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Relationship between European Corn Borer injury, Fusarium proliferatum and F. subglutinans infection and moniliformin contamination in maize

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Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1526393 since 2015-10-21T12:50:29Z

Published version:

DOI:10.1016/j.fcr.2015.07.014

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UNIVERSITÀ DEGLI STUDI DI TORINO

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21 FIELD CROP RESEARCH

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Relationship between European Corn Borer injury, *Fusarium proliferatum* and *F. subglutinans* infection and moniliformin
 contamination in maize.

- 26
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40 Acknowledgements

- 41 The authors would like to thank Fabio Carnaroglio, Mattia Ciro Mancini, Alessandro Peila,
- 42 Federico Marinaccio and Valentina Sovrani for their precious help and cooperation in the
- 43 laboratory and field work.
- 44 The research has been conducted thanks to the financial support of the Italian Ministry of
- 45 Agricultural, Food and Forestry Policies (MIPAAF), as a part of the MICOPRINCEM project
- 46 (Coordinator Dr. Maria Grazia D'Egidio, CRA QCE).

48 Abstract

The European Corn Borer (ECB), *Ostrinia nubilalis* (Hübner), plays an important role in promoting *Fusarium* infection and fumonisin production in maize kernels. Moniliformin (MON) is a mycotoxin that is usually found in association with fumonisins (FB), and as a consequence ECB is also expected to play a role in MON contamination.

53 The aim of the current study was to investigate the influence of ECB activity on MON 54 contamination and on the fungi responsible for its contamination.

A comparison has been made between maize infected naturally by insect larvae and protected maize, from 2008 to 2010 in North-West Italy; the latter was obtained by positioning an entomological net at the end of maize flowering.

The *F. proliferatum* infection of the maize grain was higher in each growing season than 58 that caused by F. subglutinans, although both species were significantly increased due to 59 the ECB damage to the maize ears. The ECB activity significantly increased the MON 60 content 26-fold in 2008 from 43 to 1137 μ g kg⁻¹, 25-fold in 2009 from < LOQ to 77 μ g kg⁻¹ 61 and 94-fold in 2010 from 6 to 564 µg kg⁻¹. The relationship between the MON content and 62 the Fusarium species producers of MON was closer for F. proliferatum (R^2 = 0.93, P < 63 0.001) than for *F. subglutinans* ($R^2 = 0.68$, P < 0.001). An *in vitro* assay was carried on in 64 65 order to assess the toxigenic capacity of different F. proliferatum and F. subglutinans strains isolated from a field experiment and artificially inoculated on a maize substrate. On 66 average, the *F. proliferatum* isolated strains showed a significantly higher (P < 0.001) 67 toxigenic capacity than the *F. subglutinans* strains. 68

In conclusion, the combination of the high toxigenic capacity of *F. proliferatum*, and its more frequent occurrence and greater intensity in the field make it possible to state that the production of MON in maize temperate areas, such as North Italy, is mainly due to *F. proliferatum* infections and is closely linked to the injury caused by ECB larvae.

- **Keywords:** maize, Moniliformin, *Fusarium proliferatum*, *Fusarium subglutinans*, European
- 74 Corn Borer

76 **Abbreviations**

ANOVA, Analysis of variance; EC, European Commission; ECB, European Corn Borer; EFSA, European Food Safety Authority; ESI, electrospray ionization; FB, fumonisins, GDD, Accumulated growing degree days; HILIC, hydrophilic interaction chromatography; HPLC, high-performance liquid chromatography; LC-MS/MS, Liquid chromatography coupled to tandem mass spectrometry detection; LOD, limit of detection; LOQ, limit of quantification; MON, moniliformin; MS, mass spectrometry detection; RSD, relative standard deviation.

85 **1. Introduction**

Maize ear rot is a common disease throughout the world that is caused by several 86 *Fusarium* species which can co-occur to give rise to the production of different mycotoxins 87 in infected kernels, with a considerable impact on human and animal health (Bottalico, 88 1998). In temperate areas, maize pink ear rot, which is mainly caused by *F. verticillioides*, 89 leads to an extensive occurrence of FB, a group of toxins among which fumonisin B₁ (FB₁) 90 has been evaluated, by the International Agency for Research on Cancer (IARC), as a 91 Group 2B carcinogen, i.e., possibly carcinogenic to humans (IARC, 2002). Regulatory 92 limits have been set for maize grain within the European Union (European Commission 93 No. 1881/2006 and EC No. 1126/2007), the USA (FDA - Food and Drug Administration, 94 2000) and other countries. 95

Moniliformin (MON) is a mycotoxin that is usually found in association with FB (D'Mello et al., 1999; Sanhueza and Degrossi, 2004). This mycotoxin is produced by *Fusarium subglutinans* (Logrieco et al., 2002) *Fusarium temperatum* (Scauflaire *et al* 2011; Scauflaire *et al* 2012) and *F. proliferatum*, which is also a FB producer. These *Fusarium* species, which refer to the *Liseola* section, could infect maize kernels in temperate areas and co-participate with *F. verticillioides* in maize ear rot (Srobarova et al., 2002).

No regulatory limits have been established about the presence of MON in food or feeds. Although its toxic effects have not yet been fully established, Jonsson et al. (2013) have recently reported a high acute toxicity of MON in rats, with the LD₅₀ value being at the same level as the most toxic *Fusarium* mycotoxins, that is, T-2 and HT-2.

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 MON in feeds and food (EFSA 2010). In addition to studies conducted to clarify the occurrence and toxicological effects, it is also of extreme importance to individuate the conditions which

110 could lead to a higher contamination of this mycotoxin, in order to understand the Best111 Management Practices that can be used to limit contamination.

The European Corn Borer (ECB), *Ostrinia nubilalis* (Hübner), is a maize pest that has an economic impact on several producing areas (Bode and Calvin, 1990; Szóke et al., 2002). Two generations of ECB larvae usually occur per year in North Italy: the first generation attacks plants during the mid to late vegetative stages and the second generation attacks during the reproductive stages (from early milk stage to maturity) (Blandino et al., 2009). Moreover, second generation larvae play a very important role in promoting Fusarium infection and mycotoxin production in maize kernels (Sobek and Munkvold, 1999).

Other *Fusarium* infection pathways are known, through seed and seedlings (systemic) or silks at flowering, although these seem to play a minor role in the final mycotoxin contamination compared to the kernel damage caused by insects (Munkvold et al., 1997).

