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Relationship between growth and mycotoxin production by *Fusarium* species, biocides and environment

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Abstract

Fusarium head blight of cereals has, in recent years, become one of the most important pre-harvest diseases worldwide. This paper examines the *in vitro* efficacy of fungicides to control *Fusarium* species in cereals and the efficacy in the field on both *Fusarium* infection of ripening ears as well as their impact on mycotoxin production. Field studies suggest that fungicides such as tebuconazole and metconazole give good control of both *Fusarium* infection of ears and control of deoxynivalenol (DON) production. However, azoxystrobin and related fungicides are less effective, and grain from treated crops has sometimes been found to have increased concentrations of DON and nivalenol. Studies of isolates of *Fusarium culmorum* from different parts of Europe showed that complex interactions occur between environmental factors, fungicide type and isolate in relation to growth inhibition and DON production. These studies confirmed the ineffectiveness of azoxystrobin and suggest that environmental stress factors, particularly water availability and temperature, and low fungicide doses may stimulate mycotoxin production by Fusaria *in vitro* and in wheat grain.

Introduction

With the intensive cultivation of monoculture cereals, the input requirements, including pesticides and fungicides, needed to maximise yield have always been significant. In the last 10 years, the increased level of colonisation and infection by *Fusarium* spp., particularly of ripening ears of both temperate and tropical cereals has attracted much attention; firstly, because of the significant effects on yield and quality of harvested grain, and secondly, because of the ability of *Fusarium* spp. to produce a wide range of mycotoxins which can enter the human and animal food chains. Table 1 lists the key *Fusarium* spp. which can be involved in head blight and ear blight of cereals and their key mycotoxins which are the trichothecenes, zearelenone (ZEN), moniliformin and the fumonisins.

A significant effort has been concentrated on the development and use of fungicides for control of *Fusarium* spp. to prevent both infection and mycotoxin production. A significant amount of screening has been carried out on a range of systemic fungicides for control of *Fusarium* spp. in cereals, particularly wheat, barley and oats, where natural contamination with deoxynivalenol (DON) and nivalenol has been found.

Table 1. Summary of important *Fusarium* species and their mycotoxins

Species	Major mycotoxins
F. culmorum	Deoxynivalenol (DON), 3-acetyl DON, 15-acetyl DON, nivalenol, fusarenone (FX), Zearalenone (ZEN)
F. graminearum	DON, 15-ADON, NIV, FX, ZEN
F. sporotrichioides	T-2 toxin, HT-2 toxin, neosolaniol (NEO), diacetoxyscirpinol (DAS), FX, ZEN.
F. poae	T-2 toxin, HT-2 toxin, NIV, DAS, FX
F. verticilloides *F. proliferatum*	Fumonisins, moniliformin, fusarin C
F. oxysporum	Moniliformin, wortmannin, fusaric acid
F. sambucinum	Sambutoxin

For example, Snijders and Perowski (1990) showed a direct relationship between natural incidence of ear blight and DON contamination of wheat kernels by *F. culmorum*. Originally, a range of fungicides, such as mixtures based on the non-systemic prochloraz, and systemic fungicides such as tridemorph, carbendazim and zineb were used in the 1980s to control foliar and ear diseases (Moss, 1985; Moss and Frank, 1985; Magan and Lacey, 1986). The expanding acreage for cultivation of bread wheats in Europe in the 1980s also resulted in an increase in head blight of ripening ears by *Fusarium* spp., similar to that observed in Canada (Sutton, 1983; Parry et al., 1995). Complex interactions between species were also observed in Canada where early infection of ripening ears by *F. poae* and *F. avenaceum* were considered to facilitate later colonization by *F. culmorum* and *F. graminearum* (Sturz and Johnston, 1983). However, while information is available on the impact of environmental factors on the germination, growth and sporulation of *Fusarium* spp. from wheat (Magan and Lacey, 1984a), and *F. moniliforme* (=*F. verticillioides*) and *F. proliferatum* from maize (Marin et al., 1995), the relationship between these factors, fungicide efficacy and effects on growth and mycotoxin production have not been examined previously. This paper will consider information that is available, and present some recent studies where the impact of water availability, temperature and interactions with fungicides on DON production were examined for a range of *F. culmorum* strains from different parts of Europe (Italy, Norway, Sweden and the U.K.).

