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Impact of Fungicide Timing on the Composition of the *Fusarium* Head Blight Disease Complex and the Presence of Deoxynivalenol (DON) in Wheat

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1. Introduction

1.1 *Fusarium* head blight: A multi-faceted agricultural problem

Fusarium Head Blight (FHB) is one of the most important diseases in wheat, caused by a complex of up to 17 *Fusarium* species. The main causal agents of FHB in Europe are *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium avenaceum*, *Fusarium poae* and *Microdochium nivale* (Audenaert *et al.* 2009; Brennan *et al.* 2003; Leonard & Bushnell 2003; Mudge *et al.* 2006; Parry *et al.* 1995). There is extensive work on the effect of FHB on grain yields of cereals. For example, in breeding programs aiming to generate resistant cultivars, yield losses have been observed ranging from 6 up to 74% (Snijders 1990; Snijders & Perkowski 1990). Symptoms of *Fusarium* occur just after anthesis. The partly white and partly green heads are diagnostic for the disease in wheat (Figure 1C). The fungus also may infect the peduncle immediately below the head, causing a brown/purplish discoloration of the stem tissue. Additional indications of FHB infection are pink to salmon-orange spore masses of the fungus often seen on the infected spikelets and glumes. Infected kernels are shriveled, lightweight and dull grayish or pinkish. These kernels sometimes are called "tomb-stones" because of their chalky, lifeless appearance. If infection occurs late in kernel development, *Fusarium*-infected kernels may be normal in size, but have a dull appearance or a pink discoloration.

Although FHB may cause wheat yield losses, the interest in FHB is primarily fuelled by the ability of *Fusarium* species to produce mycotoxins. FHB pathogens produce a diversified spectrum of mycotoxins depending on the species (Bennett & Klich 2003). Trichothecenes, zearalenon, moniliformin and fumonisins are the most important mycotoxins produced by *Fusarium* fungi. Among the trichothecenes, deoxynivalenol (DON) is the predominant mycotoxin throughout Europe and is mainly produced by *F. graminearum*. These secondary fungal metabolites can accumulate to significant doses and as such cause a serious impediment for human and animal health. Moreover, the European concern for several *Fusarium* mycotoxins has been concretized in regulations for maximum levels for human

and animal consumption. These regulations provide an extra economic motive for farmers to prevent FHB infection and mycotoxin accumulation in small grain cereals such as wheat.

The prevention of DON and *Fusarium* in wheat is not easy, since the disease is primarily associated with weather conditions during anthesis of the crop. It is generally accepted that rainfall just before and during anthesis, which is situated in the month of June, favours the FHB pathogens and can cause serious yield losses. Conidia present on crop residues reach the ears by splashed rain droplets. A recent study by Landschoot *et al.* (2011a+b) fine-tuned the influence of weather conditions. These authors demonstrated nicely that also weather conditions during the vegetative growth of the crop in winter and spring are important parameters determining the disease incidence and DON level (Table 1). This conclusion is remarkable since infection with *Fusarium* starts in the months of June and July. A possible explanation for this remarkable findings may come from the influence of weather conditions during winter on the survival of the primary inoculums in soil, weeds and crop residues. In cold winters, survival of *Fusarium* conidia is poor, resulting in a lower primary inoculums pressure in June.

Until now, no absolute FHB resistance encoded by single dominant resistance genes has been characterized in wheat. Consequently, it is difficult to implement *Fusarium* resistance into breeding programs. Two major sources for resistance have been characterized. Type I resistance stops the pathogen at the level of penetration while type II resistance is involved the inhibition of fungal spread within the infected ear (Ban & Suenaga 2000; Singh *et al.* 1995). However, the implementation of quantitative trait loci associated with resistance into commercial wheat varieties is not for tomorrow because of economic drawbacks.

A. Month	Variable	negative	Variable	positive
November	Days with frost	-0.24	75%P Air pressure	0.47
December	Days with frost	-0.53	Average Dew point	0.68
January	90%P Air pressure	-0.28	75%P Dew point	0.69
February	Days with frost	-0.53	Median Dew point	0.74
March	Days with rainfall	-0.28	Average Dew point	0.62
April	Days with rainfall	-0.65	75%P Dew point	0.51
May	Average Air pressure	-0.58	Median wind speed	0.34
June	25%P Air pressure	-0.69	Days with RH >80%	0.66
July	75%P Temperature	-0.58	Total Rainfall	0.63
B. Month	Variable	negative	Variable	positive
November	25%P RH	-0.24	75%P Air pressure	0.23
December	Median RH	-0.19	90%P Temperature	0.33
January	Days with frost	-0.28	75%P Temperature	0.35
February	Days with frost	-0.34	75%P Temperature	0.48
March	90%P Dew point	-0.23	10%P Temperature	0.39
April	Days with rainfall	-0.31	90%P Air pressure	0.36
May	Average Air pressure	-0.32	25%P Temperature	0.34
June	Average Air pressure	-0.42	25%P RH	0.34
July	10%P Temperature	-0.31	10%P RH	0.28

Table 1. A. Highest pearson correlation coefficients for weather variables (Days with Frost, Air Pressure, Total Rainfall, Relative humidity (RH) Temperature, Dew point and wind speed) and the DI, 10%P, 25%P, 75%P, 90%P, respectively mean 10%, 25%, 75%, 90% percentiles. B. Highest pearson correlation coefficients for weather variables (Days with Frost, Air Pressure, Total Rainfall, Relative humidity (RH) Temperature, Dew point and wind speed) and DON content in grain, 10%P, 25%P, 75%P, 90%P, respectively mean 10%, 25%, 75%, 90% percentiles.

Although good agricultural practices certainly help to reduce the risk for *Fusarium* epidemics, the application of fungicides remains the most important control measure to reduce *Fusarium* symptoms. Although there are a limited number of active ingredients with good control activity for FHB, the chemical control of this pathogenic disease complex remains a serious issue. The short vulnerable period of the pathogen, the fact that it is an ear pathogen, and the fact that it mainly infects under wet conditions all hamper an efficient control of the FHB complex.

1.2 The Belgian situation

In order to get a better view on the FHB problem in Flanders, a region situated in the North of Belgium, an intensive survey started in 2002. Pursuing a combined approach of symptom evaluation, DON measurement and genetic characterization of the population, a comprehensive dataset was obtained. This dataset comprised data of ten growing seasons, on at least ten locations throughout Flanders. On each location 12 cultivars of wheat were sown in a complete randomized block design with four replications. An overview of the obtained results have previously been published (Audenaert *et al.*, 2009; Landschoot *et al.*, 2011a+b) and are presented in Figure 1. From this extensive survey, several solid conclusions could be drawn.

First, it was clear that the FHB population in Flanders is very dynamic evolving from a *F. graminearum*/*F. culmorum* dominated population in 2002 and 2003 towards a *F. poae*/*F. graminearum* population in 2008 and 2009 (Figure 1A). In addition, a correlative study on all variables elucidated some clear population characteristics. First, *F. poae* was shown to be a pathogen that is often occurring in association with other members of the disease complex. This is illustrated in the heat map presented in Figure 2A where *F. poae* clearly clusters with other species such as *F. avenaceum* and *M. nivale*.

A second layer of complexity is the link between DON level and the DON-producing species *F. graminearum* and *F. culmorum*. As illustrated in Figure 1B, the presence of DON was not really correlated with the presence of DON-producing species since it clustered separately in a different branch of the tree. In addition, the presence of *F. graminearum* and *F. culmorum* was rather linked to low disease classes such as Dc1 and Dc2 while the presence of the other species was linked with the higher disease classes Dc2, Dc3 and Dc4. Finally, the presence of DON was also associated with the higher disease classes. Nevertheless, although this link was apparent, no clear linear correlation was observed between quantitative DON presence and disease symptoms (Audenaert *et al.*, 2009).

