Impact of sowing time, hybrid and environmental conditions on the contamination of maize by emerging mycotoxins and fungal metabolites

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Abstract

Mycotoxins and other fungal metabolites represent the most insidious safety risks to cereal food and the feed chain. Optimising agronomic practices is one of the main strategies adopted to minimise the contents of these undesirable substances in grain-based commodities. The aim of this study was to investigate the effect of the combination of sowing times and hybrids on the occurrence of emerging mycotoxins and fungal metabolites in maize. Field experiments were carried out in 2 sowing times (early *vs* late) and 3 maize hybrids were compared in the 2014 and 2015 growing seasons. Overall, 37 fungal metabolites produced by *Fusarium* and *Alternaria* species were detected. Apart from fumonisins type B (FBs), other metabolites produced by *Fusarium verticillioides* and *F. proliferatum,* such as fumonisins type A, fusaric acid, bikaverin and fusaproliferin, were also detected in all of the samples. Fusarin C was found in 61% of the samples. Deoxynivalenol (DON), deoxynivalenol-3-glucoside, culmorin and zearalenone, all of which are produced prevalently by *Fusarium graminearum* and *F. culmorum,* were found in all the samples. Their contents were clearly affected by the meteorological trend: the highest contamination was detected in the 2014 growing season, which was characterised by abundant rainfall and lower temperatures from flowering to maize ripening. Among the mycotoxins produced by other *Fusarium* species, aurofusarin was found to clearly be associated with DON, while moniliformin and beauvericin followed the same behaviour as the FBs.

A late sowing time significantly increased the FBs and fumonisin-associated mycotoxins in both growing seasons. The increase in contamination with the delay of sowing was more pronounced in the 2015 growing season, as the environmental conditions were less favourable to the infection of other *Fusarium* species. The effect of sowing time on DON and DON-associated mycotoxins produced conflicting results for the two growing seasons, because contamination by these metabolites depends more on the conditions that occur during maize flowering than those that occur during ripening. A clearer hybrid susceptibility was observed for these compounds. Other metabolites, such as enniatins, equisetin, T-2 and HT-2 toxins and *Alternaria* toxins, were always found in traces. The occurrence of these metabolites seems to be influenced less by the considered agronomic practices.

The results, obtained under naturally-infected field conditions, underline the key role that the sowing date and hybrid susceptibility play in influencing, in a variable way, the contamination of mycotoxins produced by different *Fusarium* species in maize subjected to different meteorological conditions. The content of mycotoxins produced by *Fusarium* spp. section *Liseola* is more directly and steadily related to late sowing time, while the contamination of mycotoxins associated to *Fusarium* spp. section *Discolor* depend more strongly on the environmental conditions at maize flowering and on hybrid susceptibility.

Introduction

Over the last decade, the increasing attention paid to safety aspects has affected cereal supply chains: the lack of adequate sanitary parameters pertaining to the presence of contaminants is actually the first cause of the decrease in the market value of cereal commodities (Pinotti and Dell'Orto, 2011). Mycotoxins and other fungal metabolites represent the most insidious safety risks for cereal food and the feed supply chain (Pinotti *et al.*, 2016). The occurrence of these compounds is a topic of great concern, because of the consequent negative health effect on humans and animals (Wild and Gong, 2010). In fact, mycotoxins, which are

secondary metabolites produced by several filamentous fungi, could cause acute or chronic toxicity in livestock and humans, including carcinogenicity and neurotoxicity, as well as developmental and reproductive toxicity, thus resulting in economic losses (Wu, 2007). Five groups of mycotoxins in particular are considered to be the most economically and toxicologically important throughout the world (Binder *et al.*, 2007): aflatoxins and ochratoxins, which are produced by the fungi of the *Aspergillus and Penicillium genera*, deoxynivalenol and zearalenone, which are mainly produced by *Fusarium graminearum* and *F. culmorum* (section *Discolor*), and fumonisins, which are mainly produced by *F. verticillioides and F. proliferatum* (section *Liseola*). Regulatory limits have been set by the European Commission (EC) to protect humans and livestock from exposure to the main mycotoxins through cereal consumption (EC, 2006a, 2007, 2011). Moreover, guidance values have been issued for the main toxins produced by *Fusarium* spp. in products intended for animal feeding (EC, 2006b).

Cereal grains, and maize in particular, are likely to be contaminated before harvesting by all of these mycotoxins (Lee and Ryu, 2017): the occurrence of these metabolites is dependent to a great extent on the co-existence of hybrid susceptibility and certain environmental factors that are favourable to fungal infection, growth and toxinogenesis, such as temperature and moisture at crucial growth stages (GS) (Lancashire *et al.*, 1991). Thus, the application of specific agronomic practices could play a crucial role in minimising the contamination levels of these compounds, by means of the control of their biosynthesis as well as their fungal infection and development during plant cultivation (Munkvold, 2003). Among the agricultural practices employed in maize cultivation, the application of an early sowing time is one of those which has shown the most important impact on the reduction in the risk of mycotoxin contamination. Several studies have shown the clear need for an early sowing time to reduce the risk of contamination by fumonisins (Blandino *et al.*, 2009a, 2009b, 2009c; Camardo Leggieri *et al.*, 2015; Parson and Munkvold, 2012), which are the main mycotoxins commonly detected in maize grain in temperate areas. In addition to fumonisins, it has also been observed that the application of a delayed sowing time may be responsible for an increase in deoxynivalenol (Blandino *et al.*, 2009b) and aflatoxin (Camardo Leggieri *et al.*, 2015) contamination in maize kernels. Thus, the choice of the proper sowing time for each geographic region and hybrid maturity probably represents the best crop technique to control the contamination of maize from the main regulated mycotoxins. However, to date, about 400 different mycotoxins have been identified in different commodities, and several of these have been found in maize (Kovalsky *et al.*, 2016). These fungal metabolites, some of which have been referred to as *emerging* (Streit *et al.*, 2013), have not yet received detailed scientific attention. The European Food Safety Authority (EFSA) needs data about the occurrence of these mycotoxins in cereals in order to evaluate the risk of exposure of the European population, and to make risk assessments (EFSA, 2010, 2014). Moreover, there is also a great interest in verifying the effect of the Good Agricultural Practices (GAP) that are normally applied in the control of the fumonisin contamination of maize on the control of contamination by other emerging mycotoxins. In this way, it will be possible to set up field programmes that will be able to minimise the overall sanitary risk (related) to maize grains. Since emerging mycotoxins can be produced by other *Fusarium* species, characterised by a different ecology from that of FB producers, as well as by other fungal species belonging to the *Alternaria*, *Penicillium* and *Aspergillus* genera, more detailed knowledge about the environmental and agronomic conditions that promote their occurrence is of great importance.

