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

21 **FIELD CROP RESEARCH**

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23 Relationship between European Corn Borer injury, *Fusarium* 24 *proliferatum* and *F. subglutinans* infection and moniliformin 25 contamination in maize.

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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.

The aim of the current study was to investigate the influence of ECB activity on MON 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 that caused by *F. subglutinans*, although both species were significantly increased due to the ECB damage to the maize ears. The ECB activity significantly increased the MON content 26-fold in 2008 from 43 to 1137 μg kg^{-1}, 25-fold in 2009 from < LOQ to 77 μg kg^{-1} and 94-fold in 2010 from 6 to 564 μg kg^{-1}. The relationship between the MON content and the *Fusarium* species producers of MON was closer for *F. proliferatum* (R^2 = 0.93, P < 0.001) than for *F. subglutinans* (R^2 = 0.68, P < 0.001). An *in vitro* assay was carried on in 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 average, the *F. proliferatum* isolated strains showed a significantly higher (P < 0.001) toxigenic capacity than the *F. subglutinans* strains.

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 Corn Borer

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.

1. Introduction

Maize ear rot is a common disease throughout the world that is caused by several *Fusarium* species which can co-occur to give rise to the production of different mycotoxins in infected kernels, with a considerable impact on human and animal health (Bottalico, 1998). In temperate areas, maize pink ear rot, which is mainly caused by *F. verticillioides*, leads to an extensive occurrence of FB, a group of toxins among which fumonisin B_1 (FB_1) has been evaluated, by the International Agency for Research on Cancer (IARC), as a Group 2B carcinogen, i.e., possibly carcinogenic to humans (IARC, 2002). Regulatory limits have been set for maize grain within the European Union (European Commission No. 1881/2006 and EC No. 1126/2007), the USA (FDA - Food and Drug Administration, 2000) and other countries.

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 LD_{50} 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

could lead to a higher contamination of this mycotoxin, in order to understand the Best 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 and its isogenic counterparts conducted in Central Europe, have also reported a significant correlation between the percentage of ECB damaged ears and the MON concentration. Unfortunately, at the moment, no data are available on the effect of ECB on MON contents in warmer maize cultivation areas, where the pressure and damage caused to ears and kernels by this insect are higher. Moreover, so far, it has not been clearly reported, in the literature, which *Fusarium* species is the most responsible for MON contamination in temperate areas or what relationship exists between these species and ECB activity.

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.

2. Materials and Methods

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 FB_1 and FB_2 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 FB_1 and FB_2, 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).

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 sandy-medium 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. 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.

No foliar insecticides were applied to the experimental field or to an approximately 20 ha 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 and severity at harvest) were collected by hand from each plot at the end of maturity at a grain moisture content of between 23 and 27%, and shelled using an electric sheller. The kernels from each plot were mixed thoroughly to obtain a random distribution; 4 kg samples were then taken and dried at 60°C for 3 days to analyze the MON and FB 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 a ZM 200 Ultra Centrifugal Mill (Retsch GmbH, Haan, Germany) and reduced to 1 kg 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 aliquot of 100 g, randomly sampled, was used in the molecular analysis, while the remaining 400 g were employed in the chemical analysis.

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

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 7-class 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.

2.4. Mycological measurements

2.4.1 Fungal ear rot incidence and severity

Fungal ear rot incidence was calculated as the percentage of ears per plot with symptoms, while 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 corresponds 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., 2009). The ear rot severity scores were converted to percentages of ears exhibiting symptoms and each score was replaced with the mid-point of the interval.

