Role of the European corn borer (Ostrinia nubilalis) on contamination of maize with 13 Fusarium mycotoxins

FOOD ADDITIVES & CONTAMINANTS: PART A

TITLE: THE ROLE OF EUROPEAN CORN BORER (*OSTRINIA NUBILALIS*) ON THIRTEEN FUSARIUM MYCOTOXIN CONTAMINATION IN MAIZE.

Running title: INSECT INJURIES AND EMERGIN MYCOTOXIN IN MAIZE

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ABBREVIATIONS

AUR, aurofusarin; BEA, beauvaricin; BIK, bikaverin; BUT, butenolide; CULM, culmorin; DON, deoxynivalenol; DON-3-G, deoxynivalenol-3-glucoside; ECB, European Corn Borer; EFSA, European Food Safety Authority; EQU, equisetin; FA, fusaric acid; FUMs, fumonisins; FUS, fusaproliferin; GDD, Accumulated growing degree days; LOD, limit of detection; LOQ, limit of quantification; MON, moniliformin; MS, mass spectrometry detection; ZEA, zearalenone.

INTRODUCTION

Mycotoxins are secondary metabolites, which are toxic to humans and animals and could result in illnesses and economic losses (Steyn 1995). They are produced by several fungal species and could affect agricultural commodities. Among these, cereals are the most contaminated (Placinta et al. 1999) and in particular maize, which, in temperate areas, could be affected by fungal ear rot caused by several *Fusarium* species (Logrieco et al. 2002).

Five mycotoxin classes are considered to be largely economically and toxicologically important in grain in several areas throughout the world: aflatoxins and ochratoxin, produced by the genus fungi *Aspergillus* and *Penicillium*, deoxynivalenol (DON), zearalenone (ZEA) and fumonisins (FUMs), mainly produced by *Fusarium* spp. (Atkins & Norman 1998).

Mycotoxin contamination in maize depends on the co-existence of host susceptibility and environmental conditions favourable to fungal infection, growth and toxinogenesis (Munkvold 2003). Moreover, the severity of fungal ear rot caused by *Fusarium* spp. can be closely correlated to insect injury, in particularly to ear damage caused by Lepidoptera borers (Avantaggiato et al. 2003; MarÍn et al. 2012). European corn borer (ECB), *Ostrinia nubilalis*, is the main maize pest in Central and South Europe, and it has been shown to promote *Fusarium verticillioides* and *F. proliferatum* infection in maize grains, well-known fungal producers of FUMs (Sobek & Munkvold 1999). second generation ECB feeding activity is crucial in maize grain FUM occurrence: damaged ears can suffer from contamination of these mycotoxins at a 40 times higher rate than healthy ones (Alma et al. 2005); the injuries produced on kernels during ripening appear to be the most important infection pathway in North Italy (Masoero et al. 1999).

73 Several studies have established that the control of ECB clearly affects FUM levels in
74 maize kernel at harvesting; this has been demonstrated through the use of methods such
75 as insecticide treatment (Folcher et al. 2009; Blandino et al. 2009a), biological control with
76 parasitoids (Dowd 2003) and genetic control involving GMO Bt technology (Ostry et al.
77 2010).
78 Although FUMs are the most common mycotoxins found in maize grain in temperate
79 areas, they are only one group of the approximately 400 mycotoxins known to date
80 (Berthiller et al. 2013). These other mycotoxins, which have not yet received a detailed
81 scientific attention, are commonly indicated as “novel” or “emerging” (Streit et al. 2013).
82 The European Food Safety Authority (EFSA) is currently working on establishing a
83 scientific opinion on the risks to public health related to the presence of emerging
84 mycotoxins in feeds and food (EFSA, 2010). Obviously, there is a need to obtain more
85 information on the occurrence of these mycotoxins in the most important cereal areas in
86 the EU, especially in maize which is one of the cereals most prone to several fungal
87 infections and development during ripening. Moreover, there is also a greater interest in
88 individuating the field conditions that could lead to a higher contamination of these
89 mycotoxins. Better knowledge of the conditions that promote their occurrence is essential
90 in order to set up Good Agricultural Practices (GAP) to minimize their occurrence.
91 The aim of this study was to investigate the role of ECB injuries on maize ears on the
92 contamination of emerging mycotoxins in maize. This information could help to individuate
93 which of them could be reduced by applying strategies to minimize FUM occurrence
94 through the control of this insect.