The aim of this study was to investigate the effect of environmental conditions, related to different growing seasons, of the sowing times and of the type of hybrids with different maturity lengths on the occurrence of emerging mycotoxins and fungal metabolites in maize.

Materials and methods

Chemicals

Methanol and acetonitrile (both LC gradient grade) were purchased from J.T. Baker (Deventer, The Netherlands); ammonium acetate (MS grade) and glacial acetic acid (p.a.) were obtained from Sigma–Aldrich (Vienna, Austria). Water was purified successively by means of reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France).

All the fungal metabolite standard solutions were stored at −20°C, and were brought to room temperature before use.

Experimental design and samples

The effect of the sowing date on emerging mycotoxin occurrence in maize was studied, during the 2014 and 2015 growing seasons, in two experimental areas in North-West Italy, characterised by a sandy-loam soil (Typic Hapludalfs according to the USDA classification). The experiment was conducted in two places: the first experimental year at Buriasco (44° 51' N, 7° 26' E; altitude 301 m), whereas the second year at Chivasso (44° 12' N, 7° 55' E; altitude 183 m). Daily temperatures and precipitations were measured at a meteorological station located near the experimental areas.

The compared treatments, in both growing seasons, under natural conditions, were factorial combinations of: 2 sowing dates [an early sowing date (at the beginning of April) and a late sowing date (in the middle of May)]; 3 maize hybrids with different lengths of maturity [Pioneer P1208 belonging to FAO maturity class 500 (medium-early maturity hybrid - 125 relative days to maturity); Monsanto DKC6815 belonging to FAO maturity class 600 (full season maturity hybrid - 130 relative days to maturity) and Pioneer PR32B10 belonging to FAO maturity class 600 (full season maturity hybrid - 132 relative days to maturity)].

The treatments were assigned to experimental plots using a completely randomised block design, with three replications. The plot size was 10 x 3 m, and each plot consisted of 4 rows. The plot alleys, orthogonal to the maize rows, were one meter wide.

Conventional agronomic techniques were adopted for the field experiments in both growing seasons. Briefly, the previous crop was maize, and mechanical sowing was carried out after an autumn ploughing (30 cm) and disk harrowing to prepare a suitable seedbed. The crop density was approximately 78,000 plants per hectare. All the plots received the same amount of nutrients: 300, 100 and 100 kg ha^{-1} of N, P$_2$O$_5$ and K$_2$O, respectively. Irrigation was carried out by means of the furrow surface method in order to prevent drought stress until the end of the dough stage (GS 87). Weed control was conducted, at a pre-emergence stage, with mesotrione (0.15 kg AI ha^{-1}), S-metolachlor (1.25 kg AI ha^{-1}) and terbuthylazine (0.75 kg AI ha^{-1}) (Lumax®, Syngenta Crop Protection S.p.A., Milan, Italy). All the plots were sprayed at GS 75 with pyrethroid lambda-cyhalothrin insecticide (Karate® Zeon, Syngenta Crop Protection S.p.A., Milan, Italy) at 0.019 kg AI ha^{-1}

using a self-propelled ground sprayer (Eurofalcon E140®, Finotto, Italy). The treatment was performed, according to the growth stage, in the middle of July for the early sowing date and in the first decade of August for the late sowing date, in order to minimise the ear injuries caused by the activity of the European Corn Borer (ECB – *Ostrinia nubilalis*, Hübner). The sowing, harvest and the insecticide application dates for each growing season are reported in Table 1.

Ears were collected by hand from 6 m^2 (2 rows x 4 m) of each plot at harvest maturity in order to quantify the grain yield (adjusted to a 14% moisture content), and to obtain a representative grain sample for the mycotoxin analyses. The ears were shelled using an electric sheller, and kernels from each plot were mixed thoroughly to obtain a random distribution. Samples (4 kg) were then taken, dried at 60°C for 3 days and ground using a ZM 200 Ultra Centrifugal Mill (Retsch GmbH, Haan, Germany), fitted with a 1 mm aperture sieve to obtain a whole meal, which was then used directly for the extraction and the analysis of the mycotoxins.

The moisture content of the wet maize grain was determined by means of a Dickey-John GAC 2000 grain analysis meter (Dickey-John Corp. Auburn, IL, USA), using the supplied program.

Entomological and mycological measurements

The ECB damage severity and fungal ear rot severity were calculated on 20 randomly sampled ears from each plot at harvest maturity. The ECB damage severity was calculated as the percentage of kernels per ear with injuries due to larva activity. A scale of 1 to 7 was used to quantify the percentage interval of surfaces exhibiting visible kernel damage due to larva activity: 1=no injuries; 2=1-5%; 3=6-10%; 4=11-20%; 5=21-35%; 6=36-60%; 7 > 60% (Blandino *et al.,* 2009c).

The fungal ear rot severity was calculated as the percentage of symptomatic kernels per ear. A scale of 1 to 7 was used to quantify the percentage interval of surfaces exhibiting visible symptoms of the disease: 1=no symptoms; 2=1-3%; 3=4-10%; 4=11-25%; 5=26-50%; 6=51-75%; 7>75% (Blandino *et al.,* 2009c). The ECB damage severity and ear rot severity scores were then converted to percentages of ears exhibiting symptoms, and each score was replaced with the mid-point of the interval.

Multi-mycotoxin liquid chromatography-mass spectrometry analysis

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 in Sulyok *et al.* (2006).

Detection and quantification were performed using a QTrap 5500 LC–MS/MS System (Applied Biosystems, Foster City, CA, USA), equipped with a TurboIonSpray electrospray ionisation (ESI) source and a 1290 Series UPLC System (Agilent,

Waldbronn, Germany). Chromatographic separation was performed at 25°C in 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 in Malachova *et al.* in 2014. The results of the mycotoxin concentrations were corrected for apparent recoveries.

Statistical analyses

The normal distribution and the homogeneity of the variance were verified by means of the Kolmogorov–Smirnov normality test and the Levene test, respectively.

All the recorded parameters were compared through an analysis of variance (ANOVA), in which the sowing date and the hybrids were set as independent variables. ANOVA was conducted separately for each growing season. The Ryan-Einot-Gabriel-Welsh Q (REGWQ) test on treatment means was performed for multiple comparison purposes. The fungal metabolite concentrations were transformed using the y'=ln(x+1) equation to normalise the residuals.

