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Reduction of *Fusarium* head blight and deoxynivalenol in wheat with early fungicide applications of prothioconazole

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Abstract

Numerous studies have identified the benefit of fungicides applied at flowering (Zadoks Growth Stage (GS) 59-69) in the reduction of *Fusarium* head blight (FHB) and the reduction of deoxynivalenol (DON) in harvested wheat grain. Two experiments were performed to identify the ability of prothioconazole (Proline®, Bayer CropScience) at three timings to reduce FHB and resulting DON in harvested grain of wheat. Prothioconazole (150 g ha^{-1}) was applied to plots of wheat at GS31, 39 and 65 in a full factorial design. Plots were inoculated with *Fusarium*-infected oat grain at GS30 and mist-irrigated at GS65 to encourage head blight development. Plots were assessed for head blight symptoms at GS77 and harvested grain was analysed for yield, specific weight, thousand grain weight and DON. Factorial ANOVA identified prothioconazole applications at each timing resulted in significant reductions in FHB and DON. The control achieved with combinations of spray timings was additive with no significant interactions. The control of FHB at GS31, GS39 and GS65 was 50, 58 and 83% respectively. The reduction in FHB achieved by all three timings combined was 97% compared to the fully untreated control plots. The reduction of DON after application of prothioconazole at GS31, GS39 and GS65 was 27, 49 and 57% respectively. The application of prothioconazole at all three timings achieved 83% reduction of DON compared to the fully untreated control plots. These experiments have determined, for the first time, significant additional

head blight disease control and mycotoxin reduction with applications of a fungicide before flowering.

Keywords: Proline®, yield, specific weight, thousand grain weight, fungicide timing, mycotoxins.

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Introduction

Fusarium head blight (FHB) is an important disease of small grain cereals as it results in decreased yield, reduced grain quality (specific weight and thousand grain weight), processing quality and the presence of fusarium mycotoxins in harvested grain (Parry et al. 1995). The disease can be caused by several pathogens; the dominant ones are *Fusarium graminearum (*teleomorph *Gibberella zeae), F. culmorum, F. poae, F. avenaceum, Microdochium nivale* and *M. majus*. The *Fusarium* species produce a wide range of mycotoxins, the most important ones in wheat are deoxynivalenol (DON) and zearalenone. Both these mycotoxins are produced by *F. graminearum* and *F. culmorum*. Surveys have indicated that fusarium mycotoxins are common contaminants of wheat, however they usually occur at low concentrations (Edwards 2009). High concentrations can occur when weather conditions are conducive, in particular wet weather from flowering to harvest (Edwards 2009). DON causes reduced feed intake, reduced weight gain and vomiting in farm animals (Anon. 2004a). Nausea, vomiting, diarrhoea, abdominal pain, headache, dizziness and fever have been reported when high concentrations of DON were consumed by humans.

The European Commission set legislative limits for the fusarium mycotoxins including DON in cereal grains and cereal-based products intended for human consumption (Anon. 2006c). The maximum limit for DON in unprocessed wheat is 1250 μg kg^{-1}; this limit applies to wheat placed on the market for processing for human consumption. Maximum limits are set on unprocessed cereals to avoid highly contaminated cereals entering the food chain and to encourage all measures to minimise fusarium mycotoxin contamination to be taken during the field stages of the production chain. The European Commission has also set guideline limits for fusarium mycotoxins in animal feed (Anon. 2006b). The lowest guidance limits have been set for pig feed owing to the high proportion of cereals in pig feed and their higher sensitivity to fusarium mycotoxins. The DON guidance value for complimentary and complete feedingstuffs for pigs is 900 μg kg^{-1}. The legislation states that growers should use Good Agricultural Practice (GAP) to reduce mycotoxins in cereals. The principles of GAP were detailed in a Commission Recommendation (Anon. 2006a) which advises integrated control measures including crop rotation (avoiding host crops as previous crop), cultivation (ploughing to bury crop debris), choice of variety (planting *Fusarium* resistant varieties) and crop

management to minimise plant stress, maintain plant nutrient balance and minimise lodging. The recommendation states that preventative measures should be used, and if necessary, application of fungicides can be used to control toxigenic *Fusarium* species.

