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RESEARCH PAPERS

Evaluation of ear rot (*Fusarium verticillioides*) resistance and fumonisin accumulation in Italian maize inbred lines

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Summary. Mycotoxin contamination of maize (*Zea mays* L.) grain is a global threat to the safety of both human food and animal feed. Hence, the development of maize genotypes with reduced mycotoxin accumulation in grain is of major importance. In order to find maize germplasm sources of resistance to Fusarium ear rot, 34 Italian and six public inbred lines were evaluated by means of artificial inoculation in field experiments during 2009 and 2010. Relationships between ear rot and fumonisin concentration in the ears were investigated. Primary ears were challenged with a mixture of two *Fusarium verticillioides* isolates from Northern Italy, through kernel inoculation, and ear rot severity was assessed.The average number of visibly infected kernels per ear, after inoculation, ranged from 2 to 68 in 2009 and from 0 to 120 in 2010. Fumonisin concentrations in the inoculated ears were greater than in the experimental controls for both years. Variability was found between the inbred lines: fumonisin accumulation ranged from 0.56 to 240.83 mg kg^{-1} in 2009 and from 1.09 to 190.60 mg kg^{-1} in 2010. In both years, six inbred lines showed high fumonisin content (≥100 mg kg^{-1}), while the other genotypes were almost equally split into two groups, low (≤10 mg kg^{-1}) and medium (from 11 to 100 mg kg^{-1}) fumonisin content. The number of infected kernels after artificial inoculation correlated with fumonisin concentration both in 2009 ($r = 0.94$; $P \leq 0.01$) and 2010 ($r = 0.67$; $P \leq 0.01$). Additionally, the percentage of internally infected kernels correlated positively with fumonisin concentration ($r = 0.37$; $P \leq 0.01$) and with the number of infected kernels ($r = 0.29$; $P \leq 0.05$). This research has demonstrated that Italian maize germplasm is a valid source of resistance to Fusarium ear rot. Furthermore, there is a strong association of visible *Fusarium* symptoms with fumonisin concentration, suggesting that selection in maize for reduced visible moulds should reduce the risk of mycotoxin contamination.

Key words: mycotoxin, fungal pathogen, artificial inoculation, genetic variability, *Zea mays* L.

Abbreviations: NIK, number of infected kernels at the inoculation point; DSR, disease severity rating; FBs, fumonisins; FB_1, fumonisin B_1; FB_2, fumonisin B_2; FB_3, fumonisin B_3; IKI, internal kernel infection.

Introduction

Mycotoxin contamination of maize grain (*Zea mays* L.) is a global threat to safety both for human food and animal feed (Balazs and Schepers, 2007). Mycotoxins are secondary metabolites produced by fungi, which may be toxic or have other debilitating effects on living organisms (Castegnaro and McGregor, 1998; CAST, 2003). New regulations for the allowable mycotoxin limits in food and feed have been put in place in many countries. The binding European Union regulations on toxin contamination for human consumption and recommendations for animal feeding (Commission Recommendation 2006; Commission Regulation 2007), have forced renewed interest in breeding efforts for resistance to toxigenic fungi as the preferred method for control of mycotoxin contamination.

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The primary causal organism of Fusarium ear rot in most maize-growing areas of Southern Europe is the toxigenic fungus *F. verticillioides* (Logrieco *et al*., 2002; Battilani *et al*., 2008; Covarelli *et al*., 2011, 2012). This pathogen causes losses in grain yield and quality, due to the contamination of grain by mycotoxins, primarily fumonisin B_1 ($FB_{1)}$ (Munkvold and Desjardins, 1997; De Curtis *et al*., 2011; Parsons and Munkvold, 2012). In Italy, maize is a major crop for animal feed, for direct human consumption and as a source for commercial products. Approximately 10 million tons of maize are produced each year, mostly in the Po valley (Northern Italy). Very high levels of fumonisin (FB_s) contamination were recorded in this area in 2004–2006 (Battilani *et al*., 2008) and in 2006–2008 (Berardo *et al*., 2011).