Relationship between fungicides, *Fusarium* spp. and mycotoxin production in cereals

Studies by Moss and Frank (1985) found that concentrations of tridemorph influenced production of T-2 toxin by *F. sporotrichioides.* At low concentrations (6–8 $\mu g\,ml^{-1}$), growth of *F. sporotrichoides* was enhanced while T-2 toxin and diacetoxyscirpenol (DAS) production was reduced. Higher fungicide concentrations (30–50 $\mu g\,ml^{-1}$) inhibited growth by >50% but stimulated formation of T-2 toxin five-fold. The same fungicide enhanced the production of aflatoxin by *Aspergillus flavus* (Badii and Moss, 1988). In contrast, the benzothiazole derivative, tricyclazole, completely inhibited aflatoxin production at concentrations only partially inhibiting growth (Fernando and Bean, 1986).

Treatment of *F. graminearum* infection of kernels of maize with maneb resulted in good control of both pathogen growth and ZEN production at 50 $\mu g\,ml^{-1}$ *in situ* and *in vitro* (Draughton and Churchville, 1983). More recently, Hasan (1993) compared the effects of dicloran, iprodione and vinclozolin and found that, at 500 $\mu g\,ml^{-1}$, the former eliminated DAS production and that 250 $\mu g\,ml^{-1}$ was sufficient to inhibit ZEN synthesis. In contrast, 500 $\mu g\,ml^{-1}$ of vinclozolin failed to prevent ZEN being produced by *F. graminearum*. Matthies and Buchenauer (1996) screened a range of fungicides in common use in Germany and found that of benomyl, thiabendazole, prochloraz, tebuconazole, tridemorph and fenopropimorph, the latter two had no effects on monoacetyl deoxynivalenol (3-AcDON) production in pure culture. The others inhibited production of the mycotoxins at 0.5–1.0 $\mu g\,ml^{-1}$. Tubiconazole induced a four-fold increase in the mycotoxin concentration at only 0.1 $\mu g\,ml^{-1}$. Similar results were obtained with *F. culmorum* at this concentration, whereas difenoconazole had no effect on growth, but increased the production of the same mycotoxin at 25°C, but not at 11°C (De Mello et al., 1998). D'Mello et al. (1998) also compared resistant and sensitive strains of *F. culmorum* to difenoconazole. Overall, the sensitive strains produced 3-AcDON in the presence of difenoconazole at 0.1 $\mu g\,ml^{-1}$, but less rapidly in resistant strains. It is possible that fungicides act as an additional stress factor stimulating synthesis of mycotoxins as a defence response. Recent studies have shown that metaconazole and tebuconazole not only inhibited growth but also caused marked morphological and cytochemical alterations of the hyphae when 20 $\mu g\,ml^{-1}$) fungicide was added to media (Kang et al., 2001a,b). They demonstrated that DON was localized in the cell walls, cytoplasm and mitochondria and vacuoles of the hyphae and that labelling density, using immunogold labelling, was significantly less dense than in untreated control hyphae of *F. culmorum*.

Very few experiments have considered interactions between fungicide concentration and environmental factors, particularly water availability and temperature, which to a large extent determine *Fusarium* ear blight of ripening ears. Surprisingly, no detailed studies of the efficacy of fungicides on mycotoxigenic *Fusarium* spp. have taken these abiotic interactions into account. This has been considered in studies with fumonisin-producing species and post-harvest preservatives such as propionic and sorbic acids (Marin et al., 1998; 1999).

Recent field studies were carried out to examine the efficacy of fungicide applications on *Fusarium* head

blight of cereals and on mycotoxin production. For example, work by Milus and Parsons (1994) suggested that levels of mycotoxins may be increased by the application of fungicides. Ellner (2000) carried out field trials with azole fungicides (tebuconazole, metconazole) in two seasons in Germany and found that control of head blight and reductions in the levels of DON did not exceed 50%. Recent work by Jennings et al. (2000) and by Simpson et al. (2001) demonstrated complex interactions between the type of fungicide used and effects on colonization by *Fusarium* spp. and mycotoxin production. For example, tebuconazole was quite effective against *F. culmorum* and *F. avenaceum*, monitored both as number of diseased ears produced and using quantitative PCR to assess pathogen biomass. However, azoxystrobin had little effect on *F. culmorum* and *F. avenaceum*, but gave significant control of *Microdochium nivale* var. *majus*. Mixtures of azoxystrobin/prochloraz and azoxystrobin/fluquinconazole were less effective against *F. culmorum* than against *M. nivale*. Higher DON productions were found in plots treated with azoxystrobin (5.2 and 10.4 $\mu g\ ml^{-1}$ respectively) in both 1998 and 1999, and in 1999 with difenoconazole (11.2 $\mu g\ ml^{-1}$). They suggested that complex interactions occur between the differential impact of the fungicides, resulting in interactions between *M. nivale* and other *Fusarium* spp. which could result in this enhanced accumulation of DON. Siranidou and Buchenauer (2001) applied the fungicides two days prior to or two days post-inoculation with *F. culmorum* to ears of wheat and found that both tebuconazole and metconazole reduced disease on spikes of winter wheat by 60–70% and DON content by 50–70%. However, chlorothalonil, prochloraz and benomyl gave no disease control. Interestingly, azoxystrobin reduced disease incidence on the spikes but DON concentrations increased. Edwards et al. (2001) developed a quantitative PCR method to quantify trichothecene-producing *Fusarium* species based on primers from the trichodiene synthase gene (Tri 5). They were able to quantify a range of *Fusarium* head blight pathogens in relation to fungicide control. Metaconazole and tebuconazole gave good control of head blight and DON production, while azoxystrobin was ineffective. A good correlation was obtained between trichothcene-producing species and DON production in grain, but no correlation was found between *Fusarium* head blight and DON. They hypothesized that fungicides affected the *Fusarium* species in the head blight complex by altering the proportion of trichothcene-producing species in the infected ears and not by altering DON production. This method does, however, provide a tool for examining the interaction between *Fusarium* species and biotic and abiotic stress factors including fungicide applications.