Numerous studies have been conducted to evaluate the efficacy of fungicides to reduce fusarium head blight and resultant DON in harvested wheat (Boyacioglu et al. 1992; Edwards et al. 2001; Ellner 1997; Ioos et al. 2005; Mennitti et al. 2003; Simpson et al. 2001). These studies have highlighted that azole fungicides have the best efficacy, although it is important to note that the efficacy reported is highly variable between different azole fungicides and can be variable between experiments (Beyer et al. 2006). Tebuconazole is the most tested triazole and has been the industry standard for many years. Metconazole was introduced in 1994, and was shown to have similar efficacy to tebuconazole (Edwards et al. 2001; Ioos et al. 2005). More recently, prothioconazole was introduced in 2004, and this triazolinthione had the greatest inhibitory activity against *F. graminearum in vitro* of the fungicides tested (Klix et al. 2007) and has been shown to have high efficacy in field experiments (Paul et al. 2008).

Field experiments of fungicide efficacy against head blight have been conducted using either natural inoculum or inoculation. Experiments may also utilise irrigation systems to ensure conditions are conducive for head blight during the flowering period for infection to occur. Experiments using natural inoculum are usually conducted in fields with high disease pressure, for example following maize and minimum tillage, to maximise the probability of severe disease occurring. By spraying spores of *Fusarium* spp. at flowering followed by irrigation, severe disease can be ensured. Application of spores at flowering does not mimic natural infection, as the spores all arrive on the host crop at a single time, and such an application can not be used to test fungicides applied earlier in the growing season. An intermediate form of inoculation is the application of *Fusarium* inoculated grain to the experiment earlier in the season. The *Fusarium* on the inoculated grain sporulates over a long period of time, thus mimicking the natural inoculum present on the ground.

Fusarium spp. cause three diseases on small grain cereals, these are seedling blight, foot rot and head blight. Seedling blight is caused by *Fusarium* present on infected seed or within surrounding soil. This disease can result in pre- and post-emergence death and diseased seedlings, as seedling grow this infection can develop

into foot rot, a disease of the stem base. It is believed that inoculum for head blight can be from these two diseases, which occur earlier in the season or from crop debris, weeds and soil (Parry et al. 1995). The traditional timing for fungicides to control head blight has been at flowering (Zadoks et al. (1974) Growth Stage 59-69) when the infection primarily occurs. This is typically the third fungicide spray for wheat in the UK, designated T3. The early fungicide applications are at first or second node detectable (GS31-32; T1) and flag leaf fully emerged (GS39; T2). As part of a preliminary study (results not shown) using oat-grain-inoculum applied at stem extension (GS30), several fungicide programs with or without prothioconazole at T1 and T2 appeared to reduce head blight and DON. However, the spread of inoculum between plots may have reduced the ability of prothioconazole at T1 and T2 to reduce the disease and DON within these small plot experiments. It was therefore decided to conduct a full factorial design plus/minus prothioconazole at all three timings to maximise the statistical strength of the experiment to detect significant differences between treatments at each timing and any interaction between applications at each timing; and to use guard plots to minimise the spread of *Fusarium* inoculum between experimental plots.

The aim of these experiments was to measure the efficacy of prothioconazole (150 g ha^{-1}) applied as the formulated product Proline® (Bayer CropScience) at three timings (T1, T2 and T3) to reduce fusarium head blight incidence and DON content of harvest grain and to increase yield and grain quality.