Development of plants able to withstand damage caused by fungal pathogens has been a significant challenge for maize breeders. Although selection eliminates genotypes particularly susceptible to diseases, cultivated hybrids frequently show serious fungal infections (Munkvold, 2003a; Balconi *et al*., 2010). The genetic modification of maize, either through plant breeding or transgene mediated, represents one potential way to reduce exposure to mycotoxins in food and feed, through increased resistance to fungal infection and/or reduced toxin production in maize tissues (Munkvold, 2003b).

Sources of resistance to Fusarium ear rot have been identified (Gendloff *et al*., 1986), but they are polygenic in nature and difficult to incorporate into hybrids (Zhang *et al*., 2006). Research efforts have included the mapping of quantitative traits loci (QTL) and marker-assisted selection. This approach was difficult to implement, however, QTL accounted for no more than 44% of the phenotypic variance for Fusarium ear rot resistance (Perez-Brito *et al*., 2001). Although lines showing reduced mycotoxin accumulation in the grain have been identified, additional sources of resistance are currently being sought (Robertson-Hoyt *et al*., 2007; Henry *et al*., 2009; Löffler *et al*., 2010a; Lanubile *et al*., 2011). The maize germplasm maintained at Consiglio per la ricerca e la sperimentazione in agricoltura, Unità di ricerca per la maiscoltura (CRA-MAC) contains over 5,000 accessions, including local populations and inbreds, synthetic populations, public lines and genetic stocks. The large number of populations and ecotypes and their genetic variability of grain composition provides interesting material for the identification of genotypes with good nutritional value and safety characteristics (Berardo *et al*., 2009; Alfieri *et al*., 2012).

A set of 34 Italian and six public inbred lines were evaluated in 2009 and 2010 in Bergamo (Northern Italy), with the aim to find new sources of genetic variability to improve the nutritional quality of maize genotypes and their resistance to pathogens. A preliminary survey was undertaken to test their susceptibility to *F. verticillioides* through the artificial inoculation of kernels in field trials and the evaluation of ear rot severity and FB contamination.

Materials and methods

Maize inbred lines

A set of 40 (34 Italian and six public) inbred lines was evaluated. The lines used in this study, together with their origins and average kernel weights, are listed in Table 1. Most lines have vitreous orange kernels, a trait characteristic of traditional Italian germplasm from which they were selected. Lines Lo2, Lo3, Lo5, Lo17, Lo18, Lo20, Lo21, Lo33, Lo465, Lo491, Lo577, Lo578, and Lo589 belong to the "Nostrano dell'Isola" group, which includes several populations that were previously commonly grown in Northern Italy. This group has optimal agronomic, technological and organoleptic characteristics, but is very heterogeneous in terms of plant height, cycle length and kernel weight. Kernel weight for the lines ranges 96 mg (Lo578) to 185 mg (Lo491). The lines Lo43, Lo67, Lo93, Lo249 and Lo441 belong to the early flowering "Scagliolo" type, still grown in Northern Italy because of good grain quality from this type. Line Lo51 ("Bianco Oderzo") is the only line with white vitreous kernels (231 mg) included in this group; white maize is typical of this area of Italy, and has recently been becoming of increasing interest for the feed industry. Line Lo58cmsC is a "Marano" line with cytoplasmic male sterility; when multiplied using the corresponding line with normal fertility, it produces small kernels (141 mg) with a typical dark orange colour. The "Cinquantino" types, Lo387 (113 mg) and Lo435 (104 mg), are among the earliest genotypes, flowering on average of 4 d before Scagliolo. Lo404, "Sacra Famiglia", is a medium-late flowering genotype with semi-vitreous round kernels (179 mg). Lines Lo186, Lo241, Lo352, Lo446, Lo452 and Lo457 derived from crosses among

Table 1. List of the maize inbred lines investigated in this study, their origins and average kernel weights (mg).