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 reverse osmosis and 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). Stock solutions of each analyte were prepared by dissolving the solid substance in acetonitrile (preferably), acetonitrile/water 1:1 (v/v), methanol, methanol/water 1:1 (v/v) or water. Twenty-three combined working solutions were freshly prepared prior to the spiking experiments by mixing the stock solutions of the corresponding analytes, and then conducting a further dilution in a neat solvent. All the solutions were stored at −20°C and were brought to room temperature before use.

Experimental

The effect of ECB larva feeding activity on emerging mycotoxin contamination in maize kernels was studied from 2008 to 2010 in North-West Italy at Carmagnola (44° 50’ N, 7° 40’ E; altitude 245 m), in a sandy-medium textured soil (Typic Udifluvents).

In each year, the natural maize ear infestation by the insect larvae was compared with the protection of the infestation, obtained by positioning an entomological net at the end of maize flowering [Growth stage (GS) 69, Lancashire et al. 1991] in order to avoid ECB ovideposition.

120 The ECB natural infestation and artificial protection treatments, were assigned to
121 experimental units using a completely randomized block design with 3 replicates. Each
122 plot consisted of 4 rows 0.75 cm apart and 4 m long. The plot alleys, orthogonal to the
123 maize rows, were one meter wide.
124 The entomological net was characterized by a mesh size of 1 mm, and it was placed on a
125 steel structure with the following dimensions: 4.20 m long and wide, 3.80 m. high. The
126 edge of the net was buried, to prevent the entrance of adult insect while the plants within
127 the net were carefully checked for possible the first generation attack. If the plants
128 presented the typical leaf injuries caused by first generation ECB larvae , they were cut at
129 the bottom and removed from the plots.
130 No foliar insecticides were applied to the experimental field or to an approximately 20 ha
131 area around the field to control ECB or other insects during the entire growing period.
132 The ECB flight activity was monitored by means of a cone trap, which was placed outside
133 the experimental plots, and baited with sex pheromone (E:Z=97:3) to attract males and
134 with phenylacetaldehyde (PAA) for females. The sex pheromones and PAA dispenser
135 were replaced each 15 and 30 d, respectively. The adults were removed from the trap and
136 counted every 1-2 d. Studies were carried out each year on the commercial dent corn
137 hybrid Syngenta NX7444 (FAO rating 600; 130 days). The normal agronomic growing area
138 technique was adopted. Briefly, the previous crop was maize, and the field was ploughed
139 each year. The crop density was approximately 75.000 plants per hectare and the
140 experiment field received 250, 90 and 100 kg ha^{-1} of N, P and K, respectively each year.
141 Irrigation was applied at flowering and during ripening to maintain the water-holding
142 capacity between 33 and 200 kPa. Weeds were controlled with metolachlor and
143 terbutilazine in pre-emergence and sulcotrione and nicosulfuron in post-emergence. The
144 sowing and harvest dates, and the ECB flight peak are reported in table 1 for each year.

145 At the end of maturity, 30 randomly selected ears were collected by hand in each plot and
146 shelled using an electric sheller. The ears were collected at a grain moisture content of
147 between 23 -27%. The kernels from each plot were mixed thoroughly to obtain a random
148 distribution; 4 kg samples were then taken to analyze the mycotoxin content and dried at
149 60°C for 3 days.
150
151 **Entomological and mycological measurements**
152 The ECB damage incidence was calculated as the percentage of ears per plot with kernel
153 injuries or apical and basal tunnels in the cob due to larva activity. The ECB damage
154 severity was calculated as the percentage of kernels per ear with injuries due to larvae
155 activity. A scale of 1 to 7 was used in which each numerical value corresponds to a
156 percentage interval of surfaces exhibiting visible kernel damage due to larva activity
157 according to the following schedule: 1 = no injuries, 2 = 1-5%, 3 = 6-10%; 4 = 11-20 %, 5 =
158 21-35%, 6 = 35-60%, 7 > 60% (Blandino et al. 2009a).
159 The fungal ear rot incidence was calculated as the percentage of ears per plot with
160 symptoms, while the fungal ear rot severity was calculated as the percentage of kernels
161 per ear with symptoms. A scale of 1 to 7 was used in which each numerical value
162 corresponds to a percentage interval of surfaces exhibiting visible symptoms of the
163 disease according to the following schedule: 1 = no symptoms, 2 = 1-3 %, 3 = 4-10%; 4 =
164 11-25 %, 5 = 26-50%, 6 = 51-75%, 7 > 75% (Blandino et al. 2009a). The ECB damage
165 severity and ear rot severity scores were converted to percentages of ears exhibiting
166 symptoms and each score was replaced with the mid-point of the interval.
167

