

Effect of fungicide application to control *Fusarium* head blight and 20 *Fusarium* and *Alternaria* mycotoxins in winter wheat (*Triticum aestivum* L.)

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Abstract

Azole fungicides have been reported to be the most effective active substances in the control of *Fusarium* Head Blight (FHB) and in the reduction of the main mycotoxins that occur in cereal grain, such as deoxynivalenol (DON). Four field experiments have been conducted in North West Italy, over a period of 2 growing seasons, in order to evaluate the effect of azole fungicide (prothioconazole) applications on the prevalence of emerging mycotoxins in common winter wheat under naturally-infected field conditions. Wheat samples have been analysed by means of a dilute-and-shoot multi-mycotoxin LC-MS/MS method. Twenty fungal metabolites were detected: enniatins, aurofusarin, moniliformin, equisetin, DON, deoxynivalenol-3-glucoside, culmorin, bikaverin, beauvericin, fumonisins, fusaric acid, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, nivalenol, zearalenone, decalonectrin, butenolide, tentoxin, alternariol and alternariol methyl ether. The most abundant fungal metabolites were DON and culmorin, with an average contamination in the untreated control of 1,360 µg/kg and 875 µg/kg, respectively, in the growing season with the highest disease pressure (2011-2012). On average, the results have shown that the fungicide application significantly reduced the enniatins (from 127 µg/kg to 46 µg/kg), aurofusarin (from 62 µg/kg to 21 µg/kg), moniliformin (from 32 µg/kg to 16 µg/kg), tentoxin (from 5.2 µg/kg to 2.5 µg/kg) and equisetin (from 0.72 µg/kg to 0.06 µg/kg) contents in all the experiments. However, DON, deoxynivalenol-3-glucoside and culmorin were only significantly reduced in the growing season with the highest disease pressure. The other fungal metabolites were mainly found in traces in the untreated plots. These results, which have been obtained in different environmental and agronomic conditions, have underlined for the first time that the fungicide usually applied to control the FHB and DON content, also consistently reduces the main emerging mycotoxins of winter wheat in temperate areas.

Keywords: prothioconazole, deoxynivalenol, enniatins, moniliformin, aurofusarin

1. Introduction

The fungal diseases that affect wheat ears (*Triticum aestivum* L.) cause worldwide yield losses and promote the production of mycotoxins in cereal grains, as well as the contamination of wheat-derived feed and food products, with a negative impact on their safety and quality. *Fusarium* Head Blight (FHB) is the most widespread wheat ear disease, it causes total or partial premature ear senescence and consequently reduces both crop yields and grain quality. Several varieties of fungi, including *Microdochium nivale*, and different *Fusarium* species

could most probably be involved in promoting this disease (Champeil *et al.*, 2004). Among the *Fusarium* species, *Fusarium graminearum* and *Fusarium culmorum* are the most important FHB agents, and are the main causes of the production and the accumulation of type-B trichothecene deoxynivalenol (DON).

DON is responsible for serious mycotoxicosis in humans and animals. Several reports have suggested that the presence of DON in human food raises potential serious safety issues, particularly anorexia and vomiting (Pestka and Smolinski, 2005). Since DON is the most prevalent

toxin in crops used for food and feed consumption throughout the world (Mishra *et al.*, 2013), it has gained global attention over the last decade and regulatory limits have been set by the European Commission (EC, 2006, 2007), to protect humans from this and other mycotoxin exposure through cereal grain consumption (Regulation EC No. 1881/2006 and EC No. 1126/2007, with a limit of 1,250 µg/kg in unprocessed cereals other than durum wheat, oats and maize).

The presence of DON is closely linked to rainy climates or hot and humid conditions during the phenological phases between earing and milk ripeness (Van der Fels-Klerx *et al.*, 2013). A rainy event, close to flowering, accounts for more than 70% of the DON contamination present at the harvest (Hooker *et al.*, 2002). Moreover, *F. graminearum* has been found to dominate in regions with warm and humid conditions, whereas *F. culmorum* has been associated with cool, wet and humid conditions (Bottalico and Perrone, 2002).

The most important strategies to minimise DON occurrence in winter wheat, in areas characterised by a probable high FHB infection, are related to the use of preventive agronomic practices to reduce the pathogen inocula in field through crop rotation and soil tillage, and to the use of resistant varieties, following an integrated approach that addresses all the possible risk factors (Blandino *et al.*, 2012). However, in climatic conditions conducive to fungal diseases, the above preventive measures could be insufficient, and direct control, through the use of fungicide application, is necessary (Mesterházy *et al.*, 2003; Ransom and McMullen, 2008). Thus, in temperate areas, fungicide application from heading to anthesis is one of the most widely diffused practices in order to minimise the negative effects of these fungal diseases on wheat (Mesterházy *et al.*, 2003; Ransom and McMullen, 2008).

Fungicides containing triazole, imidazole or triazolothione active ingredients, which inhibit the biosynthesis of ergosterol, have proved to be the most active molecules for the control of FHB infection and the consequent DON contamination (Paul *et al.*, 2008), as they lead to a clear increase in grain yield, when the disease is present.