Principal component analysis (PCA) was carried out to investigate the relationships between the main detected mycotoxins. All the data obtained in the two growing seasons were joined together before the PCA was performed. The data were preliminarily standardised by subtracting the means and dividing the results by the standard deviations for each variable.

SPSS for Windows, version 22.0 statistical package (SPSS Inc., Chicago, IL, USA), was used for the statistical analyses.

Results

Different meteorological trends were observed during the two growing seasons (Table 2). The 2014 growing period showed more abundant rainfall and a lower temperature (expressed as growing degree days, GDDs) than the 2015 growing season during maize ripening (from July to September). In 2014, most abundant rainfall was observed in July, close to the flowering stage of the first sown maize. The high GDDs observed from June to September 2015 led to an anticipated maize harvest in September, whereas in 2014 harvesting took place one month later (Table 1).

ANOVA showed a significant effect of sowing date on the grain yield and grain moisture content for both of the growing seasons: late planting resulted in a 14% lower grain yield and in a 22% higher moisture content than the early one (Table 3). The full season maturity hybrids DKC6815 and PR32B10 (FAO maturity class 600) produced in a significantly higher grain yield than P1208 (FAO maturity class 500) in both growing seasons. The interaction between sowing date and hybrid was never significant.

Table 1. Sowing, insecticide application and harvesting date of the maize hybrids compared in the 2014 and 2015 growing seasons.

Growing season	Sowing type	Sowing date	Flowering date	Insecticide application date	Harvesting date
2014	Early	April 3	7 July	July 22	October 2
	Late	May 21	2 August	August 10	October 20
2015	Early	April 3	5 July	July 15	September 7
	Late	May 11	26 July	August 1	September 23

In the 2015 growing season, the ECB severity was higher in the hybrids sown later, whereas the fungal ear rot severity was higher in the late sowing than in the early one in both growing seasons. An increasing ECB severity trend was observed moving from the early maturity hybrid to the late maturity one; the same trend was also observed for fungal ear rot severity in 2015, while the DKC6815 hybrid showed significantly higher disease symptoms than the other hybrids in 2014. The interaction between sowing date and hybrid was never significant, except for fungal ear rot severity in 2015.

Overall, considering the two growing seasons and the field conditions obtained from the combination of the sowing time and the hybrids, 34 mycotoxins and fungal metabolites produced by *Fusarium* species were detected: fumonisin B (FBs: sum of FB_1, FB_2, FB_3, FB_4, FB_6), fumonisin A (FAs: sum of FA_1, FA_2), fusarin

C, fusaric acid (FA), bikaverin (BIK), fusaproliferin (FUS), deoxynivalenol and its derivatives (DON: sum of deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol), deoxynivalenol-3-glucoside (DON-3-G), nivalenol (NIV), culmorin and its derivatives (CULM: sum of culmorin, 5-hydroxyculmorin, 15-hydroxyculmorin, 15-hydroxyculmoron), zearalenone (ZEA: sum of zearalenone, α-zearalenol, β-zearalenol, zearalenone-4-sulphate), butenolide (BUT), aurofusarin (AUR), moniliformin (MON), beauvericin (BEA), enniatins (ENNs: sum of ENN B, ENN B_1, ENN B_2), equisetin (EQU), T-2 toxin and HT-2 toxin.

The B fumonisins were the most abundant metabolites, with an average contamination of 5593 and 10993 µg kg^{-1} in the 2014 and 2015 growing seasons, respectively. The most abundant compound was fumonisin B_1 (65% of the total fumonisins), followed by fumonisin B_2 (16%), B_3 (9%) and B_4 (8%). Fumonisin B_6 was only

Table 2. Total rainfall, rainy days and growing degree days () measured near the experimental areas from April to October in both the 2014 and 2015 growing seasons.

Growing season	Month	Rainfall (mm)	Rainy days (n)	GDDs° (°C)
2014	April	71	12	163
	May	91	13	210
	June	102	12	324
	July	153	18	337
	August	50	9	345
	September	132	11	277
	October	24	9	187
	July - September	335	38	959
2015	April	71	10	162
	May	76	11	257
	June	64	14	356
	July	21	2	444
	August	80	15	378
	September	64	10	252
	October	160	19	133
	July - September	164	27	1074

GDDs, growing degree days. °Accumulated growing degree days for each month using a 10°C base. Source: Agrometeorological Network of Piedmont - Piedmont Region - Assessorato Agricoltura - Settore Fitosanitario, sezione di Agrometeorologia".

Table 3. Effect of sowing date and hybrid maturity length on grain yield, grain moisture, European corn borer severity and fungal ear rot severity at harvest.

Growing season	Source of variation	Treatment	Grain yield (t ha^{-1})	Grain moisture (%)	ECB severity° (%)	Fungal ear rot severity# (%)
2014	Sowing date	Early	17.5[a]	25.7[b]	2.3[a]	6.4[b]
		Late	14.8[b]	35.0[a]	2.4[a]	10.4[a]
		P (F)	0.001	<0.001	0.900	<0.001
	Hybrid	P1208	13.4[b]	29.0[b]	0.3[c]	4.2[b]
		DKC6815	16.7[a]	32.3[a]	2.1[b]	15.9[a]
		PR32B10	18.3[a]	29.8[b]	4.8[a]	5.1[b]
		P (F)	<0.001	<0.001	<0.001	<0.001
	Sowing date × Hybrid	P (F)	0.351	0.735	0.531	0.174
2015	Sowing date	Early	15.7[a]	26.6[b]	0.5[b]	0.4[b]
		Late	13.8[b]	28.5[a]	3.2[a]	4.2[a]
		P (F)	0.001	<0.001	0.001	<0.001
	Hybrid	P1208	12.3[b]	26.6[b]	0.6[b]	0.8[c]
		DKC6815	16.4[a]	28.1[a]	2.7[a]	2.6[b]
		PR32B10	15.5[a]	28.1[a]	2.4[a]	3.5[a]
		P (F)	<0.001	0.010	0.030	<0.001
	Sowing date × Hybrid	P (F)	0.884	0.268	0.113	0.001

ECB, European corn borer. °ECB severity was calculated as the percentage of kernels per ear with insect injuries, considering 20 ears per plot; #fungal ear rot severity was calculated as the percentage of kernels per ear with fungal disease symptoms, considering 20 ears per plot; [a-b]means followed by different letters are significantly different (the level of significance is shown in the table). The sowing date values are based on 9 replicates (3 hybrids x 3 replications), while the hybrid values are based on 6 replicates (2 sowing dates x 3 replications).

found in traces, while the fumonisin A (sum of A$_1$ and A$_2$) concentration was 2% of the total fumonisin contamination.