2. Fungicides to control FHB and associated mycotoxins

2.1 Fungicides to control fungal growth

Several active ingredients such as triazoles and strobilurins have been reported for their efficiency against several species of the *Fusarium* complex. Triazoles are known inhibitors of the ergosterol biosynthesis in fungi while strobilurin fungicides inhibit mitochondrial electron transport by binding on the Qo site of the cytochrome BC1 complex. Where the effectiveness of triazole fungicides against *Fusarium* spp. is a certainty, the activity of strobilurins against *Fusarium* spp. is doubtful. A considerable amount of evidence shows that strobilurins are mainly active against *M. nivale*. Laboratories around the globe have devoted considerable efforts to develop a coherent view of the activity of fungicides against the FHB causing species. A comprehensive overview is illustrated in Table 2.

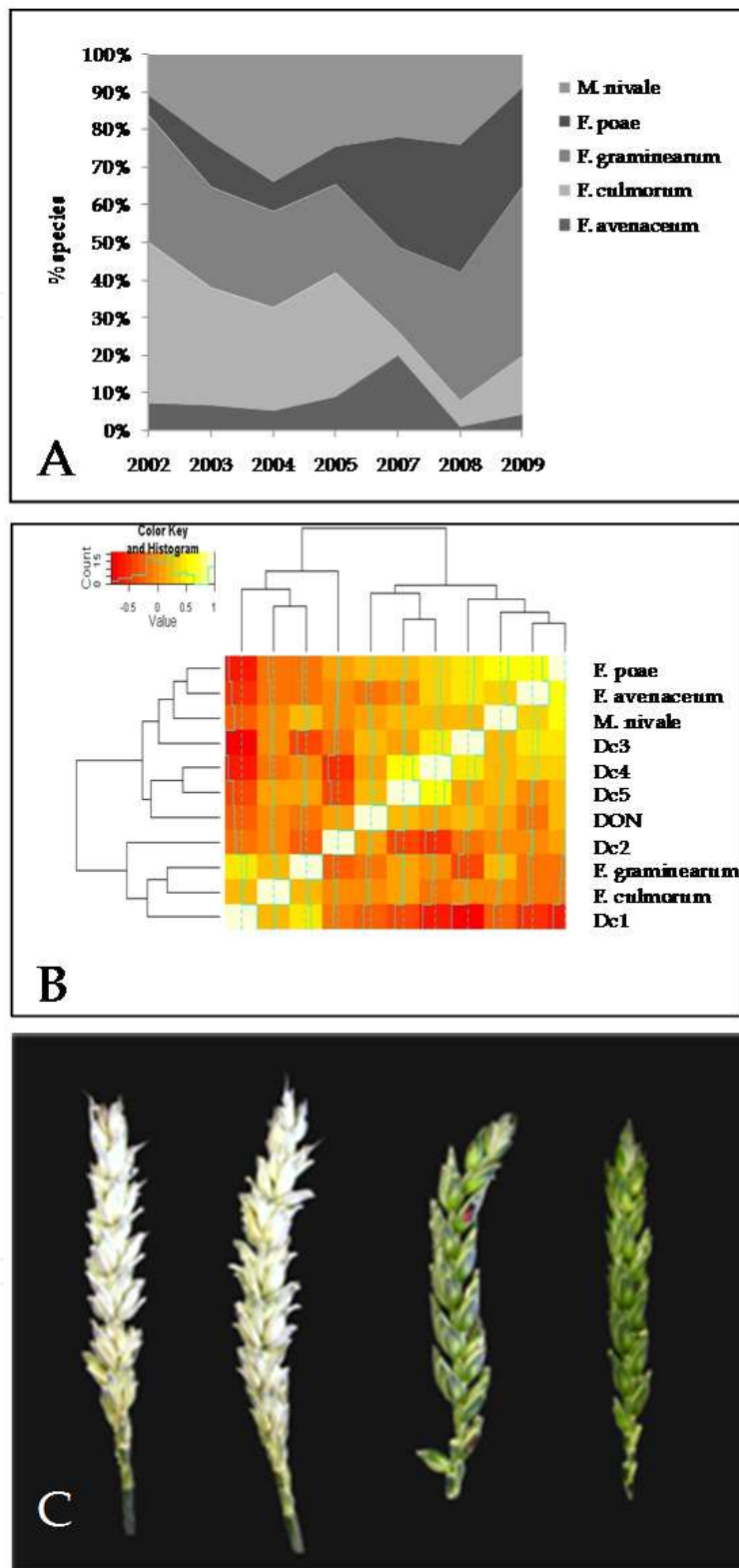


Fig. 1. Results of an extensive field survey in Flanders wheat fields on the FHB population, DON content and disease symptoms. A. shows the evolution of the population from 2002-2009. B shows the links between disease symptoms (Dc1 = disease class 1; Dc2=disease class 2; Dc3= disease class 3; Dc4 = disease class 4; Dc5 = disease class 5). C shows typical FHB symptoms at anthesis (two left ears) compared to asymptomatic ears (two right ears).

Some exceptions notwithstanding no real contradictory reports are mentioned although Zhang *et al.* (2009b) and Pirgozliev *et al.* (2002) obtained different results on the effect of azoxystrobin to control *F. graminearum* and *F. culmorum*. Possibly, these differences originate from different environmental conditions under which experiments were carried out. In line with this assumption Magan *et al.* (2002) clearly highlighted the importance of the a_w value in the efficiency of fungicides. Similarly, other researchers showed the importance of wheat cultivar and isolate aggressiveness for control of *Fusarium* using fungicides (Mesterhazy *et al.* 2003).

2.2 Fungicides to control mycotoxin production

Where the effect of fungicides on fungal outgrowth is quite straightforward, reports on the effect of fungicides on the production of mycotoxins is rather contradictory and information is fragmentary. Indeed, to date, no studies are available describing the effect of fungicides to the broad array of mycotoxins that can be produced by *Fusarium*. Most studies focus on just one or two mycotoxins.

For tebuconazole it is generally accepted that it causes a reduction in the biosynthesis or DON level (Edwards *et al.* 2001; Haidukowski *et al.* 2005; Ioos *et al.* 2005; Paul *et al.* 2008; Simpson *et al.* 2001; Zhang *et al.* 2009a) and the trichothecene nivalenol (NIV) (Ioos *et al.* 2005). Information on another triazole fungicide propiconazole is contradictory. Application of propiconazole resulted in decreased DON levels in a study by Paul *et al.* (2008), while other studies reported increased levels of DON upon propiconazole application (Magan *et al.* 2002).

Application of the triazole metconazole generally results in decreased DON levels in grain samples. This observation was corroborated by several scientific reports (Edwards *et al.* 2001; Paul *et al.* 2008; Pirgozliev *et al.* 2002). Finally for prothioconazole Paul *et al.* (2008) mentioned decreased DON levels. Consonant with this observation, Audenaert *et al.* (2010) described reduced DON levels upon application of field doses of prothioconazole in an *in vitro* assay. However, these authors added another layer of complexity in developing a coherent view on the effect of prothioconazole on DON biosynthesis. Sub lethal application of prothioconazole resulted in increased DON levels (Audenaert *et al.* 2010). This induction was shown to be orchestrated through a reactive oxygen mediated pathway. Indeed, using an *in vitro* approach the former authors succeeded to demonstrate that sub lethal application of prothioconazole results in the prompt induction of H_2O_2 which preceded the DON accumulation. In addition, elimination of H_2O_2 using catalase inhibited the production of DON.

The effect of the strobilurin fungicide azoxystrobin on DON varies from a proliferated DON biosynthesis (Zhang *et al.* 2009; Magan *et al.* 2002; Simpson *et al.* 2001; Gaurilcikiene *et al.* 2011) towards reduced DON levels (Pirgozliev *et al.* 2002). It is tempting to speculate on this observation. The fact that strobilurins often result in increased DON levels might be explained by the pathogen spectrum of strobilurin which mainly targets *M. nivale* while being less effective against *F. graminearum*. It is not unlikely that the niches that are not longer occupied by *M. nivale* are taken by *F. graminearum* which consequently lead to increased DON levels.

