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Studies on the Interactions between Fungicides, *Alternaria tenuissima, Cladosporium herbarum* and *Microdochium* spp., on Fusarium Head Blight (FHB) Development and Deoxynivalenol (DON) Concentration in Grain Caused by *Fusarium culmorum*

S.R. PIRGOZLIEV[1], R.V. RAY[2]*, S.G. EDWARDS[3], M.C. HARE[3] and P. JENKINSON[4]

[1]Monsanto Europe N.V, Brussels Office, Avenue de Tervuren 270-272, 1150, Brussels, Belgium
[2]Plant and Crop Sciences Division, School of Biosciences, The University of Nottingham, Sutton Bonington, Loughborough, LE12 5RD, UK
[3]Crop and Environment Research Centre, Harper Adams University College, Newport, Shropshire TF10 8NB, UK
[4]Sellafield Ltd, Seascale, Cumbria, CA20 1PG, UK

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Saprophytic microflora and non-toxin producing *Microdochium* spp. capable of causing Fusarium head blight (FHB) have been suggested to affect the development of FHB caused by *Fusarium* spp., the occurrence of mycotoxins and the efficacy of fungicides for the control of the disease. The effects of metconazole and azoxystrobin on the interactions between *Fusarium culmorum* and *Microdochium* spp., *Alternaria tenuissima* or *Cladosporium herbarum* on FHB symptom development, *Tri5* DNA concentration and deoxynivalenol (DON) production were studied under glasshouse conditions. Results indicated that the sequence of infection of wheat heads and the relative timing of fungicide application can significantly affect FHB severity and the resulting mycotoxin contamination of harvested grain. Introduction of *A. tenuissima, C. herbarum* or *Microdochium* spp. to wheat heads at GS 57 before inoculation with *F. culmorum* at GS 65 generally resulted in increased FHB severity, *Tri5* DNA and DON concentration in harvested grain. The greatest increases of FHB severity (266%), *Tri5* DNA (79%) and DON (152%) were observed when *Microdochium* spp. were introduced first at GS 57 and *F. culmorum* inoculation followed at GS 65. Metconazole generally reduced FHB severity, *Tri*5 DNA and DON concentration in grain but azoxystrobin was most efficient at reducing DNA of *Microdochium* spp. in grain.

Keywords: FHB, DON, qPCR, fungal interactions, wheat

Introduction

Fusarium head blight (FHB) also known as scab, is a damaging disease of yield and quality of small grain cereals caused by six major species, *Fusarium avenaceum, F. culmorum,*

* Corresponding author; E-mail: Rumiana.Ray@nottingham.ac.uk;
Phone: (+44) 115 95 16327; Fax: (+44) 115 95 16334

F. graminearum, F. poae, Microdochium nivale and *M. majus* (Parry et al. 1995). *Fusarium* spp. are also able to produce several harmful to animals and humans mycotoxins belonging to the trichothecene type A (T-2 and HT-2 toxins) or type B (deoxynivalenol, DON) (Krska et al. 2001).

Chemical control of FHB and subsequent reduction of mycotoxins have been reported frequently in the published literature as being inconsistent (Milus and Parsons 1994; Gareis and Ceynova 1994; Matthies and Buchenauer 2000; Pirgozliev et al. 2002, 2008). Fungicide performance against FHB and mycotoxin accumulation in grain are affected by fungicide formulation, time of application and differences in sensitivity of the fungal population present on the wheat heads to specific chemical treatments. Previous observations have indicated that applications of azoxystrobin effective against *Microdochium* spp. but not against *F. culmorum* resulted in increased DON concentrations in harvested wheat grain (Jennings et al. 2000; Simpson et al. 2001). Other researchers later demonstrated during *in vitro* work, that there were differences in sensitivity to triazoles (prothioconazole and tebuconazole) and to strobilurins (azoxystrobin and fluoxastrobin) among *Fusarium* spp., *Microdochium* spp. and head-colonising saprophytes (Müllenborn et al. 2008).