In temperate areas, *F. verticillioides* is generally more favoured by ECB larva feeding than other *Fusarium* species (Lew et al., 1991; Sobek and Munkvold, 1999) and the direct and indirect control of this pest is one of the main strategies adopted to minimize FB contamination (Blandino et al., 2009).

Moreover, Papst et al. (2005) and Magg et al. (2003), in comparative studies on Bt maize 126 and its isogenic counterparts conducted in Central Europe, have also reported a significant 127 correlation between the percentage of ECB damaged ears and the MON concentration. 128 Unfortunately, at the moment, no data are available on the effect of ECB on MON contents 129 in warmer maize cultivation areas, where the pressure and damage caused to ears and 130 kernels by this insect are higher. Moreover, so far, it has not been clearly reported, in the 131 literature, which Fusarium species is the most responsible for MON contamination in 132 temperate areas or what relationship exists between these species and ECB activity. 133

The aim of the current study was to investigate the influence of ECB activity on MON contamination and the fungi responsible for its contamination, in order to evaluate the

effectiveness of the insect control in managing the MON contamination. Moreover, another objective of this work was to assess which of the two *Fusarium* species, *F. proliferatum* and *F. subglutinans*, is the more responsible for MON contamination in maize cultivated in temperate areas, subjected to ECB damage.

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- 141

142 **2. Materials and Methods**

143 **2.1**. Chemicals

All the chemicals and analytical standards used for the chemical measurements were purchased from Sigma Aldrich (St. Louis, MO), or VWR (Milan, Italy). The solvents were all gradient grade or LC-MS grade.

The MON standard was purchased as sodium salt and a 93 mg L⁻¹ stock solution of MON was prepared in acetonitrile/water (84:16, v/v) and stored at 4 °C. This stock solution was used to prepare standard solutions through dilution with acetonitrile/water (84:16, v/v).

The FB₁ and FB₂ standards were purchased as a 50 mg L⁻¹ stock solutions in acetonitrile/water (50:50, v/v) and stored at -20 °C. These stock solutions were used to prepare standard solutions containing both FB₁ and FB₂, through dilution with acetonitrile/water (50:50, v/v).

All the chemicals used for the mycological measurements were purchased from Sigma (St.
 Louis, MO), Eppendorf-Germany (Hamburg, Germany), Applied Biosistems by Life
 Tecnologies (Life Technologies Italia, Monza, Italy), or PRIMM (PRIMM srl, Milan, Italy).

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161 2.2. Field Experimental Design and Samples

The effect of ECB larva feeding activity on MON contamination in maize kernels was studied from 2008 to 2010 in North-West Italy under naturally-infected field conditions in the experimental farm of the Department of Agricultural, Forest and Food Science, University of Turin located in Carmagnola (44° 50' N, 7° 40' E; altitude 245 m), in a sandymedium textured soil (Typic Udifluvents).

The natural maize ear infestation, due to the insect larvae, was compared each year with the protected maize (artificial control of ECB larvae), which was obtained by positioning an entomological net at the end of maize flowering [Growth stage (GS) 69, Lancashire et al. 170 1991] in order to avoid ECB ovideposition.

The ECB natural infestation and artificial protection treatments were assigned to experimental units using a completely randomized block design with 4 replicates. Each plot consisted of 4 rows 0.75 cm apart and 4 m long. The plot alleys, orthogonal to the maize rows, were one meter wide.

The entomological net had a mesh size of 1 mm, and it was placed on a 4.20 m long, 4.20 m wide and 3.80 m high, steel structure, in order to minimize any significant microclimatic changes. The position of the net in the field changed every growing seasons. The edge of the net was buried, to prevent adult insects from entering, while the plants within the net were carefully checked for possible first generation attack. If the plants presented any typical leaf injuries caused by first generation ECB larvae, they were cut at the bottom and removed from the plots.

182 No foliar insecticides were applied to the experimental field or to an approximately 20 ha 183 area around the field to control ECB or other insects during the entire growing period.

The normal agronomic techniques was adopted. Briefly, the previous crop was maize, and the field was ploughed each year. Studies were carried out each year on the commercial

dent corn hybrid Syngenta NX7444 (FAO maturity class 600; 130 days relative to maturity). The crop density was approximately 75.000 plants per hectare and the experiment field received 250, 90 and 100 kg ha⁻¹ of N, P and K, respectively, each year. The field was cultivated under irrigation with full length maturity hybrids, planted in each growing season in the period between the last decade of March and the first decade of April. Metolachlor and terbutilazine were applied to control weeds in pre-emergence while sulcotrione and nicosulfuron were applied in post-emergence.

Thirty randomly selected ears (included the ears used for the evaluation of ECB incidence 193 and severity at harvest) were collected by hand from each plot at the end of maturity at a 194 grain moisture content of between 23 and 27%, and shelled using an electric sheller. The 195 kernels from each plot were mixed thoroughly to obtain a random distribution; 4 kg 196 samples were then taken and dried at 60°C for 3 days to analyze the MON and FB 197 198 contents and to quantify F. verticillioides, F. subglutinans and F. proliferatum using Real Time PCR. All the amount of grains collected from each plot was completely ground using 199 200 a ZM 200 Ultra Centrifugal Mill (Retsch GmbH, Haan, Germany) and reduced to 1 kg 201 subsample by dynamically sampling the flowing flour during the grinding. This subsample was re-milled and reduced to 500 g with the same method and subsequently an aliguot of 202 100 g, randomly sampled, was used in the molecular analysis, while the remaining 400 g 203 were employed in the chemical analysis. 204

205 During all the investigated period, rainfall and temperature data have been recorded daily 206 from a weather station, located next to the experimental field.

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208 2.3. Entomological measurements

ECB damage incidence was calculated as the percentage of ears per plot with kernel injury or apical and basal tunnels in the cob due to larva activity. ECB damage severity

was calculated as the percentage of kernels per ear with injuries due to larva activity. A 7class rating scale 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., 2009). The ECB damage severity scores were converted to percentages of ears exhibiting symptoms, by using the mid-point of each class interval.