2.4.2. 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

236 dicloran cloramfenicol peptone (DCPA) and incubated at 20 °C. The *Fusarium* colonies
237 were identified after 7 to 10 days through colony and conidial morphology, as reported by
238 Nelson et al. (1983).
239
240 *2.4.3. DNA extraction from the maize samples*
241 The total DNA from the fungal reference cultures was extracted from approximately 200-
242 300 mg (wet weight) of fresh mycelium, according Henrion et al. (1992), with slight
243 modifications. The samples were crushed in liquid N_2 and immediately suspended in one
244 mL of lysis buffer (100 mM Tris-HCl, pH 8, 20 mM EDTA, pH 8, 1.4 mM NaCl, 2%
245 cetyltrimethylammonium bromide (CTAB), 1% polyvinylpyrrolidone (PVP), 1%
246 mercaptoethanol) for 60 minutes at 65°C. The proteins were denatured and removed
247 through one or more extractions by gently shaking the solution for 1 hour in ice with 1 mL
248 cloroform/isoamyl alcohol (24:1, v/v), followed by centrifugation at 17300 × g for 10
249 minutes, to separate the phases. The nucleic acids were precipitated from the aqueous
250 phase by adding 2/3 volume of isopropanol and 1/10 volume of sodium acetate 3 M (pH
251 5.2) and maintaining the sample at –20°C for 20 min. The DNA was then pelleted by
252 centrifugation at 17300 × g for 10 minutes, washed with 70% (v/v) ethanol, vacuum dried
253 briefly, resuspended in 50-150 µL of TE buffer (10 mM Tris-HCl pH 8 + 1 mM EDTA) and
254 stored at –20°C until use.
255 The flour aliquot of 100 g, obtained as previously described, was milled again in a knife
256 mill Grindomix GM 200 (Retsch Technology Gmbh, Haan, Germany). Then 10 g of re-
257 milled flour were further ground in liquid N2 and the DNA was extracted from 1 g, using the
258 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.

2.4.4. Preliminary species-specific PCR

End-Point PCR was carried out using the SUB1 (CTGTCGCTAACCTCTTTATCCA) and SUB2 (CAGTATGGACGTTGGTATTATATCTAA) primer for the detection of *F. subglutinans*; the PRO1 (CTTTCCGCCAAGTTTCTTC) and PRO2 (TGTCAGTAACTCGACGTTGTTG) primer for the detection of *F. proliferatum;* the VER1 (CTTCCTGCGATGTTTCTCC) and VER2 (AATTGGCCATTGGTATTATATATCTA) primer for the detection of *F. verticilliodes,* all designed by Mulè et al., 2004 on the *calmodulin* gene. These primers amplify respectively 631 bp for *F. subglutinans*, 585 bp for *F. proliferatum* and 578 bp for *F. verticillioides*.

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

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

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

The primers used for the detection of *F. subglutinans* were Fsub565 F (TCATTGGTATGTTGTCGCTCATG) and Fsub622A R (GTGATATGTTAGTACGAATAAAGGGAGAAC), designed on the *EF1α* gene (Nicolaisen et al., 2009; Boutigny et al. 2011). The primers Fp3-F (CGGCCACCAGAGGATGTG) and Fp4-R (CAACACGAATCGCT TCCTGAC), designed by Jurado et al. (2006) on the intergenic sequence of the ribosomal RNA gene cluster, for the detection of *F. proliferatum* and the primers VER1 and VER2, designed by Mulè et al., 2004 on the *calmodulin* gene, for the detection of *F. verticilliodes,* were used in Real Time as described by Nutz *et al*. (2011); all were synthesized by PRIMM (PRIMM srl, Milan, Italy).

Real-time PCR assays were carried out in a total volume of 25 µL, consisting of 12.5 µL 2X SYBR Green PCR Master Mix (Applied Biosystems), 600nM concentration of each primer for *F. proliferatum,* 300 nM for *F. subglutinans* and F. *verticilliodes* and 2 µL template DNA (approximately 10 - 20 ng); only for *F. subglutinans*. the reaction mixture contain also 0.5 µg μL^{-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 curves were prepared by diluting the DNA of each pathogen (approximately 121 ng μL^{-1} for *F. subglutinans,* 256 ng μL^{-1} for *F. proliferatum* and 97 ng μL^{-1} for *F. verticilliodes*) 5-, 25-, 125-, 625-, 3125-, 15625-, 78125-fold in maize DNA that was free of fungal contamination (approximately 105 ng μL^{-1}). Standard curves were generated by plotting threshold cycle values (Ct values) against the logarithm of the starting DNA quantities. The standard curves slopes were used to calculate the reaction efficiency (E) of the PCR assays. The amounts of fungal DNA obtained were normalized to the total DNA amount extracted from the meal samples.