The presence of saprophytic fungi such as *Alternaria* spp., *Botrytis* spp., *Cladosporium* spp. or the non-toxin producing *Microdochium* spp. which also infect cereal heads can influence FHB development and the mycotoxin accumulation in grain. In a range of glasshouse studies where wheat plants were inoculated with either, *A. alternata, B. cinerea* or *C. herbarum* at full head emergence (GS 59; Zadoks et al. 1974) followed by inoculation with *F. culmorum* at mid-flowering (GS 65), all saprophytes reduced FHB severity by between 46% and 78% compared with plants inoculated only with *F. culmorum* (Liggitt et al. 1997). To further elucidate the interactions between fungicides, certain saprophytes and *Microdochium* spp. on FHB development and mycotoxin production by *F. culmorum,* a series of artificially inoculated glasshouse studies were performed.

The aims of this study were (i) to evaluate the role of *A. tenuissima, C. herbarum* and *Microdochium* spp. in FHB development and DON production in wheat, (ii) to evaluate the performance of azoxystrobin and metconazole against FHB and DON production caused by *F. culmorum* in the presence of *A. tenuissima, C. herbarum* and *Microdochium* spp.

Materials and Methods

Fungicide treated seed (fludioxinil at 25 g. a.i. per 100 kg of seed, Beret Gold®, Syngenta Ltd) of the winter wheat cv. Cadenza was sown into 15 cm diameter plastic pots containing John Innes Number 2 compost at a rate of five seeds per pot. Following vernalisation, plants were grown in a glasshouse set at 22 ± 3ºC under a photoperiod of 16 h. Plants were watered daily and fed once a week with an application of foliar fertilizer (10% N, 10% P_2O_5, 27% K_2O as Phostrogen®, Phostrogen Ltd). Plants were sprayed with 0.3 l ha^{-1} Fortress® (500 g a.i. quinoxifen per litre, Dow AgroSciences) to prevent infection by powdery

mildew. Nicotine shreds (Nicotine 40% Shreds®, Dow AgroSciences) were also used according to the manufacturer's recommendation to eradicate aphid infestations.

Four *F. culmorum,* two *A. tenuisima* and *C. herbarum* and three *M. majus* and *M. nivale* isolates were sub-cultured by taking 5 mm diameter plugs of inoculum from the edges of actively growing cultures using a sterile cork borer and transferring them onto plates of Potato Dextrose Agar. Petri dishes then were incubated under darkness at 20 ± 2ºC for 14 days. In order to induce sporulation, 14-day-old cultures were then placed in an incubator under continuous near-UV light for a further 7–14 days at 20 ± 2ºC. Conidial suspensions were obtained by washing conidia from sporulating colonies using sterile distilled water. Spore concentration was determined using a haemocytometer and adjusted to the required concentration.

Artificial inoculation was achieved by spraying prepared conidial suspension onto heads using a hand-held atomizer until run-off. Metconazole (Caramba® BASF Plc, UK) and azoxystrobin (Amistar® Syngenta Ltd, UK) were applied at field rates of 1.5 (60 g a.i. l^{-1}) l ha^{-1} and 1(250 g a.i. l^{-1}) l ha^{-1} , respectively, using a precision pot sprayer carrying Lurmark 110º flat fan nozzles (03-F110, UK) with rate of 200 l ha^{-1}. Inoculation of all wheat heads with a conidial suspension of *F. culmorum* (150,000 spores per ml of water) was carried out at GS 65. Twenty-one pots were artificially inoculated with a conidial suspension of *A. tenuissima*, *C. herbarum* or *Microdochium* spp. (each at 150,000 spores per ml of water) at GS 57. Of these, seven pots received an application of metconazole at GS 59 (approx. 48 hours following inoculation) whilst another seven pots received an application of azoxystrobin at GS 59 (approx. 48 hours following inoculation). The remaining seven pots received no fungicide treatment. A further 21 pots were also artificially inoculated with *A. tenuissima*, *C. herbarum* or *Microdochium* spp. 24 hours after inoculation with *F. culmorum*. Of these, seven were sprayed with metconazole and seven were sprayed with azoxystrobin at GS 59 whilst the remaining seven pots were left unsprayed. Additionally, seven pots were treated with metconazole, seven with azoxystrobin at GS 59 and seven untreated and inoculated only with *F. culmorum* at GS 65. In all cases, heads were covered with clear polythene bags for 24 hours following inoculation to provide conditions conducive to FHB development.

