Impact of the insecticide application to maize cultivated in different environmental conditions on emerging mycotoxins

FIELD CROPS RESEARCH

Title: Impact of the insecticide application to maize cultivated in different environmental conditions on emerging mycotoxins.

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ABSTRACT

Maize can be competitively colonized by several fungi that are capable of producing a variety of mycotoxins, mainly fumonisins B (FBs), with a negative impact on maize safety and quality.

The aim of this study was to investigate the impact of the insecticide application against European Corn Borer on the contamination of “emerging” mycotoxins and other fungal metabolites co-occurring with the legislated ones in maize for human consumption in North-West Italy from 2009 to 2015.

The insecticide application on average significantly increased the yield by 5%, and significantly reduced the ECB incidence and severity and the fungal ear rot incidence and severity compared to the untreated control.

Overall, 25 *Fusarium* mycotoxins and metabolites were detected. The results underline that the use of the insecticide, the most common FBs control practice in temperate areas on food maize, also resulted in significant reductions of other mycotoxins produced by *Fusarium* spp. of the *Liseola* section. However, this practice was not generally able to reduce the contents of *Fusarium* spp. section *Discolor* and *Roseum* mycotoxins. In environmental and agronomic conditions that favor deoxynivalenol and other metabolites produced by the previous mentioned *Fusarium* spp., the insecticide treatment could even increase their contamination.

Keywords: Maize, Fumonisins, Emerging mycotoxins, *Fusarium*, Insecticide, European Corn Borer.

1. INTRODUCTION

Maize (*Zea mays* L.) is a very versatile and multipurpose cereal grain that is used throughout the world as a raw material for feed, food, industrial and energy purposes. This cereal is mainly used in Africa and South America for the preparation of traditional food, such as tortillas, arepas, couscous and porridge.

The consumption of this crop has recently increased in developed countries, as it is used as an ingredient for breakfast products, snacks, dietetic products and, in particular, for baby food and gluten-free food, whose consumption is rising (Escobar et al., 2013).

Unfortunately, this agricultural commodity can be colonized competitively by several spoilage fungi of the *Fusarium*, *Aspergillus, Alternaria* and *Penicillium* species that are capable of producing a large variety of mycotoxins as a result of fungal ear rot on maize ears (Marin et al., 2012).

Fusarium mycotoxins, which develop mainly in the field under appropriate environmental conditions, are the most common mycotoxins that contaminate maize in temperate areas (Logrieco et al., 2002). Since the same plant tissue can be colonized by various mycotoxigenic species, it is possible that several mycotoxins could co-occur in the same food or feed matrix, with the consequent possible additive or synergic toxicological effects due to their co-presence (Sanhueza and Degrossi, 2004).

Approximately 400 mycotoxins or potential risky fungal metabolites are known to date (Berthiller et al., 2007). Nevertheless, only a very limited number of these mycotoxins are subject to legislation and regular monitoring.

As far as maize is concerned, fumonisins B (FBs), aflatoxins (AFs), zearalenone (ZEA) and deoxynivalenol (DON) are the ones that are reported and monitored the most (Binder, 2007). Among them, FBs are by far the most abundant mycotoxins and constantly present over the years in Mediterranean countries as well as in Italian maize (Marin et al., 2012; Munkvold, 2003; Pietri et al., 2004; Blandino et al., 2015).

The toxicological effects of the regulated mycotoxins are well known: FB_1 was classified as possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer (IARC, 2002); AFB_1 is the most potent known hepatocarcinogen to which humans are widely exposed (group 1B) (IARC, 1993); DON is responsible for serious mycotoxicosis in humans and animals (Pestka and Smolinski, 2005); and ZEA is a non-steroidal estrogenic mycotoxin that causes hormonal effects in animals and humans (Zinedine et al., 2007).

Because of the toxicity of these substances, the European Commission (EC) has set maximum regulatory limits for different mycotoxins in unprocessed maize used for food purposes, through the EC (2007; 2010) regulations.

The other mycotoxins and fungal metabolites, which until now have not received detailed scientific attention, are commonly indicated as “novel” or “emerging” mycotoxins (Streit et al., 2013).

The European Food Safety Authority (EFSA) is currently working on establishing a scientific opinion on the risks to public health related to the presence of “emerging” mycotoxins in feeds and food (EFSA, 2010; 2014).

