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Differential control of head blight pathogens of wheat by fungicides and consequences for mycotoxin contamination of grain

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Abstract

Fusarium head blight of wheat is caused by a disease complex comprised of toxigenic pathogens, predominantly *Fusarium* spp., and a non-toxigenic pathogen *Microdochium nivale*, which causes symptoms visually indistinguishable from *Fusarium* and is often included as a causal agent of *Fusarium* head blight. Four field trials are reported here, including both naturally and artificially inoculated trials in which the effect of fungicide treatments were noted on colonisation by *Fusarium* and *Microdochium*, and on the production of deoxynivalenol (DON) mycotoxin. The pathogen populations were analysed with quantitative PCR and samples were tested for the presence of the mycotoxin DON. Application of fungicides to reduce *Fusarium* head blight gave a differential control of these fungi. Tebuconazole selectively controlled *F. culmorum* and *F. avenaceum* and reduced levels of DON, but showed little control of *M. nivale*. Application of azoxystrobin, however, selectively controlled *M. nivale* and allowed greater colonisation by toxigenic *Fusarium* species. This treatment also lead to increased levels of DON detected. Azoxystrobin application two days post-inoculation increased the production of DON mycotoxin per unit of pathogen in an artificially inoculated field trial. This result indicates the potential risk of increased DON contamination of grain following treatment with azoxystrobin to control head blight in susceptible wheat cultivars. This is the first study to show differential fungicidal control of mixed natural pathogen populations and artificial inoculations in field trials.

Introduction

Fusarium head blight (FHB) of wheat, also known as scab or *Fusarium* ear blight, constitutes a disease complex involving several *Fusarium* species. Although worldwide the principal pathogen is *F. graminearum*, in the UK and Northern Europe the predominant species is *F. culmorum* (Parry et al., 1995a), along with less prevalent species such as *F. avenaceum* and *F. poae*. *Microdochium nivale* is another causal agent of head blight of cereals which has traditionally been considered a part of the *Fusarium* head blight disease complex. It is one of the predominant pathogens of cereals in the UK and Northern Europe (Parry et al., 1995a) and causes symptoms which are visually identical to *Fusarium* head blight.

Both *F. graminearum* and *F. culmorum* produce zearalenone (ZEA) and trichothecene mycotoxins, including deoxynivalenol (DON) and nivalenol (NIV). Trichothecenes are thought to be virulence factors for *F. graminearum* infecting wheat (Proctor et al., 1995; Desjardins et al., 1996; Desjardins and Hohn, 1997) but the role, if any, of ZEA in pathogenicity is unknown. Among the other species commonly associated with FHB, *Fusarium poae* and *F. sporotrichioides* also produce trichothecene mycotoxins (including T2 and HT2) (Bottalico, 1998) whereas *F. avenaceum* produces enniatins and moniliformin (Golinski et al., 1996; Herrmann et al., 1996). In contrast, *M. nivale* varieties are not known to produce mycotoxins (Logrieco et al., 1991). The mycotoxins produced by the *Fusarium* species are of particular

significance because they pose a health risk to humans and animals if consumed and legislative limits have been set for DON in grain and grain-based products in a number of countries (Bottalico, 1998; Miller, 1994). Europe-wide limits for *Fusarium* toxins are currently being considered by the European Commission Scientific Committee on Plants (Hardy et al., 1999).

In the absence of high levels of resistance within commercial wheat varieties, most attempts to control FHB rely upon fungicide application. Although there are a number of compounds with *in vitro* activity against FHB pathogens, control of this disease in the field has often proved difficult (Milus and Parsons, 1994). There are several reports of the successful control of FHB (Fehrmann and Ahrens, 1984; Mauler-Machnik and Suty, 1997; Matthies and Buchenauer, 2000), but there are also a number of reports in which reduction of FHB has not been associated with a significant reduction of mycotoxin (Martin and Johnston, 1982). One study indicated that toxin levels were increased by fungicide application (Gareis and Ceynowa, 1994), however their study involved bulking of low numbers of small volume samples, and the tests were not fully replicated. There is also a growing concern that sub-lethal doses of particular fungicides may lead to an increase in mycotoxin production by *Fusarium* species (Milus and Parsons, 1994; D'Mello et al., 1998b). Such effects have been observed in several *in vitro* studies with some compounds inhibiting the growth of the *Fusarium* species but causing increased trichothecene production (Moss and Frank, 1985; D'Mello et al., 1998a; Matthies et al., 1999).

Part of the discrepancy between *in vitro* and *in vivo* fungicide efficacy may be due to the effects of the fungicides on non-target species. Saprophytes and minor pathogens may contribute to the suppression of more pathogenic species, by competition for space or available nutrients (Liggitt et al., 1997). For example, there may be antagonism between *M. nivale* and *F. culmorum* (Doohan et al., 1998). A similar antagonism has been observed between *Fusarium* spp. and *Microdochium bolleyi* (Reinecke et al., 1979), and a correlation has also been observed between high levels of the saprophyte *Alternaria alternata* with low levels of DON in field samples (González et al., 1999). Such findings would suggest that the removal of the non-toxin producing fungi from the ear by fungicides may have the overall effect of relieving competition and lead to the increased establishment of any toxin-producing pathogens that are less sensitive to the fungicide.