At GS 83, all heads were assessed for the severity of FHB symptoms. The total number of spikelets and the number showing necrosis or bleaching were recorded to give the percentage of infected spikelets. When ripe (GS 92), wheat heads were harvested and carefully hand threshed in order to avoid loss of any small shrivelled grain. Trichothecene producing *Fusarium* spp. and *Microdochium* spp. present in harvested grain were quantified using competitive PCR assays (Edwards et al. 2001; Glynn et al. 2007). DON concentration in grain was determined using a Ridascreen®DON Fast immunoassay (R-Biopharm Rhône Ltd, UK).

Analysis of variance was performed on all data using Genstat 5 (Release 4.1 (PC/Windows NT), Lawes Agricultural Trust, UK). Data were transformed to give a normal distribution of residuals where necessary.

Results

Analysis of variance showed that there were no significant interactions between application of fungicide and *A. tenuissima* for either FHB severity or DON. Metconazole significantly reduced FHB severity and DON in harvested grain compared with the untreated and azoxystrobin treated plants (Table 1). When *A. tenuissima* was introduced on wheat heads (GS 57) before the inoculation with *F. culmorum* (GS 65), there was a significant increase of FHB severity and DON in comparison with the *F. culmorum* alone inoculated plants or when *A. tenuissima* was introduced to wheat heads 24 hours following *F. culmorum*. There was a significant interaction between fungicide and time of introduction of *A. tenuissima* for *Tri5* DNA quantified in the grain. This dataset mirrored the other two except that azoxystrobin treatment and a late application of *A. tenuissima* resulted in a significant decrease in *Tri5* DNA concentration compared with a treatment of azoxystrobin alone or a late application of *A. tenuissima* alone.

There were significant interactions between fungicide treatment and application of *C. herbarum* for FHB severity and DON concentration in harvested grain (Table 2). These resulted from the combined effects of azoxystrobin treatment and a late application of *C. herbarum*. In fungicide untreated plants, the late introduction of *C. herbarum* significantly decreased FHB severity and DON by 50% and 33%, respectively, but this effect was not observed in the presence of an azoxystrobin treatment. Moreover, the main effects

Table 1. Effect of interactions between *A. tenuissima* and *F. culmorum* and metconazole or azoxystrobin on the severity of FHB, *Tri5* DNA and DON. Numbers in parentheses are back-transformed means

Time of inoculation or fungicide application				Arcsine % spikelets infected	Log_{10} *Tri5* DNA pg ng^{-1} of total DNA	DON mg kg^{-1}
GS 57	GS 59	GS 65	GS 65+			
		*F. c.***		25.22 (18.15)	1.52 (33.1)	56.6
	azoxystrobin	*F. c.*		25.98 (19.18)	1.73 (53.7)	49.3
	metconazole	*F. c.*		6.68 (1.35)	0.63 (4.2)	6.7
*A. t.**		*F. c.*		31.60 (27.45)	2.09 (123.0)	72.7
A. t.	azoxystrobin	*F. c.*		31.62 (27.48)	1.91 (81.2)	48.4
A. t.	metconazole	*F. c.*		16.64 (8.20)	0.96 (9.1)	30.0
		F. c.	*A. t.*	18.72 (10.30)	1.38 (23.9)	59.9
	azoxystrobin	*F. c.*	*A. t.*	20.66 (12.44)	0.74 (5.4)	35.8
	metconazole	*F. c.*	*A. t.*	7.57 (1.73)	0.87 (7.4)	6.90
P				<0.001	<0.001	<0.001
LSD Fungicide				6.46	0.43	27.1
CV				31.7	33.9	65.2
P				<0.001	<0.001	>0.05
LSD Saprophyte				6.09	0.40	25.5
CV				31.7	33.9	65.2
P				>0.05	<0.01	>0.05
LSD Fungicide *Saprophyte				7.45	0.49	31.3
CV				31.7	33.9	65.2