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219 2.4. Mycological measurements

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221 2.4.1 Fungal ear rot incidence and severity

Fungal ear rot incidence was calculated as the percentage of ears per plot with symptoms, 222 while fungal ear rot severity was calculated as the percentage of kernels per ear with 223 symptoms. A scale of 1 to 7 was used in which each numerical value corresponds to a 224 percentage interval of surfaces exhibiting visible symptoms of the disease according to the 225 following schedule: 1 = no symptoms, 2 = 1-3 %, 3 = 4-10%; 4 = 11-25 %, 5 = 26-50%, 6 = 226 51-75%, 7 > 75% (Blandino et al., 2009). The ear rot severity scores were converted to 227 percentages of ears exhibiting symptoms and each score was replaced with the mid-point 228 of the interval. 229

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231 2.4.2. Fusarium kernel infection

The evaluation of the total *Fusarium* spp (section *Liseola*) infections was carried out using 100 kernels randomly sampled from the untreated control at the dough stage (GS 85). The kernels were surface disinfested for 3 min in a 2% solution of sodium hypochlorite, then rinsed 3 times with sterile water. The kernels were placed in Petri dishes containing

dicloran cloramfenicol peptone (DCPA) and incubated at 20 °C. The *Fusarium* colonies
were identified after 7 to 10 days through colony and conidial morphology, as reported by
Nelson et al. (1983).

239

240 2.4.3. DNA extraction from the maize samples

The total DNA from the fungal reference cultures was extracted from approximately 200-241 300 mg (wet weight) of fresh mycelium, according Henrion et al. (1992), with slight 242 modifications. The samples were crushed in liquid N₂ and immediately suspended in one 243 mL of lysis buffer (100 mM Tris-HCl, pH 8, 20 mM EDTA, pH 8, 1.4 mM NaCl, 2% 244 bromide (CTAB), 1% 245 cetyltrimethylammonium polyvinylpyrrolidone (PVP), 1% mercaptoethanol) for 60 minutes at 65°C. The proteins were denatured and removed 246 through one or more extractions by gently shaking the solution for 1 hour in ice with 1 mL 247 cloroform/isoamyl alcohol (24:1, v/v), followed by centrifugation at 17300 \times g for 10 248 minutes, to separate the phases. The nucleic acids were precipitated from the aqueous 249 phase by adding 2/3 volume of isopropanol and 1/10 volume of sodium acetate 3 M (pH 250 5.2) and maintaining the sample at -20°C for 20 min. The DNA was then pelleted by 251 centrifugation at 17300 × g for 10 minutes, washed with 70% (v/v) ethanol, vacuum dried 252 briefly, resuspended in 50-150 µL of TE buffer (10 mM Tris-HCl pH 8 + 1 mM EDTA) and 253 stored at -20°C until use. 254

The flour aliquot of 100 g, obtained as previously described, was milled again in a knife mill Grindomix GM 200 (Retsch Technology Gmbh, Haan, Germany). Then 10 g of remilled flour were further ground in liquid N2 and the DNA was extracted from 1 g, using the DNeasy Plant Maxi Kit, according to the manufacturer's instruction.

The concentration of nucleic acids was determined using a Biophotometer (Eppendorf AG, Hamburg-Germany) and DNA purity was evaluated by comparing the A260/A280 and A260/A230 absorbance ratios.

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263 2.4.4. Preliminary species-specific PCR

End-Point PCR was carried out using the SUB1 (CTGTCGCTAACCTCTTTATCCA) and 264 SUB2 (CAGTATGGACGTTGGTATTATATCTAA) primer for the detection of F. 265 subglutinans; the PR01 (CTTTCCGCCAAGTTTCTTC) and PRO2 266 (TGTCAGTAACTCGACGTTGTTG) primer for the detection of F. proliferatum; the VER1 267 (CTTCCTGCGATGTTTCTCC) and VER2 (AATTGGCCATTGGTATTATATATCTA) primer 268 for the detection of F. verticilliodes, all designed by Mulè et al., 2004 on the calmodulin 269 gene. These primers amplify respectively 631 bp for F. subglutinans, 585 bp for F. 270 proliferatum and 578 bp for F. verticillioides. 271

272 Moreover, the primers subsequently used in the Real Time PCR were tested to verify high 273 specificity of the investigated *Fusarium* species.

The PCR assays were conducted in 50 µL of reaction mixtures containing 1.25 U of Tag 274 (HotMaster[™] Taq DNA Polymerase, Eppendorf-Germany), a 1X HotMaster Taq Buffer 275 with Mg²⁺, 400 nM of each primer (PRIMM srl, Milan, Italy), 150 µM of each dNTP 276 (Eppendorf-Germany), and 50 ng of total DNA (from maize). Reactions were performed 277 using the following PCR conditions: denaturation at 95 °C for 5 min; 35 cycles of 278 denaturation at 94 °C for 50 s, annealing at 56 °C for 50 s, extension at 72 °C for 1 min; 279 final extension at 72 °C for 7 min, followed by cooling at 4°C until the samples were 280 recovered. Amplification products were assessed on 1,2% agarose gel stained with Green 281 Gel Plus™(Fisher Molecular Biology-USA). 282

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284 2.4.5. Quantification of F. verticillioides, F. proliferatum and F. subglutinans using Real 285 Time PCR

The primers used for the detection of F. subglutinans were Fsub565 286 F (TCATTGGTATGTTGTCGCTCATG) Fsub622A R 287 and (GTGATATGTTAGTACGAATAAAGGGAGAAC), designed on the $EF1\alpha$ gene (Nicolaisen 288 et al., 2009; Boutigny et al. 2011). The primers Fp3-F (CGGCCACCAGAGGATGTG) and 289 Fp4-R (CAACACGAATCGCT TCCTGAC), designed by Jurado et al. (2006) on the 290 intergenic sequence of the ribosomal RNA gene cluster, for the detection of *F. proliferatum* 291 and the primers VER1 and VER2, designed by Mulè et al., 2004 on the calmodulin gene, 292 for the detection of *F. verticilliodes*, were used in Real Time as described by Nutz et al. 293 (2011); all were synthesized by PRIMM (PRIMM srl, Milan, Italy). 294

Real-time PCR assays were carried out in a total volume of 25 μ L, consisting of 12.5 μ L 287 2X SYBR Green PCR Master Mix (Applied Biosystems), 600nM concentration of each 288 primer for *F. proliferatum*, 300 nM for *F. subglutinans* and F. *verticilliodes* and 2 μ L 298 template DNA (approximately 10 - 20 ng); only for *F. subglutinans*. the reaction mixture 299 contain also 0.5 μ g μ L⁻¹ of bovine serum albumin (BSA).