Unfortunately, DON is only one of the approximately 400 mycotoxins known to date (Berthiller *et al.*, 2007). Moreover, a very limited number of these mycotoxins is subject to legislation and regular monitoring. As far as cereals are concerned, aflatoxins, fumonisins, deoxynivalenol (DON), zearalenone (ZEA) and ochratoxin A (OTA) are those most often analysed (Binder, 2007). For these reasons, the other mycotoxins, which till now have not received detailed scientific attention, are commonly indicated as 'novel' or 'emerging' mycotoxins (Streit *et al.*, 2013). There is therefore an urgent need to acquire data on the presence

and diffusion of these emerging mycotoxins in field crops, in relationship to different climatic conditions, in order to establish possible maximum limits. It is also important to investigate the effect of the agronomic strategies that are commonly applied to control DON on the occurrence of the other mycotoxins, produced by other *Fusarium* spp. or genus.

Therefore, in order to set up the Best Management Practices to minimise the risk of mycotoxins in cereal, it is of great importance to assess the efficacy of fungicide applications to control also the emerging mycotoxins, taking into account different climatic conditions. Moreover, because of the differences in fungicide sensitivity of the different *Fusarium* spp. or of the other ear-colonising saprophytic fungi, antagonist to *Fusarium* spp., such as *Microdochium* spp. or *Alternaria alternata* (Müllernborn *et al.*, 2007), it is also necessary to verify whether there could be an unbalanced modification within the mycoflora of wheat ears, which could lead to an increase in some mycotoxins. This assessment is fundamental to understand, through a holistic approach, the effect of a fungicide application on wheat sanity to control FHB and DON contamination.

In the present study, four field experiments have been conducted in North West Italy, over a period of 2 growing seasons, in order to evaluate the effect of fungicide application on the contamination of emerging mycotoxins in common winter wheat (*Triticum aestivum* L.) under naturally-infected field conditions. The aim of this study was therefore to assess and verify, through experiments conducted in different pedo-climatic conditions, whether the fungicide usually applied to control FHB and DON content, could also result in a reduction of the main emerging mycotoxins of winter wheat.

2. Materials and methods

Experimental sites and treatments

Field experiments were carried out, from 2010 to 2012, in 2 growing seasons and in 3 sites in North West Italy:

- Experiment A: at Cigliano (45° 18' N, 8° 01' E; altitude of 237 m) in the 2010-2011 growing season, in a sandy loam soil, TypicHapludalfs (USDA classification);
- Experiments B and D: at Poirino (44° 54' N, 7° 24' E; altitude 262 m) in the 2010-2011 and 2011-2012 growing seasons, respectively, in a sandy silty loam soil, TypicUdifuvents (USDA classification);
- Experiment C: at Carmagnola (44° 50' N, 7° 40' E; altitude 245 m) in the 2011-2012 growing season, in a loam soil, TypicUdifuvents (USDA classification).

The wheat cultivars were Generale (Consorzio nazionale sementi, Conselice, Italy) in experiments A and C, Bologna (S.I.S. Società Italiana Sementi, San Lazzaro di Savena, Italy)

in experiment B, and Aubusson (Limagrain Italia, Busseto, Italy) in experiment D. All the cultivars used are classified as medium susceptible to FHB.

An azole fungicide, prothioconazole [Proline[®]; Bayer, Milano, Italy; emulsifiable concentrate formulation (EC), applied at 0.250 kg of active ingredient (AI)/ha] or a prothioconazole + tebuconazole mixture [Prosaro[®]; Bayer; emulsifiable concentrate formulation (EC), applied at 0.125 kg (AI)/ha] sprayed at heading [growth stage (GS) 59] (Zadoks *et al.*, 1974) was compared in all the fields with an untreated control, under naturally-infected field conditions. The fungicides were applied at the manufacturer's recommended field rates with a 4 nozzle precision sprayer (Honda Agricultural Sprayer T-Jeet 110/04; Honda Motor Europe, Ltd., London, UK), using a fine mist at a slow walk to ensure an effective coverage. The delivery pressure at the nozzle was 324 KPa.

The treatments for each field condition were assigned to experimental units using a completely randomised block design with three replicates. The plot size was 7×2 m. The fields were seeded after an autumn ploughing (30 cm) and disk harrowing to prepare a proper seedbed, following a previous maize crop for grain. Planting was conducted in 12 cm wide rows at a seeding rate of 450 seeds/m². The weed control was conducted with mesosulfuron-metile, iodosulfuron-metil-sodium and mefenpir-dietile (Hussar Maxx[®]; Bayer) at wheat tillering (GS 31). A total of 170 kg N/ha was applied to the plots as a granular ammonium nitrate fertiliser, and was split between GS 23, 32 and 45. The sowing, fungicide application and harvesting data of each experiment are reported in Table 1.

The grain yields were obtained by harvesting the whole plot with a Walter Wintersteiger cereal plot combine-harvester (Ried, Austria). A subsample was taken from each plot to determine grain moisture and test weight (TW). The TW was determined using a Dickey-John GAC2000 grain analysis meter (Dickey-John Europe, Colombes, France), according to the supplied programme. The TW was recorded as kg/hl. The grain yield results were adjusted to a 130 g/kg moisture content. The harvested grains were mixed thoroughly, and 2 kg grain samples were taken from each plot to analyse the mycotoxin content.