* *A. tenuissima*, ** *F. culmorum*

were highly significant and the same as for *A. tenuissima*. Thus, an application of metconazole resulted in lower FHB severity and DON in harvested grain compared with the untreated control and the azoxystrobin treated plants, and the early application of *C. herbarum* significantly increased FHB severity and DON in harvested grain. Application of metconazole resulted in a significant decrease of 58% of *Tri*5 DNA compared to untreated and azoxystrobin treated plants. Plants that received an application of *C. herbarum* either before or after the application of *F. culmorum* also had significantly lower *Tri5* DNA.

There were significant interactions between fungicide and time of inoculation with *Microdochium* spp. for FHB severity and DON concentration (Table 3). However, the main effects were highly significant and trends were similar for all three parameters measured. Metconazole significantly reduced FHB severity, *Tri5* DNA concentration and DON concentration by more than 50% compared with untreated and azoxystrobin treated plants. Early application of *Microdochium* spp. had the opposite effect, resulting in a 266% increase in FHB severity, a 152% increase in *Tri5* DNA concentration and a 79% increase in DON concentration compared with fungicide untreated plants or plants with later *Microdochium* inoculation. Observed interactions were related to increases in measured parameters when an early application of azoxystrobin was followed by inoculation with *Microdochium* spp.

Table 2. Effect of interactions between *Cladosporium herbarum* and *F. culmorum* and application of metconazole or azoxystrobin on the severity of FHB, *Tri5* DNA and DON. Numbers in parentheses are back-transformed means

Time of inoculation or fungicide application				Arcsine % spikelets infected	Log_{10} *Tri5* DNA pg ng^{-1} of total DNA	DON mg kg^{-1}
GS 57	GS 59	GS 65	GS 65+			
		*F. c.***		25.22 (18.15)	1.52 (33.11)	56.6
	azoxystrobin	*F. c.*		25.98 (19.18)	1.73 (53.70)	49.3
	metconazole	*F. c.*		6.68 (1.35)	0.63 (4.26)	6.7
*C. h.**		*F. c.*		32.65 (29.10)	1.24 (17.37)	76.1
C. h.	azoxystrobin	*F. c.*		26.55 (19.97)	1.03 (10.71)	56.3
C. h.	metconazole	*F. c.*		10.79 (3.50)	0.50 (3.16)	15.2
		F. c.	*C. h.*	17.62 (9.16)	0.77 (5.88)	38.2
	azoxystrobin	*F. c.*	*C. h.*	22.42 (14.54)	0.89 (7.76)	40.8
	metconazole	*F. c.*	*C. h.*	7.29 (1.61)	0.51 (3.23)	9.30
P				0.001	<0.001	0.001
LSD Fungicide				5.15	0.30	15.43
CV				26.9	34.5	39.3
P				0.001	<0.05	<0.05
LSD Saprophyte				4.85	0.28	14.55
CV				26.9	34.5	39.3
P				<0.05	>0.05	<0.05
LSD Fungicide *Saprophyte				5.94	0.34	17.82
CV				26.9	34.5	39.3

* *C. herbarum*, ** *F. culmorum*

Table 3. Effect of interactions between *Microdochium* spp. and *F. culmorum* and application of metconazole or azoxystrobin on the severity of FHB, *Tri5* DNA and DON. Numbers in parentheses are back-transformed means