Little is known about the toxicological effects of these compounds, some of which could be potentially toxic to humans and livestock. In addition, limited information is known about the synergistic or additive toxic effects related to the co-presence of different mycotoxins, both emerging and regulated. Furthermore, although fungal ear

93 rot in maize depends on climatic factors and the susceptibility of a hybrid to the
94 disease, it has been demonstrated that *Fusarium* ear rot is closely correlated to
95 insect injury, and in particular to ear damage caused by Lepidoptera borers
96 (Avvantaggiato et al., 2003).
97 European corn borer (ECB), *Ostrinia nubilalis*, is the main maize pest in Central and
98 South Europe, and it has been shown to promote *Fusarium verticillioides* and *F.*
99 *proliferatum* infection in maize grains, which are well-known fungal producers of FBs
100 (Sobek and Munkvold, 1999; Ostry et al., 2010). Moreover, several studies have
101 established that the control of ECB clearly affects FBs levels in maize kernels at
102 harvesting, since the injuries produced by this pest on kernels during ripening appear
103 to be the most important infection pathway in North Italy (Alma et al., 2005;
104 Camardo-Leggieri et al., 2015). In 2015, Blandino et al. reported that ECB promotes
105 contamination by the other mycotoxins produced by *Fusarium* spp. of the *Liseola*
106 section in temperate areas, in the same way as for FBs, while it does not affect those
107 produced by *Fusarium* spp. of the *Discolor* and *Roseum* sections.
108 Since genetic control involving GMO *Bt* technology is forbidden in Italy, as well as in
109 other European countries, the control of ECB is usually conducted through the use of
110 insecticide treatments in order to protect maize and maize-based food products from
111 FBs contamination (Folcher et al., 2009; Blandino et al., 2009a; Mazzoni et al.,
112 2011).
113 At present, insecticide treatments against ECB in maize and an early sowing date are
114 the two main crop techniques required in the chain agreements between the food
115 processing industry and farmers in Northern Italy (Vanara et al., 2005).

Although insecticide application is a practice widely used in the maize food chain to control the occurrence of FBs, there is still a lack of information about the effect of this strategy on contamination by emerging mycotoxins and other fungal metabolites. Therefore, the aim of this study was to investigate the impact of the insecticide application on the control of emerging mycotoxins and other fungal metabolites co-occurring with the legislated ones in raw maize used for human consumption in North-West Italy over 7 growing seasons under field conditions.

2. MATERIALS AND METHODS

2.1 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 reverse osmosis, using a Milli-Q plus system from Millipore (Molsheim, France). Fungal metabolite standards were obtained 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).

2.2 Experimental design and samples

Field trials were carried out, from 2009 to 2015, over 7 growing seasons and in 2 sites in North-West Italy:

- Trials A, B, C and E: at Cardè, (44° 44' N, 07° 28' E; altitude 258 m) in the 2009, 2010, 2011 and 2012 growing seasons, respectively, in a deep and fertile sandy soil, Typic Eutrochrepts (USDA classification);
- Trials D, F, G, H and I: at Carmagnola (44° 50’ N, 7° 40’ E; altitude 245 m) in the 2011, 2012, 2013, 2014 and 2015 growing seasons, respectively, in a loam soil, Typic Udifluvents (USDA classification).

During all the investigated period, rainfall and temperature data were recorded daily from a weather station, located next to the experimental field.

Studies were conducted using maize hybrids that are suitable in the food chain for the production of both flaking grits and meal: Pioneer P1543 (FAO rating 600; 130

147 days) in trials A, B, C, D and E and Pioneer P1547 (FAO rating 600; 130 days) in
148 trials F, G, H and I.
149 An insecticide was applied in each experiment (growing season × location) to
150 minimize the ear injuries caused by ECB activity, and it was compared with an
151 untreated control, grown under naturally-infected field conditions.
152 The insecticides used were: alpha-cypermethrin (pyrethroid) [Contest®, formulation:
153 water dispersible granules, BASF, Italy, applied at 0.044 kg of active ingredient (AI)
154 ha^{-1}] in trials A, B, C and E and a lambda-cyhalothrin (pyrethroid) + chlorantraniliprole
155 (diamide) mixture [Ampligo®, formulation: suspension concentrate, capsule
156 suspension, Syngenta Crop Protection S.p.A., Italy, applied at 0.015 and 0.030 kg
157 (AI) ha^{-1}, respectively] in trials D, F, G, H and I.
158 The ECB flight activity was monitored by means of a cone trap placed outside the
159 experimental plots, baited with sex pheromone (E:Z=97:3) to attract males and with
160 phenylacetaldehyde (PAA) for females. The sex pheromones and PAA dispenser
161 were replaced every 15 and 30 days, respectively. Adults were removed from the
162 trap and counted every 1-2 days. The insecticide was applied during the milk stage,
163 [growth stage (GS) 75] (Zadoks et al., 1974), after noting the presence of the ECB
164 flight peak, by means of a self-propelled ground sprayer (GT7, Grim), according to
165 the conditions described in Blandino et al. (2009a). The sowing, flowering, harvest
166 and the insecticide application dates are reported in Table 1 for each field trial.
167 The treatments were assigned to experimental units, using a completely randomized
168 block design with 3 replicates. Each plot consisted of 12 rows of a length of 25 m
169 spaced 0.75 m apart, and separated by two untreated buffer rows on either side. The
170 plot alleys, orthogonal to the maize rows, were 1 m wide.