Fusarium Head Blight symptoms

FHB incidence and severity were recorded for each plot, by carrying out visual evaluations of the disease at the soft dough stage (GS 85). FHB head blight incidence was calculated as the percentage of ears with symptoms when 200 ears per plot were analysed. FHB severity was calculated as the percentage of kernels per ear with symptoms. A scale of 1 to 7 was used in which each numerical value corresponded to a percentage interval of surfaces exhibiting visible symptoms of the disease according to the following schedule: 1 = 0-5%, 2 = 5-15%, 3 = 15-30%; 4 = 30-50%, 5 = 50-75%, 6 = 75-90%, 7 = 90-100% (Parry *et al.*, 1995). The FHB severity scores were converted into percentages of the ear exhibiting symptoms, replacing each score with the mid-point of the interval.

Multi-mycotoxin LC-MS/MS analysis

A 2 kg representative sample of grain from each plot was ground using a ZM 200 Ultra Centrifugal Mill (Retsch GmbH, Haan, Germany) fitted with a 1 mm aperture sieve, and the resulting whole meal was used directly for the extraction. Five g representative sub-samples of the milled material were extracted using 20 ml of a mixture of acetonitrile:water:acetic acid (79:20:1, v/v/v). After extraction, the samples were centrifuged, diluted 1:1 and injected as described in detail by Sulyok *et al.* (2006). Five replicas of the 5 g ground wheat samples, free of or with very low levels of the detected mycotoxins, were spiked in order to evaluate the recovery rate of the analytical method for the different mycotoxins. Standards of fungal metabolites were obtained either as gifts from various research groups or from the following commercial sources: Biopure Referenzsubstanzen GmbH (Tulln, Austria), Sigma-Aldrich (Vienna, Austria), Iris Biotech GmbH (Marktredwitz, Germany), Axxora Europe (Lausanne, Switzerland) and LGC Promochem GmbH (Wesel, Germany). The average recovery percentages of the detected fungal metabolites were: 120.0% for alternariol (AOH), 113.9% for alternariol methyl ether (AME), 80.9% for aurofusarin (AUR), 100.3% for beauvericin (BEA), 95.7% for bikaverin (BIK), 86.3% for butenolide (BUT), 99.7% for culmorin (CULM), 103.5% for decalonectrin (DEC), 112.9% for DON, 100.2% for deoxynivalenol-3-glucoside

Table 1. Main trial information on the field experiments conducted on winter wheat in North West Italy in the 2010-2012 period.

Experiment	Growing season	Site	Sowing date	Fungicide application	Harvest date
A	2010-2011	Cigliano	30 October 2010	13 May 2011	1 July 2011
B	2010-2011	Poirino	5 November 2010	6 May 2011	29 June 2011
C	2011-2012	Carmagnola	21 October 2011	23 May 2012	11 July 2012
D	2011-2012	Poirino	24 November 2011	15 May 2012	4 July 2012

(DON-3G), 110.4% for 3-acetyldeoxynivalenol (3-ADON), 108.0% for 15-acetyldeoxynivalenol (15-ADON), 92.8% for enniatins (ENNs = ENN A, ENN A₁, ENN B, ENN B₁, ENN B₂), 200.1% for equisetin (EQU), 71.1% for fusaric acid (FA), 72.0% for fumonisins (FB₁, FB₂, FB₃), 95.7% for moniliformin (MON), 86.2% for nivalenol (NIV), 126.8% for tentoxin (TENT) and 117.0% for ZEA.

The results of the fungal metabolites concentrations were corrected on the basis of the recovery rate. Detection and quantification were performed with a QTrap 5500 LC-MS/MS System (Applied Biosystems, Foster City, CA, USA), equipped with a TurbolonSpray electrospray ionization (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini® C18-column, 150×4.6 mm i.d., 5 µm particle size, equipped with a C18 security guard cartridge, 4×3 mm i.d. (all from Phenomenex, Torrance, CA, USA). The chromatographic and mass spectrometric parameters of the investigated analytes were described by Sulyok *et al.* (2007) and Malachova *et al.* (2014). The applied multi-mycotoxin method was previously subjected to proficiency tests (Malachova *et al.*, 2014).

Statistical analysis

The normal distribution and homogeneity of variances were verified by performing the Kolmogorov-Smirnov normality test and the Levene test, respectively. An analysis

of variance (ANOVA) was conducted separately for each experiment in order to evaluate the effect of the fungicide application on grain yield, TW, FHB incidence and severity, and DON contamination, using a completely randomised block design. Moreover, ANOVA was utilised to compare the emerging mycotoxin content, using a completely randomised block design, in which the fungicide treatment was the independent variable and the experiment (different growing seasons and sites) was the random factor. Instead, when the interaction between the fungicide treatment and the experiment was significant, the ANOVA was applied separately for each experiment. The fungal metabolites concentrations were transformed using the $y' = \ln(x+1)$ equation to normalise the residuals. The SPSS for Windows, Version 20.0 statistical package (SPSS Inc., Chicago, IL, USA), was used for the statistical analysis.

3. Results

Weather conditions

The two growing seasons showed different meteorological trends from the beginning of the stem elongation stage to harvesting (Table 2). Frequent rainfall occurred in 2011, at the beginning of stem elongation (March) and at the end of ripening (June). Moreover, the rainfall was low close to the anthesis stage (May) in both experiments conducted in the 2010-11 growing season (A and B). Instead, in 2012, rainfall was concentrated close to the end of the stem elongation

Table 2. Total rainfall, rainy days, relative humidity and growing degree days (GDDs) from March to June 2010-2012 in the research sites.