Although this kind of research is mainly carried out on the mycotoxin DON, the focus is also shifted to other mycotoxins. Gaurilcikiene *et al.* (2011) demonstrated increased T-2 levels upon azoxystrobin application. A similar result was obtained for NIV (Ioos *et al.* 2005).

TRIAZOLES					
	species	effect on species	mycotoxin	effect on mycotoxin	reference
Tebuconazole	<i>F.graminearum</i>	↓	DON	↓	Zhang <i>et al.</i> (2008)
	<i>Fusarium spp.</i>	↓	DON	↓	Edwards <i>et al.</i> (2001)
	<i>F. culmorum</i>	↓	DON	↓	Simpson <i>et al.</i> (2001)
	<i>F. avenaceum</i>	↓	ND	ND	Simpson <i>et al.</i> (2001)
	<i>M. nivale</i>	—	NR	NR	Paul <i>et al.</i> (2008)
	<i>Fusarium spp.</i>	↓	DON	↓	Haidukowski <i>et al.</i> (2005)
	<i>F. culmorum</i>	↓	NIV	—	Ioos <i>et al.</i> (2005)
Propiconazole	<i>F. culmorum</i>	↓*	DON	↑	Magan <i>et al.</i> (2002)
	<i>Fusarium spp.</i>	↓	DON	↓	Paul <i>et al.</i> (2008)
Metconazole	<i>F. culmorum</i>	↓	DON	↓	Pirgozliev <i>et al.</i> (2002)
	<i>F. graminearum</i>	↓	DON	↓	Pirgozliev <i>et al.</i> (2002)
	<i>Fusarium spp.</i>	↓	DON	↓	Edwards <i>et al.</i> (2001)
	<i>Fusarium spp.</i>	↓	DON	↓	Paul <i>et al.</i> (2008)
Prothioconazole	<i>Fusarium spp.</i>	↓	DON	↓	Paul <i>et al.</i> (2008)
	<i>F. graminearum</i>	↓	DON	↑	Audenaert <i>et al.</i> (2010)
STROBILURINS					
Azoxystrobin	<i>F. graminearum</i>	—	DON	↑	Zhang <i>et al.</i> (2008)
	<i>F. culmorum</i>	—	DON	↑	Magan <i>et al.</i> (2002)
	<i>F. culmorum</i>	↓	DON	↓	Pirgozliev <i>et al.</i> (2002)
	<i>F. graminearum</i>	↓	DON	↓	Pirgozliev <i>et al.</i> (2002)
	<i>M. nivale</i>	↓	NR	NR	Simpson <i>et al.</i> (2001)
	<i>Fusarium spp.</i>	↑	DON	↑	Simpson <i>et al.</i> (2001)
	<i>Fusarium spp.</i>	—	DON	—	Edwards <i>et al.</i> (2001)
	<i>F. poae</i>	↑	T2	↑	Gaurilcikiene <i>et al.</i> (2011)
	<i>F. culmorum</i>	↑	DON	↑	Gaurilcikiene <i>et al.</i> (2011)
	<i>F. poae</i>	↓	T2	↑	This work
	<i>F. culmorum</i>	—	NIV	—/↑	Ioos <i>et al.</i> (2005)
OTHERS					
Carbendazim	<i>F. graminearum</i>	—	DON	—	Zhang <i>et al.</i> (2008)
Thiram	<i>F. graminearum</i>	—	DON	—	Zhang <i>et al.</i> (2008)
Quintozene	<i>F. verticillioides</i>	↓	Fum	↓	Falcao <i>et al.</i> (2011)
Fludioxynil+metalaxy1-N	<i>F. verticillioides</i>	↓	Fum	↓	Falcao <i>et al.</i> (2011)

Table 2. Effect of several fungicides on *Fusarium* spp. and corresponding mycotoxin production. ↑: proliferated growth/production; ↓: reduced growth/production; —: no effect; NR: not relevant; ND: not detected;*: effect dependent on the a_w value.

Finally, some other fungicides namely carbendazim and thiram were tested for their efficiency to reduce DON in grain samples. However, no clear effect was observed (Zhang *et al.* 2009). A nice study with *F. verticiloides* showed decreased fumonisin levels upon application of respectively quintozene and fludioxynil+metalaxyl-N.

Although the above mentioned examples are not meant to provide a complete and extensive literature review on the use of fungicides against FHB, they clearly demonstrate that the infield control of FHB symptoms does not completely cover control of mycotoxin production. We can conclude that when fungicides are not sprayed optimally, conditions which might be conducive for mycotoxin production might be created in the field. This conclusion will hopefully encourage further research in this scientific field.

3. Effect of fungicides on the fungal metabolome

Recently, interest in the effect of fungicides on the fungal metabolome has increased. Primarily fueling this interest in the interaction between sub lethal fungal concentrations and the fungus is that in practice, fungicidal treatments cannot always be carried out under optimal conditions. Consequently, the fungicide concentrations encountered by the pathogen are often lower than one would expect.

3.1 Short term effects

When *Fusarium* encounters fungicide concentrations that are not lethal, a complex spectrum of metabolic changes occurs. The full range of these metabolic changes is still not well dissected although the first steps have been taken to use genome wide approaches to disentangle transcriptional changes in *Fusarium* upon fungicide treatments (Liu *et al.* 2010).

Although the majority of these metabolic changes remain elusive, a fast growing number of papers focus on the oxidative stress induced by fungicides. In an *in vitro* approach it was demonstrated that exposing *F. graminearum* to sub lethal doses of prothioconazole resulted in proliferated production of DON. In addition, an increase in H₂O₂ which preceded the DON accumulation was observed. Addition of catalase, an H₂O₂ scavenger, resulted in loss of DON production. Similar results were obtained in a study using *F. graminearum* and *M. nivale*. This study provided evidence that H₂O₂ was produced by *F. graminearum* and *M. nivale* upon azoxystrobin application (Kaneko & Ishii 2009). However, this phenomenon is possibly isolate or species dependent. In a study by Covarelli *et al.* (2004), tebuconazole was shown to have a negative effect on the expression of the Tri5 gene, an indication for DON biosynthesis in *F. culmorum*.

3.2 Long term effects

The ability of fungi to adapt to stress is pivotal to their survival in the environment, and this adaptation ability is one of the key factors leading to mutations or adaptations that can give rise to more aggressive crop pathogens in an agricultural setting. In a recent study, evidence was brought forward showing that the initial efficacy of the triazole epoxiconazole eroded resulting in increasing EC₅₀ values with a factor of approximately 1.4 (Klix *et al.* 2007). An interesting study illustrated that mutations in a β -tubulin conferred resistance of *F. graminearum* to benzimidazole fungicides (Chen *et al.* 2009). In addition, a benzimidazole binding site on the β -tubulin gene was suggested to be mutated conferring strains resistant to benzimidazole fungicides such as carbendazim (Qiu *et al.* 2011). An even more interesting

observation in carbendazim resistant *F. graminearum* strains was that a proliferated production of DON was observed (Zhang *et al.* 2009b).

Typically for triazole fungicides, a slowly evolving fungicide resistance has been observed. Decreases in azole sensitivity can be caused by (i) point mutations in the target gene, (ii) overexpression of the target gene, (iii) alterations in ergosterol biosynthesis, (iv) enhanced efflux of toxic compounds, and (v) increased copy numbers of target genes or genes for efflux pumps (Becher *et al.* 2010). Similarly as in the carbendazim story, isolates displaying increased resistance to tebuconazole showed increased mycotoxin production.