Line	Origin	Kernel weight	Line	Origin	Kernel weight
Italian					
Lo2	Nostrano dell'Isola	136	Lo435	Cinquantino Bianchi	104
Lo3	Nostrano dell'Isola	175	Lo441	Scagliolo Marne	150
Lo5	Nostrano dell'Isola	151	Lo446	Lo80 x Lo71	90
Lo17	Nostrano dell'Isola	153	Lo452	Lo5^2 x Lo19	156
Lo18	Nostrano dell'Isola	153	Lo457	Lo43 x Lo58	108
Lo20	Nostrano dell'Isola	127	Lo465	Nostrano dell'Isola Finardi	169
Lo21	Nostrano dell'Isola	170	Lo491	Nostrano dell'Isola Finardi	185
Lo33	Isola basso	177	Lo514	Dente di cavallo	145
Lo43	Scagliolo	117	Lo520	ICAR 54	200
Lo51	Bianco Oderzo	231	Lo577	Nostrano maranizzato	115
Lo58cmsC	Marano	141	Lo578	Nostrano maranizzato	96
Lo67	Scagliolino G.V.	65	Lo589	Nostrano dell'Isola	122
Lo93	Scagliolino G.V.	116	Lo1264	P3394 (Cecilia)	329
Lo186	Marano x Isola Basso	122			
Lo241	Lo3 × Lo38	154	*Public*		
Lo249	Scagliolo Marne	172	A632	(Mt42×B14)×B14^3	211
Lo295	70 × 110	201	B73	Iowa Stiff Stalk Syn.	204
Lo309	King Ko	154	DSP1771	8002D/1256D	275
Lo352	Lo32 × Lo18	140	F2	Pop. Lacaune	227
Lo387	Cinquantino S.Fermo	113	MBS847	P3901 (Eva)	183
Lo404	Sacra Famiglia 43	179	W117	Minnesota 13	215

the types Nostrano, Scagliolo and Marano, showing kernel weight between 90 mg (Lo446) and 156 mg (Lo452). Five dent lines are also present in the list, including Lo295, Lo309, Lo514, Lo520, and Lo1264, with kernel weights ranging from 145 mg (Lo514) to 329 mg (Lo1264). Six public inbreds were also included as reference lines: F2 was the only flint genotype, while all the others were dent genotypes.

Field management and *Fusarium* inoculation

In 2009 and 2010 the set of inbred lines were tested at the experimental station of CRA-MAC (Bergamo, Italy, 45°68'N, 9°64'E). Experiments were conducted using randomised complete block designs with four replicates; the experimental unit was a two-row plot, 5.1 m long, thinned to 20 plants per row, with rows spaced 0.75 m apart. Fertiliser (kg ha^{-1}: N = 280, P_2O_5 = 115, K_2O = 120) and irrigation were applied during the growing season to limit drought stress. Ears were left open-pollinated. Environmental conditions, such as temperature and rainfall, were recorded at the CRA-MAC Weather-Station (Figure 1).

Two *F. verticilliodes* isolates from Northern Italy, (designated #289 and #294) were chosen for their pathogenicity and their ability to produce FBs (Paola