		Experiment A			Experiment B		
Growing season	Month	Rainfall (mm)	Rainy days (n)	GDDs ¹ (°C/d)	Rainfall (mm)	Rainy days (n)	GDDs ¹ (°C/d)
2010-2011	March	169	9	268	211	12	264
	April	59	5	474	33	3	467
	May	30	5	578	47	5	571
	June	198	14	627	252	17	599
	Flowering period ²	6	2	129	0	0	116
		Experiment C			Experiment D		
Growing season	Month	Rainfall (mm)	Rainy days (n)	GDDs ¹ (°C/d)	Rainfall (mm)	Rainy days (n)	GDDs ¹ (°C/d)
2011-2012	March	20	1	347	36	3	394
	April	148	13	365	160	14	370
	May	147	6	539	123	7	538
	June	19	2	674	38	6	680
	Flowering period ²	106	4	124	61	3	113

¹ Accumulated growing degree days for each month using a 0 °C base.

² The flowering period corresponds to the days between the first visible anthers (BBCH 61) and the end of the flowering (BBCH 69).

and anthesis in both experiments (C and D), and lower growing degree days were recorded than those of 2011.

Fusarium Head Blight symptoms

The incidence and severity of the FHB symptoms recorded during the visual evaluations were higher in the 2011-2012 growing season than in the 2010-2011 one (Table 3). According to the weather conditions during the growing season, the mean FHB incidence and severity of the untreated control were 63.0 and 14.4%, respectively, in experiments C and D (growing season 2011-2012), while these parameters were 8.7 and 1.4%, respectively, in the experiments carried out in 2010-2011 growing season. ANOVA showed a significant effect of the fungicide treatments on FHB incidence and severity in all the experiments ($P < 0.05$), except experiment B. Considering all the experiments, the azole application at heading significantly reduced the FHB incidence and severity compared to the untreated control, with an average reduction of 66 and 84%, for the two parameters, respectively.

Yield and yield components

ANOVA showed a significant effect of fungicide treatment on grain yield in all the experiments ($P < 0.05$), except experiment A (Table 3). In these experiments, the fungicide application significantly increased the yield by 12% compared to the untreated control. The fungicide treatment only significantly increased the test weight parameter by 2.5 kg/hl in experiment B, but a similar trend was observed in all the other experiments.

Mycotoxin contamination

The dilute-and-shoot multi-mycotoxin LC-MS/MS method was able to detect about 20 fungal metabolites: ENNs (ENN A, ENN A₁, ENN B, ENN B₁, ENN B₂), AUR, MON, EQU, DON, DON-3G, CULM, BIK, BEA, fumonisins (FB₁, FB₂, FB₃), FA, 3-ADON, 15-ADON, NIV, ZEA, DEC, BUT, TENT, AOH and AME.

The most abundant fungal metabolites were DON, DON-3G and CULM, with an average contamination in the untreated control of 1,360, 367 and 875 µg/kg, respectively, in the growing season with the highest disease pressure (2011-2012). Instead, in the growing season with the lowest disease pressure (2010-2011), DON, DON-3G and CULM were present with an average contamination in the untreated control of 72, 32 and 61 µg/kg, respectively. Since a significant 'experiment × fungicide treatment' interaction was recorded by ANOVA for these three mycotoxins, which are produced by the same *Fusarium* species (*F. graminearum* and *F. culmorum*), the 4 experiments were statistically analysed separately and the results are reported in Figure 1. All these mycotoxins were significantly reduced by the fungicide application ($P < 0.01$ for experiment C and $P < 0.05$ for experiment D), but only in the experiments conducted in the growing season with the highest FHB attack (2011-2012). In these experiments, on average, the fungicide treatment significantly reduced the DON, DON-3G and CULM contents by 72, 67 and 70%, respectively, compared to the untreated control.

On the other hand, ANOVA showed a significant effect of fungicide application on the occurrence of ENNs, AUR, MON, TENT and EQU, while the interaction between the fungicide treatment and the experiment was never

Table 3. Effect of fungicide application on *Fusarium* Head Blight (FHB) incidence and severity, grain yield and test weight of winter wheat. The field experiments¹ (A, B, C and D) were conducted in North West Italy in the 2010-2012 period.

Parameters	Experiment A		Experiment B		Experiment C		Experiment D	
	Untreated control	Fungicide ²	Untreated control	Fungicide ²	Untreated control	Fungicide ²	Untreated control	Fungicide ²
FHB incidence ³ (%)	8.2	2.8*	9.2	3.4	78.4	38.9***	47.6	6.2**
FHB severity ⁴ (%)	1.7	0.3*	1.1	0.4	22.7	1.2**	6.1	0.3*
Yield (t/ha)	3.1	3.1	4.0	4.5*	6.0	6.5*	7.5	8.6*
Test weight (kg/hl)	78.6	79.1	78.6	81.1*	82.8	83.7	76.3	78.9

¹ The main field experiment information is reported in Table 1. The reported data are the average of 3 replications.