Finally, for azoxystrobin, at least 27 fungal species are listed to be resistant. The majority of the resistance types are correlated with the G143A substitution in the quinol oxidation site of cytochrome b, the target for strobilurins. Also for members of the FHB complex, this type and other types of resistance towards strobilurins have been described in respectively *M. nivale* (Walker *et al.* 2009) and *F. graminearum* (Dubos *et al.* 2011). The results and examples given in the previous paragraphs clearly peeled away several layers of complexity in the chemical control of FHB. The divergence of fungicide effectiveness both at species and mycotoxin level hamper a simple control of this disease complex. Still, a better insight into the effect of fungicide application on *Fusarium* in the field is needed. European legislation for several *Fusarium* mycotoxins has been established. This legislation provides an extra economic motive for farmers to prevent FHB infection and mycotoxin accumulation in small grain cereals such as wheat.

A detailed study on the effect of fungicides at a population level will certainly contribute to new insights in the adaptive dynamics of a *Fusarium* population upon fungicide application. In addition, shifts in the population might have its consequences for the mycotoxin profiles present in these fields. In the present study, results from fungicide field trials from 2002-2010 are presented with regard to the effect of triazole and strobilurin fungicides on symptoms, population composition and the presence of the trichothecene mycotoxin DON in the field. These data provide new insights into the effect of fungicides on FHB both at a species- and mycotoxin level.

4. Experimental setup of this study

4.1 Experimental field trials

From 2002 to 2010, different field trials of winter wheat throughout Belgium were followed up for at least ten locations that were located in the most important wheat regions characterized by different growth conditions and crop husbandry measurements. The winter wheat area of Belgium is situated in the centre of the wheat growing region in Europe. Each year at each location, commercial wheat varieties were sown in a complete randomized block design with four replications. For all locations the normal crop husbandry measures were taken. Depending on the experiment, several fungicides and fungicide combinations were used and were applied at various Zadoks growth stages (GS39, GS55 up to GS65) of the crop. In this way both the effect of the active ingredient and the time of application was monitored. The wheat cultivars were sown in common crop rotation systems which lead to different previous crops, both host crops (maize or wheat) as well as non-host crops for *Fusarium* spp. (beans, sugar beets, onions or chicory).

From GS71 to GS75 the experiments were evaluated for the presence of *Fusarium* symptoms. Both the FHB incidence and the FHB severity (disease classes 1-5 with 0, 25, 50, 75 or 100 % bleached ear surface, respectively) were scored. To take into account both assessments for 100 randomly chosen ears per plot, the disease index (DI) was computed as follows: $DI = (0n_1 + 1n_2 + 2n_3 + 3n_4 + 4n_5) / 4n \times 100\%$; with "n" the number of evaluated ears and "ni" the number of ears in disease class i.

In order to assess the composition of the FHB population, wheat ears were plated on PDA medium (potato dextrose agar, Oxoid, Belgium) for further species identification. Seeds were surface-sterilized for 1 minute in 1% NaOCl, washed for 1 min with 70% EtOH, washed with distilled sterile water, dried for 5 min and subsequently put on PDA plates. After five days of incubation at 20°C, outgrowing mycelium was transferred to a new PDA plate. For species determination, five mycelium plugs randomly taken from the fully grown PDA plates were transferred to liquid GPY-broth (10 g glucose, 1 g yeast extract and 1 g peptone, Oxoid, Belgium) and incubated for five days at 20°C. After five days, mycelium was transferred to eppendorf tubes, centrifuged for 10 min at 12,000 rpm and then freeze-dried for 6 h at -10°C and 4 h at -50°C (Christ Alpha 1-2 LD Plus, Osterode, Deutschland). DNA extraction was performed as described by Audenaert *et al.* (2009), based on the CTAB (hexadecyl trimethyl ammonium bromide) method (Saghai-Marooof *et al.*, 1984). PCR for single species detection was performed in a 25 µl reaction mixture (Demeke *et al.*, 2005).

DNA amplification was performed in an Applied Biosystems GeneAmp PCR system 97000 PCR. Amplicons were separated on 1.5% (wt/vol) agarose gels stained with 0.1 µl ethidium bromide. PCR was validated by including reference strains obtained from the MUCL/BCCM collection in each PCR run: *F. graminearum* MUCL 42841; *F. culmorum* MUCL 555; *F. poae* MUCL 6114; *M. nivale* MUCL 15949; *F. avenaceum* MUCL 6130 (Audenaert *et al.*, 2009, Isebaert *et al.*, 2009).

At harvest, DON levels was analyzed by enzyme-linked immunosorbent assay (ELISA) (Veratox DON 5/5 kit Biognost - Neogen). A subsample was taken from each field out of the randomized block design. All DON results are the average of at least four DON measurements per treatment.

4.2 *In vitro* trials

In the present study, fluoxastrobin+prothioconazole was tested for its efficiency to control several field isolates of *F. poae*. The field dose of the fungicide was the point of departure for the *in vitro* assay. The field dose mounted to 0.5 g/l + 0.5 g/l for fluoxastrobin+prothioconazole, A dilution series of the fungicide was prepared to obtain a final concentration of 1 mg/l, 5 mg/l, 10 mg/l and 50 mg/l in the 24-well plates in which the assay was executed. In these wells, 250 µl of conidial suspension was added and amended with 250 µl of the fungicide. The final concentration of the microconidia was 10⁶ conidia per ml. These wells were incubated at 22°C. Two repetitions were done per dilution and the experiment was repeated two times independently in time. Control treatments consisted of 250 µl of spore suspension and 250 µl of distilled water. T-2 production kinetics were monitored using an ELISA (Veratox T-2 kit, Biognost-Neogen).

At each time point (4 h, 24 h, 48 h) after inoculation, the percentage of germinated conidia were counted. At each time point, three repetitions per treatment were counted.

5. Results

5.1 *In vitro* effect of fluoxastrobin+prothioconazole on *F. poae*

From previous work it is known that fluoxastrobin+prothioconazole provides good protection against *F. graminearum*. For several isolates it was shown that a dose of 50 mg/l of this fungicide resulted in a reduction in germination rate of 95% (Audenaert *et al.* 2010). Based on these results, we wanted to focus on the sensitivity of *F. poae* to this fungicide. *F. poae* was an underestimated species for long time since it was described being a weak pathogen. However, throughout Europe a steadily increase of this species has been observed. Yet, the basis for this increased importance remains elusive. To date research on this pathogen remains limited although research groups around the globe tend to initiate research initiatives with regard to this pathogens (Stenglein, 2009). From Figure 2 it is clear that a diversified spectrum of susceptibility can be observed in *F. poae* depending on the isolate. Most importantly, some of the isolated strains showed residual germination levels ranging from 20% up to 80% at fungicide concentrations three times the field dose (data not shown). In addition, among isolates very diverse reaction patterns were observed in the dilution series. This result highlights the high diversity of *F. poae* with regard to fungicide resistance.