Chemical Analyses

Sample Preparation and Extraction

Maize samples were ground using a ZM 200 Ultra Centrifugal Mill (Retsch GmbH, Haan, Germany) fitted with a 1 mm screen and the flour 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 five g of ground maize samples at free or at very low levels of the detected mycotoxins were spiked in order to evaluate the recovery rate of the analytical method for the different mycotoxins. The average percentages of recovery for the mycotoxins detected were: 69.4% for aurofusarin (AUR), 98.8% for beavaricin (BEA), 95.7% for bikaverin (BIK), 84.0% for butenolide (BUT), 106.7% for culmorin (CULM), 111.8% for deoxynivalenol (DON), 103.3% for deoxynivalenol-3-glucoside (DON-3-G), 200.1% for equisetin (EQU), 69.1% for fusaric acid (FA), 67.9% for fumonisins (FUMs), 101.8% for fusaproliferin (FUS), 98.7% for moniliformin (MON) and 106.9% for zearalenone (ZEA).

The results of the mycotoxin concentrations were corrected for the recovery rate.

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 an 1290 Series UPLC 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, US).

The chromatographic and mass spectrometric parameters of the investigated analytes were described by Sulyok et al. in 2007 and by Malachova et al. in 2014. The applied

193 multi-mycotoxin method was previously subjected to a regular participation in a proficiency
194 test.
195
196 **Statistical analysis**
197 The normal distribution and homogeneity of variances were verified by performing the
198 Kolmogorov–Smirnov normality test and the Levene test, respectively.
199 An analysis of variance (ANOVA) was utilized to compare the fungal ear rot incidence and
200 severity and the mycotoxin contamination separately for each year, using a completely
201 randomized block design, in which the natural presence of ECB larva feeding injuries was
202 the independent variable. The incidence and the severity values of fungal ear rot incidence
203 and severity were previously transformed using $y'=\arcsin\sqrt{x}*180/\pi$ as percentage data
204 derived from counting. The concentration of all the researched mycotoxins was
205 transformed using the $y'=\ln(x+1)$ equation to normalize the residuals.
206 Simple correlation coefficients were obtained for all the detected mycotoxin, relative to
207 each another and to ECB severity and fungal ear rot severity, by joining the data sets that
208 referred to the three growing seasons.
209 The SPSS Version 21.0 for Windows statistical package, (SPSS Inc., 2008) was used for
210 the statistical analysis.
211

RESULTS AND DISCUSSION

Meteorological data

The three growing seasons were subject of 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 2008 and 2010 years had heavy rainfall in May and June and also close to flowering, while less rainfall occurred during the spring in 2009, although it was more concentrated in July, after maize flowering. The GDDs from June to September were higher in 2009 than those in 2008 and 20120, and this led to an anticipated harvest at the beginning of September (Table 1).

ECB flight peak, damage incidence and severity

The flight activity of the first-generation moths started in the middle of July in 2008, and peaked later than in the other growing seasons (Table 1). Instead, the ECB flight activity peaked at the end of July in the 2009 growing season.

In each growing season, the ears collected in the plots protected with entomological nets were free from ECB attack, while those collected in the plots subject to natural insect attacks showed a variable damage severity that depended on the insect pressure in each growing season. The percentage of ears infested by this insect ranged from 41% to 80% in 2009 and from 81% to 93 % in 2010. The ECB pressure in 2008 was higher, with all the collected ears damaged by insect larvae. The average ECB severity observed on the ears at harvest in the naturally infested plot was 26%, 6% and 21% for 2008, 2009 and 2010, respectively.

237 **Fungal ear rot incidence and severity**

238 The ECB larva presence significantly affected the fungal ear rot incidence and severity in
239 each growing season ($P<0.01$). The artificial protection of the insect led to a reduction of
240 78%, 58% and 93% of fungal ear rot severity for the 2008, 2009 and 2010 growing
241 seasons, respectively.