Material and methods

Experimental design

A field experiment was conducted in 2007/08 and repeated in 2008/09. Experimental plots (4 x 12 m) were separated by guard plots (6 x 12 m). Winter wheat, cv. Solstice was sown and grown according to standard farm practice in Shropshire, UK. The experiments were designed as a split plot randomised block with eight treatments replicated four times. The design was a full factorial design of untreated and 0.6 l ha^{-1} Proline® (ai prothioconazole 250 g l^{-1}) treated plots at three timings: T1 (GS 31; first node detectable), T2 (GS39; flag leaf fully emerged) and T3 (GS65; mid-anthesis). The rate of Proline® applied at each timing was 0.75 of the recommended single dose. Treatments are listed in Table 1. T1 and T2 treatments were fully randomised (whole plot) and T3 treatments were randomised between sub-plots (2 x 12 m). All

fungicides were applied in 200 l ha^{-1} water using an 'AZO' knapsack sprayer with 110° flat fan nozzles. All guard plots received a robust fungicide regime containing prothioconazole to minimise spread of inoculum between treated plots. Treated plots were mist irrigated for 17 hours each day (05:00-22:00) for five days from 1 day after the T3 fungicide was applied to optimise conditions for FHB infection.

Artificial inoculation

Three isolates each of *F. graminearum* (Fg75/11, Fg113, Fg2001/169) and *F. culmorum* (Fc2001/158, Fc2001/152, Fc103) were taken from the culture collection at Harper Adams University College. Isolates were sub-cultured on fresh potato dextrose agar (PDA, Merck KGaA, Germany) and after 5 days growth at room temperature used to seed 500 ml of potato dextrose broth (PDB, Merck) in 2-litre flasks. Flasks were shaken twice a day by hand for 5 days. One kg of oats were added to 100 ml of deionised water in a 400 x 550 mm autoclave bag, soaked for 1 hour at room temperature and then autoclaved for 1 h at 121°C. One hundred ml of inoculated PDB was used to inoculate each bag of sterilised oat grains. Bags were gently mixed to distribute the inoculum and incubated for 2 weeks at ca. 20°C. Inoculated oat grains were mixed together to produce a composite inoculum and treated plots were inoculated with 19 g m^{-2} of oat grain inoculum at GS 30 (stem extension).

Disease assessment

Head blight assessments were completed on all plots at late milk (GS 77). Incidence of FHB was calculated as number of infected heads per square metre based on counts conducted in ten quadrats (33 cm^{-2}) within each plot. Data was converted to % FHB incidence based on average number of heads per square metre.

Yield assessment

At maturity, each plot was harvested using a plot combine and the moisture content and total grain yield recorded. Yield was adjusted to tonnes ha^{-1} at 15% moisture content. One kilogram grain samples were taken for determination of grain quality parameters; thousand grain weight (TGW) and specific weight (SW). Grain samples were then milled (ZM100 mill with 1 mm screen, Retsch UK Ltd, Leeds), mixed in a tumbler mixer and laboratory samples removed for DON analysis.

DON analysis

DON was quantified using a DON FAST ELISA kit (R-Biopharm Rhone, Glasgow) according to the manufacturer's instructions. Eight grams of flour were extracted in 40 ml deionised water.

Statistical analysis

The statistical package used for all data analysis was Genstat (Version 12, Lawes Agricultural Trust, Rothamsted, UK). Percentage FHB incidence was logit transformed and DON data log10 transformed to obtain normally distributed residuals. Data from both experiments was first analysed by split-plot analysis of variance (ANOVA) with year as whole plot and treatment as sub-plot. This identified if there was a significant difference between years, treatments and an interaction between year and treatment. Both experiments were then analysed together using a split plot factorial (T1*T2*T3) ANOVA with a block structure of block nested within year; T1*T2 as whole plots and T3 as sub-plots.

Results

There was no significant difference in TGW between the two field experiments conducted in 2007/08 and 2008/09, for all other parameters measured there was a highly significant difference ($p<0.001$) between the two experiments (Table 2). For FHB disease incidence the predicted mean for 2008 and 2009 was 0.7 and 15% respectively. There was a corresponding impact on DON, yield and SW (Table 2). Treatment differences were highly significant ($p<0.001$) for all parameters. There was a significant interaction between year and treatment for yield ($p=0.029$) and SW ($p=0.037$) but not for FHB incidence, DON or TGW. As the interactions were either not significant or were much less significant than the main effects then the treatment differences were broadly consistent between years. When each experiment was analysed by ANOVA the residual mean squares were similar, it was therefore acceptable to analyse the datasets for both years together (block nested within year) and these results are presented (Table 3). Factorial analysis identified that there was no significant interactions between fungicide timings for any parameter measured.