In addition to FBs, other metabolites produced by *Fusarium verticillioides* and *F. proliferatum,* such as FA, BIK and FUS, were detected in all of the samples, with an average contamination of 400 µg kg^{-1}, 237 µg kg^{-1} and 551 µg kg^{-1}, respectively. The occurrence of fusarin C was found in 61% of the samples, with an average contamination of 75 µg kg^{-1}.

CULM, DON, DON-3-G and ZEA were detected in all of the samples in the 2014 growing season, with an average contamination of 5239 µg kg^{-1}, 3669 µg kg^{-1}, 1412 µg kg^{-1} and 1508 µg kg^{-1}, respectively. In the 2015 growing season, these metabolites ranged from 44% to 89% of the cases, with an average contamination of 447 µg kg^{-1}, 274 µg kg^{-1},103 µg kg^{-1} and 41 µg kg^{-1}, respectively for CULM, DON, DON-3-G and ZEA. Other mycotoxins produced by *F. graminearum* and *F. culmorum,* such as NIV and BUT, were on average found at lower concentrations.

As far as the modified mycotoxins are concerned, the ratio between 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol and DON in all of the compared situations was 22 and 3%, respectively. The DON-3-G/DON ratio (on average 40%) was similar for both of the growing seasons and sowing date, but resulted to be higher in full season maturity hybrids than the medium-early one. The hydroxylated forms of culmorin (5-hydroxyculmorin, 15-hydroxyculmorin, 15-hydroxyculmoron) accounted for 67% of the total CULM, and their ratio was stable in the compared experimental trials.

Among the mycotoxins produced by other *Fusarium* species, MON and BEA had been produced by *Fusarium* species fungi belonging to both the *Liseola* (*F. proliferatum* and *F. subglutinans*) and *Roseum* sections (*F. avenaceum*). MON and BEA were found in similar contents in both growing seasons, with an average contamination of 264 µg kg^{-1} and 125 µg kg^{-1}, respectively. On the other hand, like DON, the AUR contamination was different in the two growing seasons. Concentrations of 15348 µg kg^{-1} and 413 µg

kg^{-1} were observed in the 2014 and 2015 growing seasons, respectively. EQU, which is produced by *F. equiseti*, was found in 97% of the cases, with an average content of 62 µg kg^{-1}, while T-2 and HT-2 toxins, which are produced by *F. sprorotrichioides*, *F. langsaethiae* and *F. poae*, were found in 36% of the samples, with an average concentration of 20 µg kg^{-1} (sum of the T-2 and HT-2 toxins), and a similar content of both toxins. Finally, ENNs were found at very low contamination levels in all the samples of the compared production situations.

Among the toxins produced by the *Alternaria* species, alternariol (AOH), alternariol methyl ether (AME) and tentoxin (TENT) were found in traces in both growing seasons in less than 30% of the samples.

In addition to the meteorological conditions, the concentrations of mycotoxins and fungal metabolites were clearly influenced by the sowing times and by the type of hybrid considered. In both growing seasons, a late sowing time significantly increased all the mycotoxins produced by *F. verticillioides* and *F. proliferatum* (Table 4). On average, the content of FBs, FAs, Fusarin C, FA, BIK and FUS in the 2014 growing season resulted to be 2.1, 2.3, 2.9, 1.7, 2.1 and 2.8 times higher in the late sowing than in the early one. The contamination of all these toxins increased more (from 4 to 8 times) in the late sowing time in 2015. As far as the type of hybrid is concerned, the full season maturity hybrids, and particularly the PR32B10 hybrid, which is characterised by the longest cycle, often resulted in a significantly higher content of FBs, FAs, Fusarin C, BIK and FUS than the medium-early maturity hybrid (P1208). Only for FA contamination a negative relationship with hybrid precocity was not observed clearly in both growing seasons. In 2014, the interaction between sowing date and hybrid for all these metabolites was never significant. However, a significant interaction was observed for FBs, FAs and Fusarin C in 2015. As far as the content of FBs is concerned, the lowest contamination was observed in DKC6815, in the early sowing time, whereas a significantly higher contamination was observed in the

Table 4. Effect of sowing date and hybrid maturity length on the occurrence of mycotoxins produced mainly by *Fusarium verticillioides* and *Fusarium proliferatum*.

Year	Source of variation	Treatment	FBs T	N (µg kg^{-1})	FAs T	N (µg kg^{-1})	Fusarin C T	N (µg kg^{-1})	FA T	N (µg kg^{-1})	BIK T	N (µg kg^{-1})	FUS T	N (µg kg^{-1})
2014	Sowing date	Early	8.1[b]	3572	4.1[b]	67	3.4[a]	68	5.0[b]	159	4.8[b]	139	5.7[b]	367
		Late	8.8[a]	7614	4.9[a]	155	4.7[a]	118	6.1[a]	466	5.6[a]	294	6.6[a]	1025
		P (F)	0.013	-	0.002	-	0.091	-	<0.001	-	0.003	-	0.011	-
	Hybrid	P1208	8.4[a]	4910	4.1[b]	64	3.7[a]	76	5.4[a]	321	5.0[a]	196	5.3[b]	250
		DKC6815	8.0[b]	3538	4.3[b]	81	3.9[a]	90	5.4[a]	244	5.1[a]	184	6.6[a]	841
		PR32B10	8.9[a]	8332	5.1[a]	187	4.7[a]	112	5.8[a]	372	5.5[a]	269	6.6[a]	998
		P (F)	0.033	-	0.002	-	0.462	-	0.148	-	0.294	-	0.003	-
	Sowing date × Hybrid	P (F)	0.621	-	0.405	-	0.674	-	0.053	-	0.777	-	0.968	-
2015	Sowing date	Early	7.0[b]	2030	2.8[b]	45	<LOQ[b]	<LOQ	3.8[b]	105	4.4[b]	98	1.3[b]	62
		Late	9.4[a]	19956	5.5[a]	400	3.3[a]	111	6.7[a]	871	5.8[a]	419	6.4[a]	751
		P (F)	<0.001	-	<0.001	-	<0.001	-	<0.001	-	<0.001	-	<0.001	-
	Hybrid	P1208	7.7[b]	2552	3.6[b]	40	<LOQ[b]	<LOQ	6.0[a]	643	4.5[b]	97	2.8[b]	141
		DKC6815	7.6[b]	11055	3.4[b]	281	2.5[a]	75	4.1[b]	403	5.3[a]	337	3.4[b]	500
		PR32B10	9.2[a]	19371	5.4[a]	347	2.5[a]	91	5.7[a]	418	5.5[a]	342	5.3[a]	579
		P (F)	0.002	-	<0.001	-	<0.001	-	0.037	-	0.020	-	0.023	-
	Sowing date × Hybrid	P (F)	0.001	-	<0.001	-	<0.001	-	0.059	-	0.134	-	0.086	-