Conventional agronomic techniques were adopted for the field experiments in all of the growing seasons. Briefly, the previous crop was always maize, mechanical sowing was carried out after an autumn ploughing (30 cm) and disk harrowing was conducted to prepare a suitable seedbed. The crop density was approximately 75,000 plants ha^{-1}. All of the plots received the same amount of nutrients: 300, 100 and 100 kg ha^{-1} of N, P_2O_5 and K_2O, respectively. Irrigation was carried out by means of the sprinkling method in order to avoid any 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., Italy). The ears were collected by hand from 4.5 m^2 (2 rows × 3 m) in each plot at the end of maturity, at a grain moisture content of between 23-27%. A sub-sample of 30 ears was used to evaluate ECB and fungal ear rot incidence and severity. All the collected ears were shelled using an electric sheller to obtain grain samples. The grain yield results were adjusted to 14% of moisture content. The kernels in each plot were mixed thoroughly to obtain a random distribution, and 1 kg was taken to measure the moisture content by means of a Dickey-John GAC2000® grain analysis meter (Dickey-John Corp. Auburn, IL, USA).

Samples (5 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 whole meal, which was thoroughly homogenized by mixing and then used directly for the extraction and the analysis of the mycotoxins.

2.3 Entomological and mycological measurements

The ECB damage incidence was calculated as the percentage of ears per plot with kernel injuries or apical and basal tunnels in the cob due to larva activity. 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 in which each numerical value corresponded to a percentage interval of surfaces exhibiting visible kernel damage due to larva activity, according to the following schedule: 1 = no injuries, 2 = 1-5%, 3 = 6-10%; 4 = 11-20 %, 5 = 21-35%, 6 = 35-60%, 7 > 60% (Blandino et al., 2008).

The fungal ear rot incidence was calculated as the percentage of ears per plot with symptoms, while the fungal ear rot 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 = no symptoms, 2 = 1-3 %, 3 = 4-10%; 4 = 11-25 %, 5 = 26-50%, 6 = 51-75%, 7 > 75% (Blandino et al., 2008). The ECB damage severity and ear rot severity scores were converted to percentages of ears exhibiting symptoms, and each score was replaced with the mid-point of the interval.

2.4 Multi-mycotoxin LC-MS/MS analysis

Representative 5 g 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 by a mixture of acetonitrile/water/acetic acid 20 + 79 + 1 (v + v + v) and injected, as described in detail by Sulyok et al. (2006).

Detection and quantification were performed with a QTrap 5500 LC–MS/MS System (Applied Biosystems, Foster City, CA), equipped with a TurboIonSpray 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, the USA). The chromatographic and mass spectrometric parameters as well as all the validation parameters of the analytical method (limit of detection, LOD; limit of quantification, LOQ; recovery; matrix effect; etc.) related to the investigated analytes were described by Malachova et al. (2014). Quantification was performed on the basis of an external calibration, and the results were corrected for apparent recoveries determined in the maize. Fumonisins A were semi-quantified using the response of FB_2. The accuracy of the method was verified by participating in proficiency testing schemes organized by BIPEA (Gennevilliers, France), with 160 out of the 168 results submitted for maize and maize-based feed exhibiting a z-score of between -2 and 2.