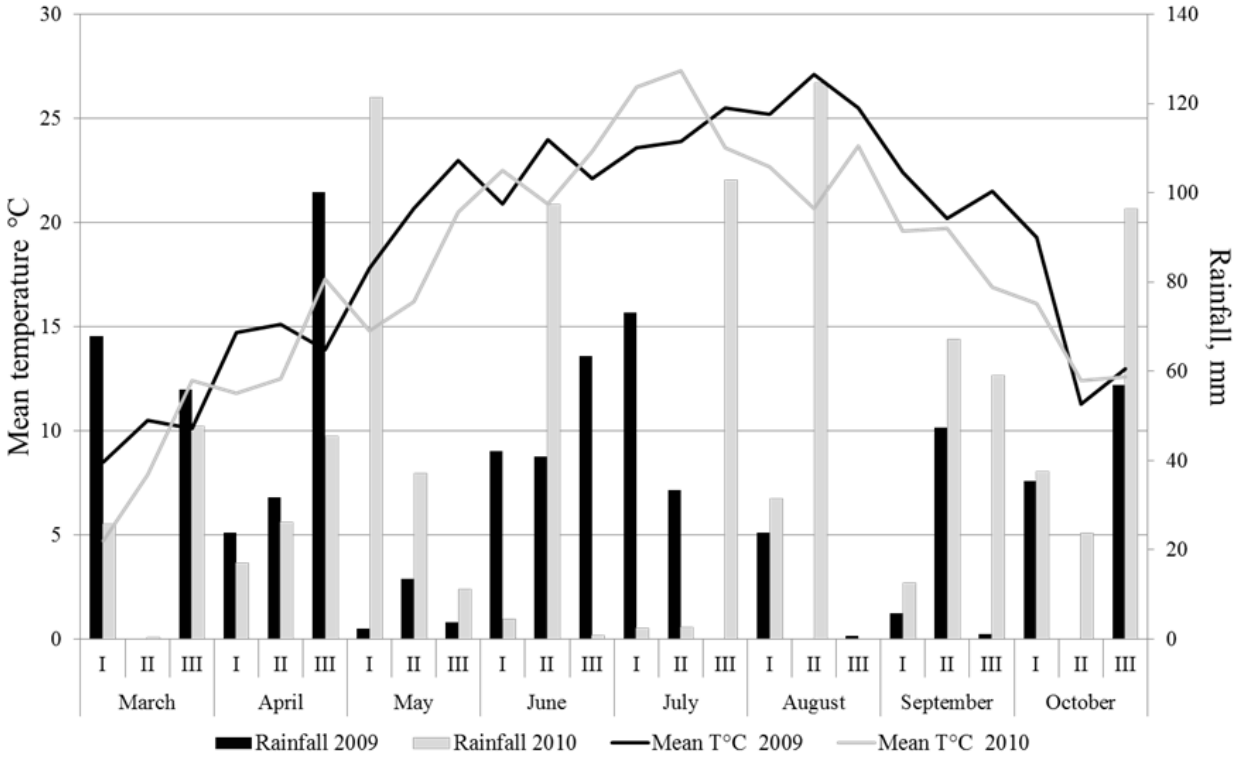

Figure 1. Environmental data (mean temperature, rainfall) recorded at CRA-MAC in 2009 and 2010.

Battilani, personal communication). The isolates were maintained in Potato Dextrose Agar (PDA) plates; spores were harvested from 15-d-old cultures as follows: each plate surface was washed with 12 ml of sterile distilled water (SDW) and the spore suspension obtained was adjusted (with SDW) to the final concentration of 10^6 spores mL^{-1}, after counting with a Bürker haemocytometer.

In each field plot, primary ears presenting the same development stage were chosen for inoculation. Ten primary ears of each inbred line were inoculated at 15 d after mid-silking, through kernel inoculation with fresh spore suspensions. Negative controls consisted of ten sterile water-inoculated and ten non-inoculated primary ears, chosen in the same sub-plot as the *Fusarium* inoculated ears. The kernel inoculation involved wounding the husk of each ear, and three kernels in the centre of the ear, by stabbing with a stainless fork, which was dipped in spore suspension, or in sterile water prior to wounding, following the methods of Reid *et al.* (1996a) for obtaining reproducible infections.

Ear evaluations

At maturity, when kernel moisture was less than 20%, ears were manually harvested and after hand de-husking, the severity of *F. verticillioides* infection was measured as follows: i) for *Fusarium* and water-inoculated ears, the number of kernels showing visible symptoms of infection, such as rot and mycelium, around the point of inoculation (number of infected kernels, NIK) was counted; ii) for non-inoculated primary control ears a rating scale (% of kernels with visible symptoms of infection, such as rot and mycelium growth) was used as follows: Disease Severity Rating, DSR, ranging from 1 = 0%, 2 = 1–3%, 3 = 4–10%, 4 = 11–25%, 5 = 26–50%, 6 = 51–75%, 7 = 76–100% of visibly infected kernels/ear (Reid *et al.*, 1996a). After visual inspection, the ears from each plot were dried, shelled, and the kernels bulked.

Kernel samples

For each sample, the kernels obtained from ten ears were dried at 40°C and thoroughly mixed before

sub-sampling to ensure homogeneity. From these pre-samples, sorted samples for laboratory analyses were obtained by applying standard procedures (Berardo *et al*., 2005). One sample was for fungal analysis, a second was for chemical and fumonisin analyses (after grinding with a Retsch laboratory mill to pass a 1.0 mm sieve), and a third sample was preserved at -20°C.