Strobilurins are a relatively new group of fungicidal compounds. One recently introduced strobilurin product for use on cereals is azoxystrobin (Amistar; Zeneca Agrochemicals, Fernhurst, Surrey, UK; Mercer and Ruddock, 1998). This fungicide has been shown to be effective against *M. nivale* and saprophytic fungi found on the wheat heads (Bertelsen et al., 1999) while being less effective against *F. culmorum* (Faure and Declercq, 1999). How this differential fungicidal action affects the mixed FHB population has not been previously studied in the field. This is what we hope to show in the research presented here.

Advances in molecular diagnostic assays for FHB pathogens now permit the specific detection and relative quantification of the major FHB pathogens, including *F. culmorum, F. graminearum, F. avenaceum, F. poae* and *M. nivale* (Nicholson et al., 1996; Nicholson et al., 1998; Turner et al., 1998; Parry and Nicholson, 1996). Quantification of the level of individual species within a mixed population represents a revolution in the study of disease complexes. The effects of fungicide treatment on competing populations can now be determined. Using these techniques, we provide evidence from naturally and artificially inoculated field trials for the differential control of toxigenic *Fusarium* species and non-toxigenic *M. nivale*, by strobilurin and other fungicides and for the consequences for toxin accumulation in grain. This is the first case where differential effects of fungicides on the head blight disease complex are shown in field trials.

Materials and methods

Field trials (natural inoculum)

Plots of the winter wheat cultivar Riband were sown at Morley Research Centre (MRC), Norfolk in October of 1996 and grown according to normal agronomic practice. Selected plots were treated with azoxystrobin (see Table 1), using the full recommended rate, at mid-anthesis (Growth Stage 65 (GS 65)) (Zadoks et al., 1974). Adjacent plots were left untreated. Ears were taken from both plots at GS 90, and separated into grain and chaff. DNA was extracted and analysed for the presence of *F. graminearum, F. culmorum*, *F. avenaceum* and *M. nivale* var. *majus* and *M. nivale* var. *nivale*, by quantitative PCR as described below. Two ears were harvested from each of these plots and

Table 1. Active ingredients, source and application rates of fungicides used

Fungicide	Active ingredients	Full application rate (g a. i. Ha^{-1})	Supplier
Amistar	Azoxystrobin	250	Zeneca Agrochemicals, Fernhurst, Surrey, UK
Folicur	Tebuconazole	250	Bayer UK Ltd. Bury St Edmonds, UK
Flamenco	Fluquinconazole	250	Aventis, Hauxton, Cambridge, UK
Flamenco plus	Fluquinconazole Prochloraz	250 450	Aventis, Hauxton, Cambridge, UK
Amistar + Flamenco	Azoxystrobin Fluquinconazole	250 250	See above
Amistar + Flamenco plus	Azoxystrobin Fluquinconazole Prochloraz	250 250 450	See above
Amistar + Sportak	Azoxystrobin Prochloraz	250 450	See above

between 10 and 20 grains or pieces of chaff were surface sterilised and placed onto Petri dishes containing PDA and incubated at 20 °C for 6 days. Isolations were made from the resultant fungal colonies, and these were analysed microscopically for the presence of macroconidia of *Fusarium* species.

Wheat (cv Rialto) was grown at Spalding, Lincolnshire, in 1998 in four replicates of three treatment blocks; tebuconazole, azoxystrobin and untreated control. The fungicides and their dosages are given in Table 1. The plants were cultivated according to normal agronomic practice and fungicides were applied at GS 65. Visual disease assessment was made at GS 77 as percentage of ears showing disease symptoms. Grain was sampled at harvest (GS 90) and a 50 g representative sample was milled. DNA was extracted from 4 g of the milled material and analysed by quantitative PCR for the pathogens, *Fusarium culmorum, F. avenaceum, F. graminearum, Microdochium nivale* var. *nivale*, and *M. nivale* var. *majus*. A representative 200 g sample of grain was milled and 10 g of milled material was analysed for DON toxin content as described below.

Wheat (cv Rialto) was grown at Wisbech, Cambridgeshire in 1998. Three replicates were planted of six treatments; azoxystrobin, at half and full recommended rate, tebuconazole, at half and full recommended rate, azoxystrobin plus tebuconazole, each at half recommended rate (Table 1), and untreated control. Cultivation, fungicide application, sampling and analysis were carried out as described above.

Production of inoculum for controlled inoculation trials

Five isolates were cultured for each of three species inoculated (*F. avenaceum*: cc39, cc40, cc76, cc32, cc33; *F. culmorum*: cc69, cc52, cc100, cc42, cc57; *M. nivale* var. *majus*: cc114, cc115, cc65, cc60, cc8). Infected wheat grain for the ground level inoculation was produced as follows. For each isolate, 2 kg of grain was autoclaved at 121 °C for 15 min and inoculated with 50 ml of a spore suspension containing 10^5 spores/ml. Grain bags inoculated with either *F. culmorum* or *F. avenaceum* isolates were incubated at 25 °C and those with *M. nivale* var. *majus* at 18 °C for 21 days. The bags were shaken every 3 days to prevent grain sticking together. Prior to inoculation of the plots all the infected grain was combined and mixed thoroughly.