² Fungicide treatment at heading: experiments A and C = prothioconazole (formulation: EC, 0.250 kg active ingredient/ha); experiments B and D = prothioconazole + tebuconazole mixture (formulation: EC, 0.125 kg active ingredients/ha). *, **, *** = level of significance of ANOVA. * = $P(F) < 0.05$, ** = $P(F) < 0.01$, *** = $P(F) < 0.001$.

³ FHB incidence was calculated as the percentage of ears with FHB damage, considering 200 ears per sample.

⁴ FHB severity was calculated as the percentage of kernels per ear with FHB damage, considering 200 ears per sample.

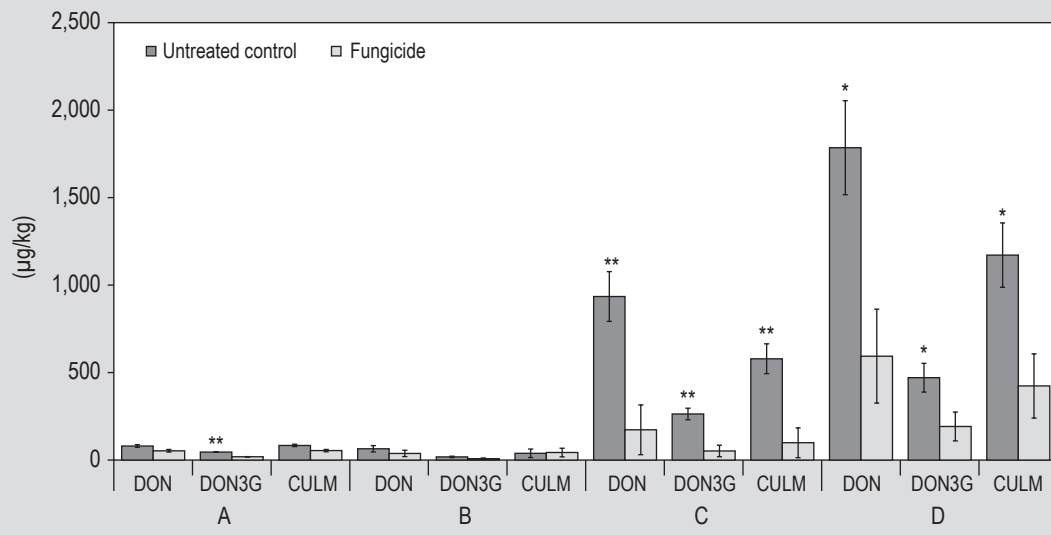


Figure 1. Effect of fungicide application on deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON-3G) and culmorin (CULM) contaminations in 4 field experiments carried out in North West Italy in the 2010-2012 period. The main field experiment information is reported in Table 1. *P* (F) = level of significance of ANOVA; * *P*<0.05, ** *P*<0.01.

Table 4. Effect of fungicide application on enniatins (ENNs), aurofusarin (AUR), moniliformin (MON), tentoxin (TENT) and equisetin (EQU) contamination^{1,2} in winter wheat grain. The field experiments were conducted in North West Italy in the 2010-2012 period.

Source of variation	Factor	ENNs		AUR		MON		TENT		EQU	
		T	N (µg/kg)	T	N (µg/kg)	T	N (µg/kg)	T	N (µg/kg)	T	N (µg/kg)
Experiment ³	A	3.7	51	4.1	66	1.3	4	0.91	1.61	0.28	0.58
	B	3.4	73	1.9	7	2.1	17	1.25	2.72	0.04	0.04
	C	4.0	69	2.7	22	2.8	25	1.19	2.51	0.29	0.40
	D	4.8	144	4.1	69	3.8	49	2.13	8.13	0.29	0.42
Treatment ⁴	Untreated control	4.6	127	3.9	62	3.0	32	1.65	5.20	0.42	0.72
	Fungicide	3.4	46	2.6	21	2.1	16	1.14	2.57	0.06	0.06
	<i>P</i> (F) ⁵	*		*		*		**		*	
Treatment × Experiment	<i>P</i> (F) ⁵	NS		NS		NS		NS		NS	

¹ The reported values of the experiment factor are based on 6 replications (2 treatment × 3 repetitions), while the values of the treatment factor are based on 12 replications (4 experiment × 3 repetitions).

² The reported mycotoxin contamination means are transformed [T; $y' = \ln(x + 1)$] and not transformed (N) values.

³ The main field experiment information is reported in Table 1.

⁴ Fungicide treatment at heading: experiments A and C = prothioconazole (formulation: EC, 0.250 kg active ingredient/ha); experiments B and D = prothioconazole + tebuconazole mixture (formulation: EC, 0.125 kg active ingredients/ha).

⁵ *P* (F) = level of significance of ANOVA. * = *P* (F)<0.05, ** = *P* (F)<0.01, NS = not significant.

significant (Table 4). On average, the fungicide application significantly reduced the ENNs (from 127 to 46 µg/kg, *P*=0.036), AUR (from 62 to 21 µg/kg, *P*=0.032), MON (from 32 to 16 µg/kg, *P*=0.036) TENT (from 5.20 to 2.57 µg/kg, *P*=0.002) and EQU (from 0.72 to 0.06 µg/kg, *P*=0.050) contents. Compared to the untreated control, the average reduction recorded was 64, 66, 50, 51 and 92% for ENNs, AUR, MON, TENT and EQU, respectively.