The Real-time PCR reactions were performed in triplicate on all the samples and were performed on an ABI 7500 Real-Time PCR System (Applied Biosystems) using three different cycling protocols. The first, for *F. proliferatum*, was 2 min at 50 °C; 10 min at 95 °C; 40 cycles of 15 s at 95 °C and 1 min at 62 °C. The second, for *F. subglutinans*, was: 2 min at 50 °C; 4 min at 95 °C; 35 cycles of 35 s at 95 °C, 32 s at 64 °C, 32 s at 72°C and 5 min at 72°C. The third, for *F. verticilliodes*, was 2 min at 50 °C; 10 min at 95 °C; 40 cycles of 50 s at 94 °C, 50 s at 60 °C, 1 min at 72°C and 5 min at 72°C.

All cycling protocols were followed by a dissociation curve analysis at 60 to 95 °C; the fluorescence was measured in the first and the second protocol during the annealing phase; in the third during the extension phase.

In order to test for linearity and the presence of inhibitors in the pathogen DNA, standard 310 curves were prepared by diluting the DNA of each pathogen (approximately 121 ng μ L⁻¹ 311 for *F.* subglutinans, 256 ng μ L⁻¹ for *F.* proliferatum and 97 ng μ L⁻¹ for *F.* verticilliodes) 5-, 312 25-, 125-, 625-, 3125-, 15625-, 78125-fold in maize DNA that was free of fungal 313 contamination (approximately 105 ng μL^{-1}). Standard curves were generated by plotting 314 threshold cycle values (Ct values) against the logarithm of the starting DNA quantities. The 315 standard curves slopes were used to calculate the reaction efficiency (E) of the PCR 316 assays. The amounts of fungal DNA obtained were normalized to the total DNA amount 317 318 extracted from the meal samples.

Real-time PCR reactions were performed in triplicate for each sample.

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2.5. In vitro MON production by means of different monoconidial strains of *F. verticillioides, F. proliferatum and F. subglutinans*

An *in vitro* assay was performed in order to analyze the toxigenic capacity of the fungal monoconidial strains isolated from the field experiment. Five *F. proliferatum*, five *F. subglutinans* and five *F. verticilliodes* strains were randomly chosen from all the isolated strains and they were grown for a week on potato dextrose agar (PDA) at 25°C in Petri dishes in the dark.

Reference Cultures (*Fusarium proliferatum*, KSU D-4854, Kansas State University; *Fusarium subglutinans*, KSU E-0990, Kansas State University and *F. verticilliodes*, KSU A-0999, Kansas State University) were instead grown for DNA extraction on potato dextrose broth (PDB) in 100 mL Erlenmeyer flasks at room temperature on a rotary shaker. The

332 mycelium was harvested after 14 days by means of filtration and then rinsed in distilled 333 water.

A commercial maize lot was ground using a ZM 200 Ultra Centrifugal Mill (Retsch GmbH, Haan, Germany) and the flour was used as a substrate for the *Fusarium proliferatum*, *Fusarium subglutinans* and *F. verticilliodes* strains. The grain was preliminary tested by means of LC-MS/MS in order to correct the results and to conduct the assays using a meal in which the MON content was as low as possible.

The meal was autoclave sterilized and moistened with 25 g of sterile water in 35 g of milled kernels; the Petri dishes (\emptyset 9 cm) were then inoculated with the selected *Fusarium* strains, using 5 mycelium plugs of 6 mm², taken from one-week-old colonies actively growing on PDA at 25°C in the dark. Three replicates were performed for each fungal strain and 6 Petri dishes were prepared for each replicate. Six Petri dishes without mycelium plugs were also produced as a control.

All the Petri dishes were incubated at 25°C in the dark for 20 days and the flour was then exsiccated in a dry kiln for 36 hours at 65°C and for 12 hours at 50°C before being ground again in order to homogenize the mycelium with the meal. These samples were tested for the MON content by means of LC-MS/MS analysis, and *F. subglutinans*, *F. proliferatum* and *F. verticilliodes* were quantified using Real Time PCR.

The toxigenic capacity of the different fungal strains was expressed as the ratio between the MON content and the fungal DNA, normalized for the total DNA.

352

353 2.6. Chemical Analyses

354 2.6.1. Sample Preparation and Extraction

For MON extraction 25 g of maize flour was extracted by mechanical shaking at 100 rpm for 1 h (shaker mod. M102-OS, MPM Instruments, Milan, Italy) with 100 mL

acetonitrile/water (84:16, v/v). The extracts were filtered through Whatman[®] no. 1 filters
 (Brentford, UK) and subjected to clean-up and purification.

For FB₁ and FB₂ extraction 50 g of maize flour was extracted by mechanical shaking at 100 rpm for 20 min (shaker mod. M102-OS, MPM Instruments, Milan, Italy) with 100 mL methanol/water (80:20, v/v). The extracts were filtered through Whatman[®] no. 1 filters (Brentford, UK), diluted 1:5 with Dulbecco's Phosphate Buffer Saline (PBS, 1X solution, Sigma Aldrich, St. Louis, MO), filtered again through Whatman[®] glass microfiber filters, Grade 934-AH[®] (Brentford, UK) and subjected to clean-up and purification.

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366 2.6.2. Clean-up

The clean-up method for MON was performed with MycoSep[®] 240 Mon clean-up columns 367 (Romer Labs[®], Tulln, Austria). The clean-up procedure (Scarpino et al., 2013) was 368 adapted from the Romer Labs[®] procedure. The cleanup MycoSep[®] columns were pushed 369 into test tubes containing 5 mL of the sample extracts, and the extracts were allowed to 370 filter upwards through the packing material of the columns. The interferences adhered to 371 the chemical packing in the columns and the purified extracts, containing MON, passed 372 373 through the columns. The purified extracts (1.5 mL) were transferred to HPLC vials and analyzed by means of LC-MS/MS, according to the method described below. 374

The clean-up method for FB (FB₁ and FB₂) was performed with immunoaffinity columns (IA) FumoniTestTM WB Columns (VICAM_®, USA). Five mL of the sample extracts were loaded and passed through the columns and after a washing step with 2.5 mL of PBS 1 X solution FB were recovered and eluted with 2 mL of methanol. The purified extracts were transferred to HPLC vials and analyzed by means of LC-MS/MS, according to the method described below.

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382 2.6.3. LC-MS/MS Analysis

LC-MS/MS analysis was carried out on a Varian 310 triple quadrupole mass spectrometer
 (Agilent, Italy) equipped with an electrospray ionization ESI source, a 212 LC pump, a
 ProStar 410 AutoSampler and dedicated software.