2.5 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 in order to evaluate the effect of the insecticide application on the ECB incidence and severity, fungal ear rot incidence and severity, grain yield and mycotoxin content, using a completely randomized block design, in which the insecticide treatment was the independent variable and the trial (combination of growing seasons and sites) was the random factor ($\alpha = 0.05$). In

order to report the effect of insecticide application on the control of ECB and fungal development in each considered production situation, the ECB incidence and severity and the fungal ear rot incidence and severity were analyzed separately for each trial, using a completely randomized block design, in which the insecticide treatment was the independent variable ($\alpha = 0.05$).

The incidence and the severity values of fungal ear rot incidence and severity were previously transformed using $y'=\arcsin\sqrt{x}*180/\pi$ as percentage data derived from counting. The mycotoxin concentrations were transformed using the $y'=\ln(x+1)$ equation to normalize the residuals.

The SPSS for Windows, Version 24.0, statistical package (SPSS Inc., Chicago) was used for the statistical analysis.

3. RESULTS

3.1 Meteorological data

The seven growing seasons showed clearly different meteorological trends, as far as both rainfall and temperature (expressed as growing degree days, GDDs) from flowering to harvesting are concerned (Table 2). The 2013 year suffered from heavy rainfall in July, close to maize flowering. Moreover, in 2013, abundant rainfall was also recorded during the spring (96 mm in March and 144 mm in April) and this led to the sowing date being delayed to May, with a consequent late harvesting date in November. Overall, the 2014 year was the wettest year, and had the highest number of rainy days, in particular during flowering. Moreover, it was characterized by cooler weather and the lowest GDDs. Conversely, the 2009 year suffered from very abundant rainfall during ripening, in particular in September, and this delayed the harvest until the middle of October. The 2010, 2011, 2012 and 2015 years were the driest and warmest years during the summer, and very little rainfall was recorded. This led to harvest dates occurring in September or at the beginning of October. The 2015 year was the driest and warmest year during ripening, with the lowest rainfall and GDDs and, as a result, the earliest harvest date at the beginning of September. In the 2011 and 2012 years, the Carmagnola site (trials D and F) showed less rainfall and higher temperatures than the Cardè site (trials C and E).

3.2 Entomological and mycological data

Table 3 summarizes the mean ECB incidence and severity as well as the mean fungal ear rot incidence and severity recorded for each trial for the untreated control and the insecticide application of the ears collected in each sampling year.

On average, the insecticide application significantly reduced the ECB incidence and severity ($P < 0.001$). The effect of insecticide application on ECB control for each considered production situation is reported in Figure 1. Only in trial E was the ECB severity not significantly reduced by the insecticide application. The insecticide application led to reductions of 73%, 81%, 81%, 63%, 41%, 83%, 91%, 62% and 87% of the ECB severity for the A, B, C, D, E, F, G, H and I trials, respectively.

The effect of insecticide application on fungal ear rot incidence and severity varied over the considered production situations (Figure 2). The insecticide application led to significant reductions of 74%, 76%, 65%, 77%, 74% and 80% of fungal ear rot severity for the A, B, D, E, F, G and I trials, respectively. Conversely, in the H trial, the fungal ear rot severity was increased by 8% after the insecticide application, although the difference was not significant.

3.3 Grain yield

ANOVA showed a significant effect of insecticide treatment on the grain yield in all of the trials ($P = 0.046$) (Table 3). In the 9 trials conducted over 7 growing seasons, the insecticide application on average significantly increased the yield by 5%, compared to the untreated control.

3.4 Mycotoxin contamination

The dilute-and-shoot multi-mycotoxin LC-MS/MS method was able to detect 25 *Fusarium* mycotoxins: aurofusarin (AUR), beauvericin (BEA), bikaverin (BIK), butenolide (BUT), culmorin (CULM), DON, deoxynivalenol-3-glucoside (DON-3-G), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), equisetin

(EQU), fusaric acid (FA), fumonisin A_1, fumonisin A_2, FBs (FB_1, FB_2, FB_3, FB_4), fusaproliferin (FUS), fusarin C, moniliformin (MON), nivalenol (NIV), ZEA, zearalenone-4-sulphate (ZEA-4-S), alpha-zearalenol (α-ZEAol) and beta-zearalenol (β-ZEAol).

The contamination of maize by mycotoxins mainly produced by *Fusarium* spp. of the *Liseola* (FBs, FA, FUS, fusarin C, BEA, BIK and MON) and *Gibbosum* (EQU) sections is reported in Table 4. No significant effects of the interaction between the insecticide treatment and trials were observed.