For head inoculation, conidia of *F. culmorum* and *F. avenaceum* were produced from cultures grown on sucrose nutrient agar (SNA (Nirenberg, 1981), KH_2PO_4 1 g, KNO_3 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCL 0.5 g, glucose 0.2 g, sucrose 0.2 g, Technical agar No. 3 20 g, distilled water 1 l), incubated at 25 °C under near UV for 15 days. Conidia of *M. nivale* var. *majus* were produced from cultures grown on Oxoid potato dextrose agar (PDA) at 18 °C under near UV for 15 days.

Field trial (artificial inoculation at ground level, GS 31)

The spring wheat cultivar Promessa was grown at Central Science Laboratory, Sand Hutton, York, sown in 1998. Plots were inoculated at growth stage 31 by spreading 2 kg of inoculum, a mixture of *F. culmorum*, *F. avenaceum* and *M. nivale* var. *majus* infected grain, throughout each 1.5×4 m plot. Controlled misting activated by humidity sensors in the plots maintained a minimum plot humidity of 70%. Misting was achieved using an Eindor overhead misting system (Access Irrigation Ltd, Northampton), from mid-anthesis for 5 days. Misting was switched off during fungicide application (during the third day of misting). Seven fungicide treatments were

applied: fluquinconazole; fluquinconazole with prochloraz; tebuconazole; azoxystrobin; azoxystrobin with prochloraz; azoxystrobin with fluquinconazole; and azoxystrobin with fluquinconazole and prochloraz, all at full recommended rates (Table 1), with an untreated control. Spray was applied using a single nozzle Oxford precision sprayer (MDM Engineering Ltd, Hampshire) using a fine mist at a slow walk to ensure effective coverage of heads. Each treatment was repeated in triplicate. Visual disease score (% ear affected by *Fusarium* symptoms) was assessed at growth stage 85 (soft dough), and at harvest all grain was collected from each plot using a single-ear thresher to ensure effective collection of all grain. A 50 g representative sample of grain from each plot was freeze-dried and milled and DNA was extracted from 4 g of the milled material. This was analysed for fungal DNA of the inoculated pathogens by quantitative PCR. A 200 g representative sample of grain was milled and 10 g of the milled material was analysed for DON and ZEA toxin content as described below.

Fungicide trial (artificial inoculum to ear, GS 65)

The spring wheat cultivar Promessa was grown at the Central Science Laboratory, Sand Hutton, York, in 1998. Plots were inoculated with a conidial suspension of a mixture of *F. culmorum*, *F. avenaceum* and *M. nivale* var. *majus* applied to the ear at mid-anthesis (19.4 °C and 86% humidity). Conidia were combined to give a concentration of 10^5 spores/ml for each species and 200 ml of the spore suspension applied to each 1.5×4 m plot using an Oxford precision sprayer (MDM Engineering Ltd, Hampshire). Controlled misting activated by humidity sensors in the plots was used as above to maintain a minimum plot humidity of 70%. Fungicides and untreated controls were as above. Sprays were applied as above. Fungicides were applied three days post inoculation, during which time misting was switched off. Each treatment was repeated in triplicate. Sampling and mycotoxin analysis was carried out as detailed for the field trial with artificial inoculation at ground level (GS 31).

DNA extraction and quantification

DNA was extracted from wheat ears using the CTAB (hexadecyltrimethyl-ammonium bromide) buffer method described by Simpson et al. (2000). DNA was quantified according to the SYBR Green method described by Hopwood et al. (1997).

Diagnostic and quantitative PCR

PCR amplification conditions used in detection and quantification of fungi within plant samples was carried out according to the protocols detailed in Nicholson et al. (1996). The PCR primers used were specific to *M. nivale* var. *majus* and var. *nivale* (MnM2 F/R; Y13N F/R; Nicholson and Parry, 1996), *F. culmorum* and *F. graminearum,* (Fc01 F/R; Fg16 F/R; Nicholson et al., 1998) and *F. avenaceum* (Fa-U16 F/R; Turner et al., 1998). Quantification of fungi was according to the quantitative PCR method described by Nicholson et al. (1998). An aliquot of the extracted DNA from each sample was assayed in triplicate for the quantification of *F. graminearum, F. culmorum, F. avenaceum, M. nivale* var. *nivale* and *M. nivale* var. *majus*. The amount of each pathogen was expressed as a percentage of the total DNA content (plant plus fungus) of each sample.

Analysis for deoxynivalenol (DON) content

DON analysis was carried out using a Veratox DON quantitative vomitoxin test (Adgen diagnostic systems, Ayr, UK), according to the manufacturer's instructions. This test detects DON, 15-acetyl-DON and 3-acetyl-DON with equal sensitivity. However, it does not detect Triacetyl-DON or NIV. ZEA was analysed using a Veratox quantitative ZEA test (Adgen diagnostic systems, Ayr, UK), according to the manufacturer's instructions.