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

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

mycelium was harvested after 14 days by means of filtration and then rinsed in distilled 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 (Ø 9 cm) were then inoculated with the selected *Fusarium* strains, using 5 mycelium plugs of 6 mm^2, 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.

2.6. Chemical Analyses

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

357 acetonitrile/water (84:16, v/v). The extracts were filtered through Whatman® no. 1 filters
358 (Brentford, UK) and subjected to clean-up and purification.
359 For FB_1 and FB_2 extraction 50 g of maize flour was extracted by mechanical shaking at
360 100 rpm for 20 min (shaker mod. M102-OS, MPM Instruments, Milan, Italy) with 100 mL
361 methanol/water (80:20, v/v). The extracts were filtered through Whatman® no. 1 filters
362 (Brentford, UK), diluted 1:5 with Dulbecco's Phosphate Buffer Saline (PBS, 1X solution,
363 Sigma Aldrich, St. Louis, MO), filtered again through Whatman® glass microfiber filters,
364 Grade 934-AH® (Brentford, UK) and subjected to clean-up and purification.
365
366 *2.6.2. Clean-up*
367 The clean-up method for MON was performed with MycoSep® 240 Mon clean-up columns
368 (Romer Labs®, Tulln, Austria). The clean-up procedure (Scarpino et al., 2013) was
369 adapted from the Romer Labs® procedure. The cleanup MycoSep® columns were pushed
370 into test tubes containing 5 mL of the sample extracts, and the extracts were allowed to
371 filter upwards through the packing material of the columns. The interferences adhered to
372 the chemical packing in the columns and the purified extracts, containing MON, passed
373 through the columns. The purified extracts (1.5 mL) were transferred to HPLC vials and
374 analyzed by means of LC-MS/MS, according to the method described below.
375 The clean-up method for FB (FB_1 and FB_2) was performed with immunoaffinity columns
376 (IA) FumoniTest™ WB Columns (VICAM®, USA). Five mL of the sample extracts were
377 loaded and passed through the columns and after a washing step with 2.5 mL of PBS 1 X
378 solution FB were recovered and eluted with 2 mL of methanol. The purified extracts were
379 transferred to HPLC vials and analyzed by means of LC-MS/MS, according to the method
380 described below.
381

382 *2.6.3. LC-MS/MS Analysis*

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

386 For MON analysis LC separation was performed on a 100 mm × 2.1 mm i.d., 3.5 µm, 100
387 Å ZIC®-HILIC (Merck, SeQuant, Milan, Italy) column. The mobile phase consisted of water
388 buffered with 100 mM ammonium formate (pH 6.4) (A) and acetonitrile (B) delivered at 200
389 µL min^{-1}. The chromatographic and mass spectrometric parameters of the investigated
390 analytes were described by Scarpino et al. in 2013. The deprotonated molecule (*m/z* =
391 97.0) was fragmented to its product ion (*m/z* = 41.0) and used for quantification and
392 identification purposes.

393 The average percentage of recovery for MON was: 81% (Relative Standard Deviation,
394 RSD%: 9%). The results of the MON concentrations were corrected for the recovery rate.

395 The limit of detection (LOD) and the limit of quantification (LOQ) were 1 µg kg^{-1} and 4 µg
396 kg^{-1}, respectively.

397 For FB analysis LC separation was performed on a 50 mm × 2.1 mm i.d., 5 µm, Varian
398 Pursuit 5 (Agilent, Italy) C18 column. The mobile phase consisted of water acidified with
399 0,1% CH_3COOH (A) and acetonitrile acidified with 0,1% CH3COOH (B) delivered at 200
400 µL min^{-1} for 15 min. Mass spectrometric analyses were performed in the positive-ion
401 mode. For FB_1 MS detection the protonated molecule (m/z = 722.5) was fragmented to its
402 product ions (m/z = 334.2 and m/z = 352.3) and used for quantification and identification
403 purposes.