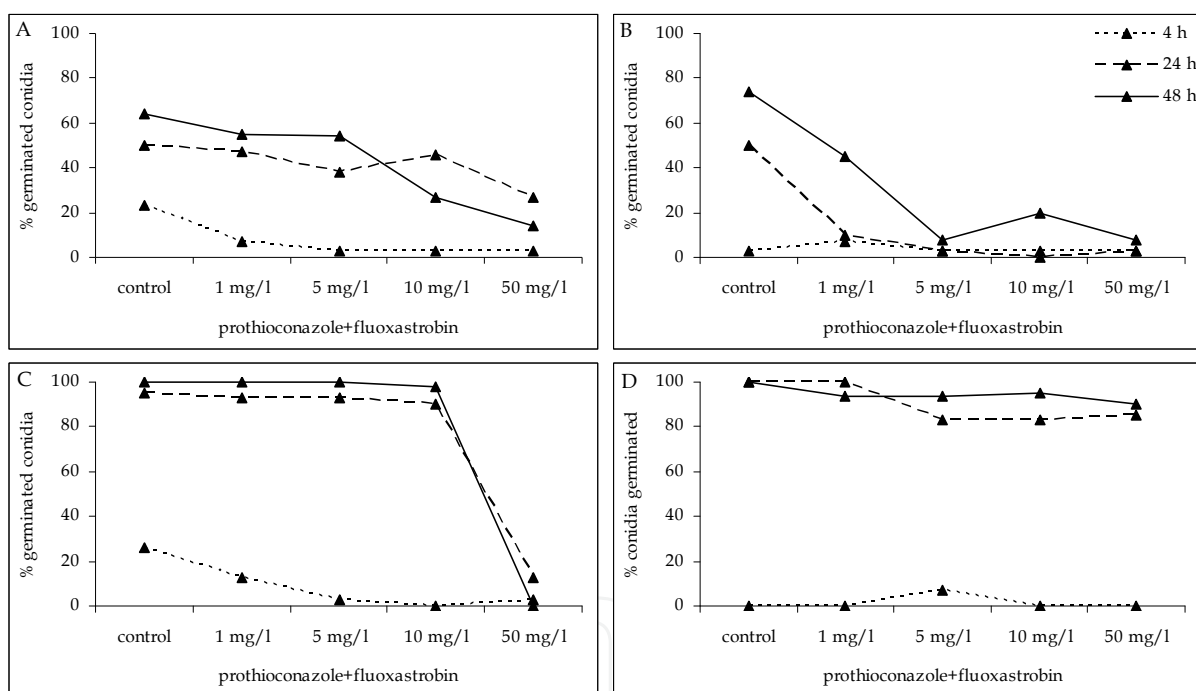


Fig. 2. Effect of a dilution series of fluoxastrobin+prothioconazole on the conidia germination of four different *F. poae* isolates (A,B,C, and D). Each panel shows the germination of a different isolate.

The results obtained in this work are rather contradictory with what has been described in literature. Generally it is accepted that *F. graminearum* is more resistant to fungicides than *F. poae*. Using several fungicides such as diphenconazole, tebuconazole, iprodione,... it was demonstrated that the sensitivity of *F. graminearum* was lower compared to *F. poae* (Hudec, 2007; Mullenborn *et al.* 2008). Till now, we have no clear explanation for this discrepancy, however, possibly the isolate, the incubation temperature, the culture conditions might influence the sensitivity response in both species.

In a second step, we wanted to investigate the interaction between stress induced by prothioconazole+fluoxastrobin and the toxin production by the *F. poae* isolates. Surprisingly, using an LC-MSMS approach, the *F. poae* isolates were characterized as T-2 chemotype. In addition, several other mycotoxins were produced such as diacetylscirpenol and nivalenol (data not shown). The ability of *F. poae* to produce T-2 is rather exceptional. Indeed, the ability of *Fusarium* to produce T-2 toxin has been described to be mainly restricted to *F. langsethiae* and *F. sporotrichoides*. A reason for this apparent discrepancy originates from the fact that no structural genetic information is available regarding the toxic metabolome of *F. poae*. Therefore, majority of the studies use artificial media in search for toxins of *F. poae*. However, recent research in our laboratory clearly illustrates that *F. poae* does not produce toxins on all media. There is some evidence that the nitrogen source and eventual amino acids or polyamines could play a role in the induction of T-2 production by *F. poae*. In Figure 2, the production kinetics of T-2 upon prothioconazole+fluoxastrobin is shown.

Similar to the results on the conidia germination (Figure 3), the effect of the fungicide prothioconazole+fluoxastrobin differed clearly depending on the isolate. Some isolates did not show a consistent proliferated T-2 production upon fungicide stress (Figure 3 A and C) others did (Figure 3B). Remarkably, the isolate that was extremely resistant to the fungicide prothioconazole+fluoxastrobin also showed extremely high basal levels of T-2 production which even increased upon fungicide application. Although these results are very preliminary, they pinpoint T-2 production as a possible protective mechanism upon fungicide application. Similar results were previously obtained with DON. Using a *tri5* knockout mutant, it was demonstrated that DON-negative mutants of *F. graminearum* became hypersusceptible to fungicide application (Audenaert *et al.*, 2011).

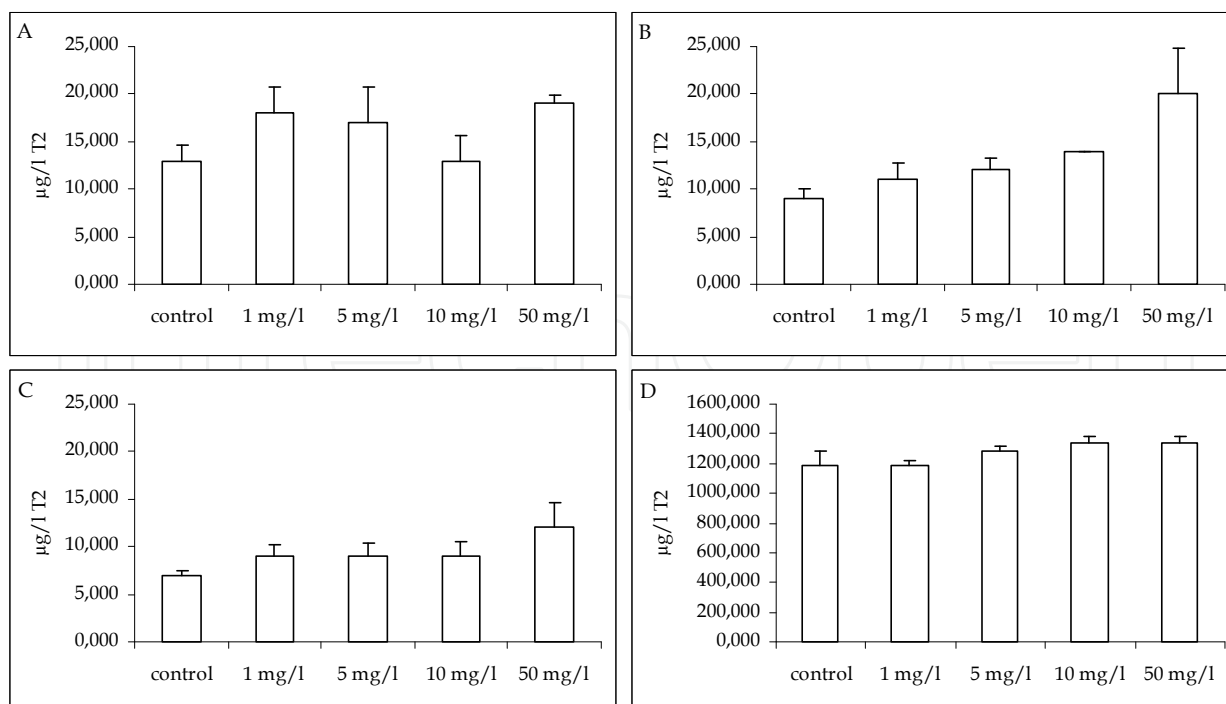


Fig. 3. Effect of sub lethal prothioconazole+fluoxastrobin concentrations on T-2 production on four different isolates (A,B,C and D).

5.2 Control of FHB by fungicide application at growth stage GS55

5.2.1 Effect of fungicides on DON content

In order to peel away the layers of complexity regarding control of *Fusarium* and corresponding DON contamination, a fungicide trial using prothioconazole, epoxyconazole+metconazole and prothioconazole+tebuconazole was set up during the growing seasons of 2007-2008, 2008-2009 and 2009-2010. Pursuing a combined approach of symptom scoring and DON measurement, we aimed to disentangle the effect of triazole fungicides on *Fusarium* development and mycotoxin production. For all treatments, fungicide applications resulted in a clear reduction in symptom development which was the same for all tested active ingredients (data not shown).

More interesting is the effect of these treatments on the DON level. In Figure 4, the effect of triazole application on the DON levels is displayed. By these results, evidence is brought forward demonstrating that the efficiency of triazole fungicides to reduce DON levels is depending on the background level of DON observed in the control treatments. Under conditions of low DON levels, fungicides do not result in decreases in DON level. On the contrary, several fungicide treatments resulted in increased DON levels compared to the control. This detrimental effect of fungicide treatments with respect to DON content has previously been described by other authors (Magan *et al.*, 2002).