242

243 **Mycotoxin contamination**

244 The FUM, FUS, MON and BEA contaminations were significantly affected by the ECB
245 larva feeding activity on the maize ears in all the considered growing seasons (Table 4).
246 The occurrence of BIK and FA was significantly increased by the ECB presence,
247 compared to the protected plots, but only in the 2008 and 2010 growing seasons. On
248 average, considering the data obtained in the three growing seasons, the presence of ECB
249 damage increased the content of FUMs from 995 to 4694 μg kg^{-1}, MON from 22 to 673 μg
250 kg^{-1}, FUS from 17 to 1089 μg kg^{-1}, BIK from 58 to 377 μg kg^{-1} , BEA from 6 to 177 μg kg^{-1}
251 and FA from 21 to 379 μg kg^{-1}.

252 These data underline how the ECB feeding activity on the maize ears clearly increased not
253 only the occurrence of FUMs, but also that of all the other main mycotoxins produced by
254 *Fusarium* spp. of *Liseola* section (Table 5).

255 These results confirm the important link between the infection and development of some
256 fungal species and ECB activity in the damage of maize ears (Sobek & Munkvold 1999;
257 Dowd 2003). ECB larvae are vectors of *Fusarium* spp.; they cause entry wounds and carry
258 fungal inoculum from the plant surface to the ears, promote ear rot disease development
259 and lead to a clear increase in total mycotoxin contamination. Munkvold et al. (1997)
260 reported that ECB larvae consistently led to an important increase in maize ear rot from *F.*
261 *verticillioides, F. proliferatum* and *F. subglutinans*, all species of *Liseola* section, while the

262 effect on other *Fusarium* species was limited. Reviewing the effect of Bt maize, Ostry et al.
263 (2010) reported that in 19 out of 23 studies the genetically modified crop resistant to the
264 insect was less contaminated with *Fusarium* mycotoxins than the conventional control
265 hybrid. This reduction can be mainly be related to the lower FUM content observed in the
266 kernels.
267 However, the collected data clearly show that ECB injuries play an important role in
268 promoting other *Fusarium*-toxins. As far as the different mycotoxins produced by *Liseola*
269 section from FUMs is concerned, a relationship with ECB feeding on maize ears had only
270 previously been reported for MON. Lew et al. (1991), Magg et al. (2002) and Papst et al.
271 (2005) reported a mean reduction of this mycotoxin through the ECB control of between 49
272 and 71%. To the authors' knowledge, the present study is the first work to attest the close
273 relationship between ECB damage on maize ear and FUS, BIK, BEA and FA.
274 Although all these mycotoxins resulted to be closely linked to the ECB activity, the risk
275 intensity of contamination in the considered growing season changed in a different ways.
276 The role played by the ECB larvae in increasing FUMs was higher in the 2010 year (+43
277 times), and this was followed by 2008 (+13 times) and 2009 (+5 times). Only BIK,
278 produced mainly from *F. verticilliodies* (Busman et al. 2012; Lazzaro et al. 2012), showed
279 similar behavior to FUMs, while FUS and FA, resulted in a higher growth in the naturally
280 infested plots in the 2008 experiments (+79 and 25 times, respectively). FUS and FA were
281 both mainly produced by *F. proliferatum* (Jestoi 2008; Shimshoni et al. 2013). The MON
282 occurrence in maize grain in the 2009 and 2010 growing season was increased
283 remarkably by ECB (48 and 93 times, respectively), as this mycotoxin was only found in
284 traces in the insect protected plot. On the other hand, in 2008, the insect protected plot
285 showed an average contamination of 57 μg kg^{-1}, which was increased25 times in the ears
286 naturally infected by the insect.