FBs, sum of fumonisin B$_1$, B$_2$, B$_3$, B$_4$, B$_6$ (LOQ=limit of quantification=2.4 µg kg^{-1}); FAs, sum of fumonisin A$_1$, A$_2$ (LOQ=2.4 µg kg^{-1}); fusarin C (LOQ=4.8 µg kg^{-1}); FA, fusaric acid (LOQ=1.6 µg kg^{-1}); BIK, bikaverin (LOQ=8 µg kg^{-1}); FUS,fusaproliferin (LOQ=40 µg kg^{-1}). The mycotoxin contamination means reported are transformed [T; y'= ln (x + 1)] and non-transformed (N) values. [a,b]Means followed by different letters are significantly different (the level of significance is shown in the table). The sowing date values are based on 9 replicates (3 hybrids x 3 replications), while the hybrid values are based on 6 replicates (2 sowing dates x 3 replications).

late sowing period for both DKC6815 and PR32B10 than for P1208 (Figure 1). In both growing seasons, the sowing date also clearly affected the mycotoxin contents produced by *F. graminearum* and *F. culmorum* (Table 5). During the 2014 growing season, which was characterised by abundant rainfall and cold temperatures during the flowering stage of the early planted maize, the early sowing date resulted in a significantly higher contamination of DON (+53%), DON-3-G (+64%), NIV (+3 times), CULM (+42%), ZEA (+22%) and BUT (+35%) than for the late sowing date. Conversely, in 2015, the early sowing significantly reduced the DON, DON-3-G, CULM, ZEA and BUT contents by 78, 85, 84, 88 and 84%, respectively, compared to the late one. In the same year, no significant difference was observed for NIV for the compared sowing dates. As far as hybrid susceptibility is concerned, the DKC6815 cultivar showed the highest contamination level for all these mycotoxins and metabolites in both growing seasons, with the exception of NIV in 2015. No significant differences were reported for DON-3-G, NIV, ZEA or BUT in the 2015 growing season for the full season maturity hybrids. No significant difference was observed between the medium-early maturity hybrid, P1208, and the full season maturity hybrid, PR32B10, for any of the mycotoxins, with the exception of DON-3-G in 2014 and ZEA in 2015.

The interaction between sowing date and hybrid for these metabolites was never significant, with the exception of NIV in 2014 and CULM in the 2015 growing season. In particular, as shown in Figure 2, the CULM content in the 2015 growing season significantly increased with the late sowing time, but only for the P1208 hybrid.

As far as the mycotoxins and fungal metabolites produced by other *Fusarium* species are concerned, the sowing date significant-

ly affected the contamination of AUR, MON, BEA and the T-2 and HT-2 toxins (Table 6). The late sowing in 2015 increased the AUR, MON and BEA contents by 8.9, 3.3 and 11.4 times, respectively. Conversely, in 2014, the MON, T-2 and HT-2 contents were significantly higher in the early sowing time than in the late one. The occurrence of ENNs and EQU was never affected by the sowing time. As far as hybrid susceptibility is concerned, a significantly
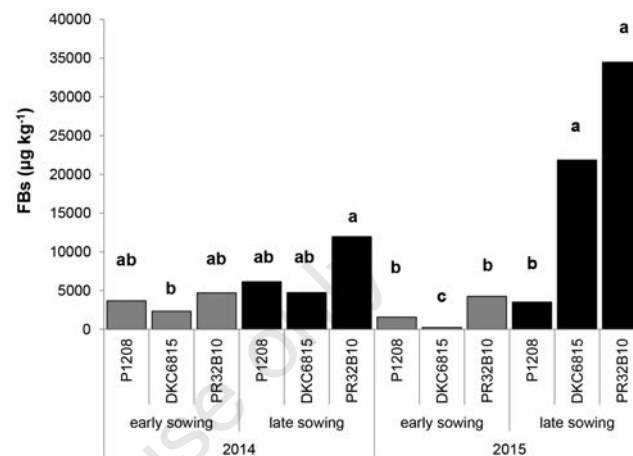
Figure 1. Effect of interaction between sowing date and hybrid maturity length on fumonisin B contamination. Means followed by different letters within each year are significantly different (P<0.01). The reported data are the means of 3 replications.

Table 5. Effect of sowing date and hybrid maturity length on the occurrence of mycotoxins produced mainly by *Fusarium graminearum* and *Fusarium culmorum*.

Year	Source of variation	Treatment	DON T	N (μg kg^{-1})	DON-3-G T	N (μg kg^{-1})	NIV T	N (μg kg^{-1})	CULM T	N (μg kg^{-1})	ZEA T	N (μg kg^{-1})	BUT T	N (μg kg^{-1})
2014	Sowing date	Early	8.2[a]	4443	7.1[a]	1758	2.9[a]	29.1	8.5[a]	6159	7.0[a]	1660	5.8[a]	338
		Late	7.6[b]	2896	6.4[b]	1066	1.2[b]	7.2	8.0[b]	4319	6.4[b]	1356	5.4[b]	250
		P (F)[b]	0.016	-	0.004	-	0.001	-	0.046	-	0.050	-	0.004	
	Hybrid	P1208	7.5b	2251	5.8[c]	387	1.1[b]	4.4	7.9[b]	3359	5.7[b]	508	5.5[b]	242
		DKC6815	8.8[a]	6818	8.0[a]	3157	3.5[a]	37.8	9.2[a]	9724	8.1[a]	3491	6.0[a]	425
		PR32B10	7.4[b]	1939	6.4[b]	692	1.6[b]	12.2	7.7[b]	2634	6.2[b]	526	5.3[b]	215
		P (F)	<0.001	-	<0.001	-	0.001	-	0.001	-	<0.001	-	<0.001	-
	Sowing date × Hybrid	P (F)	0.366	-	0.351	-	0.039	-	0.384	-	0.151	-	0.121	-
2015	Sowing date	Early	1.4[b]	99	1.7[b]	26	0.5[a]	1.9	3.1[b]	123	1.1[b]	9	3.0[b]	34
		Late	4.0[a]	450	4.7[a]	179	<LOQ[a]	<LOQ	6.3[a]	772	3.7[a]	73	5.0[a]	214
		P (F)	0.036	-	0.001	-	0.184	-	<0.001	-	<0.001	-	0.001	-
	Hybrid	P1208	1.1[b]	30	1.8[b]	20	0.3[a]	1.2	3.5[b]	286	1.1[b]	5	3.1[b]	44
		DKC6815	5.5[a]	670	4.4[a]	202	<LOQ[a]	<LOQ	6.1[a]	840	3.4[a]	72	4.9[a]	238
		PR32B10	1.4[b]	123	3.4[ab]	87	0.4[a]	1.6	4.5[b]	217	2.7[a]	46	4.0[ab]	89
		P (F)	0.013	-	0.024	-	0.616	-	0.006	-	0.007	-	0.030	-
	Sowing date × Hybrid	P (F)	0.963	-	0.753	-	0.616	-	0.038	-	0.489	-	0.773	-