Internal kernel infection (IKI)

Fifty kernels, randomly chosen from each sample, were surface-sterilized in a 1% sodium hypochlorite solution for 2 min and subsequently in a 90% ethanol solution for 2 min and rinsed in sterile water. The samples were dried on paper, under a sterile hood, plated (five kernels/plate) on a *Fusarium* selective Dichloran-rose bengal agar medium (King *et al*., 1979) and incubated at 25°C using an 8 h light photoperiod. Seven d after plating, the number of kernels showing visible *Fusarium* mycelium were recorded and the fungi were identified according to the key of Nelson *et al*. (1983). Data of *Fusarium* incidence were averaged over ten replicates.

ELISA technique for fumonisin analysis

Extraction procedures and use of an ELISA kit for FB analysis were as described by Berardo *et al*. (2011). To obtain each sample solution for assay, 25 mL of methanol/water (70:30, v/v) was added to 5 g of ground kernel sample. The mixture was then shaken vigorously for 3 min on a shaker and the extract was filtered through a Whatman no. 1 filter. The samples were tested with the Ridascreen FBs ELISA test kit (R-Biopharm), which detects total FBs (FB_1, FB_2, and FB_3) at concentrations as low as 0.025 mg kg^{-1}. Data of FB content were averaged over two replicates.

Statistical analyses

The effects of treatments (i.e. non-inoculated vs. water-inoculated vs. *Fusarium*-inoculated) and genotypes on visual ear rot severity rating and fumonisin concentration were evaluated for each year separately, using a split-plot model with treatment as the main plot and genotypes as subplots. Statistical analyses (ANOVA) and calculation of simple correlation coefficients (r) between parameters were carried out with the MSTAT-C program (Michigan State University, East Lansing, MI, USA).

Results

Environmental conditions, artificial inoculation, fumonisin accumulation

Environmental conditions (mean temperature, rainfall) recorded in 2009 and 2010 at the CRA-MAC Weather Station are reported in Figure 1. Rainfall was more abundant in 2010 (995.2 mm) than in 2009 (723.2 mm), especially during late July and August. In contrast, the mean temperature in 2010 was consisitently lower than in 2009, with the exception of the period from late June until late July.

The average NIK around the inoculation points of the 40 inbred lines analysed, inoculated either with *F. verticillioides* or with sterile water, in both 2009 and 2010, as assessed at maturity, is reported in Figure 2. For both years, a significantly greater mean NIK resulted from inoculation with *F. verticillioides* spores (14 in 2009 and 10.9 in 2010), compared to sterile water inoculations (2 in 2009 and 3 in 2010).

The average FB content for ears inoculated with *F. verticillioides*, in comparison with the sterile water-inoculated controls, in 2009 and 2010 is illustrated in Figure 3. For both years FB concentrations in the inbred lines inoculated with fungal spore suspension were similar (44.31 ± 73.40 mg kg^{-1} in 2009 and 43.88 ± 49.36 mg kg^{-1} in 2010), and significantly greater than in water-inoculated samples (4.48 ± 6.62 mg kg^{-1} in 2009 and 10.89 ± 17.59 mg kg^{-1} in 2010).

Visual evaluation of non-inoculated primary ears indicated, on average, similar scores in 2009 (DSR 2 ± 0.8) and 2010 (DSR 2 ± 1.1); the data suggested that only 1–3% of kernels/ear showed visible symptoms of infection, such as rot and mycelium growth. The average FB concentration of these samples was 7.25 ± 17.76 mg kg^{-1} in 2009 and 9.6 ± 19.02 mg kg^{-1} in 2010.

Responses to *Fusarium verticillioides* infection and FB accumulation in inbred lines

In Table 2 NIK values and FB concentrations, determined at maturity during 2009 and 2010, are reported for the 40 *Fusarium*-inoculated inbred lines. The relative rankings of the inbred lines for both parameters are also included. All data reported rep-

Table 2. Mean number of infected kernels at the inoculation point (NIK) and fumonisin concentration (FBs, mg kg^{-1}) for the 40 maize inbred lines evaluated after *F. verticillioides* kernel inoculation (2009 and 2010).