287 BEA, produced by *F. verticillides, proliferatum* and *subglutinans* (Sanhueza & Degrossi
288 2004; Jestoi 2008) showed a more stable relationship with the ECB activity throughout the
289 3 growing seasons.
290 It has been reported that, in temperate areas, *F. verticillioides* is more favoured by ECB
291 larva feeding than other *Fusarium* species (Lew et al. 1991; Munkvold et al. 1999). In the
292 present field experiment, the content of both FUS and MON, on average increased more
293 after the ECB activity than FUMs. *F. proliferatum*, after *F. verticillioides*, is the most
294 predominant *Fusarium* species found in maize and a high fumonisin producer (Bacon &
295 Nelson, 1994), but it can also produce, as previous mentioned, a wide range of other
296 mycotoxins. This mycotoxin synthesis is clearly affected by the environmental conditions,
297 especially the temperature, which could influence both the growth rates of the fungi (Marín
298 et al., 2001) and mycotoxin production (Samapundo et al. 2005) These data suggest that
299 ECB and other insect activities could also affect the predominance of different *Fusarium*
300 spp., thus leading to a changed mycotoxin accumulation in the maize kernel .
301 In 2008, EQU (*F. equiseti*, section *Gibbosum*) was also increased significantly by ECB
302 activity on the maize ears, on average from 0.3 to 34 μg kg^{-1}, while in 2009 and 2010,
303 although the differences were not significant, a similar trend was observed. Analyzing
304 single maize kernels, Mogensen et al. (2011) reported that, in South Africa, EQU was not
305 clearly linked to FUM occurrence.
306 The DON, DON-3-G, ZEA, CULM, AUR and BUT contents, produced by *Fusarium* spp. of
307 *Discolor* and *Roseum* sections, for each year were not affected significantly by the
308 presence of ECB larva injuries on the maize ears (Table 6). These data confirm the other
309 results obtained in similar environmental condition on DON (Masoero et al. 1999; Blandino
310 et al. 2009b) and ZEA (Bakan et al. 2002; Saladini et al. 2008), where F. *verticillioides* was
311 the predominant species. However, since in environments where maize is more prone to

312 DON contamination a significant effect of ECB infestation has also been observed for this
313 mycotoxin (Valenta et al. 2001; Papst et al. 2005), it is possible to suppose that the
314 *Fusarium* spp. of *Discolor* and *Roseum* sections also takes advantage of the entry holes
315 produced by ECB larval feeding in the areas and years in which this species finds more
316 favourable climatic conditions for its development and when there is no competition from
317 other *Fusarium* spp. of *Liseola* section. Moreover, as also reported by Folcher et al.
318 (2010), competition occurs among the *Fusarium* species that produce FUMs and
319 trichothecens, and the control of ECB could change the relative competition capacity
320 during maize ripening. Although the differences were not significant, in 2010 only the grain
321 from the protected plots resulted contaminated by DON, while the occurrence of this
322 mycotoxin was under the LOQ in unprotected plots.
323 Table 7 reports the correlation coefficients and the significances between all the
324 mycotoxins recorded, and their relationships with ECB severity and fungal ear rot severity.
325 FUMs show the highest correlation to ECB and fungal ear rot severity. As far as the link
326 between ECB and mycotoxins in the kernel is concerned, a highly significant correlation
327 can be observed for BEA, BIK, MON, FUS FA and EQU: the coefficient of correlation for
328 this mycotoxin and ECB severity is reduced according to the reported order. All these
329 mycotoxins are result significantly correlated to FUMs: the highest relationship is found for
330 BIK (r = 0.904), and this is followed by BEA (r = 0.878), MON (r = 0.855), FUS (r = 0.845),
331 FA (r = 0.734) and EQU (r = 672). The correlation coefficient of the other mycotoxins with
332 the severity caused by ECB larvae is always lower than 0.40. The occurrence of DON-3-G,
333 ZEA, CULM and AUR is closely related to DON contamination, although the level of
334 correlation between the mycotoxins produced by *Fusarium* of the *Discolor* and *Roseum*
335 sections is lower than that observed for the toxins produced by the *Liseola* section. A
336 significant correlation between ZEA and AUR with FUS can be observed, which is

337 probably related to the lower FUS content recorded in the 2009 growing season, which
338 corresponds to the very low content of both these other mycotoxins.
339 The occurrence of other mycotoxins, such as toxins T2 and HT2 or aflatoxins, was never
340 detected in protected and unprotected plots. The climatic and agronomic conditions did not
341 favour the infection and the development of producing fungi.
342 In conclusion, this research, which to the authors' knowledge is the first to analyze the
343 influence of ECB on the most diffused emerging mycotoxins in maize in temperate areas
344 at the same time, offers a further contribution towards determining the strategies that can
345 be adopted to minimize the overall toxin risk for this crop. The results collected clearly
346 suggest that, as for FUMs, the application of a strategy that is able to reduce ECB damage
347 on maize is the most effective solution in temperate areas to control and reduce the other
348 mycotoxins produced by *Fusarium* spp. of *Liseola* section, while it does not affect those
349 produced by *Fusarium* spp. of *Discolor* and *Roseum* sections. These results may be valid
350 for temperate areas where *Fusarium* spp. of *Liseola* section are the predominant species,
351 while in Northern countries the ECB activity could significantly affect also the
352 contamination of mycotoxin produced by *Fusarium* spp. of *Discolor* and *Roseum* sections
353 In non Bt maize fields cultivated in areas with a high ECB pressure, the control of the
354 second generation larvae of this insect could be achieved mainly through preventive
355 control practices, such as an early planting time or through direct control by means of
356 insecticide applications (Blandino et al. 2008). However, since the ecology of the
357 producing *Fusarium* species is slightly different, it will be necessary to verify the real
358 efficacy of these practices on reducing these compounds in comparison to the reference
359 mycotoxins, which, in temperate areas, are FUMs, and to verify their interaction with other
360 crop techniques and pedo-climatic conditions.
361