For MON analysis LC separation was performed on a 100 mm × 2.1 mm i.d., 3.5 µm, 100 Å ZIC[®]-HILIC (Merck, SeQuant, Milan, Italy) column. The mobile phase consisted of water buffered with 100 mM ammonium formate (pH 6.4) (A) and acetonitrile (B) delivered at 200 µL min⁻¹. The chromatographic and mass spectrometric parameters of the investigated analytes were described by Scarpino et al. in 2013. The deprotonated molecule (*m/z* = 97.0) was fragmented to its product ion (*m/z* = 41.0) and used for quantification and identification purposes.

393 The average percentage of recovery for MON was: 81% (Relative Standard Deviation,

RSD%: 9%). The results of the MON concentrations were corrected for the recovery rate.

The limit of detection (LOD) and the limit of quantification (LOQ) were 1 μ g kg⁻¹ and 4 μ g kg⁻¹, respectively.

For FB analysis LC separation was performed on a 50 mm × 2.1 mm i.d., 5 μ m, Varian Pursuit 5 (Agilent, Italy) C18 column. The mobile phase consisted of water acidified with 0,1% CH₃COOH (A) and acetonitrile acidified with 0,1% CH3COOH (B) delivered at 200 μ L min⁻¹ for 15 min. Mass spectrometric analyses were performed in the positive-ion mode. For FB₁ MS detection the protonated molecule (m/z = 722.5) was fragmented to its product ions (m/z = 334.2 and m/z = 352.3) and used for quantification and identification purposes.

For FB₂ MS detection the protonated molecule (m/z = 706.5) was fragmented to its product ions (m/z = 318.0 and m/z = 336.0) and used for quantification and identification purposes.

The average percentage of recovery for FB was: 75% (Relative Standard Deviation, RSD%: 8%). The results of the FB concentrations were corrected for the recovery rate. The limit of detection (LOD) and the limit of quantification (LOQ) were 2 μ g kg⁻¹ and 8 μ g kg⁻¹, respectively.

411

412 2.6.4. Calibration

Ten different MON concentrations between 0.93 and 930 μ g L⁻¹, were prepared for calibration in acetonitrile/water (84:16, v/v). A linear regression was used to obtain the regression curve.

Ten different FB concentrations between 1 and 10000 μ g L⁻¹, were prepared for calibration in acetonitrile/water (50:50, v/v). A linear regression was used to obtain the regression curve.

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420 **2.7.** *Statistics*

421 The normal distribution and homogeneity of variances were verified by performing the 422 Kolmogorov–Smirnov normality test and the Levene test, respectively.

An analysis of variance (ANOVA) was utilized to compare the fungal ear rot incidence and 423 severity, the Fusarium kernel infection, the F. verticillioides, the F. proliferatum and 424 subglutinans DNA quantification and the MON and FB contamination separately for each 425 year, using a completely randomized block design, in which the natural presence of ECB 426 larva feeding injuries was the independent variable. The incidence and the severity values 427 of fungal ear rot, the Fusarium kernel infection, and the F. verticillioides, the F. proliferatum 428 and F. subglutinans DNA quantification were previously transformed 429 usina y'=arcsine $\sqrt{x^*180/\pi}$. The concentration of MON and FB was transformed using the 430 y'=ln(x+1) equation to normalize the residuals. 431

Linear regression analysis was performed for the relationship between the *F. proliferatum* DNA, the *F. subglutinans* DNA, the sum of *F. proliferatum* and *F. subglutinans* DNA and the MON concentration in 24 maize field samples over the three year growing season period (2008-2010). Since the x-values were free to vary and subject to error, the Reduced Major Axis Model II linear regression analysis method was applied to better fit the data (Ludbrook, 2012).

Pearson correlation coefficients were obtained for MON and FB, relative to each another and to ECB incidence and severity, fungal ear rot incidence and severity, *Fusarium* kernel infection, *F.* verticillioides, *F. proliferatum* and *F. subglutinans* by joining the data sets that referred to the three growing seasons.

An analysis of variance (ANOVA) was used to compare the toxigenic capacity of isolated *F. proliferatum* and *F. subglutinans* strains. In order to apply ANOVA, the toxigenic capacity values were subjected to a rank transformation, a valid alternative bridging parametric and non-parametric analysis proposed by Conover and Iman (1981) when parametric model assumptions do not hold. An REGWQ test was subsequently used as a Post-hoc test.

The SPSS Version 21.0 for Windows statistical package, (SPSS Inc., 2012) was used for the statistical analysis.

450

451 **3. Results**

452 **3.1.** Meteorological data

The three growing seasons showed remarkably different meteorological trends as far as 453 454 both rainfall and temperature (expressed as growing degree days, GDDs) from flowering to harvesting are concerned (Table 1). The years 2008 and 2010 were characterized by 455 low GDDs and high rainfall in May and June, and also close to flowering. Heavy rainfall 456 457 occurred, in particular during the 2010 growing season, from the early milk stage to the harvest. The year 2009 instead had less rainfall during the spring, although it was more 458 concentrated in July, after maize flowering. The average GDDs from June to September 459 were higher in 2009 than those in 2008 and 2010, and this led to an early harvest at the 460 beginning of September (Table 1). 461

462

463 **3.2.** ECB damage

Table 2 summarizes the mean ECB incidence and severity recorded for each growing 464 season for the natural infestation of ears collected for each sampling year. The ears 465 collected in the plots protected with entomological nets were free from ECB attack, in each 466 growing season, while those collected in the plots subject to natural insect attacks showed 467 a variable damage severity that depended on the insect pressure in each growing season. 468 The samples collected in the 2008 and 2010 growing seasons showed more higher ECB 469 damage, while the ECB severity recorded in 2009 was more than 3 times lower than in 470 2008 and 2010 (Table 2). 471

472

473

475 **3.3.** *Mycological measurements in the field samples*

The presence of ECB larvae significantly affected the fungal ear rot incidence and severity in each growing season (Table 3). The ECB attack increased the fungal ear rot severity 22-fold and 153-fold in the 2008 and 2010 growing seasons, respectively. Conversely, in 2009, the year characterized by the lowest ECB pressure, the fungal ear rot severity only increased 3-fold. The *Fusarium* kernel infection was significantly higher in the natural infected ears in each growing season, according to the ECB attack (Table 3).