Statistical analysis

Where necessary, data were transformed prior to analysis due to non-independence of mean and variance. All statistical analyses were carried out using Minitab version 10.1 (1994, Minitab Incorporated, State College, PA, USA). General linearised ANOVA was used to assess experimental vsariability, to confirm normal distribution of the transformed data, and to indicate statistically significant differences between treatments. Pairwise comparisons of treatments were then made using Fisher's (protected) least significant difference test (Steel and Torrie, 1981).

Results

Field trials (natural inoculum)

The fungal DNA content of chaff of untreated ears from Morley Research Station was *Fusarium avenaceum*

Table 2. Percentage of pathogen DNA detected in samples of Riband wheat grown at Morley Research Station in 1997 with natural infection, and isolation of *Fusarium* spp. from segments of two sampled ears (% from which *Fusarium* was cultured)

Plot	*M. nivale* var. *majus*	*M. nivale* var. *nivale*	*F. avenaceum*	*F. culmorum*	*F. graminearum*	Isolation of *Fusarium* spp.
Untreated chaff	5.00	3.80	7.90	0.05	0.00	0.0
Untreated grain	0.13	0.14	0.34	0.02	0.00	11.7
Azoxystrobin chaff	0.69^{-}	1.05^{-}	19.7	1.73^{+}	0.25	48.8
Azoxystrobin grain	0.13	0.24	1.94	0.11^{+}	0.01	41.3

$^{-}$significantly lower than untreated control plot ($P < 0.05$).
$^{+}$significantly higher than untreated control plot ($P < 0.05$).

(7.90% of the total extracted DNA), *M. nivale* var. *majus,* (5.00%) and var. *nivale,* (3.80%) (Table 2). These species were also detected in the untreated grain at low levels. Very low levels of *F. culmorum* were detected in the untreated material and *F. graminearum* was not detected. In contrast, in the ears treated with azoxystrobin, the chaff contained significantly less *M. nivale* ($P < 0.05$) and the level of *F. culmorum* was significantly greater in both the chaff and grain than in the untreated ears ($P < 0.05$). The level of *F. avenaceum* was also higher in both the chaff and grain (19.7 and 1.94% of total DNA), and *F. graminearum,* absent in untreated ears, was present in both chaff and grain from azoxystrobin treated ears. Of the samples with the highest level of fungal contamination it was noted that over 20% of the DNA extracted was fungal pathogen in origin. *Fusarium* spp. were not isolated from the untreated chaff samples, and were isolated from only 11% of the samples of untreated grain, whereas *Fusarium* was cultured from 48% of the chaff and 43% of the grain samples from the azoxystrobin plots.

In the field trail from Spalding there was a low incidence of visual disease symptoms, with 9.1% of untreated ears, 3.4% of azoxystrobin treated ears and 5.4% of tebuconazole treated ears showing disease. The untreated plots contained moderate levels of *M. nivale* var. *nivale* (1.01% of total DNA) (Figure 1a) and low levels of *F. avenaceum* (0.35%) and *F. graminearum* (0.27%). Following tebuconazole treatment both *M. nivale* var. *nivale* and *F. avenaceum* were significantly reduced ($P < 0.05$ and $P < 0.01$ respectively). The reduction of *F. graminearum* was not significant. In the azoxystrobin treated samples the *M. nivale* var. *nivale* was significantly reduced ($P < 0.05$) but *F. avenaceum* was not reduced ($P > 0.20$). The reduction of *F. graminearum* was not significant ($P > 0.05$). DON mycotoxin was detected in only two samples, one from the untreated control

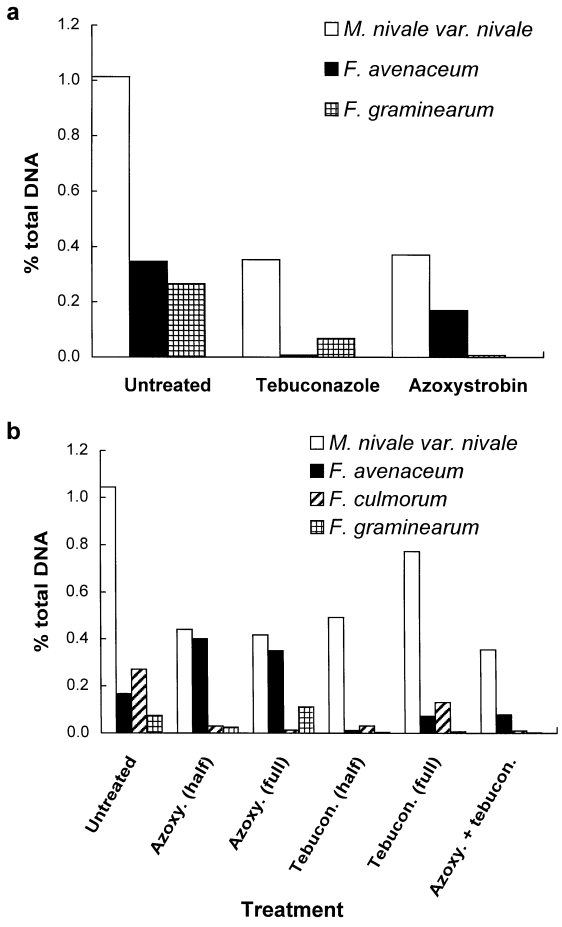

Figure 1. Amount of pathogen DNA (as % of total DNA extracted), detected in grain harvested from field trails of wheat, grown in (a) Spalding and (b) Wisbech and treated with fungicides at full or half the recommended application rate.