Table 3 shows the p-values and prothioconazole predicted mean values as percentage differences compared to the untreated control for each fungicide application timing.

Each application of prothioconazole significantly ($p<0.05$) reduced the incidence of FHB at GS 77 and DON at harvest. The most effective timing was T3 with 83% and 57% reduction respectively (Table 3) and the cumulative benefit of three applications of prothioconazole resulted in the greatest observed reductions of 97% and 83% respectively compared to the untreated controls (Figure 1). These values are close to the calculated cumulative reduction based on the individual T1, T2 and T3 reductions presented in Table 2 (96% and 84% respectively).

Overall yield, specific weight (SW) and thousand grain weight (TGW) were as expected considering the severity of FHB in the untreated controls. Factorial analysis identified that all fungicide treatments resulted in a highly significant ($p<0.001$) increases in yield, SW and TGW, except for SW ($p=0.050$) and TGW ($p=0.005$) with a T1 application (Table 3). Again, benefits of prothioconazole were cumulative resulting in greatest increases in yield, SW and TGW from the application of prothioconazole at all three timings. Compared to the untreated control the application of prothioconazole at all three timings resulted in a yield increase of 4.6 ton ha^{-1} (98%). The greatest contribution to increased yield was from the T2 application (26%). Compared to the untreated control the application of prothioconazole at all three timings resulted in an increase of SW of 11.4 kg hl^{-1} (19%) and an increase in TGW of 13.4 g (38%).

Discussion

As part of a preliminary study (results not shown) using oat-grain-inoculum at GS30, several fungicide programs with or without prothioconazole at T1 and T2 appeared to reduce head blight and DON in 2006 but not in 2007. These studies were conducted in a standard small plot (2 x 12 m) randomised block design. The difference observed between 2006 and 2007 may have been due to large differences in rainfall. In 2006, the period from T1 application to end of flowering was relatively dry with only 86 mm of rainfall of which only 8 mm fell during flowering. In the following year over the same period there was 172 mm rainfall, including 64 mm over 3 days during flowering. Rainfall events are known to result in splash dispersal of *Fusarium* conidia (Jenkinson and Parry 1994) and stimulate release of *Gibberella zeae* ascospores (Paulitz 1996). It was therefore concluded that in 2007, the high rainfall resulted in

any reduction in head blight inoculum as a result of T1 and T2 fungicide sprays were masked due the dispersal of inoculum between plots during subsequent periods of high rainfall. It was therefore decided to modify the experimental design to include guard plots to minimise spread of inoculum between treated plots. As the inoculum pressure at flowering would be the same for fungicide programs with the same T1 and T2 treatments, the treatments were paired together to provide whole plots treated with combinations of plus/minus prothioconazole at T1 and T2 and subplots plus/minus prothioconazole at T3. This reduced the number of guard plots required and therefore increased the potential size of guard plots for a given experimental area available.

The use of inoculated oat grain as inoculum applied early in the wheat growing season and the use of guard plots to minimise the spread of inoculum between plots allowed the successful identification of the benefit of prothioconazole applied at all three timings. The reduction in head blight and DON was greatest from an application of prothioconazole at T3 and least at T1. Prothioconazole resulted in 83% reduction of head blight and 57% reduction of DON in harvested grains with a single application of three quarter dose at GS65 (T3 timing). This compares well with previous studies, where on average tebuconazole and metconazole applied at full rate, resulted in 58% (n=7 experiments) and 60% (n=24 experiments) DON reduction respectively (Beyer, 2006). The timing of application is critical at flowering with a drop in fungicide efficacy as the fungicide is applied further away from the timing of infection (Pirgozliev et al. 2008).

Two previous studies using controlled environment and glasshouse experiments indicated that early applications of fungicides could reduce FHB and in one study, DON (Greenfield and Rossall 2000; Hutcheon and Jordan 1992). However, statistical analysis was not presented and the studies were not repeated. In a field experiment on disease control of leaf spot and head blight with natural infection, there was no significant reduction of FHB with tebuconazole applied at GS39 but there was a significant reduction in DON (40%) in one year out of three (Wiersma and Motteberg 2005).