DON, sum of deoxynivalenol (LOQ=limit of quantification=1.2 μg kg^{-1}) and deoxynivalenol derivatives: 3-acetyldeoxynivalenol (LOQ=1.2 μg kg^{-1}) and 15-acetyldeoxynivalenol (LOQ=1.2 μg kg^{-1}); DON-3-G, deoxynivalenol-3-glucoside (LOQ=0.8 μg kg^{-1}); NIV, nivalenol (LOQ=1.2 μg kg^{-1}); CULM=sum of culmorin (LOQ=8 μg kg^{-1}) and culmorin derivatives: 5-hydroxyculmorin (LOQ=40 μg kg^{-1}), 15-hydroxyculmorin (LOQ=4 μg kg^{-1}), 15-hydroxyculmoron (LOQ=24 μg kg^{-1}); ZEA, sum of zearalenone (LOQ=0.12 μg kg^{-1}) and zearalenone derivatives: α-zearalenol (LOQ=0.8 μg kg^{-1}), β-zearalenol (LOQ=1.2 μg kg^{-1}), zearalenone-4-sulphate (LOQ=1.6 μg kg^{-1}); BUT, butenolide (LOQ=5.6 μg kg^{-1}). The mycotoxin contamination means reported are transformed [T; y'= ln (x + 1)] and non-transformed (N) values. [a-c]Means followed by different letters are significantly different (the level of significance is shown in the table). The sowing date values are based on 9 replicates (3 hybrids × 3 replications), while the hybrid values are based on 6 replicates (2 sowing dates × 3 replications).

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higher AUR content was found in DKC6815 in 2014, while EQU was significantly lower in P1208 than in the full season maturity hybrids. A significantly higher contamination of MON was found in PR32B10 than P1208 in 2015. No significant differences between hybrids were observed for BEA, ENNs or for the T-2 and HT-2 toxins. The interaction between sowing date and hybrid was never significant for any of these metabolites.
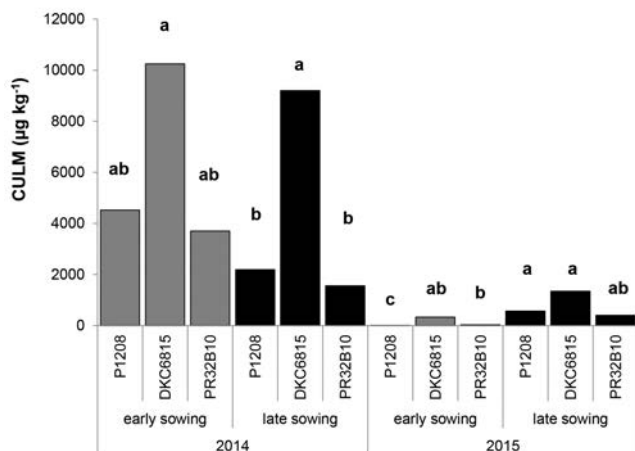
PCA was carried out to investigate the relationships between the main detected mycotoxins, as it allows the maize samples to be clustered according to similar mycotoxin contaminations. The results of the PCA performed with the main metabolites detected in the two growing seasons were used to produce a loading plot in which the two first principal components explained 43% and 33% of the total variations of the samples (Figure 3). It can be assumed

Figure 2. Effect of interaction between sowing date and hybrid maturity length on culmorin and culmorin derivates (5-hydroxy-culmorin, 15-hydroxyculmorin and 15-hydroxyculmoron) contamination. Means followed by different letters within each year are significantly different (P<0.01). The reported data are the means of 3 replications.
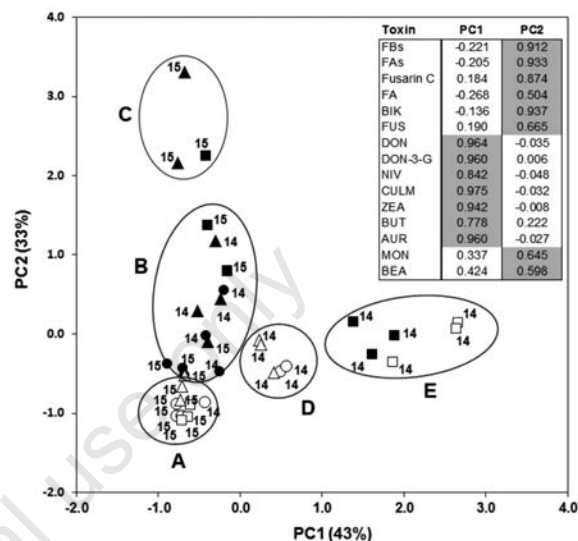

Figure 3. Score plots of maize samples (N=36) marked according to the hybrid type (circle: P1208 hybrid; square: DKC6815 hybrid; triangle: PR32B10 hybrid), sowing date (white: early sowing; black: late sowing date) and growing season (14=2014, 15=2015). The loadings (highlighted values >|0.5|) of each parameter are reported with the two first principal components.

Table 6. Effect of sowing date and hybrid maturity length on the occurrence of mycotoxins produced by other *Fusarium species*.