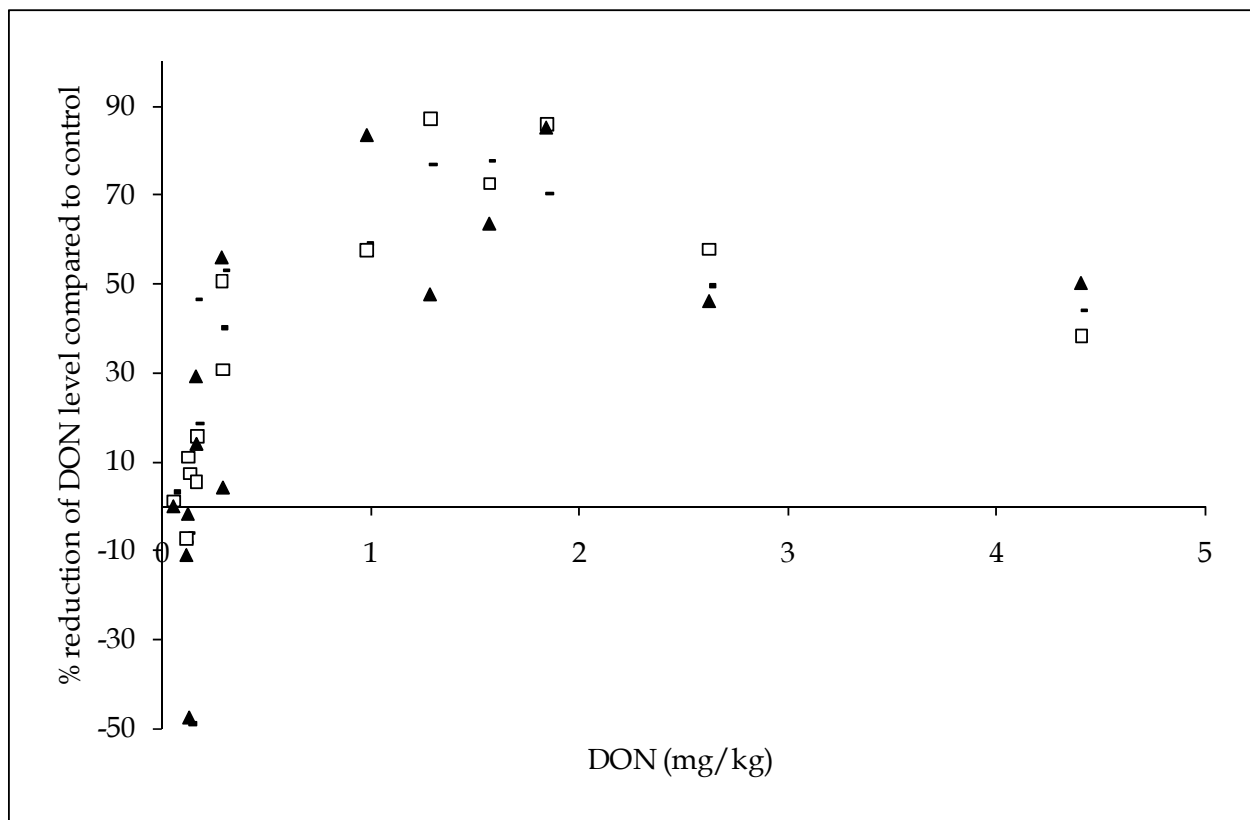


Fig. 4. Levels of DON after application of prothioconazole (▲), epoxyconazole+metconazole (□) and prothioconazole+tebuconazole (■) in function of the DON levels present in the untreated control fields. All data points are the result of four independent repetitions.

Surprisingly, our experimental field trials show similar efficiencies in function of the DON concentration for the three triazoles: prothioconazole, epoxyconazole+metconazole and prothioconazole+tebuconazole applied at GS55 were all the most efficient at DON concentrations between 1 mg/kg up to 2 mg/kg while the efficiency reduced to about 50 % for higher DON concentrations.

These results suggest that although fungicides have been described to be very effective to control *Fusarium* symptoms in the field, their efficiency to reduce DON seems to be limited. In addition, Figure 4 illustrates the usefulness of fungicides for fields with DON levels that are situated around the DON threshold values set by the European Commission: with reductions of the DON level from 50% to 90% this implies that samples that would exceed the European threshold limits drop below these limits when triazole fungicides are applied.

5.2.2 Effect of fungicides on the population structure

Besides the effect of fungicide application on FHB symptoms and DON levels, the impact of fungicides on the population constitution was monitored as well. The five predominant species in Flanders wheat fields were monitored i.e. *F. graminearum*, *F. poae*, *F.culmorum*, *F. avenaceum* and *M. nivale*. Results from these field trials clearly subscribe that fungicide application clearly influences the species distribution within the population (Figure 5).

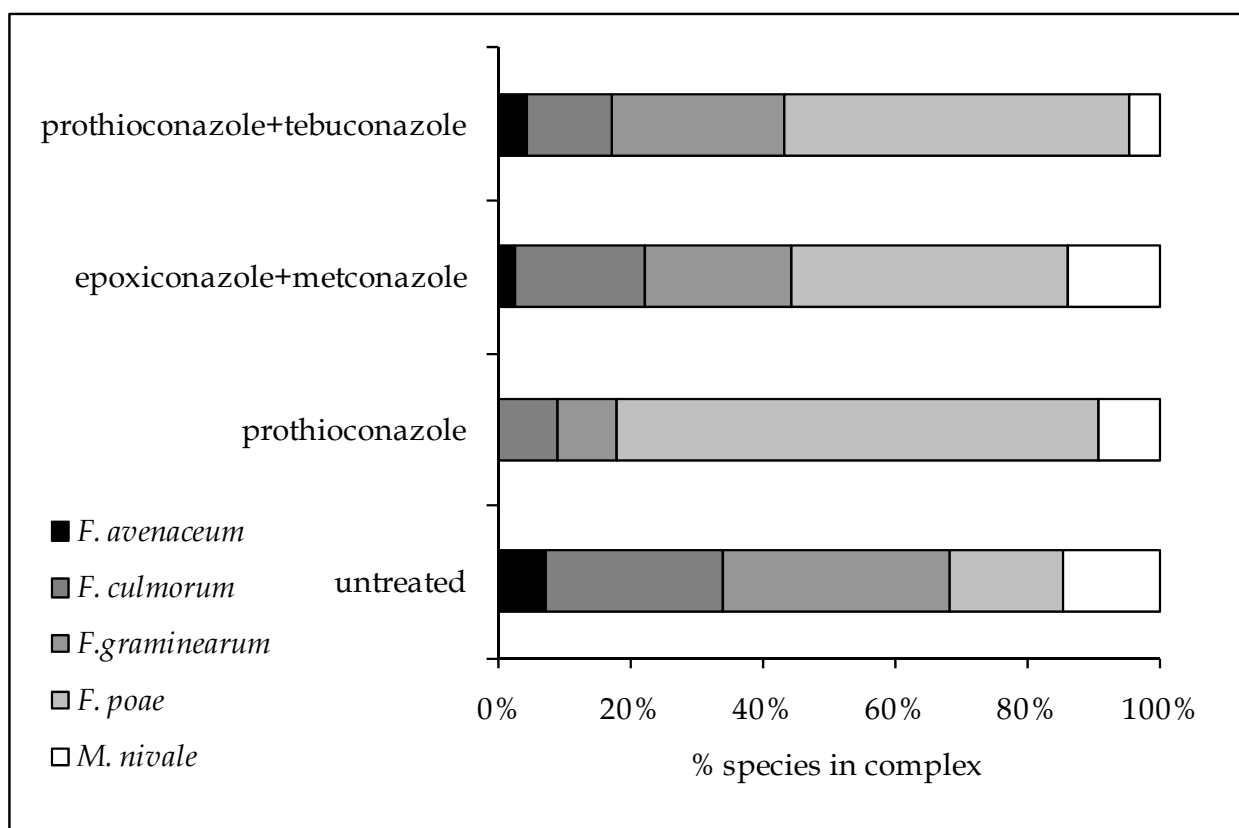


Fig. 5. Effect of prothioconazole, epoxiconazole+metconazole and prothioconazole+tebuconazole on the population of *Fusarium* species.