Fumonisin, FA, ZEA and NIV were found at low contamination levels, while ANOVA showed significant 'experiment × fungicide' interactions for DON, DON-3G and CULM. The effect of fungicide application on fumonisin, FA, ZEA and NIV contents is shown in Table 5 for each experiment. Fumonisin and FA were significantly reduced after the fungicide application (*P*<0.05) in experiment D, which was conducted in the growing

Table 5. Effect of fungicide application on fungal metabolites contamination in winter wheat grain. The field experiments¹ (A, B, C and D) were conducted in North West Italy in the 2010-2012 period.

Fungal metabolites ²	Experiment A		Experiment B		Experiment C		Experiment D	
	Untreated control (µg/kg)	Fungicide ³ (µg/kg)	Untreated control (µg/kg)	Fungicide ³ (µg/kg)	Untreated control (µg/kg)	Fungicide ³ (µg/kg)	Untreated control (µg/kg)	Fungicide ³ (µg/kg)
FB	5.58	8.11	6.24	9.77	5.73	8.69	12.81	8.82*
FA	<LOQ	<LOQ	9.99	<LOQ	5.24	<LOQ	16.78	3.30*
BEA	0.63	0.48	1.45	0.63	0.11	0.04	0.31	0.16
BIK	<LOQ	<LOQ	2.70	1.84	<LOQ	<LOQ	<LOQ	<LOQ
ZEA	1.32	<LOQ*	0.71	<LOQ	1.60	<LOQ	4.38	0.52*
NIV	0.75	<LOQ	<LOQ	<LOQ	0.68	<LOQ	1.13	<LOQ
3-ADON	<LOQ	<LOQ	<LOQ	<LOQ	6.67	<LOQ	9.84	5.06
15-ADON	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BUT	3.33	1.98	<LOQ	<LOQ	5.48	<LOQ	6.11	3.10
DEC	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1.29	0.89
AOH	2.68	1.46	<LOQ	<LOQ	0.28	<LOQ	<LOQ	<LOQ
AME	0.53	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.13	<LOQ

¹ The information of different field experiments is reported in Table 1. The reported data are the average of 3 replications. * = level of significance of ANOVA. * = $P(F) < 0.05$.

² FB = fumonisins (LOQ = limit of quantification = 0.70 µg/kg); FA = fusaric acid (LOQ = 0.70 µg/kg); BEA = beauvericin (LOQ = 0.006 µg/kg); BIK = bikaverin (LOQ = 0.06 µg/kg); ZEA = zearalenone (LOQ = 0.20 µg/kg); NIV = nivalenol (LOQ = 0.20 µg/kg); 3-ADON = 3-acetyldeoxynivalenol (LOQ = 0.20 µg/kg); BUT = butenolide (LOQ = 1.10 µg/kg); DEC = decalonectrin (LOQ = 0.06 µg/kg); AOH = alternariol (LOQ = 0.10 µg/kg); AME = alternariol methyl ether (LOQ = 0.10 µg/kg). Moreover, 15-ADON = 15-acetyldeoxynivalenol was also detected, but always under the LOQ (0.43 µg/kg).

³ Fungicide treatment at heading: experiments A and C = prothioconazole (formulation: EC, 0.250 kg active ingredient/ha); experiments B and D = prothioconazole + tebuconazole mixture (formulation: EC, 0.125 kg active ingredients/ha).

season with the highest FHB disease pressure (2011-2012). Moreover, ZEA was significantly reduced with the fungicide application ($P < 0.05$) in experiments A and D. As far as FA, ZEA and NIV are concerned, the fungicide application on average reduced their contents in the experiments in which no significant differences in FHB attack were observed, in comparison to the untreated control. On the other hand, fumonisin content was not affected by the azole application in experiments A, B and C.

The other fungal metabolites (BIK, BEA, 3-ADON, 15-ADON, DEC, BUT, AOH and AME) were only found in a few cases in traces, but mainly in the untreated plots (Table 5). A similar contamination reduction trend was also observed for these mycotoxins, after the fungicide application.

4. Discussion

The findings of this research confirm that the application of azole fungicides could contribute to control FHB in areas characterised by a probable high infection and under climatic conditions that favour fungal diseases. These results are in agreement with several other experiments (Edwards

et al., 2001; Matthies and Buchenauer, 2000; Menniti *et al.*, 2003; Paul *et al.*, 2008) that have proved the effectiveness of fungicides containing triazole or triazolothione in the control of FHB infection. In particular, among the most recent fungicides, the active ingredient prothioconazole has confirmed its high capacity to reduce DON contamination in cereal grain (Haidukowski *et al.*, 2012).

Since the field experiments were conducted over two years characterised by different meteorological trends, the collected data also show that the significant efficacy of the fungicide application on DON contamination is closely linked to the pressure level of the disease. In a previous work conducted in NW Italy (Blandino *et al.*, 2011), DON contamination was reduced by the fungicide to a greater extent in the low risk agronomic and environmental conditions than in the high risk ones.