Even though different levels of contamination were recorded for the considered production situations, the insecticide application significantly reduced the FBs contamination ($P < 0.001$) in each trial, on average by 72%.

The other mycotoxins and metabolites produced by *Fusarium* spp. of the *Liseola* section, as well as EQU, showed the same behavior as the FBs: the insecticide treatment significantly reduced the FA ($P = 0.001$), FUS ($P = 0.016$), fusarin C ($P = 0.035$), BEA ($P = 0.021$), BIK ($P = 0.002$), MON ($P < 0.001$) and EQU ($P = 0.006$) contents by 70%, 64%, 50%, 72%, 72%, 58% and 64%, respectively, compared to the untreated control.

Moreover, in addition to the mycotoxins listed above in the H and I trials, conducted during 2014 and 2015, respectively, the co-occurrence of FBs with fumonisin A-series (data not shown in Table 4), N-acetyl analogs of the former ones, was also observed. Considering the sum of fumonisin A_1 and fumonisin A_2, the insecticide application reduced its contamination from 375 μg kg^{-1} to 137 μg kg^{-1} and from 246 μg kg^{-1} to 147 μg kg^{-1}, compared to the untreated control.

The effects of the insecticide application on the contamination of mycotoxins produced by *Fusarium* spp. of the *Discolor* and *Roseum* sections, such as DON,

328 DON-3-G, 3-ADON, 15-ADON, NIV, ZEA, ZEA-4-S, α-ZEAol, β-ZEAol, AUR, BUT
329 and CULM, are reported in Tables 5 and 6. No significant effects of the interaction
330 between the insecticide treatment and trials were observed for DON, DON-3-G, 3-
331 ADON, 15-ADON, AUR, BUT or CULM. A different trend was observed for NIV, ZEA,
332 ZEA-4-S, α-ZEAol and β-ZEAol after the insecticide application in experiment G
333 (2013 growing season), and the data for this growing season were therefore
334 analyzed separately. On average, the insecticide application did not significantly
335 affect the concentration in maize grain of any of the detected mycotoxins produced
336 by *Fusarium* spp. of the *Discolor* and *Roseum* sections (Tables 5 and 6). Instead,
337 when only the effect of the insecticide in the G trial carried out in the 2013 growing
338 season was considered, a significant increase of all these metabolites was recorded
339 (Table 7). In the G trial, the application of the insecticide significantly reduced ECB
340 and fungal ear rot severity, but also significantly increased the DON ($P < 0.008$),
341 DON-3-G ($P < 0.009$), 3-ADON ($P < 0.032$), 15-ADON ($P < 0.034$), NIV ($P < 0.026$),
342 ZEA ($P < 0.013$), ZEA-4-S ($P < 0.001$), α-ZEAol ($P < 0.019$), β-ZEAol ($P < 0.007$) and
343 AUR ($P < 0.003$) contents by 72%, 79%, 66%, 63%, 78%, 79%, 94%, 75%, 83% and
344 89%, respectively, compared to the untreated control.
345

4. DISCUSSION

The effect of the insecticide application on FBs contamination has been well documented in literature, and the obtained result is in agreement with previous studies (Folcher et al., 2009; Blandino et al., 2009a). Focusing on the trials with the highest levels of FBs contamination, that is, E, F, G, H and I trials, in which the concentrations in the untreated control were above the maximum regulatory limit (4000 μg kg^{-1}) established by the EC (2007), the insecticide was always able to reduce the contamination to below this limit. The only exception in the considered production situation was the H trial conducted in the 2014 growing season, which was extremely cold and rainy from flowering to harvest, where the insecticide application reduced the FBs contamination from 13192 μg kg^{-1}, in the untreated control, to 4707 μg kg^{-1} after the treatment. As reported by Blandino et al. (2009a) these results confirm that the optimum application timing, as well as a greater persistence of the pyrethroid insecticides applied, represent important technical factors to improve the effectiveness of ECB and FBs contamination control. Conversely, increasing the number of insecticide applications is not economically favorable and could have an effect on non-target biota, in particular on the natural enemies of ECB, and this would lead to an increase in other pests. Two or more applications with pyrethroids could cause secondary infestations of the mite *Tetranichus urticae* Koch and an outbreak of maize aphids (Saladini et al., 2008; Ayyappath et al., 1996).