(0.01 ppm) and one from the azoxystrobin treated plots (0.02 ppm).

In the field trial from Wisbech 15% of untreated ears showed symptoms of disease. This was reduced

Table 3. Mean and standard error for visual disease scores (percentage ear affected) and thousand grain weight (g) from artificially inoculated field trials. Values in each column with the same superscript are not significantly different ($P > 0.05$)

Application	Ground level inoculum (GS 31)		Ear inoculum (GS 65)	
	Visual score	Yield	Visual score	Yield
Azoxystrobin	1.4 ± 0.6^{ab}	54.5 ± 0.7^{bc}	15.2 ± 7.1^{a}	53.1 ± 0.2^{abc}
Tebuconazole	3.6 ± 1.0^{ab}	52.9 ± 0.1^{ab}	4.1 ± 2.4^{a}	49.8 ± 1.4^{a}
Fluquinconazole	4.8 ± 1.5^{b}	55.7 ± 0.9^{bcd}	14.9 ± 3.5^{a}	51.5 ± 1.9^{ab}
Fluquinconazole + Prochloraz	2.5 ± 0.9^{ab}	54.7 ± 1.2^{bc}	5.5 ± 2.8^{a}	54.42 ± 1.3^{bc}
Azoxystrobin + Fluquinconazole	2.0 ± 0.2^{ab}	57.3 ± 0.8^{cd}	7.2 ± 3.9^{a}	55.1 ± 1.1^{bc}
Azoxystrobin + Fluquinconazole + Prochloraz	0.7 ± 0.4^{a}	56.5 ± 0.4^{cd}	5.6 ± 3.0^{a}	55.3 ± 1.8^{b}
Azoxystrobin + Prochloraz	1.7 ± 0.7^{a}	58.4 ± 0.5^{d}	6.1 ± 4.6^{a}	53.4 ± 0.3^{abc}
Untreated	3.8 ± 2.0^{ab}	50.9 ± 1.9^{a}	13.4 ± 4.1^{a}	52.0 ± 0.9^{abc}

to 4–5% in samples treated with azoxystrobin, 7–8% in the plots treated with tebuconazole and 4% in plots treated with both fungicides together. The untreated plots contained moderate levels of *M. nivale* var. *nivale* and low levels of *F. avenaceum, F. culmorum* and *F. graminearum*. *M. nivale* was reduced by all fungicide treatments ($P < 0.01$), with the exception of the full rate tebuconazole treatment (Figure 1b). No treatment gave significant control of *F. avenaceum* or *F. culmorum*, relative to the untreated control. *F. graminearum* was controlled by all treatments containing tebuconazole ($P < 0.01$). Low levels of DON toxin were detected in the samples from this trial (ranging from 0.0 to 0.4 ppm). The highest value for DON contamination (0.4 ppm) was in one of the untreated control plots. DON was present in 5 of the 6 samples treated with azoxystrobin (mean 0.11 ppm) and only one of the six samples treated with tebuconazole (mean 0.03 ppm). Low levels of DON were also detected in two of the three samples treated with tebuconazole and azoxystrobin together, however these values were not statistically different ($P > 0.05$).

Field trial (artificial inoculum at ground level, GS 31)

Relatively low visual disease levels were recorded and are shown in Table 3. The values ranged from 0.72% ear affected (azoxystrobin plus fluquinconazole plus prochloraz treated ears) to 4.8% (fluquinconazole treated plots), with the untreated ears at 3.85% affected. Samples treated with azoxystrobin, alone (1.35%) or mixed (0.72–1.97%), had a lower visual disease score than the other treatments, but this observation was not statistically significant. All treatments, with the exception of tebuconazole, gave a significant yield benefit (Table 3). The untreated plots contained *F. culmorum* (0.60% of total DNA), *F. avenaceum* (0.12%) and *M. nivale* var. *majus* (0.20%) (Figure 2). Treatment with azoxystrobin significantly reduced *M. nivale* var. *majus* ($P < 0.05$), as did all treatments containing azoxystrobin mixed with another compound ($P < 0.01$) (Figure 2). Treatments with azoxystrobin and azoxystrobin with fluquinconazole resulted in an increased amount of *F. avenaceum* and *F. culmorum* relative to the untreated ears, but these results were not statistically significant ($P > 0.05$). No DON or ZEA was detected in samples from the ground level inoculated plots.

