterile distilled water. Tissue pieces were blotted on sterile tissue paper and split longitudinally in two. One piece was placed on potato dextrose agar containing streptomycin sulphate ($100\,\mu g\,ml^{-1}$), neomycin ($50\,\mu g\,ml^{-1}$) and chloramphenicol ($50\,\mu g\,ml^{-1}$), and the other on the same medium supplemented with $10\,\mu g\,ml^{-1}$ benomyl (Benlate fungicide, DuPont UK Ltd, 50% w/w a.i.). Since *M. nivale* populations are largely resistant to benomyl (Locke et al., 1987) and *F. avenaceum* is slightly more 'tolerant' to the fungicide than the faster-growing *F. culmorum*, this process allows the separation of the species present in isolations from multiple infections. Isolated fungi were identified by colony colour and morphology and by macroconidium morphology according to Booth (1971) with additional guidelines from D. Brayford (International Mycological Institute, Kew, UK, personal communication). The numbers of tillers with single infections by each of the three target pathogen species, *F. culmorum, F. avenaceum* and *M. nivale*, were recorded for each sample. Also recorded were the numbers of each combination of double infection and the numbers of tillers infected with all three species.

Statistical methods

Two levels of association between the three species were investigated. The first was at the site level, i.e., whether the occurrence of one species was geographically related to the other two species. The second was at the stem level, i.e., whether the occurrence of one species was related to the presence/absence of the other two species on the same stem on a site where more than one species was found. Chi-square test was used to determine whether there was significant association/interaction between species at the first level.

Log-linear models were used to test the independence of the three *Fusarium* species in the stem rot complex within each sampling site at the stem level assuming a Poisson sampling. In this context, Poisson sampling means that a fixed amount of effort was used to sample the wheat stems, which were then categorised into cells in the table depending on the presence/absence of three species. Specifically, it assumed that neither the total number of individual species nor the total occurrence of all species was known before the sampling took place. The present sampling indeed met these two criteria.

In the log-linear model, each count in the individual cell was treated as a Poisson response variable, which was then regressed on independent variables/factors. In the present study, there were three factors, i.e., three species (*F. culmorum, F. avenaceum* and *M. nivale*), each with two levels: present or absent. The full model, with all the interactions (one three-way and three two-way interactions), was the saturated model. The test statistic for independence of two or three species equals the sum of squared Pearson residuals from the log-linear model without the specific interaction term to be tested. This test statistic has a chi-square distribution with one degree of freedom. This test was done for all four interaction terms in turn. This process of fitting log-linear models for testing each interaction was done for all those sample sites where there were at least two species present.

Since the results from fitting the log-linear model showed that at no sites were there more than one significant interaction term and that for all the sites the three-way interaction was not significant, the Chi-square test and Fisher's exact test were also applied to determine whether two species interactions were significant. Chi-square test is an approximate test and its accuracy increases with the sample size. Fisher's exact test, as the name indicates, provides an exact probability for

the observed data, but its application is restricted to two-way interactions.

Logistic regression analysis (Cox and Snell, 1989), which is based on the logit transformation of the proportion (p) of stems infected ($\ln(p/(1-p))$) at each site, was used to assess the relationship between temperature and the incidence of each fungal species. In this analysis, the number of stems with each species at each site was assumed to be distributed binomially. All the statistical analyses were done using Genstat™ (Payne et al., 1993).

Results

General results

Figure 2 shows the histogram of the disease incidence over all the samples for the three species. Of three species, *F. culmorum* had the highest incidence with an average of 50.5% sampled stems infected, whereas *F. avenaceum* had the lowest incidence with average 12.5% stems infected: incidence for *M. nivale* was 37.1%. There were 12, 20 and 71 samples that did not contain *F. culmorum*, *M. nivale* or *F. avenaceum*. The frequency data, the number of stems infected with each species at individual sites, cannot be fitted satisfactorily to Poisson distributions for all three species. The variance and mean ratios were 5.7, 4.9 and 4.5 (for a Poisson distribution, the ratio is expected to be 1; a Poisson distribution is expected for random distributed counts data) for *F. culmorum*, *M. nivale* and *F. avenaceum*, respectively, indicating extreme aggregation of diseased stems within a sample site. A negative binomial distribution fitted satisfactorily only to the data for *F. avenaceum*, whereas none of common discrete distributions fitted *F. culmorum* and *M. nivale* data satisfactorily because of excess of observed infected stems in the upper level of incidence (Figure 2).