Nevertheless, as extensively reported by the North American literature (Munkvold and Hellmich, 1999; Hammond et al., 2003; Wu, 2006) the *Bt* hybrid adoption, through the high pest protection that it confers, has effectively given the highest FBs

370 contamination control in comparison to a non-transgenic maize leading to economic,
371 human and animal health benefit. These aspects should be taken into account on
372 national-level policy makers of the EU who have not yet allowed *Bt* maize adoption in
373 the food chain. In this scenario the use of insecticides remains a necessary
374 component of good agricultural practices in several European countries in order to
375 protect maize and maize-based food products from FBs contamination.
376 It is however extremely important to consider the presence and the level of
377 contamination of all the other mycotoxins that could contaminate raw maize together
378 with FBs, and which co-occur with the legislated ones, in order to establish the
379 overall risk of mycotoxin contamination in raw maize used for human consumption,
380 and consequently in maize food products.
381 The other mycotoxins and metabolites produced by *Fusarium* spp. of the *Liseola*
382 section, as well as EQU, showed the same behaviour as the FBs. To the best of the
383 authors' knowledge, this is the first study that has investigated and reported the effect
384 of the insecticide application on the contamination of the previous mentioned
385 mycotoxins in maize grains.
386 As far as the toxicological relevance of the other mycotoxins co-produced with FBs
387 by *Fusarium* spp. of the *Liseola* section is concerned, particular attention should be
388 paid to fusarin C, which was found in the G, H and I trials at concentrations of 479 µg
389 kg^{-1}, 3516 µg kg^{-1} and 736 µg kg^{-1}, respectively, in the untreated control. Although
390 fusarin C is known to be unstable (Gelderblom et al., 1983), it has been reported to
391 be the cause of a high incidence of human oesophageal cancer in South Africa and
392 China (Cheng et al., 1985; Marasas et al., 1979). It has also been shown to have
393 mycoestrogenic properties (Sondergaard et al., 2011), mutagenic activity
394 (Gelderblom et al., 1984) and several immunosuppressive effects comparable with

those of aflatoxin B1 and sterigmatocystin intoxications (Cantalejo et al., 1999). Even though it was classified by IARC in 1993 as being part of group 2B, as a potential carcinogen for humans together with FB1 and FB2, it has not yet been considered in any legislation.

Regulatory limits also have not been established concerning the presence of MON in food or feeds. Although its toxic effects have not yet been fully established, Jonsson et al. (2015) have recently reported a high acute toxicity of MON in rats, with the LD_{50} value at the same level as that of T-2 and HT-2 toxins, the most toxic *Fusarium* mycotoxins. Fusaric acid has a broad spectrum of action, and it is considered to be related directly to the severity of damping off, vascular wilt and root rot diseases of numerous vegetable crops (Bacon and Hinton, 1996; Stipanovic et al., 2011). In addition to the critical roles in plant pathogenesis, fusaric acid can be mildly toxic to mammals, and can increase the overall toxicity of other mycotoxins that synergistically interact with the toxicity of other naturally co-occurring mycotoxins (Chamberlain et al., 1993; Bacon et al., 1996). BEA has been found to affect the electromechanical and physiological properties of isolated smooth and heart muscle preparations (Lemmens-Gruber et al., 2000). It is a specific cholesterol acyltransferase inhibitor (Tomoda et al., 1992), is toxic to several human cell lines (Logrieco et al., 2002) and can induce apoptosis and DNA fragmentation (Ojcius et al., 1991). FUS has been found to be toxic in mammalian cells (human B lymphocytes) (Logrieco et al., 1996) and causes teratogenic effects in chicken embryos (Ritieni et al., 1997). In 1998, Van der Westhuizen et al. reported that fumonisin A-series, like FBs, also have the ability to inhibit sphingosine N-acyltransferase.