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TABLES

Table. 1

Main trial information and natural ECB infestation recorded for each year; field experiments conducted at Carmagnola (TO) in the 2008 - 2010 period.

Year	Sowing date	Harvest date	ECB flight peak date	ECB incidence[a] (%)	ECB severity[b] (%)
2008	April 16	October 6	August 7	100.0	25.7
2009	April 10	September 14	July 27	60.0	5.6
2010	April 2	October 10	August 1	88.9	20.8

[a] ECB incidence was calculated as the percentage of ears with symptoms, based on 3 replications of 30 ears each.

[b] ECB severity was calculated as the mean percentage of kernels with symptoms per ear, based on 3 replications of 30 ears each.

537 **Table 2**

538 Total rainfall, rainy days, relative humidity and growing degree days (GDD 10s) from June
539 to October 2008-2010 at the research site

540

Growing season	Month	Rainfall (mm)	Rainy days (n°)	GDD 10s[a] (°C d^{-1})
2008	May	121	16	204
	June	95	17	304
	July	63	8	382
	August	52	6	372
	September	57	8	228
	October	30	5	151
	May-October	418	60	1641
2009	May	30	10	292
	June	26	7	341
	July	121	8	391
	August	56	11	404
	September	62	8	273
	October	54	6	163
	May-October	349	50	1864
2010	May	117	12	214
	June	192	11	332
	July	37	8	420
	August	116	11	354
	September	51	12	240
	October	105	9	120
	May-October	618	63	1680

541

542 [a] Accumulated growing degree days for each month using a 10°C base.

543 **Table. 3**

544 Effect of ECB infestation on fungal ear rot incidence and severity; field experiments
545 conducted at Carmagnola (TO) in the 2008 - 2010 period.

546

Year	ECB infestation	Fungal ear rot incidence[a]		Fungal ear rot severity[b]	
		T	N (%)	T	N (%)
2008	Natural	84.9	97.7	25.1	18.0
	Artificial control	32.2	28.7	5.6	1.0
	P (F)[c]	**0.001**		**< 0.001**	
	sem[d]	8.3		1.7	
2009	Natural	49.6	57.8	9.6	2.8
	Artificial control	19.3	11.1	4.0	0.6
	P (F)	**0.004**		**0.010**	
	sem	7.4		1.7	
2010	Natural	70.7	88.9	23.4	15.9
	Artificial control	10.0	4.4	1.6	0.1
	P (F)	**< 0.001**		**< 0.001**	
	sem	7.7		1.6	

547

548 [a] Fungal ear rot incidence was calculated as the percentage of ears with symptoms, based on 3 replications of 30 ears
549 each.

550 [b] Fungal ear rot severity was calculated as the mean percentage of kernels with symptoms per ear, based on 3
551 replications of 30 ears each.

552 The reported fungal ear rot incidence and severity means are transformed (T; $y'=\arcsin\sqrt{x}*180/\pi$) and not transformed
553 (N) values.

554 [c] The level of significance of ANOVA is reported in the table.

555 [d] sem: standard error of mean

556

557

558

559 **Table. 4**

560 Effect of ECB infestation on the contamination of mycotoxin produced by *Fusarium* spp. of
561 *Liseola* and *Gibbosum* sections; field experiments conducted at Carmagnola (TO) in the
562 2008 - 2010 period.