Field trial (artificial inoculation to ear, GS 65)

Direct inoculation to the ear facilitated the development of higher visual disease scores than in the ground level inoculum trial. Visual scores (given in Table 3) ranged from $4.1\% \pm 2.4$ (mean and standard error) ear affected in the tebuconazole treated ears, to $15.55\% \pm 7.1$ of the azoxystrobin treated ears, with the untreated control having $13.35\% \pm 2.4$ of each ear affected. However, there was a high level of variability in the data and none of the fungicide treatments gave a significant reduction of the visual symptoms recorded. No treatment

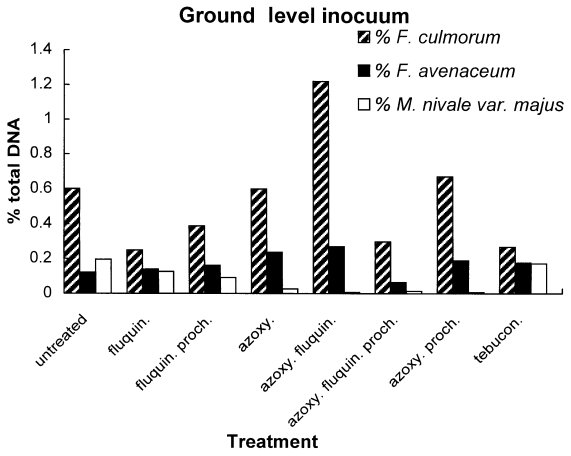

Figure 2. Amount of pathogen DNA (as % of total DNA extracted), detected in grain harvested from field trials of wheat, grown in Sand Hutton, York, inoculated with infected grain spread on the ground at GS 31 and treated with fungicides.

showed a significant effect on yield over the untreated plots (Table 3). Levels of fungal DNA were also higher than in ground inoculated plots, with *F. culmorum* as the major pathogen (4.16% of total extracted DNA) (Figure 3A). *F. avenaceum* and *M. nivale* var. *majus* were also present on the untreated ears at low levels (Figure 3B). Tebuconazole provided the most effective control of *F. culmorum* ($P < 0.01$) although fluquinconazole with prochloraz (2.05%) and azoxystrobin with fluquinconazole and prochloraz also gave a significant reduction of *F. culmorum* ($P < 0.05$) (Figure 3A). Treatment with azoxystrobin alone did not control *F. culmorum* ($P > 0.10$) nor when mixed with either prochloraz ($P > 0.10$) or fluquinconazole ($P > 0.05$). No treatment significantly controlled *F. avenaceum* ($P > 0.05$), levels of which were increased by treatment with fluquinconazole or azoxystrobin ($P < 0.10$) compared to the untreated control. *M. nivale* var. *majus* was significantly reduced by treatment with azoxystrobin ($P < 0.01$), azoxystrobin with fluquinconazole ($P < 0.01$) and by azoxystrobin with prochloraz ($P < 0.05$), but was not reduced by treatment with tebuconazole ($P > 0.20$).

The mean level of DON in the untreated control was 3.13 ppm (Figure 3A). This was significantly reduced in the plots treated with tebuconazole (1.38 ppm, $P < 0.05$) and was lowest in the plots treated with azoxystrobin plus fluquinconazole and prochloraz (0.32 ppm, $P < 0.01$). In contrast, the plots treated with fluquinconazole or with azoxystrobin

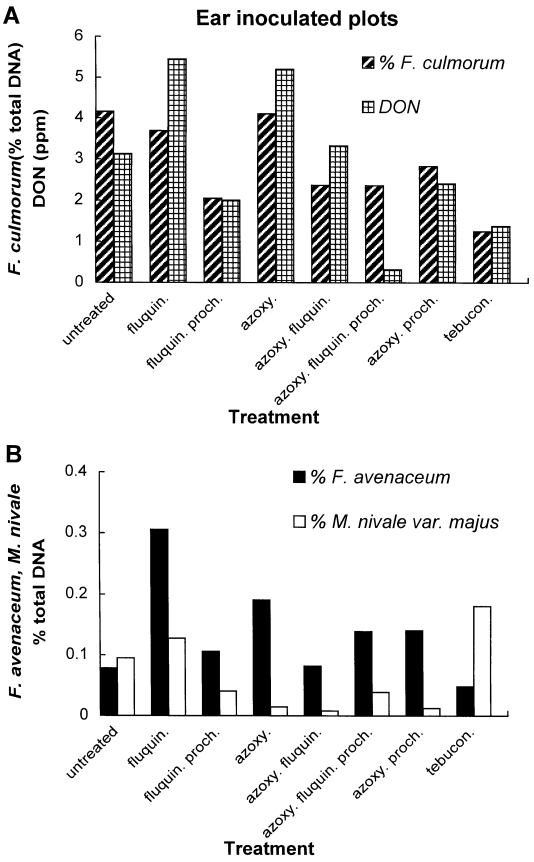

Figure 3. Amount of pathogen DNA (as % of total DNA extracted), and DON mycotoxin (ppm) detected in grain harvested from field trials of wheat, grown in Sand Hutton, York, inoculated by direct spray of conidial suspension at GS 65 and treated with fungicides.

alone contained significantly more DON ($P < 0.05$) than the untreated control (5.45 ppm and 5.20 ppm, respectively) (Figure 3A). This increase was despite there being no significant increase in the amount of *F. culmorum* in these samples (Figure 3A). No ZEA was detected in samples from this trial.