419 The data of the present study clearly confirm that the contamination of mycotoxins
420 produced by *Fusarium* spp. of the *Discolor* and *Roseum* sections in temperate maize
421 growing areas depends to a great extent on environmental conditions that favor the
422 infection and development of these fungal species, such as abundant rainfall and low
423 temperatures at maize flowering or during the ripening stage. Thus, an abundant
424 occurrence of these metabolites, in particular DON and ZEA, in maize is generally
425 associated with wet and cool growing seasons (Doohan et al., 2003), or with a late
426 planting and harvesting time and the cultivation of a late maturity hybrid (Blandino et
427 al., 2009a; Lauren et al., 2007).
428 Together with DON and ZEA, their associated metabolites, which are usually referred
429 to as “masked mycotoxins”, that is, DON-3-G, 3-ADON, 15-ADON, ZEA-4-S, α-ZEAol
430 and β-ZEAol, have also been detected. DON-3-G is a phase II plant metabolite of the
431 *Fusarium* mycotoxin DON (Berthiller et al., 2013), which could be hydrolyzed in the
432 digestive tract of mammals, thus contributing to the total dietary DON exposure of
433 individuals (Berthiller et al., 2011). The acetylated derivatives of DON, 3-ADON and
434 15-ADON are usually considered as fungal derived metabolites and, as described in
435 previous studies (Pinton et al., 2012; Berthiller et al., 2013). 3-ADON has been found
436 to be less toxic than DON, which is less toxic than 15-ADON. Thus, 3-ADON and 15-
437 ADON together with DON-3-G should be considered an additional contributing factor
438 of the total dietary exposure to DON, and the Joint European Commission FAO/WHO
439 Expert Committee (JECFA) together with the Codex Alimentarius Commission (CAC)
440 are currently working on this problem (JECFA, 2010; CAC, 2011). Moreover, the
441 higher toxicity of 15-ADON should be also taken into account.
442 α-ZEAol and β-ZEAol are phase I plant metabolites of ZEA, and the reduction
443 metabolism of the parent mycotoxin results in an activation of the derived molecule

444 with a higher toxicity level, especially for α-ZEAol. The risk of hyperestrogenic effects
445 of α-ZEAol has been underestimated because α-ZEAol, which is a more estrogenic
446 derivative of ZEA, is often not determined or regulated (Berthiller et al., 2013).
447 In the present experiment, the insecticide application did not reduce the
448 concentration in maize grain of any of the detected mycotoxins produced by
449 *Fusarium* spp. of the *Discolor* and *Roseum* sections. Nevertheless, the insecticide
450 application led to an increase in DON contamination to above the maximum
451 regulatory limit (1750 μg kg^{-1}, EC 2007), that is, from 1501 μg kg^{-1} to 1898 μg kg^{-1} in
452 trial A (2009 growing season) and from 1842 μg kg^{-1} to 6565 μg kg^{-1} in trial G (2013).
453 Similarly, ZEA contamination increased above the maximum regulatory limit (350 μg
454 kg^{-1}), from 234 μg kg^{-1} to 1144 μg kg^{-1} in trial G. Moreover, in the H trial (2014) the
455 DON and ZEA contaminations in the untreated control were already much higher
456 than the maximum regulatory limits, with values of 20146 μg kg^{-1} and 2629 μg kg^{-1},
457 respectively. This effect could be probably due to the environmental conditions that
458 favored the *F. graminearum* infection at flowering, before the ECB feeding.
459 An increase in these fungal metabolites, due to the insecticide application was
460 previously reported in North Italy after a 3-year experiment on DON by Blandino et al.
461 (2009b), but only in the wettest growing season and when a full maturity hybrid was
462 considered. On the other hand, Folcher et al. (2009), in an experiment conducted in
463 France, reported a significant reduction in trichothecenes content after the insecticide
464 application, while no significant differences were observed between the control and
465 insecticide treatments for ZEA and its metabolites. Similarly, in Canada (Schaafsma
466 et al., 2002), in an environment with a strong predominance of *F. graminearum*
467 compared to *F. verticillioides* and consequently low FBs contamination levels, a
468 reduction in DON contamination was recorded in *Bt* maize.

To the best of the authors' knowledge, no other information about an increasing trend, due to the insecticide applications on DON associated mycotoxins, are available.

Considering the overall mycotoxin contamination, FBs were found to be the most abundant fungal metabolites in all the trials, with the exception of the A, G and H trials conducted in the 2009, 2013 and 2014 growing seasons, respectively, in which the most abundant metabolite was DON. In environments and agronomic conditions where maize is more prone to *Fusarium* section *Discolor* infection, the risk associated with their mycotoxin contamination could increase as a consequence of a reduction in competition with the *Fusarium* spp. of the *Liseola* section due to ECB control. As also reported by Folcher et al. (2010), competition occurs among the *Fusarium* species that produce FBs and trichothecens, and the control of ECB could change the relative competition capacity of these species during maize ripening. This could result in a reduction of certain mycotoxins and the simultaneous increase of others, and this process has been named the "flora inversion" phenomenon. As far as the cultivation of maize in temperate areas is concerned, this effect could be risky in production situations in which the contamination of fungal metabolites produced by *F. graminearum* and *culmorum* is favored.