Mycotoxin[a]	ECB infestation	Year					
		2008		2009		2010	
		T	N (µg kg^{-1})	T	N (µg kg^{-1})	T	N (µg kg^{-1})
FUMs	Natural	9.8	21038	7.0	1306	9.9	22502
	Artificial control	7.3	1598	5.3	249	6.2	528
	P (F)[b]	**0.007**		**0.042**		**0.001**	
	sem[c]	0.7		0.8		0.6	
FUS	Natural	7.8	2537	5.2	227	5.6	503
	Artificial control	3.3	32	0.0	< LOQ	2.2	20
	P (F)	**0.001**		**0.010**		0.069	
	sem	0.7		1.0		2.0	
MON	Natural	7.2	1413	4.8	122	5.7	485
	Artificial control	3.3	57	1.1	3	1.4	5
	P (F)	**0.016**		**0.001**		**0.012**	
	sem	1.4		0.6		1.4	
BIK	Natural	6.5	665	3.8	56	6.0	411
	Artificial control	4.6	117	3.2	27	3.2	28
	P (F)	**0.014**		0.382		**0.002**	
	sem	0.6		1.0		0.5	
BEA	Natural	5.8	438.9	3.2	25.9	4.5	66.1
	Artificial control	2.6	15.9	0.4	0.5	1.0	2.4
	P (F)	**0.014**		**< 0.001**		**0.018**	
	sem	1.1		0.3		0.8	
FA	Natural	6.7	847	3.4	35	5.5	254
	Artificial control	1.7	33	1.4	10	2.4	21
	P (F)	**0.027**		0.138		**0.046**	
	sem	2.0		1.6		1.5	
EQU	Natural	3.3	33.7	1.2	3.6	2.1	53.8
	Artificial control	0.2	0.3	0.9	2.4	0.6	1.7
	P (F)	**0.009**		0.749		0.419	
	sem	0.9		1.2		2.3	

563

564 [a] fumonisins (FUMs), fusaproliferin (FUS), moniliformin (MON), bikaverin (BIK), beauvericin (BEA), fusaric acid (FA) and
565 equisetin (EQU). The reported contamination means are transformed [T; y'= ln (x + 1)] and not transformed (N) values.
566 [b] The level of significance of ANOVA is reported in the table.
567 [c] sem: standard error of mean

568 **Table. 5**

569 Main producing mycotoxin *Fusarium* species detected in the maize samples.

Mycotoxin	Produced by	Section	References
Fumonisins (FUMs)	*F. verticillioides* *F. proliferatum*	Liseola	Logrieco et al. 2002 Sanhueza et al. 2004
Moniliformin (MON)	*F. subglutinans* *F. proliferatum*	Liseola	Sanhueza & Degrossi 2004 Battilani et al. 2009
Fusaproliferin (FUS)	*F. proliferatum* *F. subglutinans*	Liseola	Logrieco et al. 1996 Jestoi 2008
Bikaverin (BIK)	*F. verticillioides*	Liseola	Busman et al. 2012 Lazzaro et al. 2012
Beauvericin (BEA)	*F. subglutinans* *F. proliferatum* *F. verticillioides*	Liseola	Sanhueza & Degrossi 2004 Jestoi 2008
Fusaric Acid (FA)	*F. proliferatum* *F. verticillioides*	Liseola	Bacon et al. 1996 Shimshoni et al. 2013
Equisetin (EQU)	*F. equiseti*	Gibbosum	Wheeler et al. 1999 Streit et al. 2013
Deoxynivalenol (DON)	*F. graminearum* *F. culmorum*	Discolor	Bottalico 1998 Rasmussen et al. 2012
Deoxynivalenol-3-glucoside (DON-3-G)	Phase II plant metabolite of DON ("Masked mycotoxin")		Rasmussen et al. 2012 Berthiller et al. 2013
Zearalenone (ZEA)	*F. graminearum* *F. culmorum*	Discolor	Logrieco et al. 2002 Garrido et al. 2012
Culmorin (CULM)	*F. graminearum* *F. culmorum*	Discolor	Pedersen & Miller 1999 Streit et al. 2013
Aurofusarin (AUR)	*F. avenaceum* *F. graminearum* *F. culmorum*	Discolor / Roseum	Uhlig et al. 2006 Streit et al. 2013
Butenolide (BUT)	*F. graminearum* *F. culmorum*	Discolor	Wang et al. 2009 Streit et al. 2013

Table. 6

Effect of ECB infestation on the contamination of mycotoxin produced by *Fusarium* spp. of *Discolor* and *Roseum* sections; field experiments conducted at Carmagnola (TO) in the 2008 - 2010 period.