404 For FB_2 MS detection the protonated molecule (m/z = 706.5) was fragmented to its
405 product ions (m/z = 318.0 and m/z = 336.0) and used for quantification and identification
406 purposes.

407 The average percentage of recovery for FB was: 75% (Relative Standard Deviation,
408 RSD%: 8%). The results of the FB concentrations were corrected for the recovery rate.
409 The limit of detection (LOD) and the limit of quantification (LOQ) were 2 µg kg^{-1} and 8 µg
410 kg^{-1}, respectively.
411
412 *2.6.4. Calibration*
413 Ten different MON concentrations between 0.93 and 930 µg L^{-1}, were prepared for
414 calibration in acetonitrile/water (84:16, v/v). A linear regression was used to obtain the
415 regression curve.
416 Ten different FB concentrations between 1 and 10000 µg L^{-1}, were prepared for calibration
417 in acetonitrile/water (50:50, v/v). A linear regression was used to obtain the regression
418 curve.
419
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.
423 An analysis of variance (ANOVA) was utilized to compare the fungal ear rot incidence and
424 severity, the *Fusarium* kernel infection, the *F.* verticillioides, the *F. proliferatum* and
425 *subglutinans* DNA quantification and the MON and FB contamination separately for each
426 year, using a completely randomized block design, in which the natural presence of ECB
427 larva feeding injuries was the independent variable. The incidence and the severity values
428 of fungal ear rot, the *Fusarium* kernel infection, and the *F.* verticillioides, the *F. proliferatum*
429 and F. *subglutinans* DNA quantification were previously transformed using
430 $y'=\arcsin\sqrt{x}*180/\pi$. The concentration of MON and FB was transformed using the
431 $y'=\ln(x+1)$ equation to normalize the residuals.

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.

3. Results

3.1. Meteorological data

The three growing seasons showed remarkably different meteorological trends as far as 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 low GDDs and high rainfall in May and June, and also close to flowering. Heavy rainfall 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 concentrated in July, after maize flowering. The average GDDs from June to September were higher in 2009 than those in 2008 and 2010, and this led to an early harvest at the beginning of September (Table 1).

3.2. ECB damage

Table 2 summarizes the mean ECB incidence and severity recorded for each growing season for the natural infestation of ears collected for each sampling year. The ears collected in the plots protected with entomological nets were free from ECB attack, in each growing season, 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 samples collected in the 2008 and 2010 growing seasons showed more higher ECB damage, while the ECB severity recorded in 2009 was more than 3 times lower than in 2008 and 2010 (Table 2).

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

476 The presence of ECB larvae significantly affected the fungal ear rot incidence and severity
477 in each growing season (Table 3). The ECB attack increased the fungal ear rot severity
478 22-fold and 153-fold in the 2008 and 2010 growing seasons, respectively. Conversely, in
479 2009, the year characterized by the lowest ECB pressure, the fungal ear rot severity only
480 increased 3-fold. The *Fusarium* kernel infection was significantly higher in the natural
481 infected ears in each growing season, according to the ECB attack (Table 3).
482 As far as DNA quantification is concerned, the *F. proliferatum* occurrence significantly
483 increased in the natural infection conditions of 29-fold, 15-fold and 21-fold in the 2008,
484 2009 and 2010 growing seasons, respectively, compared to the artificial control. The
485 insect activity on the ears increased the infection of *F. subglutinans* of 23-fold, 19-fold and
486 25-fold in the 2008, 2009 and 2010 growing seasons, respectively, although its effect was
487 only significant in the first two years (Table 3). The ECB activity significantly increase *F.*
488 *verticillioides* infection in all the growing seasons.
489

490 ### *3.4. FB and MON contamination in the field samples*

491 The MON contamination was affected significantly by the ECB larva feeding activity on the
492 maize ears in all the considered growing seasons ($P = 0.002$, 2008; $P = 0.002$, 2009; $P <$
493 0.001, 2010) (Table 3). The presence of ECB damage increased the MON content 26-fold
494 in 2008 from 43 to 1137 μg kg^{-1}, 25-fold in 2009 from ~~3~~ < LOQ to 77 μg kg^{-1} and 94-fold in
495 2010 from 6 to 564 μg kg^{-1}. Moreover, as reported in Table 3 the ECB larva feeding
496 activity on the maize ears significantly affected the FB contamination in the years with the
497 higher ECB pressure, 2008 and 2010.
498 As far as the relationships between the major *Fusarium* species producers of MON and
499 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.