As far as DNA quantification is concerned, the *F. proliferatum* occurrence significantly increased in the natural infection conditions of 29-fold, 15-fold and 21-fold in the 2008, 2009 and 2010 growing seasons, respectively, compared to the artificial control. The insect activity on the ears increased the infection of *F. subglutinans* of 23-fold, 19-fold and 25-fold in the 2008, 2009 and 2010 growing seasons, respectively, although its effect was only significant in the first two years (Table 3). The ECB activity significantly increase *F. verticillioides* infection in all the growing seasons.

489

490 3.4. FB and MON contamination in the field samples

The MON contamination was affected significantly by the ECB larva feeding activity on the maize ears in all the considered growing seasons (P = 0.002, 2008; P = 0.002, 2009; P < 0.001, 2010) (Table 3). The presence of ECB damage increased the MON content 26-fold in 2008 from 43 to 1137 μ g kg⁻¹, 25-fold in 2009 from 3 < LOQ to 77 μ g kg⁻¹ and 94-fold in 2010 from 6 to 564 μ g kg⁻¹. Moreover, as reported in Table 3 the ECB larva feeding activity on the maize ears significantly affected the FB contamination in the years with the higher ECB pressure, 2008 and 2010.

As far as the relationships between the major *Fusarium* species producers of MON and mycotoxin contamination are concerned, the amount of *F. proliferatum* DNA (Fig. 1), the

amount of *F. subglutinans* DNA (Fig. 2) and the sum of the amounts of the 2 previous *Fusarium* species DNA (Fig. 3) showed a significant linear relationship with the MON content.

503 Moreover, as reported in Table 4, MON and FB contamination, fungal ear rot incidence 504 and severity, *Fusarium* kernel infection, *F.* verticillioides, *F. proliferatum* and *F.* 505 *subglutinans* were significantly correlated to each other and to ECB damage parameters, 506 incidence and severity.

As can be seen in Fig. 1 and Fig. 2, the relationship between the MON content and the DNA of the *Fusarium* species producers of MON was closer to *F. proliferatum* (r = 0.96, P < 0.001) than to *F. subglutinans* (r = 0.83, P < 0.001). Moreover, as shown in Fig. 3, the sum of the DNA of the 2 previous *Fusarium* species did not significantly improve the relationship with the MON content (r = 0.97, P < 0.001), but respect to the relationship between the MON content and the DNA of the only *F. subglutinans* r value was slightly improved.

514

515 **3.5**. In vitro MON production by monoconidial strains of F. verticillioides, F. 516 proliferatum and F. subglutinans.

The results related to the toxigenic capacity of the different strains of *F. proliferatum* and *F. subglutinans* tested during the *in vitro* assay are summarized in Table 4 Table 5. As can be seen in this table, the different strains have significantly different toxigenic capacity (P < 0.001).

On average, the *F. proliferatum* strains showed a significantly higher (P < 0.001) toxigenic capacity than the strains of *F. subglutinans*. Two strains of *F. proliferatum* (n° 1 and 2) showed the highest toxigenic capacity, which was significantly higher than all of the analyzed *F. subglutinans* strains. Only *F. subglutinans* strains 6 and 7 showed a

525 comparable toxigenic capacity with the other *F. proliferatum* strains, while two *F.* 526 *subglutinans* strains (9 and 10) showed an extremely low toxigenic capacity towards this 527 mycotoxin. On the other hand, the 5 *F. verticillioides* strains tested in this assay have 528 never produced MON (the contamination was always under the LOQ).

530 **4. Discussions and Conclusions**

The results related to the MON content in maize fields sampled over 3 growing seasons show that the contamination of this mycotoxin is promoted by a complex and integrated system in which the meteorological conditions, the ECB activity and the *Fusarium* species all play important roles.

The collected data underline a clear increase, due to ECB feeding activity on the maize ears, not only of the FB content, as widely reported in literature, but also of the MON content, a mycotoxin, which like FB, is produced by *Fusarium* spp. of the *Liseola* section. Thus, the relationship between MON contamination and ECB injuries is extremely high in temperate areas.

The results of the present work point out a significant effect of ECB larva activity on the 540 occurrence of both F. proliferatum and F. subglutinans species. Munkvold et al. (1997) and 541 Gatch and Munkvold (2002) reported an important increase in maize ear and stalk rot 542 caused by F. verticillioides, F. proliferatum and F. subglutinans, all of which are species of 543 the Liseola section, following ECB larva feeding damage. In a field experiment conducted 544 in Austria, Lew et al. (1991) reported that the increase in MON contamination in maize 545 ears damaged by ECB was related to a higher colonization of the grains by F. 546 subglutinans. 547

Although MON contamination has been shown to be closely linked to ECB activity, the meteorological and climatic conditions are also contributing factors that have the effect of changing the intensity of the MON contamination. The average MON content was higher in the 2008 and 2010 growing seasons, years characterized by lower GDD from the flowering to the end of ripening than 2009. Furthermore, in the 2010 growing season, the MON occurrence in maize grain was increased remarkably by ECB (93-fold), as this mycotoxin was only found in traces in the insect protected plot. In 2008, the insect

protected plot showed an average contamination of 43 µg kg⁻¹, which increased 26-fold in the ears naturally infected by the insect. On the other hand, in the 2009 experiment, with a lower natural ECB pressure and warmer and drier climatic conditions from flowering to harvest, the occurrence of MON was the lowest, in both the damaged and undamaged ears.

As reported by Marin et al. (2001) and by Samapundo et al. (2005), this mycotoxin synthesis is affected to a great extent by the temperature and by rainfall, which could influence both the growth rates of the fungi and the mycotoxin production. Lew et al. (2001) observed that a significant increase in *F. proliferatum* infection occurred in the nineties in Austria, due to changed climatic conditions, with milder and more humid winters followed by drier and warmer summers, which favored the progress of *F. proliferatum*.