Logit regression showed that the percentage of variation in disease incidence within a sampling site accounted for by weather condition was very small, especially for *M. nivale* and *F. avenaceum*. Temperature was summarised either from 100 days before harvest or from February to harvest. There were no indications from these data that the incidences of the three species at the time of sampling were significantly differently affected by temperature.

Association/interaction between species at the site level

Table 1 gives the summary of species coexistence at the site level. Of the 260 sampling sites, *F. culmorum*, *M. nivale* and *F. avenaceum* were found in 248, 239

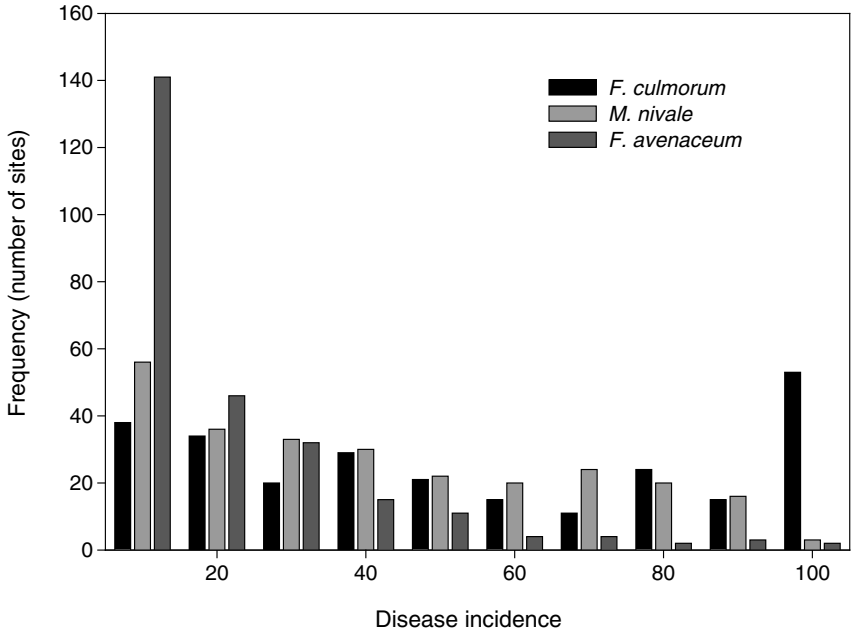

Figure 2. Histograms of the sampling sites with different incidence levels of *F. culmorum, M. nivale* and *F. avenaceum*.

Table 1. Summary of the coexistence of *F. culmorum, F. avenaceum* and *M. nivale* at site level

Species	Number of sites with the species (out of 260)	Species combination	Expected number of sites with the combination assuming no interactions	Observed
F. avenaceum (Fa)	185	Fa/Fc	176	178
F. culmorum (Fc)	248	Fc/Mn	228	228
M. nivale (Mn)	239	Fa/Mn	170	169
		Fa/Fc/Mn	162	163

Table 2. Summary of statistical analysis of species coexistence of *F. culmorum* (Fc), *F. avenaceum* (Fa) and *M. nivale* (Mn) on the individual stem-base of wheat at each site

Species	No of sites out of 260 with the species	Number of site with significant interaction		
		LL	Fisher	χ^2
Fa × Fc	178	25	10	25
Fc × Mn	228	13	5	14
Fa × Mn	169	5	0	8

LL – Log-linear method; Fisher – Fisher's exact test; χ^2 – Chi-square test.

and 185 sites, respectively. The observed number of sites with various combinations of three species was extremely close to the expected under the assumption of independence between species (Table 1). For example, assuming independence, both *F. culmorum* and *F. avenaceum* were expected to occur in 176 out of 260 sites, comparing the observed 178 sites. All three species were expected to occur in 162 out 260 sites under the assumption of independence, compared to the observed value of 163 sites.

Association between species on the same stem

Results from fitting log-linear models showed that at none of sites were there significant three-way interactions between the species. Furthermore, there was no more than one significant two-way interaction at any given site.