It is not clear how fungicide applications before head emergence could reduce head blight and subsequent DON. For the T1 application the likely mechanism is in the reduction of *Fusarium* on the young crop, particularly the dead outer leaf sheaths and on surrounding crop debris and soil. At T2, most fungicide is deposited on the upper leaf canopy. At this timing the fungicide may reduce the number of *Fusarium*

spores on the leaf surfaces. There is evidence of natural suppression of FHB from saprophytic microflora (Liggitt et al. 1997); prothioconazole may benefit the competitors of *Fusarium* spp. due to its high inhibitory activity towards this genus (Klix et al. 2007). As prothioconazole is systemic and the T2 application is applied within 7-14 days of head emergence, there may also be some direct inhibition of infection from prothioconazole translocated to the wheat heads from the T2 application.

In contrast, some strobilurin fungicides have been shown to increase DON when applied during flowering (Simpson et al. 2001). This may be due to the disruption of natural suppression of *Fusarium* spp. by other head blight pathogens (*Microdochium* spp.) (Jennings et al. 2000) and saprophytic microflora (Liggitt et al. 1997). Ellner (2006) reported that applications of some strobilurins before flowering (GS33-55) could also result in an increase in DON.

Increases in grain quality were also greatest after a T3 application of prothioconazole and least from a T1 application. This correlation would indicate that the increase is grain quality was associated with the reduction in head blight. The greatest increase in yield was associated with the T2 application of prothioconazole. As the greatest control of FHB was from the T3 timing, this would indicate that the control of other pathogens was a major contributing factor to the yield benefit from application of prothioconazole at T2. This is likely to be partially due to the control of foliar pathogens, such as *Septoria tritici*, on the flag leaf, as this leaf is the predominant source of yield potential in wheat (Milne et al. 2007).

There was a large difference in the severity of head blight between the two years even though the experimental design was unchanged. This is likely to be due to differences in environmental conditions during key crop growth stages for head blight infection. As well as flowering, when both experiments were mist irrigated, key environmental conditions are during the spring when spore production occurs and the week before flowering when spores can be dispersed onto the emerging wheat heads (De Wolf et al. 2003). In late spring of 2009 it was observed that oat grains were covered with large perithecia, indicating that conditions had been conducive for ascospore production by *Gibberella zeae*. These perithecia were more prolific and larger than observed in the previous year. Two other factors may have increased the disease pressure in 2009. Firstly, in the week before flowering there were 5 days with rainfall greater than 5 mm whereas in 2008 there was no rainfall in the same period.

Secondly, the average temperature during mist irrigation in 2009 was 16.3°C compared to 13.4°C in 2008. The optimum temperature for *F. graminearum* growth is 25°C (Brennan et al. 2003). Under the high disease pressure which occurred in 2009 a higher rate of prothioconazole would be required to reduce the DON concentration at harvest to below the legal limit of 1250 μg kg^{-1}.

The ideal method to control crop diseases is through host resistance. Resistance to *Fusarium* head blight is polygenic and several resistance loci are closely linked to negative agronomic traits (Bai and Shaner 2004). Consequently the availability of economically viable varieties with partial resistance to head blight is limited in many wheat growing regions of the world. At least for the short to medium term, fungicides will continue to play a key role in the reduction of fusarium mycotoxins in small grain cereals. This study has provided clear evidence that application of prothioconazole early in fungicide programs (ie before head emergence) can have a significant contribution to reducing head blight and subsequent DON contamination of harvested grain. The benefit of prothioconazole applied at each timing was additive, with control achieved from all three timings was 97% reduction of FHB and 83% reduction of DON. Growers should therefore consider *Fusarium*-active fungicides within all application timings as part of an integrated control strategy. Earlier fungicide applications will be particularly beneficial when weather conditions at flowering are not conducive to application of a head blight fungicide at the optimum timing.