Mycotoxin[a]	ECB infestation	Year					
		2008		2009		2010	
		T	N (µg kg^{-1})	T	N (µg kg^{-1})	T	N (µg kg^{-1})
DON	Natural	3.9	305.0	3.3	93.2	0.1	0.1
	Artificial control	1.5	24.9	0.1	0.1	2.1	130.2
	P (F)[b]	0.375		0.116		0.374	
	sem[c]	3.4		2.3		87.6	
DON-3-G	Natural	3.3	138.7	2.0	12.6	1.3	14.2
	Artificial control	4.6	104.5	0.2	0.3	3.9	74.8
	P (F)	0.482		0.165		0.155	
	sem	2.5		1.4		2.1	
ZEA	Natural	3.4	94.1	-	< LOQ	0.8	3.0
	Artificial control	1.3	3.1	-	< LOQ	2.5	28.6
	P (F)	0.135				0.303	
	sem	1.6				2.0	
CULM	Natural	4.7	188.0	2.3	29.9	2.5	68.8
	Artificial control	4.1	66.4	0.0	< LOQ	5.4	251.5
	P (F)	0.544		0.147		0.133	
	sem	1.1		1.8		2.2	
AUR	Natural	5.9	2046.1	1.7	21.7	1.9	10.3
	Artificial control	3.8	50.1	0.6	1.0	3.9	89.8
	P (F)	0.227		0.427		0.153	
	sem	2.2		1.8		1.6	
BUT	Natural	3.1	32.5	1.3	6.7	2.5	37.7
	Artificial control	1.5	11.8	0.4	0.6	4.9	173.3
	P (F)	0.241		0.374		0.150	
	sem	1.7		1.2		1.9	

[a] deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON-3-G), zearalenone (ZEA), culmorin (CULM), aurofusarin (AUR) and butenolide (BUT). The reported contamination means are transformed [T; y'= ln (x + 1)] and not transformed (N) values.

[b] The level of significance of ANOVA is reported in the table.

[c] sem: standard error of mean

Table. 7

Correlation matrix between ECB and fungal ear rot severity and mycotoxin contamination in maize kernels.

Correlation	ECB severity	Fungal ear rot severity	FUMs	FUS	MON	BIK	BEA	FA	EQU	DON	DON-3-G	ZEA	CULM	AUR
Fungal ear rot severity	**0.975****													
FUMs	**0.893****	**0.911****												
FUS	**0.786****	**0.782****	**0.845****											
MON	**0.830****	**0.814****	**0.855****	**0.859****										
BIK	**0.831****	**0.870****	**0.904****	**0.866****	**0.839****									
BEA	**0.876****	**0.849****	**0.878****	**0.917****	**0.945****	**0.861****								
FA	**0.821****	**0.808****	**0.734****	**0.767****	**0.827****	**0.793****	**0.812****							
EQU	**0.704****	**0.672****	**0.612****	0.451	**0.557***	**0.576***	**0.623****	**0.576***						
DON	0.203	0.104	0.141	0.413	0.180	0.107	0.362	0.062	0.323					
DON-3-G	-0.029	-0.100	0.067	0.318	0.031	0.119	0.175	0.009	-0.149	**0.581***				
ZEA	0.375	0.305	0.346	**0.511***	0.246	0.392	0.363	0.299	0.224	**0.472***	**0.700****			
CULM	0.187	0.133	0.285	0.454	0.121	0.265	0.274	0.213	0.092	**0.481***	**0.842****	**0.740****		
AUR	0.397	0.304	0.361	**0.519***	0.308	0.355	0.469	0.253	0.219	**0.654****	**0.783****	**0.864****	**0.769****	
BUT	0.181	0.174	0.221	0.367	0.112	0.220	0.140	0.361	0.043	0.182	**0.542***	**0.619****	**0.767****	0.453

fumonisins (FUMs), fusaproliferin (FUS), moniliformin (MON), bikaverin (BIK), beauvericin (BEA), fusaric acid (FA), equisetin (EQU), deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON-3-G), zearalenone (ZEA), culmorin (CULM), aurofusarin (AUR) and butenolide (BUT).

(*) = correlation significant at P ≤ 0.05; (**) correlation significant at P ≤ 0.01. The aata reported in the table are Pearson product-moment correlation coefficients.