Table 2 gives the summary of analysis on association of species on the same stem. In 25 out of 178 sites, significant interactions between *F. culmorum* and *F. avenaceum* were detected by the log-linear model analysis, compared to 13 out of 228 sites and 5 out 169 sites for *F. culmorum* × *M. nivale* and *F. avenaceum* × *M. nivale*, respectively. Similar results were obtained from the Chi-square testing (Table 2). Almost all of the sites with significant interactions identified by log-linear modelling coincided with those identified by Chi-square testing. Most of significant interactions were of competition type, i.e., presence of one species inhibited the presence of another on the same stem.

In contrast, there were fewer sites with significant interactions from Fisher's exact testing (Table 2): 10 out of 178 and 5 out of 228, and zero were significant for *F. culmorum* × *F. avenaceum*, *F. culmorum* × *M. nivale* and *F. avenaceum* × *M. nivale*, respectively. The sites with significant interactions identified by the Fisher's testing were a subset of those identified by log-linear modelling or Chi-square test.

Discussion

This study showed that the conclusions on the independence of species, causing the *Fusarium* stem rot complex, critically depended on the analytical method used. Applying both log-linear model and Chi-squared analyses resulted in similar conclusions, indicating a much greater number of sites with significant interactions than the Fisher's exact test. Since the Fisher's test gives the exact probability, its results might be expected to be more reliable than the other two methods. The main reason for this difference between three analytical methods is likely to be the relatively restricted number of stems sampled per site.

The survey results strongly indicate that *F. culmorum*, *F. avenaceum* and *M. nivale* are independently distributed across the sampling sites, with logistic regression analysis showing no differential effects of temperature sum on the incidence of the three species at the time of sampling. If there were differential effects of temperature on the three species, it would be expected that the species would not be independently distributed across the sites. It may also be possible that the temperature range across the sites in the survey year was not great enough to detect differential

effects on the three pathogen species. Alternatively, other factors such as local agronomy and/or fungicide treatments may have confounded some individual site data. When data from a number of sites in a particular region (e.g. a county) have been pooled, weak correlations between incidence and temperature sum were obtained for *M. nivale* (Pettitt et al., 1996) and for *F. graminearum* (Smiley and Patterson, 1996).

This study showed no evidence of competition or codependence between *F. culmorum* and *M. nivale.* However, this may be the result of a combination of the normal temporal sequence of infections leading to *M. nivale* infections becoming established and reaching their peak before significant activity of *F. culmorum*, and the inability of *M. nivale* to exclude *F. culmorum* from infected tissues. It may be that these two species are able to colonise the same host tissues because of differing requirements from the colonised tissues. However, when *F. culmorum* is the primary coloniser of new substrates, including winter wheat stems, it is usually extremely efficient at excluding subsequent potential colonisers (Cook, 1970). It would therefore be useful to carry out sequential inoculations of wheat plants with *F. culmorum* and *M. nivale* to determine the effects of each species as a primary coloniser on the incidence of subsequent infections, before finally discounting the existence of competition between these two species in wheat stem-bases.

The only evidence of competition in this study was between *F. culmorum* and *F. avenaceum*, with significant competition, as identified by Fisher's test, occurring in 10 (ca. 5.6%) out of the 178 sites were both of these species are present. This may reflect the fact that *F. avenaceum* and *F. culmorum* are more closely related than *F. culmorum* and *M. nivale* (Booth, 1971) and are closer together in terms of temperature and moisture requirements for infection and pathogenicity (Colhoun and Park, 1964; Pettitt and Parry, 2001). Whilst competition between *F. culmorum* and *F. avenaceum* probably results from their similar infection and colonisation requirements, *F. avenaceum* is generally a far weaker pathogen of UK winter wheat crops (Colhoun et al. 1968). The techniques used in this first investigation of the interdependence of the main *Fusarium* species involved in the stem base rot complex of wheat were limited to confirming the presence or absence of individual species on individual tillers. Further work is now needed to confirm the tentative conclusions of this study and this will probably be best achieved using new molecular techniques that will allow quantification of individual pathogen biomass as well as confirmation of their presence. Competitive PCR and, more recently, 'real-time' PCR assays have been developed for quantifiable detection of *F. graminearum* and *F. culmorum* (Nicholson et al., 1998; Schilling et al., 1996), *M. nivale* var. *majus* and var. *nivale* (Nicholson et al., 1996), and *F. avenaceum* (Turner et al., 1998; Schilling et al., 1996). These assays should provide the means to dissect the *Fusarium* stem rot complex into its component parts and determine the prevalence and role of each component in disease development and under various conditions.

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