Some studies have identified the environmental conditions which allow *F. culmorum* and *F. graminearum* to infect ripening ears of cereals (Jennings and Turner, 1996; Lacey et al., 1999; Savard et al., 2000). However, it is surprising that very few studies have examined the interaction between fungicides, environmental conditions and growth, and interactions between key *Fusarium* species. Furthermore, little is known of the variation in sensitivity between species of mycotoxigenic strains of *F. culmorum* in Europe. Few studies have examined the impact that interacting environmental conditions and fungicides might have on pathogen growth and mycotoxin production in infected plants and grain.

Impact of environmental factors on growth mycotoxigenic strains of *F. culmorum* from different parts of Europe

Recent studies in the Applied Mycology Group, Cranfield University, U.K. have examined the ecology of mycotoxigenic strains of *F. culmorum* from different parts of Europe. Studies have included a comparison of the water relations of the strains, assessments of the *in vitro* efficacy of fungicides used in cereals under different environmental regimes and their ability to control pathogen growth and mycotoxin production in wheat grain.

Initial studies showed that between strain differences (three strains each) from an individual country were, to a large extent, statistically non-significant (data not shown). Subsequent studies were carried out with one strain from each country. Figure 1 compares the effect of water activity (a_w) and temperature on representative strains of *F. culmorum* from Italy, Norway, Sweden and the U.K. Isolates behaved relatively similarly at 25 °C, with growth occurring down to 0.92 a_w. At 15 °C, growth was reduced by about 40–50%, but with a similar tolerance to a range of water availabilities. There were no significant differences between strains from the different countries at 25 °C but a strain from Norway appeared to grow significantly better than the others at 15 °C. These results, obtained on a milled wheat grain agar, were similar to those obtained earlier in studies comparing the water relations of *Fusarium* spp. from U.K. wheat (Magan and Lacey, 1984b).

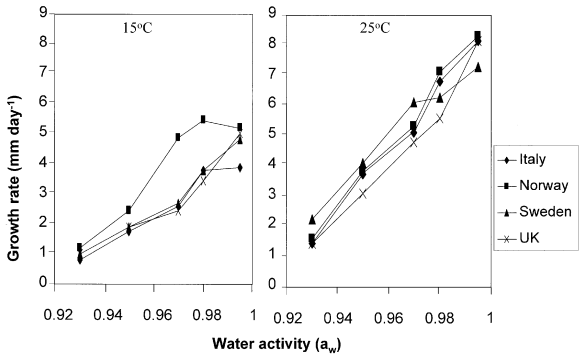

Figure 1. Comparison of water relations and growth of *F. culmorum* isolates from different European countries *in vitro* on a milled wheat agar medium.

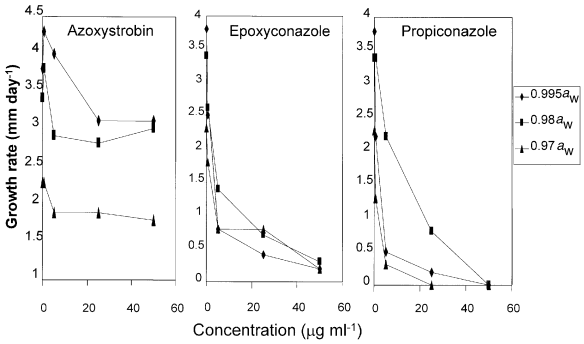

Figure 2. *In vitro* efficacy of three fungicides against a mycotoxigenic isolate of *F. culmorum* at 15 °C and three different water availability conditions (as indicated on the right axis).