Time of inoculation or fungicide application				Arcsine % spikelets infected	Log_{10} *Tri5* DNA pg ng^{-1} of total DNA	DON mg kg^{-1}
GS 57	GS 59	GS 65	GS 65+			
		*F. c.***		18.7 (10.27)	0.53 (3.38)	16.2
	azoxystrobin	*F. c.*		18.7 (10.33)	0.49 (3.09)	15.4
	metconazole	*F. c.*		12.2 (4.53)	0.26 (1.81)	7.9
*M.**		*F. c.*		37.8 (37.56)	0.95 (8.91)	40.8
M.	azoxystrobin	*F. c.*		22.9 (10.51)	0.72 (5.24)	25.4
M.	metconazole	*F. c.*		13.4 (5.37)	0.42 (2.63)	12.3
		F. c.	*M.*	14.6 (6.35)	0.45 (2.81)	18.1
	azoxystrobin	*F. c.*	*M.*	18.9 (10.49)	0.48 (3.01)	21.3
	metconazole	*F. c.*	*M.*	8.90 (2.39)	0.14 (1.38)	3.20
P				<0.001	<0.01	<0.001
LSD Fungicide				6.65	0.20	8.0
CV				33.6	38.1	40.2
P				<0.001	<0.01	<0.05
LSD *Microdochium*				6.27	0.19	7.5
CV				33.6	38.1	40.2
P				<0.01	>0.05	<0.05
LSD Fungicide * Microdochium				7.68	0.23	9.2
CV				33.6	38.1	40.2

* *Microdochium* spp., ** *F. culmorum*

Table 4. Effect of interactions between *Microdochium* spp. and *F. culmorum* and application of metconazole or azoxystrobin on DNA of *Microdochium* spp. in grain. Numbers in parentheses are back-transformed means

Time of inoculation or fungicide application				Log_{10}
GS 57	GS 59	GS 65	GS 65+	*Microdochium* spp. DNA
	azoxystrobin	*F. c.***		0.26 (1.81)
	metconazole	*F. c.*		0.50 (3.16)
*M.**		*F. c.*		1.32 (20.89)
M.	azoxystrobin	*F. c.*		0.22 (1.65)
M.	metconazole	*F. c.*		1.30 (19.95)
		F. c.	*M.*	0.73 (5.37)
	azoxystrobin	*F. c.*	*M.*	0.26 (1.81)
	metconazole	*F. c.*	*M.*	0.73 (5.37)
P				<0.001
LSD				0.54

* *Microdochium* spp., ** *F. culmorum*

DON concentrations quantified by ELISA were found to be within published range of DON quantified in wheat spikes by GC-MS (Peiris et al. 2011). In our laboratory, the Ridascreen®DON Fast immunoassay used in this study was previously validated against

UKAS accredited GC-MS analysis. There was a good agreement between results from both analyses and a strong positive relationship between quantified data ($R^2 = 0.8$).

Quantification of DNA of *Microdochium* spp. in grain showed that there was significantly more of this species when plants were inoculated early with *Microdochium* spp. at GS 57 and left untreated or treated with metconazole following inoculation (Table 4). Azoxystrobin was most efficient at reducing *Microdochium* spp. in grain.

Discussion

This study has demonstrated that early colonisation of wheat heads with *A. tenuissima, C. herbarum* or *Microdochium* spp. at GS 57 before infection with *F. culmorum* resulted in an increased FHB severity and greater DON concentration in harvested grain. Under natural conditions, conidia of *A. tenuissima* or *C. herbarum* are known to infect wheat heads post GS 75 once wheat heads have began to ripen (Clark et al. 2008). Inoculations with *A. tenuissima* and *Microdochium* spp. resulted in the increase of *Tri5* DNA indicating that FHB severity and DON were due to biomass accumulation of *F. culmorum*. In contrast, whilst *C. herbarum* inoculation also resulted in greater FHB severity and DON concentration in harvested grain, there was no change in *Tri5* DNA indicating that metabolic activity rather than increase in fungal biomass was responsible for elevated disease severity and mycotoxin concentration.