The relative effects of azoxystrobin and tebuconazole fungicide applications were compared across all field trials by relating the amount of each pathogen present in treated plots to that in the untreated control from each trial. Figure 4 shows the effect of these treatments on levels of the non-toxin producing *Microdochium* species and the toxigenic Fusaria over the four trials reported. Tebuconazole treatment gave significant control of *Fusarium* ($P < 0.001$) but had no significant effect on levels of *Microdochium*

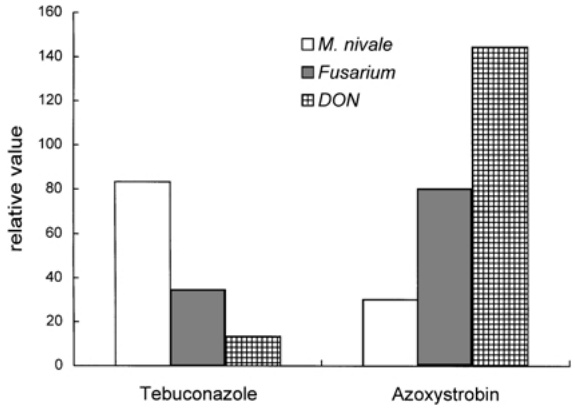

Figure 4. Amount of all *Microdochium nivale* and *Fusarium* spp. and the level of DON mycotoxin detected in the samples treated with tebuconazole and azoxystrobin fungicides (applied at full recommended rates), across four field trials relative to the untreated control in each trial. (Untreated control = 100)

($P > 0.20$). In contrast, the azoxystrobin treatments effectively controlled *Microdochium* ($P < 0.01$) but did not significantly reduce levels of *Fusarium* ($P > 0.20$). The values for DON were not significantly different, since DON was not detected in all trials.

Discussion

The study of population changes within a disease complex requires the development of assays that can specifically detect and quantify the pathogens, many of which may be closely related. The recently developed specific quantitative PCR assays for the most prevalent plant pathogens involved in head blight (Nicholson et al., 1996; 1998; Parry and Nicholson 1996; Turner et al., 1998) are invaluable for these studies. Because these assays permit the specific quantification of different disease components within a mixed pathogen population it is possible to study how a fungicide application affects the disease complex in the field situation. Without such assays this research could not be achieved.

Analysis of the field plots from Morley Research Station in 1997 (Table 2) showed that a change had occurred in the pathogen population on wheat ears, following the application of azoxystrobin. *Microdochium nivale* was selectively controlled while *Fusarium* spp., absent or at low levels on the untreated ears, were markedly increased following azoxystrobin treatment.

The naturally inoculated field trials from 1998 showed the same general pattern of population change following fungicide treatment (Figure 1). Treatment with azoxystrobin tended to reduce *M. nivale* while leaving the toxigenic Fusaria, predominantly *F. avenaceum*, largely unaffected. In contrast, tebuconazole generally reduced *Fusarium* more than it did *Microdochium.* This change in population was previously noted by Faure and Declercq (1999), in wheat inoculated with *Fusarium* spp. and *Microdochium nivale* and treated with fungicides. On analysis with PCR-based assays and from culturing isolates from samples, they found that treatment with tebuconazole (applied as Horizon) resulted in high *Microdochium* and low *Fusarium* levels. Grain from azoxystrobin-treated ears (applied as Amistar), contained high levels of *Fusarium* and only low or moderate *Microdochium*, as did the untreated control.

The distribution of pathogens detected in naturally inoculated trials reported here were consistent with the findings of a UK-wide survey in 1998 by the HGCA (Turner et al., 1999). From 53 crops surveyed, they reported a predominance of *M. nivale* (present in 94% of samples) with *F. avenaceum*, *F. graminearum*, *F. poae* and *F. culmorum* affecting 43, 40, 34 and 21% of samples, respectively. The data from uninoculated trials reported here were highly variable, and statistically significant results were rarely observed because of the generally low level of disease present.

Artificial inoculation (Figures 2 and 3) led to a greater predominance of *F. culmorum* over *M. nivale*, which was predominant in the naturally inoculated trials (Figure 1). The same artificial inoculum applied at two different growth stages gave rise to quite different proportions of the pathogens contaminating the grain in each case. Infection from the ground level inoculum, applied early in the season (GS 31), was still reliant on natural transmission of the pathogens to the ear. This gave some variability in the levels of disease, and did not result in detectable levels of DON mycotoxin. *M. nivale* is homothallic, and therefore may be more able to produce air dispersed ascospores (Parry et al., 1995b), rather than splash dispersed asexual conidia as is the case with *F. culmorum*. However, this should not have been a major factor since the inoculum contained a mixture of isolates. *M. nivale* has a lower optimal temperature for growth than *F. culmorum* (Nakajima and Abe, 1990; Parry et al., 1995a). Hence in the trial reported here inoculated at GS 31 with infected grain applied at ground level, *M. nivale* may have been able to colonise the developing ear earlier in the season than the Fusaria. Direct spraying of the pathogens onto the

ear at mid-anthesis provided conditions better suited for the rapid colonisation of the true Fusaria, and there was a greater predominance of *F. culmorum* in the GS 65 inoculated trial (Figure 3).