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

3.5. In vitro MON production by monoconidial strains of F. verticillioides, F. 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).
529

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 occurrence of both *F. proliferatum* and *F. subglutinans* species. Munkvold et al. (1997) and Gatch and Munkvold (2002) reported an important increase in maize ear and stalk rot caused by *F. verticillioides*, *F. proliferatum* and *F. subglutinans*, all of which are species of the *Liseola* section, following ECB larva feeding damage. In a field experiment conducted in Austria, Lew et al. (1991) reported that the increase in MON contamination in maize ears damaged by ECB was related to a higher colonization of the grains by *F. subglutinans*.

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 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, Castellá et al., 1999) than that of *F. proliferatum* (Temperature = 30°C, Marin et al., 1995). 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 European areas, while it is substituted by *F. subglutinans* in central areas (Logrieco et al., 2002). As reported in literature, *F. proliferatum* is widely present in Italy (Logrieco et al.,1995) and in nearby European countries (Lević et al., 1997; Srobárová, 1997), in association with *F. verticillioides*, but the occurrence of *F. proliferatum* has rarely been recorded in Austria (Krüger, 1989; Krska et al., 1997), Croatia (Jurjević et al., 1997), Slovakia (Piecková and Jesenská, 1997), Hungary (Szécsi, 1994) and Poland (Kostecki et al., 1995) to the advantage of *F. subglutinans*. A remarkable presence of *F. temperatum*, which is able to produce MON, has recently been reported in Belgium by Scauflaire *et al.* (2012); but, because of the low number of strains examined, the authors underlined the

581 necessity of extending their screening before assessing the importance of this new
582 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.
587 The results obtained in the current study underline that *F. proliferatum* could be the main
588 agent responsible for MON production in maize grain in the temperate maize growing
589 areas, such as in North-West Italy, where there is a high ECB pressure. In the *in vitro*
590 study on *F. proliferatum*, the collected strains showed a lower infection growth rate on the
591 maize substrate compared to those of *F. subglutinans*, but the toxigenicity capacity of the
592 *F. proliferatum* strains was about 3600 times higher. This finding appears to be in contrast
593 with what Logrieco et al. (2002) reported, that is, that the toxigenicity of *F. proliferatum* in
594 Europe seemed comparable with that of *F. subglutinans*, considering the *Fusarium*
595 species from maize ear rot. However, Logrieco et al. (1995) reported that some strains of
596 *F. proliferatum* from pre-harvest maize ear rot in Italy were able to produce very large
597 quantities of MON on autoclaved maize. In fact, as underlined by our results the *F.*
598 *proliferatum* strain 1 was able to produce a very large amount of MON compared to the
599 others and this behavior could be due to the natural variability among strains of this
600 species. Several authors report the presence in the natural population of *F. proliferatum* of
601 strains with different capabilities in MON synthesis (Logrieco and Bottalico 1988; Logrieco
602 et al., 1995; Vesonder et al., 2000).
603 The *F. proliferatum* infection of maize grain in the field experiments was higher than that
604 caused by *F. subglutinans* in each growing season. Moreover, the relationship between
605 the MON content and the DNA quantification of the *Fusarium* species producers of MON
606 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 considered experimental conditions, and its more frequent occurrence and greater intensity in the field make it possible to state that the production of MON in the temperate maize areas, such as the North Italian maize areas investigated during this work, is mainly due to *F. proliferatum* infections. Thus, owing to the close link between MON contamination in kernels and injuries caused by ECB larvae on maize ears, any of the strategies that can control this insect could also reduce the contamination of this mycotoxin. Further studies are required to evaluate whether an the integrated field program that is able to minimize the FB content in maize could also lead to a comparable control of MON.