DON production by *Fusarium* spp. has been shown to play an important role in the virulence of these species (Bai et al. 2001) and it has been suggested that toxigenic fungi may produce more toxins under stress, i.e. competition for resources (Xu et al. 2007a). This is also in agreement with further evidence of competition between isolates of different *Fusarium* species provided from a series of experiments conducted to investigate FHB development, fungal growth and mycotoxin accumulation in wheat heads following inoculation with one or more toxigenic *Fusarium* pathogens (Xu et al. 2007b). In these experiments, mycotoxin production increased dramatically in the co-inoculations (1000-fold) compared with single species inoculations. Results from our study demonstrated that an interaction also exists between *F. culmorum* and *Microdochium* spp. since the introduction of *Microdochium* spp. prior to the artificial inoculation of heads with *F. culmorum* increased *Tri*5 DNA by 79% and DON content by 152%. It could be suggested therefore that where *Microdochium* spp. were already established on wheat heads and infection with *F. culmorum* occurred later, due to competition for space and resource with the species already present, *F. culmorum* produced more DON. The corresponding increase of FHB symptoms by 266% in this inoculation combination was probably due to symptoms caused by *Microdochium* spp. in addition to *F. culmorum* or due to the elevated DON concentration.

In general, metconazole treatment resulted in a significant decrease in FHB severity and DON concentration in harvested grain compared with the no fungicide control and azoxystrobin treated plants. Azoxystrobin applications to control FHB caused by mixed species inoculum including *Microdochium* and *Fusarium* have been previously associated with an elevated DON concentrations in grain, possibly due to the elimination of the com-

petition between *Fusarium* and *Microdochium* or other saprophytic species on wheat heads (Jennings et al. 2000; Simpson et al. 2001). Results from our study partially support this, since an increase in FHB development and DON concentration in grain was observed where azoxystrobin was applied after the introduction of *Microdochium* spp. to wheat heads at GS 57. Furthermore, the azoxystrobin treatment reduced *Microdochium* DNA most, possibly facilitating increased biomass and production of DON by *F. culmorum*. Azoxystrobin has also been shown to suppress *C. herbarum* growth on wheat leaves (Bertelsen et al. 2001). In our study, higher DON concentrations were measured where applications of azoxystrobin before or after introduction of *C. herbarum* or *A. tenuissima* were made, however, these increases were not statistically different at the 5% significance level. In contrast, metconazole was consistently effective in reducing FHB symptoms, *Tri*5 DNA and DON concentration in grain where *C. herbarum* or *A. tenuissima* were used. The fungicide was less efficacious where *Microdochium* spp. were included and was applied as curative spray after the introduction of *Microdochium* spp.

Both *A. tenuissima* and *C. herbarum* increased FHB symptoms development when introduced at GS 57 before inoculation with *F. culmorum* at GS 65 by more than 20%. This is contrary to previous work showing 63% and 78% reductions in FHB severity, respectively, when *C. herbarum* and *A. alternata* were introduced onto wheat heads at GS 59, before the introduction of *F. culmorum* at GS 65 (Liggitt et al. 1997). More recent study has failed to show any significant effects of inhibition of mycelial growth of several *Fusarium* spp. including *F. culmorum* by *A. alternata in vitro* (Müllenborn et al. 2008). Such discrepancies may be explained by differences in the growth rates of the species and isolates used and/or experimental conditions or inoculum loads in the separate studies.

The sequence of infections occurring on the wheat heads and the selective activity of fungicides can have a major effect on the interactions between fungal species populating the wheat head and thus influence disease control and mycotoxin accumulation in wheat grain. This effect was most pronounced where *Microdochium* spp. were present either before or after fungicide applications. Whilst this study clearly indicates that other fungi can affect FHB development and mycotoxin production by *F. culmorum* it should be made clear that the results reported here were derived from a single experimental series under a controlled environment thus further confirmation should be sought via repeated independent experimentation. Ultimately, knowledge about timing and sequence of infection of wheat heads with *Fusarium* and *Microdochium* spp. could potentially allow for a more efficient and targeted approach to FHB control.

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