In fields treated with prothioconazole at GS55, *F. poae* became the predominant species whereas in the untreated fields, the population was initially dominated by *F. culmorum* and *F. graminearum*. This phenomenon was to a lesser extent also observed in the other treatments with prothioconazole+tebuconazole and epoxiconazole+metconazole. This result is in concordance with the *in vitro* experiments shown in Figure 1 which already suggested that a considerable portion of the *F. poae* isolates possesses a considerable level of resistance towards triazole fungicides. Finally, it was consistently surprising that this shift within the population towards an increase of *F. poae* was mainly at the expense of *F. culmorum*. Surprisingly, application of triazole fungicides did not result in a consistent increase of *M. nivale* in the population. *M. nivale* has previously been described for its resistance towards triazole fungicides but this could not be pinpointed in the present field study. No clear explanation for this observation can be found.

5.3 Fungicide application timing and control of FHB

5.3.1 Effect of fungicide timing on FHB symptoms and DON content

Another layer of complexity in developing a coherent view of the effects of fungicide application versus *Fusarium* and its mycotoxins is the timing of the application. In order to investigate the effect of timings for chemical control of *Fusarium*, several triazole fungicides were applied at different growth stages during wheat growth. One series of trials involved a *Fusarium* treatment at GS55 and GS65. Figure 5A clearly illustrates that application of triazole fungicides at GS65 does not result in reduced *Fusarium* symptoms. At the other hand, application of triazole fungicides at GS55 clearly reduces the impact of *Fusarium* at the level of the symptoms. The results for the concomitant DON levels are slightly different. Although chemical control of *Fusarium* at GS65 is inefficient at the level of symptom development, a consistent effect was observed at the level of DON (Figure 6C). This result came as a surprise since several authors report that suboptimal fungicide application can result in proliferated DON production (Audenaert *et al.*, 2010; Magan *et al.*, 2002). We assume that this late fungicide application at GS65 and at the normal application rate comes too late to avoid symptom development, however this application is still in time to decrease DON levels to some extent. It has been described by several authors that DON is a crucial virulence/pathogenicity factor at later stages of infection to facilitate migration of the pathogen in the ear (Mudge *et al.* 2006).

In a second series of time trials, application of prothioconazole was performed at GS39, GS65 and a combination at GS39+GS65. Results are presented in Figure 6B+D. Similar as in Figure 6A, application of prothioconazole at GS65 results in a small decreased number of symptoms, although this reduction was not significant. Remarkably, application of prothioconazole at GS39 resulted in a significant decrease in *Fusarium* symptoms. In addition, a combined application at GS39+GS65 had a synergistic effect at the level of symptoms. For DON, results were quite similar. Application of prothioconazole at GS39 resulted in reduced DON levels compared to untreated plots and plots treated at GS65. A combined application of prothioconazole did not result in further reduction of the DON level. This result confirms that the ability of fungicides to reduce DON levels in *Fusarium* infected fields is limited to reductions of about 50%.

Scientific research on the effects of fungicide timing with regard to *Fusarium* symptom development and DON levels are scarce. Previously, Wiersma and Motteberg (2005) reported

GS60 as ideal for control of FHB. In addition, using tebuconazole, these authors checked the efficiency to control FHB and DON levels but regarding *Fusarium* symptoms, they could not come up with solid conclusions regarding the timing of application. For the efficiency of tebuconazole to reduce DON levels, applications at GS39 and GS60 performed equally.

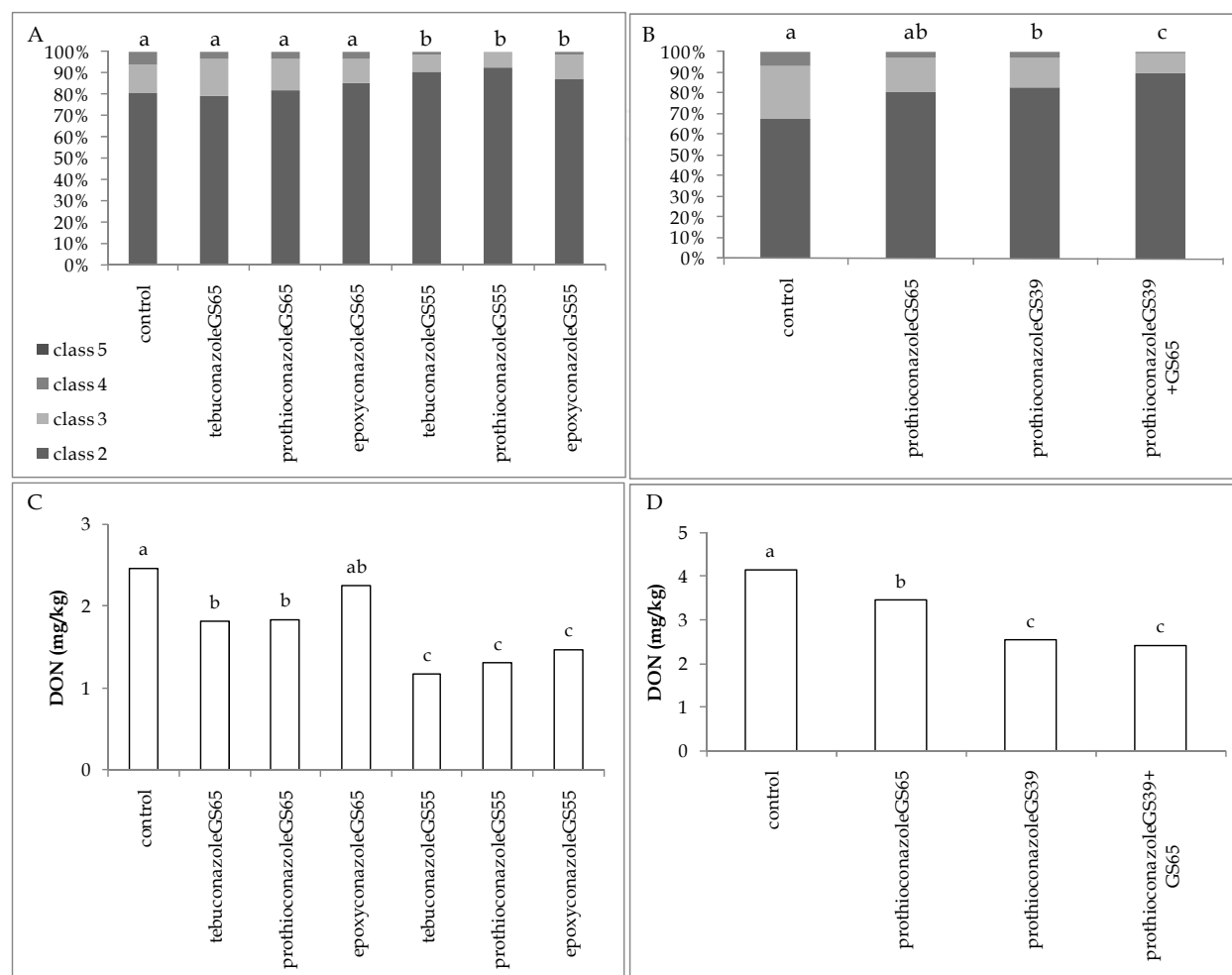


Fig. 6. Effect of timing of fungicide application on *Fusarium* symptom development and DON content. A+C: effect of prothioconazole, tebuconazole and epoxyconazole applied at GS65 and GS55 on symptoms (A) and DON level (C). B+D: effect of prothioconazole applied at GS39, GS65 and GS39+GS65 on *Fusarium* symptom development (B) and DON level (D).