Both of the *Fusarium* species responsible for MON contamination can be found throughout 566 567 the world, but as reported by Doohan et al. (2003), the optimum temperature for the growth of F. subglutinans in laboratory conditions is lower (Temperature = 15-25°C, 568 569 Castellá et al., 1999) than that of *F. proliferatum* (Temperature = 30°C, Marin et al., 1995). 570 As far as the European distribution and prevalence of the two different *Fusarium* species that cause MON production is concerned, F. proliferatum is more common in southern 571 European areas, while it is substituted by *F. subglutinans* in central areas (Logrieco et al., 572 2002). As reported in literature, F. proliferatum is widely present in Italy (Logrieco et 573 al., 1995) and in nearby European countries (Lević et al., 1997; Srobárová, 1997), in 574 association with *F. verticillioides*, but the occurrence of *F. proliferatum* has rarely been 575 recorded in Austria (Krüger, 1989; Krska et al., 1997), Croatia (Jurjević et al., 1997), 576 Slovakia (Piecková and Jesenská, 1997), Hungary (Szécsi, 1994) and Poland (Kostecki et 577 al., 1995) to the advantage of *F. subglutinans*. A remarkable presence of *F. temperatum*, 578 which is able to produce MON, has recently been reported in Belgium by Scauflaire et al. 579 (2012); but, because of the low number of strains examined, the authors underlined the 580

necessity of extending their screening before assessing the importance of this new
 species among the MON producer *Fusaria* species.

583 Until now, it has been assumed that the MON contamination of maize in Europe is mainly 584 associated with an *F. subglutinans* infection (Lew et al., 1991; Kostecki et al., 1995; 1997); 585 no other works that have investigated in field conditions the relationship between *F.* 586 *proliferatum* infection and MON production are present in the literature.

The results obtained in the current study underline that *F. proliferatum* could be the main 587 agent responsible for MON production in maize grain in the temperate maize growing 588 areas, such as in North-West Italy, where there is a high ECB pressure. In the in vitro 589 study on F. proliferatum, the collected strains showed a lower infection growth rate on the 590 maize substrate compared to those of *F. subglutinans*, but the toxigenicity capacity of the 591 F. proliferatum strains was about 3600 times higher. This finding appears to be in contrast 592 593 with what Logrieco et al. (2002) reported, that is, that the toxigenicity of *F. proliferatum* in Europe seemed comparable with that of F. subglutinans, considering the Fusarium 594 595 species from maize ear rot. However, Logrieco et al. (1995) reported that some strains of F. proliferatum from pre-harvest maize ear rot in Italy were able to produce very large 596 quantities of MON on autoclaved maize. In fact, as underlined by our results the F. 597 proliferatum strain 1 was able to produce a very large amount of MON compared to the 598 others and this behavior could be due to the natural variability among strains of this 599 species. Several authors report the presence in the natural population of *F. proliferatum* of 600 strains with different capabilities in MON synthesis (Logrieco and Bottalico 1988; Logrieco 601 et al., 1995; Vesonder et al., 2000). 602

The *F. proliferatum* infection of maize grain in the field experiments was higher than that caused by *F. subglutinans* in each growing season. Moreover, the relationship between the MON content and the DNA quantification of the *Fusarium* species producers of MON was closer for the *F. proliferatum* species than for *F. subglutinans*.

In conclusion, the combination of the high toxigenic capacity of F. proliferatum, in the 607 considered experimental conditions, and its more frequent occurrence and greater 608 intensity in the field make it possible to state that the production of MON in the temperate 609 maize areas, such as the North Italian maize areas investigated during this work, is mainly 610 due to F. proliferatum infections. Thus, owing to the close link between MON 611 contamination in kernels and injuries caused by ECB larvae on maize ears, any of the 612 strategies that can control this insect could also reduce the contamination of this 613 mycotoxin. Further studies are required to evaluate whether an the integrated field 614 program that is able to minimize the FB content in maize could also lead to a comparable 615 control of MON. 616

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769	new $C_{21}H_{38}N_2O_6$ metabolite phytotoxic to Lemna minor L. J. Nat. Toxins 9(2), 103-
770	112.

TABLES

774	Table. 1.	Total	rainfall,	rainy	days,	relative	humidity	and	growing	degree	days	(GDD)	from
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June to October 2008-2010 at the research site.

Year	Month	Rainfall (mm)	Rainy days	GDD ^ª (°C d ⁻¹)
2008	Мау	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	Мау	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	Мау	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

^a Accumulated growing degree days for each month using a 10°C base.

Source: weather station in the experimental farm of the Department of Agricultural, Forest and FoodScience, University of Turin located in Carmagnola.

Table 2. Natural ECB infestation recorded per year, for the field experiments conducted at

782 Carmagnola (TO) in the 2008 - 2010 period.

Year	ECB incidence ^a	ECB severity ^b
2008	100.0	25.7
2009 2010	88.9	20.8

783

^a ECB incidence was calculated as the percentage of ears with symptoms, based on 4 replications of 30 ears
 each.

^b ECB severity was calculated as the mean percentage of kernels with symptoms per ear, based on 4
 replications of 30 ears each.

789 **Table 3.** Effect of ECB infestation on fungal ear rot incidence and severity, *Fusarium* kernel infection, *F. verticillioides*, *F. proliferatum*

and *F. subglutinans* occurrence and FB (FB₁ + FB₂) and MON contaminations; field experiments conducted at Carmagnola (TO) in the

791 2008 - 2010 period.

Year	ECB infestation	Fungal ea inciden	ar rot ce ^c	Fungal seve	ear rot rity ^d	<i>Fusal</i> kerr infec	rium nel tion	<i>F. ver</i> inf	<i>ticillioides</i> ection ^e	<i>F. pr</i> in	<i>oliferatum</i> fection ^e	F. sui inf	bg <i>lutinans</i> ection [®]	I	FB	Π	MON
		т	N (%)	т	N (%)	т	N (%)	т	N (Normalized DNA)	т	N (Normalized DNA)	т	N (Normalized DNA)	т	Ν (μg kg-1)	т	N (µg kg⁻¹)
2008	Natural Artificial	86.2	98.3	27.9	22.0	65.4	82.4	15.6	75.0	14.6	68.3	6.4	12.6	8.5	4817	7.0	1137
	control	32.9	29.8	5.6	1.0	41.6	44.1	5.96	12.76	2.29	2.32	1.20	0.54	7.6	2036	3.3	43
	$P\left(F ight)^{a}$	< 0.001***		< 0.001*	***	< 0.001*	***	0.006**	k	0.004**	e	< 0.001	***	0.001	**	0.002	**
	sem ^b	5.4		2.1		4.3		4.06		4.64		0.82		0.3		0.7	
2009	Natural	48.0	55.0	8.8	2.4	59.7	71.2	3.76	4.61	3.70	4.76	2.26	1.71	7.2	1745	4.1	77
	control	21.1	13.3	4.5	0.7	25.6	20.3	0.83	0.25	0.87	0.31	0.51	0.09	5.5	298	1.4	<loq<sup>f</loq<sup>
	$P\left(F ight)^{a}$	< 0.001***		0.014*		0.012*		0.013*		0.043*		0.019*		0.090		0.002	**
	sem ^b	5.4		1.5		11.8		0.80		1.12		0.53		0.9		0.5	
2010	Natural Artificial	73.4	91.7	22.9	15.3	56.3	68.7	15.55	84.58	9.48	31.85	1.99	2.54	8.8	7024	6.2	564
	control	7.5	3.3	1.2	0.1	32.3	29.3	3.44	4.54	2.00	1.54	0.56	0.10	5.7	644	1.6	6
	$P\left(F ight)^{a}$	< 0.001***		< 0.001'	***	0.009**		0.037*		0.031*		0.298		0.016	*	< 0.00)1***
	sem ^b	5.6		1.4		7.9		6.29		3.67		1.81		1.3		0.6	

The reported data are the averages of 4 replications.