Following inoculation at mid-anthesis, the least visual disease was observed in plots treated with tebuconazole, but no fungicide reduced the visual disease score significantly ($P > 0.05$). This result contrasts with the visual disease observations in the field trial inoculated at GS 31 where azoxystrobin treatments were the most effective. It was noted, however, that the former was more predominantly affected by *Fusarium* spp. than *M. nivale*, and tebuconazole has been shown to be more effective against *Fusarium* than *Microdochium* (Mauler-Machnik and Suty 1997; Mesterházy and Bartók, 1997; Faure and Declercq, 1999).

In the artificially inoculated trials fluquinconazole plus prochloraz, azoxystrobin plus fluquinconazole and prochloraz, and azoxystrobin plus prochloraz were moderately effective against *Fusarium*, measured by the presence of pathogen DNA. This can reasonably be attributed to the inclusion of prochloraz in these formulations, since prochloraz has been demonstrated to be effective against *Fusarium* (Hutcheon and Jordan, 1992; Doohan et al., 1999; Matthies and Buchenauer, 2000).

The detection of DON in grain samples from the uninoculated field trials, although at low levels, was of some concern. The highest levels of DON were generally detected in the azoxystrobin treated plots, despite only small amounts of *F. culmorum* or *F. graminearum* being present. The mycotoxin levels detected in the trial inoculated at mid-anthesis also indicated an increase in DON following application of fluquinconazole or azoxystrobin, despite there being no significant increase of *F. culmorum* (Figure 3). An increase in the level of DON per unit of fungus in the azoxystrobin treated ears, compared with the tebuconazole treated ears indicates that in some cases this fungicide treatment can have a stimulatory effect on the production of DON from *F. culmorum*. Increased production of mycotoxin following fungicide application has been noted previously in field trials by Milus and Parsons (1994), who reported that several fungicides, including tebuconazole, failed to control head blight symptoms and some treatments gave a 50% increase in DON contamination. A similar field trial by Gareis and Ceynowa (1994) noted that tebuconazole treatment did control the symptoms of head blight but led, in some cases, to an increase in the amount of NIV mycotoxin detected by up to 16 fold. However, this was based on a comparison of toxin levels detected in a single, inoculated, treated sample with very low levels of toxin detected in a single, inoculated, untreated control sample. This effect of tebuconazole on either DON or NIV production has not been reported elsewhere. Studies *in vitro* (Matthies et al., 1999) have shown that the fungicide dose applied may influence production or accumulation of mycotoxins. Fungicides, including tebuconazole and fluquinconazole were shown to reduce both the growth and mycotoxin production in *F. graminearum* when applied at high concentration, but at sub-lethal levels these fungicides enhanced mycotoxin production. Matthies and Buchenauer (2000) did not, however, note this effect in the field, and there is no evidence of tebuconazole treatment causing a stimulation of DON production in the present trials.

The wheat varieties used in the studies reported here are all susceptible to FHB. It is possible that a variety with resistance to the disease would not show increased disease and toxin levels. However, the majority of commonly grown wheat varieties do not have good resistance to FHB (McMullen et al., 1997; Mesterházy et al., 1999).

The four field trials reported here are not replicates since they differ greatly in design and treatments. However, all four studies show a general agreement of the effects of tebuconazole and azoxystrobin on the FHB population and on DON levels. This gives an indication that the differential control of *Fusarium* and *M. nivale* is reproducible over a range of conditions. Overall the field trials presented here show that application of the strobilurin fungicide azoxystrobin can control the establishment of non-toxigenic FHB pathogens *M. nivale* var. *majus* and var. *nivale,* but allows their replacement with toxigenic *Fusarium* species (Figure 4). The fungicide appears to provide little or no control of *F. culmorum* or *F. avenaceum,* and may stimulate production of DON mycotoxin by the former, leading to more DON per unit of fungus. Toxin production by *F. avenaceum* was not measured in this study, but this species is known to produce enniatin B (Herrmann et al., 1996) and moniliformin (Golinski et al., 1996) which may also have adverse effects on the health of human and animal consumers (Langseth et al., 1998). Concern over the use of strobilurin fungicides such as azoxystrobin, and in particular the possible effect on *Fusarium* colonisation and mycotoxin production, was highlighted in a recent report by the

European Commission Scientific Committee on Plants (Hardy et al., 1999). This report indicated the need for further data to be published from the results of field studies.

Differential control of head blight pathogens by fungicides was consistently repeated here in field trials with natural and artificial inoculation, and with high and low disease levels. By creating this change in pathogen population, and by potentially stimulating the production of mycotoxins, the application of azoxystrobin fungicide where *Microdochium nivale* and *Fusarium* species are both present, greatly increases the risk of subsequent mycotoxin contamination of the grain of susceptible wheat varieties.

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