5.3.2 Fungicide timing and population structure

The timing of fungicide application to control *Fusarium* clearly had an effect on the composition of the population. When we compare the population in the untreated control of the experimental field trial presented in Figure 7 with the untreated control in Figure 4, the huge differences in population composition is obvious. This enormous elasticity of the FHB population depending on field location has previously been described by Audenaert *et al.* (2009).

Application of prothioconazole at GS55 clearly favoured *F. poae* which was not present in the control fields but which popped up in the prothioconazole treatment (Figure 7A).

For the other triazole fungicides, a consistent reduction of *F. avenaceum* was observed. In addition, the niches seemed to solely colonized by *F. graminearum*. In the other field trial, where prothioconazole was applied at GS39, GS65 and GS39+GS65, major shifts in the population were observed although no consistent changes were observed between the treatments. At first sight this might be unexpected, however both application of GS39 and GS65 can be considered to be apspecific for FHB. Therefore, the population causing disease symptoms has not yet been established (at GS39) or is already fading (GS65). This might explain the less consistent results in this experiments compared to the results obtained in the experiment where fungicides were applied at GS55 (Figure 5).

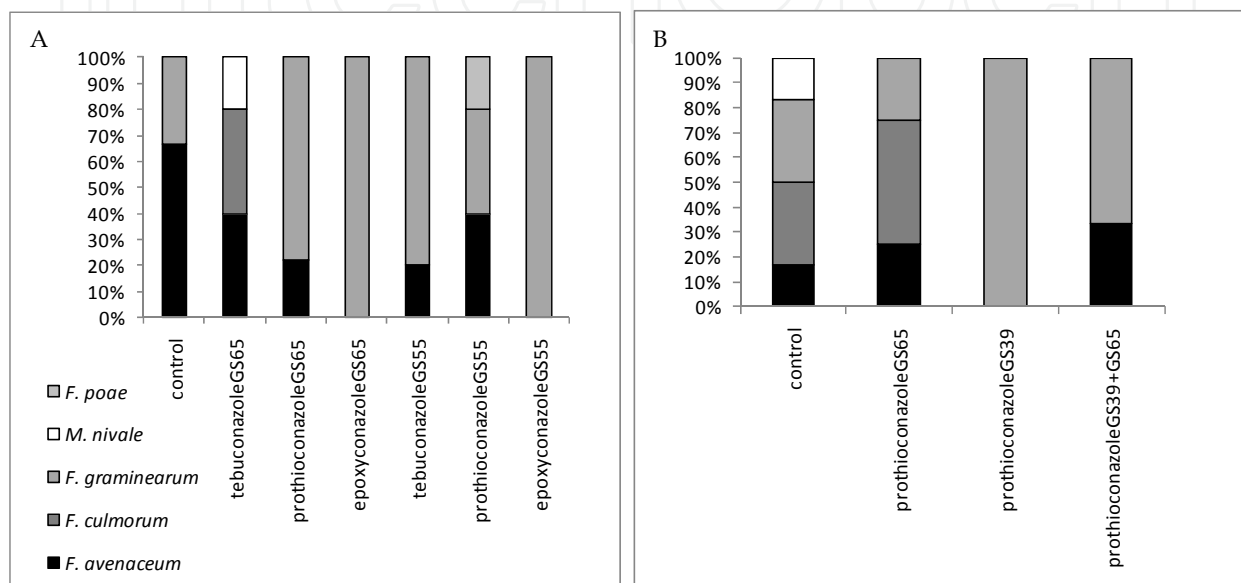


Fig. 7. Effect of timing of fungicide application on the FHB population. A: effect of prothioconazole, tebuconazole and epoxyconazole applied at GS65 and GS55. B: effect of prothioconazole applied at GS39, GS65 and GS39+GS65.

It was remarkable that the increased portion of *F. graminearum* in the fungicide treatments did not result in increased DON levels. This result provides indirect evidence that the presence of DON-producing chemotypes within the population does not necessarily result in a proliferated DON level in the field. This finding underscores the work by Landschoot *et al.* (2011a+b).

6. Conclusions

The aim of this study was to disentangle the effect of fungicide application on the FHB disease complex. Pursuing a combined approach of *in vitro* and *in vivo* field trials, some very interesting conclusions could be drawn. First, it is important to state that fungicides applied at normal rate under optimal conditions are effective in controlling FHB disease. However, working in the field often implies working under suboptimal conditions, and then, problems regarding FHB presence of concomitant mycotoxins might occur. First, it is important to highlight that not all species comprised in the FHB disease show the same susceptibility towards fungicide application. The data presented in the present work provide a compelling amount of evidence illustrating that *F. poae* is more resistant to triazoles than the other members of the disease complex. This was illustrated in *in vitro*

trials which showed that a considerable amount of isolates were able to grow at field doses of fungicide applications. Consonant with these *in vitro* trials field trials demonstrated that triazole application generally resulted in a shift in the FHB population in favour of *F. poae*.

At the level of symptom development, all tested triazole fungicides when optimally applied at GS55 resulted in similar disease symptoms reduction. When varying the timing of application, it was obvious that triazole application at GS65 came too late to efficiently reduce FHB symptoms. On the contrary, fungicide spraying at GS39 resulted in a significant reduction of disease symptoms. We suggest that these treatments reduce the primary inoculums present in the vegetative crop. A combined application at GS39+GS65 resulted in a synergistic effect of the treatments.

With regard to DON, several lines of evidence corroborate a role for timing of fungicide application. The results obtained in the present study demonstrate nicely that where complete control of FHB can be obtained at the level of disease symptoms, there seems to be some sort of threshold efficiencies that cannot be exceeded when DON levels are higher than 2 mg/kg. In all the field trials comprised in the present study, a maximum DON reduction of 50% was obtained compared to the untreated control fields. In addition, where application of triazoles was not affecting disease symptoms when applied at GS65, a minor reduction of DON levels was observed. Contrary to observations on the symptom level, no additional effect was observed when performing a combined application of triazoles at GS39+GS65 compared to single applications at GS39 or GS65.

In conclusion, this study peeled away several layers of complexity in the chemical control of FHB in wheat. We are convinced that fungicide use is an important hurdle that can be included in crop management systems to prevent FHB and concomitant mycotoxin present. However, it is clearly an oversimplification to pretend that fungicide use is the "holy grail" in the control of FHB disease. The disease is far too complex and too multifaceted to draw this conclusion. It will take all branches of scientific research to keep this problem under control. The use of wheat varieties with high levels of resistance, the use of good culture practices such as broad crop rotations and intelligent soil tillage will certainly contribute

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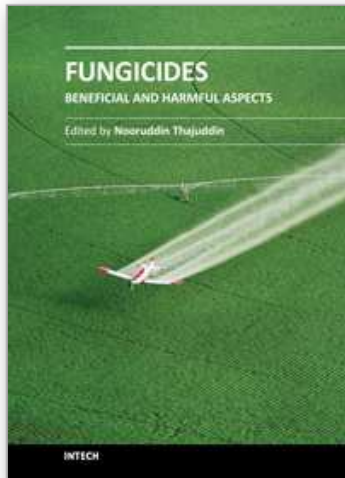
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Fungicides - Beneficial and Harmful Aspects

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Fungicides are a class of pesticides used for killing or inhibiting the growth of fungus. They are extensively used in pharmaceutical industry, agriculture, in protection of seed during storage and in preventing the growth of fungi that produce toxins. Hence, fungicides production is constantly increasing as a result of their great importance to agriculture. Some fungicides affect humans and beneficial microorganisms including insects, birds and fish thus public concern about their effects is increasing day by day. In order to enrich the knowledge on beneficial and adverse effects of fungicides this book encompasses various aspects of the fungicides including fungicide resistance, mode of action, management fungal pathogens and defense mechanisms, ill effects of fungicides interfering the endocrine system, combined application of various fungicides and the need of GRAS (generally recognized as safe) fungicides. This volume will be useful source of information on fungicides for post graduate students, researchers, agriculturists, environmentalists and decision makers.

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