In most of the environmental conditions taken into consideration in this work, the flora inversion phenomenon was never observed. This phenomenon occurred in the environmental (2013 growing season, G trial) and agronomic conditions (full maturity hybrids, late planting time and consequent ripening) that favored DON contamination. Moreover, in the G experiment the highest ECB control was achieved through insecticide application. In contrast, in the 2014 growing season, the highest DON

493 content was strongly related to the fungal silk infection and was not influenced by
494 ECB activity.
495 The data collected suggest that the flora inversion, for the main *Fusarium* species in
496 maize, could take place when their co-occurrence is more equally distributed within
497 the fungal population during ripening.
498 Moreover, since the multi-mycotoxin LC-MS/MS method was able to simultaneously
499 detect 288 fungal metabolites during the 9 experiments conducted in the present
500 study, the compounds produced by different *Fusarium* spp. from the previously
501 mentioned ones (for example T-2 and HT-2 toxins among the regulated ones), and
502 by other fungi, such as the *Aspergillus* (aflatoxins), *Penicillium* (ochratoxin) and
503 *Alternaria* species, were never detected in the untreated control. In addition, it is
504 important to underline that the insecticide treatment has never favored their
505 presence.

5. CONCLUSIONS

In conclusion, the results of these experiments, obtained under naturally-infected field conditions and conducted over 7 growing seasons, characterized by different meteorological trends and ECB pressures, underline that the use of insecticides, which is the most common practice applied in temperate areas to control FBs contamination in maize for human consumption, also results in a consistent and clear reduction of the other *Fusarium* spp. of the *Liseola* section mycotoxins that co-contaminate this crop together with FBs. Conversely, the control of ECB injuries through insecticide application did not generally affect the contents of other *Fusarium* mycotoxins, in particular those produced by the *Fusarium* spp. section *Discolor* and *Roseum*. However, in the environmental and agronomical situations that favor the infection of these fungal species, and which make them more competitive than the most widespread *F. verticillioides* and *proliferatum*, insecticide application could lead to an increase in their mycotoxin production. Thus, although the presence and the dominance of certain *Fusarium* species and therefore their mycotoxigenic outcomes may differ depending on the environmental and climatic conditions, insecticide application requires particular attention in cold and wet summer environments, especially at flowering and the last part of ripening, and in cropping system characterized by late planting and late harvesting of full maturity hybrids.

In order to minimize the risk of flora inversion in maize treated with insecticides, Good Agricultural Practices (GAP) require an integrated approach that can address all the possible risk factors in order to prevent an overall mycotoxin contamination. Thus, this study has confirmed that the cultivation of maize for the food chain requires not only a careful control of insect injuries, but also the application of other

practices which have proved to have a significant effect on minimizing all *Fusarium* toxins (Blandino et al., 2017), such as early planting and the use of hybrids that are less prone to overall fungal infections.

Hence, while awaiting the results of other toxicological researches on estimating the impact of these metabolites on human and animal health, and their possible additive or synergistic effects, the results of the present study deepen the information on the presence and on the overall risk of mycotoxin contamination in raw maize for human consumption from temperate areas, as well as suggest strategies to control their co-contamination in the field.

ABBREVIATIONS: 3-ADON, 3-Acetyldeoxynivalenol; 15-ADON, 15-Acetyldeoxynivalenol; AFs, Aflatoxins; ANOVA, Analysis of variance; AUR, Aurofusarin; BEA, Beauvericin; BIK, Bikaverin; BUT, Butenolide; CULM, Culmorin; DON, Deoxynivalenol; DON-3-G, Deoxynivalenol-3-glucoside; EC, European Commission; EQU, Equisetin; FA, Fusaric acid; FBs, Fumonisins B; FHB, Fusarium Head Blight; FUS, Fusaproliferin; GDD, Accumulated growing degree days; GS, Growth stage; HPLC, High performance liquid chromatography; JECFA, Joint Expert Committee on Food Additives; LC-MS/MS, Liquid chromatography coupled to tandem mass spectrometry detection; LOD, Limit of detection; LOQ, Limit of quantification; MON, Moniliformin; NIV, Nivalenol; PMTDI, Provisional maximum tolerable daily intake; ZEA, Zearalenone; ZEA-4-S, Zearalenone-4-sulphate; α-ZEAol, Alpha-Zearalenol; β-ZEAol, Beta-Zearalenol.

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