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771

772 **TABLES**
773

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

Year	Month	Rainfall (mm)	Rainy days	GDD[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

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

777 Source: weather station in the experimental farm of the Department of Agricultural, Forest and Food
778 Science, University of Turin located in Carmagnola.

779

780

781 **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	60.0	5.6
2010	88.9	20.8

783

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

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

788

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 (FB_1 + FB_2) and MON contaminations; field experiments conducted at Carmagnola (TO) in the 2008 - 2010 period.

Year	ECB infestation	Fungal ear rot incidence[c]		Fungal ear rot severity[d]		*Fusarium* kernel infection		*F. verticillioides* infection[e]		*F. proliferatum* infection[e]		*F. subglutinans* infection[e]		FB		MON	
		T	N (%)	T	N (%)	T	N (%)	T	N (Normalized DNA)	T	N (Normalized DNA)	T	N (Normalized DNA)	T	N (µg kg-1)	T	N (µg kg^{-1})
2008	Natural	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
	Artificial 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 (F)[a]	< 0.001***		< 0.001***		< 0.001***		0.006**		0.004**		< 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
	Artificial 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[f]
	P (F)[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	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
	Artificial 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 (F)[a]	< 0.001***		< 0.001***		0.009**		0.037*		0.031*		0.298		0.016*		< 0.001***	
	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.

[f] LOQ = Limit of Quantification of the LC-MS/MS analytical method = 4 µg 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 period.

Correlation	ECB severity	Fungal ear rot incidence	Fungal ear rot severity	*Fusarium* kernel infection	*F. verticillioides* infection	*F. proliferatum* infection	*F. subglutinans* 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**
***Fusarium* kernel infection**					0.634**	0.660**	0.699**	0.754**	0.700**
***F. verticillioides* infection**						0.584**	0.621**	0.751**	0.604**
***F. proliferatum* infection**							0.761**	0.749**	0.965**
***F. subglutinans* infection**								0.587**	0.829**
FB									0.767**

(**) correlation significant at P ≤ 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	Normalized DNA[c]	MON (µg kg^{-1})	MON / Normalized DNA[d] (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

The reported data are the averages of 3 replications.

[a] SD = standard deviation.

[b] LOD = Limit of Detection of the LC-MS/MS analytical method = 1 µg kg^{-1}.

[c] Normalized DNA = pg Fungal DNA / ng Total DNA.

[d] MON / DNA = Ratio of the MON concentration and Fungal DNA normalized for the Total DNA.

Means followed by different letters are significantly different ($P < 0.001$). The REGWQ test was used as a Post-hoc test.

FIGURES

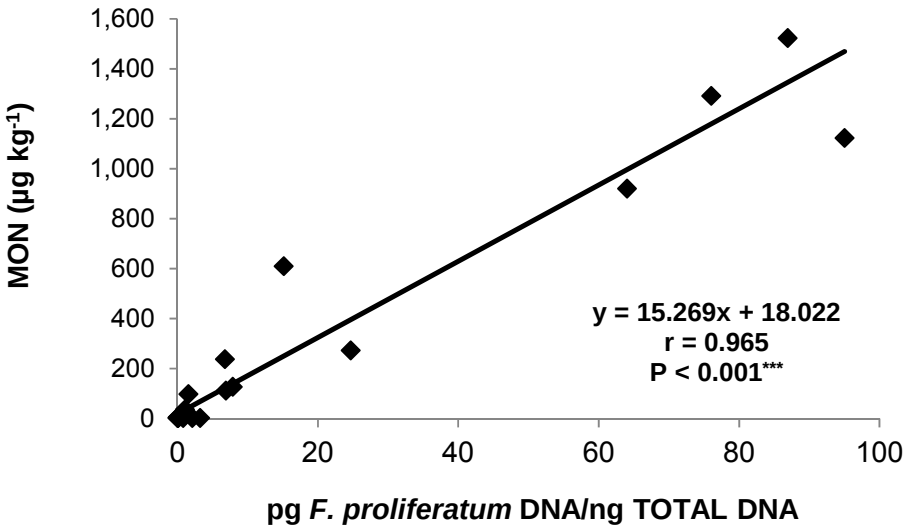

Figure 1. Relationship between the *F. proliferatum* DNA and the MON concentration in 24 maize field samples over three growing season periods (2008-2010).