Year	Source of variation	Treatment	AUR T	AUR N (μg kg^{-1})	MON T	MON N (μg kg^{-1})	BEA T	BEA N (μg kg^{-1})	ENNs T	ENNs N (μg kg^{-1})	EQU T	EQU N (μg kg^{-1})	T-2 and HT-2 toxin T	T-2 and HT-2 toxin N (μg kg^{-1})
2014	Sowing date	Early	9.3[a]	16527	5.7[a]	384	5.0[a]	186	0.1[a]	0.2	2.7[a]	63	1.9[a]	12.7
		Late	8.8[a]	14169	4.7[b]	150	5.0[a]	148	0.2[a]	0.7	3.5[a]	83	0.2[b]	0.5
		P (F)[b]	0.137	-	0.007	-	0.942	-	0.644	-	0.637	-	0.007	-
	Hybrid	P1208	8.3[b]	6229	4.9[a]	174	4.8[a]	148	<LOQ[a]	<LOQ	1.7[a]	8	1.4[a]	7.7
		DKC6815	10.4[a]	33831	5.5[a]	425	5.3[a]	219	<LOQ[a]	<LOQ	3.2[a]	95	0.9[a]	3.1
		PR32B10	8.5[b]	5984	5.2[a]	201	4.9[a]	134	0.5[a]	1.4	4.5[a]	115	0.8[a]	9.0
		P (F)	<0.001	-	0.322	-	0.418	-	0.211	-	0.022	-	0.642	-
	Sowing date × Hybrid	P (F)	0.364	-	0.060	-	0.340	-	0.802	-	0.334	-	0.316	-
2015	Sowing date	Early	2.5[b]	83	4.5[b]	120	2.0[b]	13	0.1[a]	0.1	2.7[a]	22	1.4[a]	14.4
		Late	6.0[a]	742	5.7[a]	400	4.6[a]	152	<LOQ[a]	<LOQ	3.1[a]	82	0.3[a]	1.0
		P (F)	0.002	-	0.004	-	<0.001	-	0.337	-	0.473	-	0.122	-
	Hybrid	P1208	3.1[a]	77	4.4[b]	108	2.8[a]	29	0.1[a]	0.2	2.4[a]	20	1.2[a]	14.8
		DKC6815	5.6[a]	822	5.2[ab]	287	3.1[a]	78	<LOQ[a]	<LOQ	3.3[a]	116	1.0[a]	7.9
		PR32B10	4.0[a]	339	5.6[a]	386	4.0[a]	140	<LOQ[a]	<LOQ	3.0[a]	20	0.2[a]	0.4
		P (F)	0.115	-	0.050	-	0.140	-	0.397	-	0.458	-	0.451	-
	Sowing date × Hybrid	P (F)	0.797	-	0.993	-	0.282	-	0.397	-	0.131	-	0.395	-

AUR, aurofusarin (LOQ=limit of quantification=2.4 μg kg^{-1}); MON, moniliformin (LOQ=1.6 μg kg^{-1}); BEA, beauvericin (LOQ=0.008 μg kg^{-1}); ENNs, sum of enniatins B, B$_1$, B$_2$ (LOQ=0.04 μg kg^{-1}); EQU, equisetin (LOQ=0.24 μg kg^{-1}); T-2 toxin (LOQ=0.8 μg kg^{-1}) and HT-2 toxin (LOQ=3.2 μg kg^{-1}). The mycotoxin contamination means reported are transformed [T; y'= ln (x + 1)] and non-transformed (N) values. [a,b]Means followed by different letters are significantly different (the level of significance is shown in the table). The sowing date values are based on 9 replicates (3 hybrids × 3 replications), while the hybrid values are based on 6 replicates (2 sowing dates × 3 replications).

that the first component differentiated the samples according to their toxin contents produced by *F. graminearum* and *F. culmorum* (DON, DON-3-G, NIV, CULM, ZEA, BUT) and to their AUR (loadings > |0.77|). On the other hand, the second component differentiated the samples according to their toxin contents produced by *F. verticillioides* and *F. proliferatum* (FBs, FAs, Fusarin C, FA, BIK and FUS). The contaminations of MON and BEA, which are mainly produced by *F. subglutinans* (section *Liseola*) and *F. avenaceum* (section *Roseum*), were found to be associated with FBs. When the production situations resulting from the combination of growing seasons, sowing time and hybrid were considered, five groups were identified. Group A clustered samples with an overall low content of all the mycotoxins considered in the PCA. On the contrary, the B and C group samples showed higher FB and FB-associated mycotoxin contents. These two groups only consisted of samples from both growing seasons characterised by a late sowing time. Moreover, the group C samples, which were characterised by the highest FB contamination, only referred to the full season maturity hybrids matured in the warmer and drier growing season (2015). The samples characterised by high contaminations of DON and DON-associated mycotoxins are clustered in groups D and E. All these samples refer to the 2014 growing season, which was characterised by more favourable environmental conditions to *F. graminearum* and *F. culmorum* infection. These two groups differed according to the hybrids that were clustered. In fact, the samples characterised by the highest DON and DON-associated metabolite contamination (group E) only came from the DKC6815 hybrid, which resulted more prone to *F. graminearum* and *F. culmorum* infection for both sowing times.

Discussion

The collected data indicate that the most prevalent mycotoxins and fungal metabolites in irrigated maize cultivated in temperate areas can be grouped into two main classes, depending on the environmental conditions that occur during cultivation. The main environmental conditions which influence the infection and the development of different fungal species are determined by the combination of the sowing date, the susceptibility and maturity length of the cultivated maize hybrid as well as the meteorological trends from flowering to the end of ripening of the maize kernel.

The first group of mycotoxins and fungal metabolites refers to fumonisins (FBs and FAs) and fumonisin-associated mycotoxins, such as Fusarin C, FA, BIK and FUS, all of which are produced mainly by *F. verticillioides* and *F. proliferatum*. As regards the occurrence of this class of compounds, FBs were detected in both growing seasons, and as also reported in several previous scientific works, FBs are by far the most abundant mycotoxins in Northern Italian maize (Pietri *et al.*, 2004; Blandino *et al.*, 2015). To the best of the authors' knowledge, this is the first work that has reported the co-occurrence of FBs and FAs, which are the N-acetyl analogs of FBs, in maize grain samples.

Moreover, the present study reports data on the natural content of Fusarin C, whose presence has only been reported in South Africa in selected maize kernels (Gelderblom *et al.*, 1984) and in Germany in whole maize and feed samples, but on those occasions the levels of contamination were at least 10 times lower (Kleigrewe *et al.*, 2011).