^a P (F) = ANOVA level of significance, * P < 0.05, ** P < 0.01, *** P < 0.001.

- ^b sem: standard error of mean.
- ^c Fungal ear rot incidence was calculated as the percentage of ears with symptoms, based on 4 replications of 20 ears each.
- ^{*d*} Fungal ear rot severity was calculated as the mean percentage of kernels with symptoms per ear, based on 4 replications of 20 ears each.

^e *F. verticillioides*, *F. proliferatum* and *F. subglutinans* infections were calculated as the pg Fungal DNA / ng Total DNA ratio. The amounts of fungal DNA obtained
 were normalized to the total DNA amount extracted from the meal samples.

- ^fLOQ = Limit of Quantification of the LC-MS/MS analytical method = 4 μ g kg⁻¹.
- The fungal ear rot incidence and severity, the *Fusarium* kernel infection, the *F. verticillioides*, *F. proliferatum* and *F. subglutinans* occurrence means reported are transformed (T; y'=arcsin $\sqrt{x^*180/\pi}$) and not transformed (N) values. The FB and MON contamination means reported are transformed [T; y'= ln (x + 1)] and not transformed (N) values.

Table 4. Correlation matrix between ECB incidence and severity, fungal ear rot incidence and severity, *Fusarium* kernel infection, *F.*

verticillioides, F. proliferatum and F. subglutinans infections and FB and MON contaminations in maize kernels in the 2008 - 2010

814 period.

Correlation	ECB severity	Fungal ear rot incidence	Fungal ear rot severity	<i>Fusarium</i> kernel infection	<i>F. verticillioides</i> infection	<i>F. proliferatum</i> infection	<i>F. subglutinans</i> infection	FB	MON
ECB incidence	0.970**	0.965**	0.922**	0.803**	0.798**	0.813**	0.825**	0.822**	0.849**
ECB severity		0.956**	0.967**	0.774**	0.845**	0.848**	0.863**	0.841**	0.892**
Fungal ear rot incidence			0.926**	0.838**	0.816**	0.800**	0.811**	0.849**	0.837**
Fungal ear rot severity				0.759**	0.802**	0.877**	0.893**	0.746**	0.888**
<i>Fusarium</i> kernel infection					0.634**	0.660**	0.699**	0.754**	0.700**
<i>F. verticillioides</i> infection						0.584**	0.621**	0.751**	0.604**
<i>F. proliferatum</i> infection							0.761**	0.749**	0.965**
<i>F. subglutinans</i> infection								0.587**	0.829**
FB									0.767**

815 (**) correlation significant at P \leq 0.01. The data reported in the table are Pearson product-moment correlation coefficients.

Table 5. In vitro MON production by different fungal strains of *Fusarium proliferatum* and
 F. subglutinans on maize substrates.

Fungal species	Fungal strain	FungalNormalizedMONstrainDNA ^c (μ g kg ⁻¹)		MON / Normalized DNA [⊄] (µg kg⁻¹)
		Average ± SD ^a	Average ± SD ^a	Average
Fusarium proliferatum	1	0.015 ± 0.001	699 ± 1	47383.5 a
	2	3.635 ± 0.895	2605 ± 338	738.4 a
	3	0.735 ± 0.071	154 ± 20	214.5 ab
	4	5.113 ± 1.547	411 ± 156	98.7 abc
	5	0.578 ± 0.067	< LOD ^b	1.6 cd
Fusarium subglutinans	6	139.041 ± 3.000	1360 ± 1030	9.9 bcd
	7	64.068 ± 1.612	110 ± 53	1.7 cd
	8	77.760 ± 39.253	61 ± 11	1.2 de
	9	70.399 ± 7.425	24 ± 3	0.3 ef
	10	60.826 ± 19.564	11 ± 4	0.2 f

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- 819 The reported data are the averages of 3 replications.
- 820 ^a SD = standard deviation.
- 821 b LOD = Limit of Detection of the LC-MS/MS analytical method = 1 μ g kg⁻¹.
- ^cNormalized DNA = pg Fungal DNA / ng Total DNA.
- ^d MON / DNA = Ratio of the MON concentration and Fungal DNA normalized for the Total DNA.
- 824 Means followed by different letters are significantly different (P < 0.001). The REGWQ test was used as a 825 Post-hoc test.







Figure 2. Relationship between the *F. subglutinans* DNA and the MON concentration in 24

maize field samples over three growing season periods (2008-2010).

The amount of *F. subglutinans* DNA was determined by means of real-time PCR. The MON concentration

853 was determined using an LC-MS/MS method.

Reduced Major Axis linear regression analysis (Model II linear regression analysis) was applied to fit the

data; r: Pearson product-moment correlation coefficient; P: level of significance, * P < 0.05, ** P < 0.01, *** P

- 856 < 0.001.

1,600 1,400 1,200 MON (µg kg-1) 1,000 800 600 y = 13.534x + 9.128400 r = 0.972 200 P < 0.001** 0 0 20 40 60 80 100 120 pg DNA F. proliferatum + F. subglutinans/ng TOTAL DNA



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Figure 3. Relationship between the sum of *F. proliferatum* and *F. subglutinans* DNA and the MON concentration in 24 maize field samples over three growing season periods (2008-2010).

- The amount of *F. proliferatum* and *F. subglutinans* DNA was determined by means of real-time PCR. The MON concentration was determined using an LC-MS/MS method.
- 877 Reduced Major Axis linear regression analysis (Model II linear regression analysis) was applied to fit the 878 data; r: Pearson product-moment correlation coefficient; P: level of significance, * P < 0.05, ** P < 0.01, *** P879 < 0.001.

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