As regards the contamination levels, the obtained results show that mycotoxins such as DON, DON-3G and CULM, which are mainly produced by *F. culmorum* and *F. graminearum*, were the most abundant mycotoxins in the environmental conditions of the considered field experiments. The contamination of all these mycotoxins

was higher, especially in the growing season with the highest disease pressure (2011-2012), thus confirming the close link between these *Fusarium* spp. and FHB. DON-3G, one of the several masked mycotoxins, is a phase II plant metabolite of the *Fusarium* mycotoxin DON (Berthiller *et al.*, 2013) which could be hydrolysed in the digestive tract of mammals, thus contributing to the total dietary DON exposure of individuals (Berthiller *et al.*, 2011). Moreover, data reported by Lemmens *et al.* (2005) and by Cirilini *et al.* (2014) support the hypothesis that, in resistant wheat lines, most of the DON is converted to DON-3G and this conversion mechanism actually seems to be related to their resistance to FHB. As far as the occurrence and the relationship between DON and DON-3G are concerned, the present results are supported by data from other studies which show that the DON-3G/DON ratio varies in relation to the year and genotype and can reach levels of 29% (Berthiller *et al.*, 2009) or even 70% (De Boevre *et al.*, 2012). On the other hand, the relationship between the DON and CULM contents reported in the present work is in agreement with data obtained by Ghebremeskel and Langseth (2000), which indicate that CULM and various hydroxy-culmorins (5- and 15-hydroxy-culmorin) are present in the same concentration ranges as DON in naturally contaminated grain. Moreover, although CULM and hydroxy-culmorins have shown low toxicity in several *in vitro* assays (Pedersen and Miller, 1999), they may contribute to enhancing the toxicity of DON (Ghebremeskel and Langseth, 2000).

The obtained results are also in agreement with several studies that show that: (1) *Fusarium culmorum* and *F. graminearum* are potent colonisers of wheat tissue that can compete and reduce the growth of other *Fusarium* spp. (Jones *et al.*, 1997) or other toxigenic fungi such as *Alternaria* spp. (Müller *et al.*, 2012); (2) in temperate areas, DON is the most prevalent mycotoxin in wheat (Mishra *et al.*, 2013).

As far as the effectiveness of fungicide application to control these mycotoxins is concerned, on average, azole applications at heading have reduced the DON, DON-3G and CULM contents by about 70%, compared to an untreated control. On the other hand, although the ENNs, AUR, MON, EQU, BIK, BEA, fumonisins, FA, 3-ADON, NIV, ZEA, DEC, BUT, TENT, AOH and AME have been detected at lower levels than the DON, DON-3G and CULM, the presence of some of them have been reported for the first time in the current study in naturally infected winter wheat from Southern Europe. The levels of MON, BEA and NIV are comparable to those reported by Jestoi *et al.* (2004), Uhlig *et al.* (2007, 2013) and Lindblad *et al.* (2013). ENNs have been detected at comparable or slightly lower levels, depending on which growing season was considered in the previously cited studies, while AUR, ZEA, EQU, BUT and 3-ADON are present at lower values than those found by Uhlig *et al.* (2013). The *Alternaria* toxins,

AOH and AME, are instead present at lower values than those reported by Müller and Korn (2013) and Uhlig *et al.* (2013), while tentoxin, although only present in traces, has been found for the first time in naturally contaminated winter wheat. Finally, fumonisins, FA, BIK and DEC have also been detected in traces and, until now, no evidence has been reported about their occurrence in naturally contaminated winter wheat.

As far as the effectiveness of a fungicide application to control these mycotoxins is concerned, on average, in the different field experiments conducted in the current study, the fungicide treatment has significantly reduced, by more than 50%, the occurrence of the emerging mycotoxins. The mycotoxins detected in traces, were also mainly present in the untreated plots and a similar contamination reduction trend was observed after the fungicide application.

The FHB of wheat is mainly caused by a complex of *Fusarium* species, including *F. graminearum*, *F. culmorum*, *Fusarium avenaceum*, *Fusarium poae* and *Fusarium sporotrichioides* (Parry *et al.*, 1995), and all of these *Fusarium* species are able to produce a wide range of mycotoxins. For this reason, it is necessary to take into account the distribution and predominance of FHB pathogens and the consequently produced mycotoxins which vary from year to year and which are closely linked to environmental and climatic factors (Doohan *et al.*, 2003). The competitive interaction that occurs between the application of different fungicides classes and the fungal species involved in FHB in wheat has been well documented in literature. The strobilurin fungicides have shown poor efficacy against FHB caused by toxigenic *Fusarium* spp., such as *F. culmorum*, *F. avenaceum* and *F. graminearum*, while they have ensured a significant reduction in *M. nivale*, a non-toxigenic pathogen which, unlike the *Fusarium* species, despite being involved in the symptomatology of the disease, is not able to synthesize DON (Edwards, 2004; Simpson *et al.*, 2001). Moreover, during *in vitro* studies, strobilurins have been shown to induce the production of DON (D'Mello *et al.*, 2001), while during field studies it emerged that these molecules do not seem to have a direct effect on the synthesis of the toxin (Pirgozliev *et al.*, 2002). In these previous cases, the increase in DON contamination was probably due to a reduction in the presence of *M. nivale*, which resulted in an increase in the DON-toxigenic *Fusarium* species (Pirgozliev *et al.*, 2003).

In the field experiments analysed in the present study, mycotoxins produced by several different *Fusarium* spp. have been detected together with traces of those produced by *Alternaria* spp. (Table 6). The present results show a similar contamination reduction trend after the fungicide application, not only for DON but also for almost all the mycotoxins present at different contamination levels.