Line	2009				2010			
	NIK rank[1]	NIK	FBs rank[2]	FBs (mg kg^{-1})	NIK rank[1]	NIK	FBs rank[2]	FBs (mg kg^{-1})
Italian								
Lo 2	10	3.8	14	5.84	13	3.8	24	38.05
Lo 3	18	4.6	9	4.05	24	6.8	22	33.35
Lo 5	16	4.0	19	9.08	16	4.8	25	38.45
Lo 17	7	3.2	10	4.48	19	5.4	18	22.85
Lo 18	10	3.8	8	3.90	3	1.6	4	1.87
Lo 20	23	6.4	17	8.42	25	7.0	30	53.55
Lo 21	18	4.4	6	3.77	8	2.6	8	3.85
Lo 33	21	5.2	24	15.07	17	5.0	16	10.65
Lo 43	39	64.0	40	240.83	21	5.8	31	64.55
Lo 51	40	68.0	39	222.61	35	15.8	26	40.10
Lo 58cmsC	23	6.4	27	21.37	23	6.0	28	48.35
Lo 67	34	38.0	31	41.08	6	2.4	7	3.55
Lo 93	31	15.2	34	70.15	11	3.0	13	6.95
Lo 186	37	51.0	35	175.82	38	26.4	40	190.60
Lo 241	35	44.0	38	220.99	31	10.0	20	33.20
Lo 249	28	8.0	30	28.07	40	120.0	39	180.55
Lo 295	27	7.8	11	4.87	20	5.6	14	9.30
Lo 309	3	3.0	18	9.04	10	2.8	5	2.80
Lo 352	3	3.0	25	15.66	29	9.6	23	35.65
Lo 387	10	3.8	3	1.71	6	2.4	10	5.65
Lo 404	3	3.0	2	0.88	11	3.0	15	10.20
Lo 435	2	2.4	1	0.56	8	2.6	9	4.00
Lo 441	29	9.2	22	11.20	37	24.6	37	123.20
Lo 446	30	9.6	16	7.99	13	3.8	12	6.15
Lo 452	10	3.8	7	3.79	39	37.0	19	24.90
Lo 457	38	55.0	37	213.79	15	4.0	2	1.47
Lo 465	10	3.8	23	12.72	34	15.0	32	71.10
Lo 491	20	4.8	12	5.47	26	8.4	27	47.10
Lo 514	33	16.4	32	43.10	27	8.8	29	49.05

(Continued)

Table 2. (Continued)

Line	2009				2010			
	NIK rank[1]	NIK	FBs rank[2]	FBs (mg kg^{-1})	NIK rank[1]	NIK	FBs rank[2]	FBs (mg kg^{-1})
Lo 520	26	7.4	5	3.68	32	12.6	21	33.30
Lo 577	8	3.4	15	6.16	1	0	1	1.09
Lo 578	3	3.0	13	5.52	3	1.6	3	1.53
Lo 589	16	4.0	21	10.05	21	5.8	34	93.95
Lo 1264	32	15.6	33	65.30	33	14.8	35	101.70
Public								
A632	25	7.2	29	26.36	30	9.8	33	84.95
B73	36	50.0	36	196.39	27	8.8	36	116.20
DSP1771	10	3.8	20	9.69	36	20.8	38	133.60
F2	1	2.0	28	25.19	5	2.2	11	5.90
MBS847	8	3.4	4	2.24	2	1.4	6	2.98
W117	22	5.4	26	15.74	17	5.0	17	19.10
Mean		14.0		44.32		10.9		43.88
$LSD_{0.05}$		6.1		8.50		8.1		11.24

[1]Relative ranking of inbred lines from the smallest to the greatest number of infected kernels at the inoculation point.

resent averages across replicates; in all cases, differences between replicates were not statistically significant.

For both years, significant differences between inbred lines were detected for both traits under study. In detail, NIK ranged from 2 (F2) to 68 (Lo51) in 2009, and from 0 (Lo577) to 120 (Lo249) in 2010. Five out of 40 inbred lines (Lo309, Lo404, Lo435, Lo578, F2) showed low disease severity (NIK from 0 to 3) for both years, 17 inbred lines showed medium disease severity (NIK from 3 to 18), and only one inbred line (Lo186) showed high disease severity (NIK >18). The remaining inbred lines did not show consistent results across years.