The amount of *F. proliferatum* DNA was determined by means of real-time PCR. The MON concentration 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, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

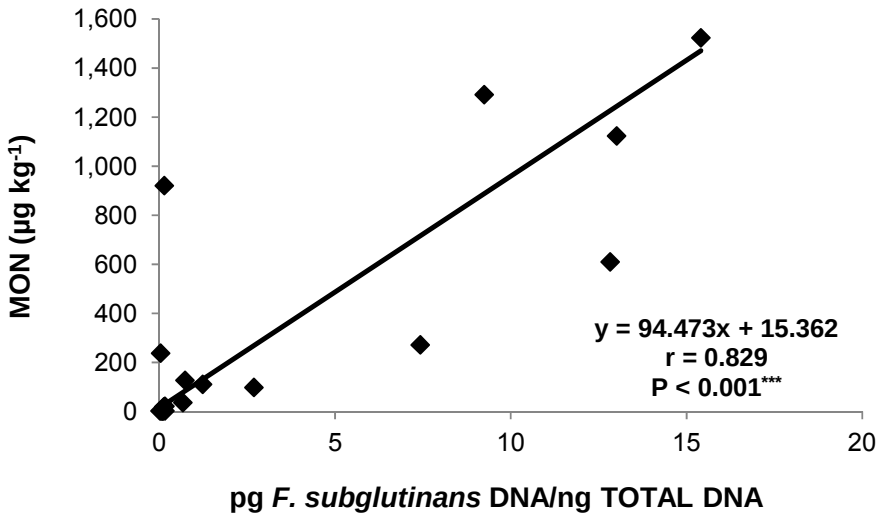

Figure 2. Relationship between the *F. subglutinans* DNA and the MON concentration in 24 maize field samples over three growing season periods (2008-2010).

The amount of *F. subglutinans* DNA was determined by means of real-time PCR. The MON concentration 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, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

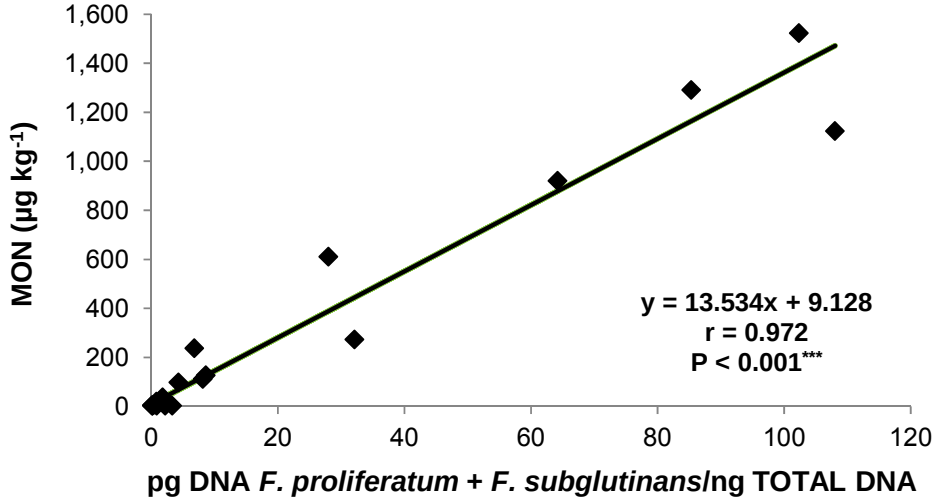

Figure 3. Relationship between the sum of *F. proliferatum* and *F. subglutinans* DNA and the MON concentration in 24 maize field samples over three growing season periods (2008-2010).

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.

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, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.