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Table 1. Factorial split-plot design for winter wheat FHB field experiment with three spray timings (T1, T2 and T3) when plots were untreated or treated with 150 g ha^{-1} prothioconazole. T1*T2 combinations were whole plots and T3 treatments were applied to sub-plots.

Treatment	Whole plot	T1 (GS31)	T2 (GS39)	T3 (GS65)
1	1	Untreated	Untreated	Untreated
2	1	Untreated	Untreated	Prothioconazole
3	2	Prothioconazole	Untreated	Untreated
4	2	Prothioconazole	Untreated	Prothioconazole
5	3	Untreated	Prothioconazole	Untreated
6	3	Untreated	Prothioconazole	Prothioconazole
7	4	Prothioconazole	Prothioconazole	Untreated
8	4	Prothioconazole	Prothioconazole	Prothioconazole

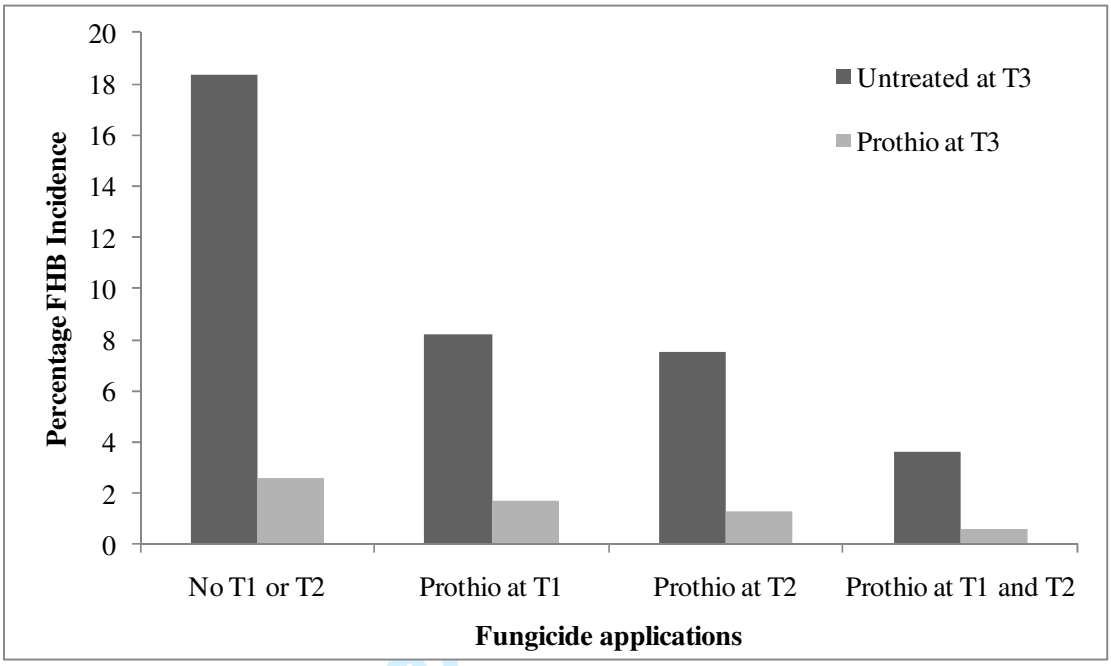
Table 2. Overall mean % FHB incidence, DON, yield, specific weight (SW) and thousand grain weight (TGW) for winter wheat field experiments conducted in 2007/08 and 2008/09. Values are back-transformed means for incidence and DON data. P-values are presented for year (n=2), treatment (n=8) and the interaction between these two main factors.

	% FHB incidence	DON (µg kg^{-1})	Yield (ton ha^{-1})	SW (kg hl^{-1})	TGW (g)
2007/08	0.7	1816	8.7	70.4	42.9
2008/09	14.8	13122	6.1	63.0	42.7
Year p-value	<0.001	<0.001	<0.001	<0.001	0.864
Treatment p-value	<0.001	<0.001	<0.001	<0.001	<0.001
Year*Treatment p-value	0.512	0.964	0.037	0.029	0.125

Table 3. Predicted mean % FHB incidence, DON, yield, specific weight (SW) and thousand grain weight (TGW) for untreated (Unt) and prothioconazole (Proth; 150 g ha^{-1}) treated plots at each fungicide application timing (T1, T2 and T3). Values are back-transformed means for incidence and DON data. P-values and percentage differences are shown in parenthesis.