Table 6. Main producing species of mycotoxins detected in the wheat samples.

Mycotoxin	Produced by	References	Mycotoxin	Produced by	References
Deoxynivalenol	<i>Fusarium graminearum</i> ; <i>Fusarium culmorum</i>	Bottalico and Perrone, 2002; Rasmussen <i>et al.</i> , 2012	Alternariol methyl ether	<i>A. alternata</i>	Müller and Korn, 2013; Prelle <i>et al.</i> , 2013
Deoxynivalenol-3-glucoside	Phase II plant metabolite of deoxynivalenol ('masked mycotoxin')	Berthiller <i>et al.</i> , 2013; De Boevre <i>et al.</i> , 2013	3/15-acetyl-deoxynivalenol	Phase I plant metabolite of deoxynivalenol ('masked mycotoxin')	Berthiller <i>et al.</i> , 2013; De Boevre <i>et al.</i> , 2013
Culmorin	<i>F. graminearum</i> ; <i>F. culmorum</i>	Pedersen and Miller, 1999; Ghebremeskel and Langseth, 2000; Streit <i>et al.</i> , 2013;	Nivalenol	<i>F. graminearum</i> ; <i>F. culmorum</i>	Bottalico and Perrone, 2002; Lindblad <i>et al.</i> , 2013
5/15-hydroxy-culmorin	<i>F. graminearum</i> ; <i>F. culmorum</i>	Pedersen and Miller, 1999; Ghebremeskel and Langseth, 2000; Streit <i>et al.</i> , 2013	Zearalenone	<i>F. graminearum</i> ; <i>F. culmorum</i>	Bottalico and Perrone, 2002; Lindblad <i>et al.</i> , 2013
Enniatins	<i>Fusarium avenaceum</i>	Bottalico and Perrone, 2002; Lindblad <i>et al.</i> , 2013	Butenolide	<i>F. graminearum</i> ; <i>F. culmorum</i>	Wang <i>et al.</i> , 2009; Streit <i>et al.</i> , 2013
Aurofusarin	<i>F. avenaceum</i> ; <i>F. graminearum</i> ; <i>F. culmorum</i>	Uhlig <i>et al.</i> , 2006; Streit <i>et al.</i> , 2013	Decalonectrin	<i>F. graminearum</i> ; <i>Fusarium sporotrichioides</i>	McCormick <i>et al.</i> , 2004
Moniliformin	<i>F. avenaceum</i> ; <i>Fusarium subglutinans</i> ; <i>F. proliferatum</i>	Jestoi, 2008; Lindblad <i>et al.</i> , 2013	Fumonisin	<i>Fusarium verticillioides</i> ; <i>F. proliferatum</i>	Palacios <i>et al.</i> , 2011; Scott, 2012; Cendoya <i>et al.</i> , 2014
Equisetin	<i>Fusarium equiseti</i>	Wheeler <i>et al.</i> , 1999; Streit <i>et al.</i> , 2013	Fusaric acid	<i>F. verticillioides</i> ; <i>F. proliferatum</i>	Bacon <i>et al.</i> , 1996; Shimshoni <i>et al.</i> , 2013
Tentoxin	<i>Alternaria alternata</i>	Prelle <i>et al.</i> , 2013; Shimshoni <i>et al.</i> , 2013	Beauvericin	<i>F. avenaceum</i> ; <i>Fusarium subglutinans</i> ; <i>F. proliferatum</i>	Jestoi, 2008; Lindblad <i>et al.</i> , 2013
Alternariol	<i>A. alternata</i>	Müller and Korn, 2013; Prelle <i>et al.</i> , 2013	Bikaverin	<i>F. verticillioides</i>	Lazzaro <i>et al.</i> , 2012

Although the present study was not set up to analyse the infection and development of fungal community in detail, the collected data, considering the final level of several fungal metabolites, show that the applied azole treatments are able to reduce both FHB infection caused by *Fusarium* spp. and the occurrence of other saprophytic fungi antagonist to *Fusarium* spp., such as *Alternaria alternata*. These results are supported by Bertelsen *et al.* (2001) and Müllenborn *et al.* (2007), which reported that, although the growth reduction of *Alternaria alternata* obtained through the use of strobilurins was stronger than that obtained through the use of triazoles, these last cited fungicides were also able to inhibit the mycelia growth of this saprophyte fungus. Thus, no evidence has emerged of an increase in any mycotoxin as a consequence of a modification in the fungal community, related to the different control capacity of the azole fungicides.

In conclusion, the results of these experiments, obtained under naturally-infected field conditions and conducted over two growing seasons characterised by extremely different meteorological trends and FHB pressures, underline that the application of an azole fungicide at heading (prothioconazole and tebuconazole), the most common FHB and DON contamination control practice applied in temperate areas, also results in a consistent and clear reduction in the other winter wheat mycotoxins.

In order to reconsider wheat protection programmes, and to take into account emerging mycotoxins, a fungicide treatment, applied to control fungal ear infection, could also contribute to improving the global sanity of this crop. Therefore, since Good Agricultural Practices require an integrated approach that addresses all the possible risk factors in order to prevent mycotoxin contamination, the role of other practices, which have proved to have a significant effect on DON control, such as soil tillage,

crop rotation and cultivar susceptibility to FHB, need to be considered to verify their effectiveness on emerging mycotoxins.

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