Although the relative concentrations of these metabolites could change according to the growing season, they all showed a similar response following the application of the compared cropping systems. In particular, the late sowing time was found to significantly increase the concentration of all these compounds in both growing seasons. In fact, the delay of sowing, and consequently of kernel ripening, may result in more favourable conditions for the development of *Fusarium* species in the final part of ripening, and may prolong the kernel dry down (Doohan *et al.*, 2003; Nielsen *et al.*, 2002). Moreover, the late sowing time resulted in a higher level of ECB damage of the ears (Blandino *et al.*, 2008), and the damage of this insect is not only clearly correlated to fumonisin occurrence (Munkvold, 2003; Blandino *et al.*, 2009a), but also to the presence of BIK, FUS, FA, MON and BEA (Blandino *et al.*, 2015). Late sowing shifts the ear development phase of the crop to a period with greater insect activities, and this produces more kernel injuries due to ECB larvae, which in turn favours a higher *F. verticillioides* and *F. proliferatum* infection (Blandino *et al.*, 2009b). Although an insecticide was applied in all the compared situations to minimise insect damage, the later sowing time in 2015 resulted in a higher level of ECB larva injury than the early one.

The increase in the content of fumonisins and other metabolites produced by *F. verticillioides* and *F. proliferatum* due to the delay of sowing was more pronounced in the 2015 growing season, which was characterised by less favourable environmental conditions for infection by other *Fusarium* species, such as *F. graminearum*, *F. culmorum* and *F. avenaceum*. This finding confirms the results on fumonisins reported by Parson and Munkvold (2012) in the U.S.A., which pointed out a higher risk for late sowings in years with dryer and warmer conditions after pollination, in which the competition between *F. verticillioides* and *F. proliferatum* and other *Fusaria* species is lower. Moreover, the risk of contamination by fumonisins and fumonisin-associated metabolites after a late sowing is higher for full season maturity hybrids than for earlier hybrids. In these production situations, the combination of late sowing time and full season maturity hybrids creates conditions in which the exposure to the ECB activity is more critical, and the development of the fungal species responsible for contamination is increased. In previous research, in which the early sowing time was employed, the contamination of fumonisins did not seem to be clearly influenced by the hybrid maturity length (Blandino and Reyneri, 2008; Camardo Leggieri *et al.*, 2015), but did seem to be influenced by the hardness or the chemical composition of the grain, which was found to influence fungal infection and development. Conversely, Pietri *et al.* (2012) reported a higher FB contamination in medium-early and full season maturity hybrids (FAO maturity classes 500 and 600) than in early maturity hybrids (FAO maturity class 300), none of which have been considered in the present study.

The second class of fungal metabolites refers to deoxynivalenol and its related metabolites. The content of acetylated forms of deoxynivalenol, DON-3-G, and of all the other fungal metabolites produced by *F. gramineaurum* and *F. culmorum* (CULM, NIV, ZEA, BUT), were found to be closely correlated to the deoxynivalenol content, as already reported for wheat (Blandino *et al.*, 2017). Moreover, AUR was also found to be correlated to DON contamination, and to follow the same trend of contamination. The high occurrence of these metabolites in maize is generally associated with wet and cool growing seasons (Doohan *et al.*, 2003). An increase in deoxynivalenol content was previously reported after the application of a late sowing date (Blandino *et al.*, 2009b) or after delaying the harvest date (Lauren *et al.*, 2007), as was also observed for the 2015 conditions. Furthermore, the data of the present study clearly underline that the contamination of these metabolites depends more on the conditions that occur during maize flowering than those that occur during ripening. The mete-

orological conditions in July 2014 made the early-planted maize more prone to fungal producer infection than the maize planted at the late sowing dates, which were characterised by a delay of flowering at the beginning of August. Moreover, DON, DON-3-G, ZEA, CULM, BUT and AUR were detected in 2014 at much higher levels of contamination than those reported in another work on a three-year study in the same growing area (Blandino *et al.*, 2015).

The importance of flowering infection on the contamination of DON and DON-associated mycotoxins can be confirmed by considering the clear susceptibility observed in the DKC6815 cultivar, compared to the other hybrids with similar (PR32B10) or different (P1208) precocity. While the resistance sources to *F. verticillioides* and fumonisin accumulations are polygenic and more difficult to incorporate in hybrids (Clements *et al.*, 2003), the genetic control of *F. graminearum* in maize hybrids is regulated by just a few genes and is closely linked to silk infection (Reid *et al.*, 1994; Chungu *et al.*, 1996).

The MON and BEA levels seemed to be connected to those of the fumonisins, as already reported by Blandino *et al.* (2015) in a work that considered maize ears with variable injuries caused by insects. Furthermore, the contamination by these metabolites is influenced less by the sowing date and the type of hybrid than FBs. This might be due to the fact that these compounds can be synthesised by several fungal species characterised by different ecologies.

Although the study compared production situations with clearly different environmental conditions, other metabolites produced by *Fusarium* species, such as ENNs, EQU, T-2 and HT-2 toxins and *Alternaria* toxins (AOH, AME, TENT), were always found in traces or at low concentrations in the maize, compared to wheat cultivated in the same growing areas (Blandino *et al.*, 2017). Thus, the occurrence of these metabolites seems to be influenced less by the compared agronomic practices. To the best of the authors' knowledge, this is the first work that has reported, although only in traces, the presence of ENNs and *Alternaria* toxins in maize grains from South European fields.

T-2 and HT-2 toxins were found more frequently in samples taken from early-sown plots, although no differences were observed among the considered hybrids. The higher T-2 and HT-2 toxin contents could be an indirect consequence of a change in the relative competition capacity among the fungal species. The application of an early sowing date and of an insecticide treatment against ECB is known to reduce FB-producer infection, and consequently an increase in the infection by less predominant fungal species may be possible.

No metabolites produced by *Penicillium* or by other fungal genera were detected in any of the compared production situations. However, it has been well documented that the contamination of maize grain by aflatoxins could occur in different growing areas and seasons from those considered in this study. Again in this case, the sowing date may have had an important effect of aflatoxin contamination; in fact, as reported in previous studies, the application of a late sowing may increase aflatoxin contamination (Wiatrak *et al.*, 2005; Camardo Leggieri *et al.*, 2015).

Conclusions

The results of these experiments, obtained under naturally-infected field conditions, underline the key role of agronomic strategies in influencing the contamination by mycotoxins pro-

duced by different *Fusarium* species in maize. The choice of the sowing date and the use of certain hybrids could shift the most critical growth stages of maize from flowering to the end of ripening, in weeks characterised by different environmental factors, and this could result in a variable selection of the fungal species that can infect and develop on maize ears. The effect of these crop techniques on the overall control of mycotoxins clearly interacts with the meteorological trend of the growing seasons, and could result in changeable behaviour of all the different compounds. These results, which need to be confirmed in other environments, lead to an improvement in knowledge on the control of these contaminants in maize cultivations, which could help in the design of integrated management strategies that are able to minimise the overall risk of mycotoxin contamination in this crop in different growing areas.

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