Spray Timing	% FHB incidence		DON ($\mu g\ kg^{-1}$)		Yield ($ton\ ha^{-1}$)		SW ($kg\ hl^{-1}$)		TGW (g)	
	Unt	Proth	Unt	Proth	Unt	Proth	Unt	Proth	Unt	Proth
T1	4.8	2.4	5715	4169	6.8	8.0	67.5	65.9	44.2	41.3
	(p<0.001; -50)		(p=0.03; -27)		(p<0.001; 18)		(p=0.05; 2.4)		(p=0.005; 7.1)	
T2	5.2	2.2	6823	3499	6.5	8.2	68.6	64.8	45.3	40.3
	(p<0.001; -58)		(p<0.001; -49)		(p<0.001; 26)		(p<0.001; 5.8)		(p<0.001; 12.4)	
T3	8.1	1.4	7482	3184	6.6	8.2	69.5	63.9	46.0	39.5
	(p<0.001; -83)		(p<0.001; -57)		(p<0.001; 24)		(p<0.001; 8.9)		(p<0.001; 16.5)	

A.



B.

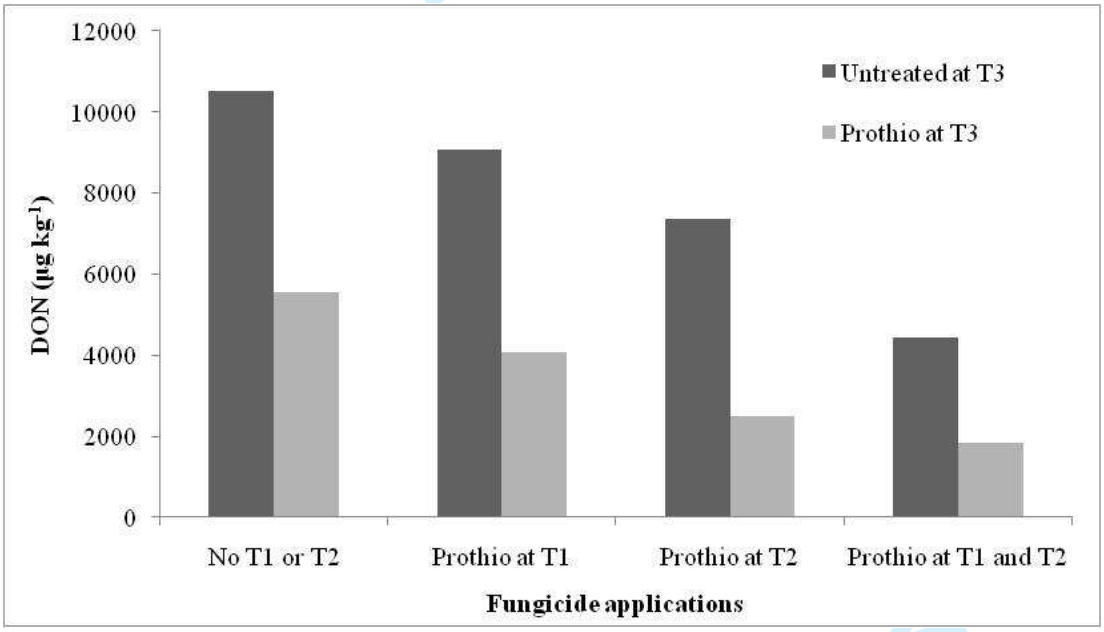


Figure 1. Back-transfomed mean percentage *Fusarium* head blight incidence (A) and DON concentration (B) for winter wheat plots untreated or treated with prothio (prothioconazole; 150 g ha^{-1}) at three fungicide application timings; T1, T2 and T3. Replication was two years x four blocks.