The efficacy of different concentrations of three different fungicides used in cereals were compared at different a_w levels. Figure 2 shows that, for a U.K. isolate epoxyconazole and propiconazole were most effective at slightly reduced a_w levels with azoxystrobin being relatively ineffective, with little difference in efficacy at 20–50 µg ml^{-1}. Figure 3 further compares the effect of the fungicides at 15 °C on a strain of *F. culmorum* from each European country examined. This again shows that, in the range 0.5–50 µg ml^{-1} and 0.97 a_w, azoxystrobin was ineffective.

Subsequently, studies were carried out on irradiated wheat grain that had retained their germination capacity. Experiments were carried out on single layers of wheat grain modified with sterile water to the required water content (Lee and Magan, 2000), and with only the two most effective fungicides. A comparison of the growth rates across layers of wheat grain showed that

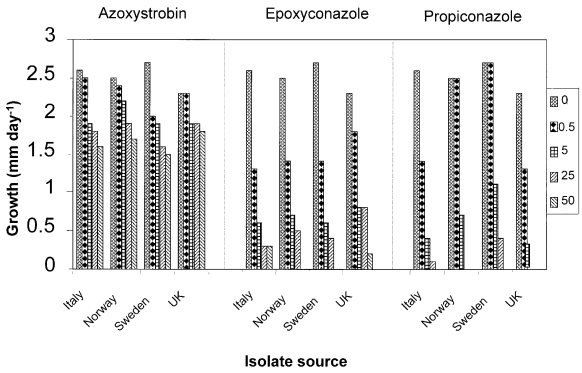

Figure 3. Comparison of the efficacy of three fungicides against growth of isolates of *F. culmorum* from four different European countries *in vitro* at 15 °C and 0.97 water activity. Concentrations of fungicides used are indicated on the right axis.

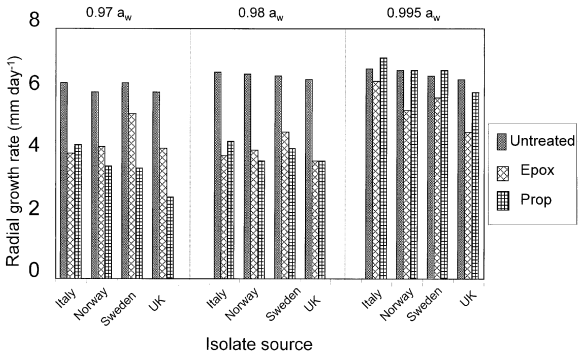

Figure 4. Effect of epoxyconazole (epox) and propiconazole (prop) at 25 µg ml^{-1} on growth of *F. culmorum* isolates at three different water availabilities on irradiated wheat grain at 25 °C.

at 25 µg ml^{-1} and freely available water, there was little effect of the fungicides on growth of the strains from the four different countries (Figure 4). However, at 0.98 and 0.97 a_w, propiconazole was more effective, reducing the growth rate by about 30–40% when compared to untreated controls under the same environmental conditions. Table 2 shows the effect of the fungicides on DON production by *F. culmorum*. The concentrations used show that there was a stimulation of DON production in the presence of the two fungicides. This points to a significant interaction between environmental conditions, especially water availability and temperature, fungicide type and concentration. To this must be added the interaction with other cereal ear mycoflora and the differential effect of fungicides on these fungal communities.

Table 2. Effect of water activity (a_w) and fungicide (25 μg ml^{-1}) on DON production by strains of *F. culmorum* from different parts of Europe grown on irradiated wheat grain with conserved germination capacity

Isolate source	a_w	Deoxynivalenol concentration (μg^{-1})		
		Control	Epoxiconazole	Propiconazole
U.K.	0.99	0.31	0.05*	0.55*
	0.98	0.72	0.88	1.23*
	0.97	1.09	1.31*	1.26*
Italy	0.99	0.67	0.41	0.88
	0.98	3.10	1.93	3.26
	0.97	5.03	20.00*	16.70*
Sweden	0.99	2.60	2.53	3.26
	0.98	19.33	5.93*	3.93
	0.97	5.03	20.00*	20.00*
Norway	0.99	3.20	3.40	2.26
	0.98	0.85	5.13*	4.73*
	0.97	1.16	20.00*	20.00*

*, indicates significantly different from the control at $P = 0.05$.

Conclusions

It is important that work now concentrates on some of the complex interactions between *Fusarium* infections, differential effects of fungicides, environmental factors and associated mycoflora which can occur on ripening ears of cereals. Furthermore, control of these fungi, and in particular, the prevention of increased concentrations of mycotoxins are essential if consumers are to be protected from toxic contaminants. Such effects are an important critical control point in the production process and more information is required for a hazard analysis critical control point system approach to preventing the entry of such mycotoxins into human and animal food chains.

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