In contrast, FB concentrations (mg kg^{-1}) ranged from 0.56 (Lo435) to 240.83 (Lo43) in 2009, and from 1.09 (Lo577) to 190.60 (Lo186) in 2010. Comparing the FB concentration of each inbred line after artificial inoculation (Table 2), ten inbred lines (Lo18, Lo21, Lo295, Lo309, Lo387, Lo435, Lo446, Lo577, Lo578, MBS847) out of 40 tested showed low amounts (from 0 to 10 mg kg^{-1}), for both 2009 and 2010, eight inbred lines showed medium amounts (from 11 to 100 mg kg^{-1}) and two genotypes (Lo186 and B73) showed high amounts of the mycotoxins (from 101 to 250 mg kg^{-1}).

Correlations between fumonisin content and phytopathological traits

Simple correlation coefficients (r) between FB content and NIK were determined in both years in the samples inoculated with *F. verticilliodes* (Figure 4, a and b). FB concentrations were positively correlated with NIK both in 2009 ($r = 0.94$; $P \leq 0.01$) and 2010 ($r = 0.67$; $P \leq 0.01$). Additionally, in a set of these samples ($n = 66$) the Internal Kernel Infection (IKI) trait was positively correlated with FB concentration ($r = 0.37$; $P \leq 0.01$) and with NIK [$r = 0.29$; $P \leq 0.05$], as outlined in Table 3.

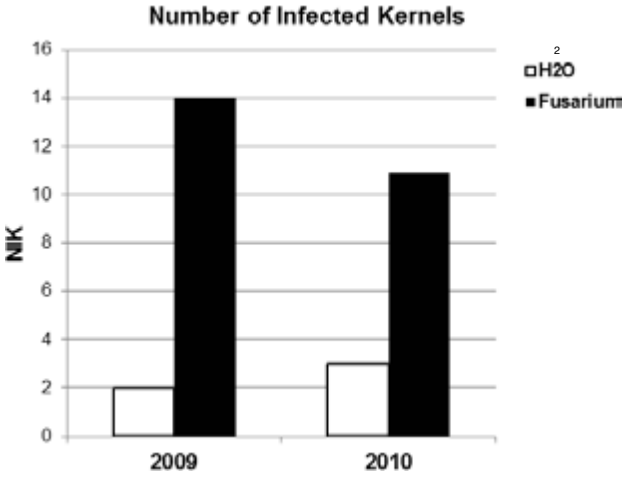

Figure 2. Average Number of Infected Kernels (NIK) for 40 inbred maize lines inoculated with sterile water (□ H_2O) and with *F. verticillioides* (■ *Fusarium*) in 2009 (LSD $_{0.01}$: 0.82) and 2010 (LSD $_{0.01}$: 2.73) at CRA-MAC.

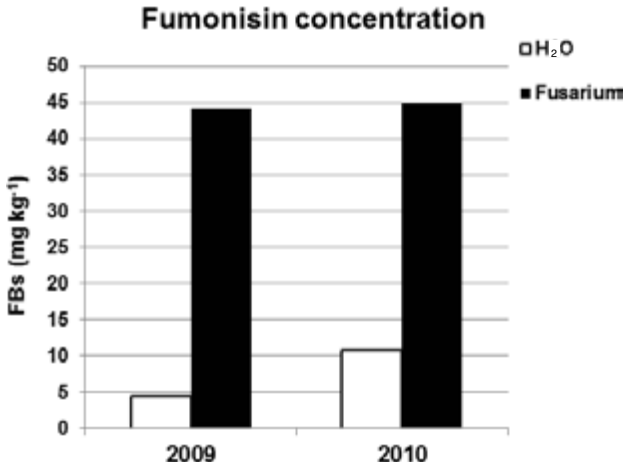

Figure 3. Average fumonisin concentrations (FBs mg kg^{-1}) for the 40 inbred maize lines inoculated with sterile water (□ H_2O) and with *F. verticillioides* (■ *Fusarium*) in 2009 (LSD $_{0.01}$: 4.71) and 2010 (LSD $_{0.01}$: 24.46) at CRA-MAC.

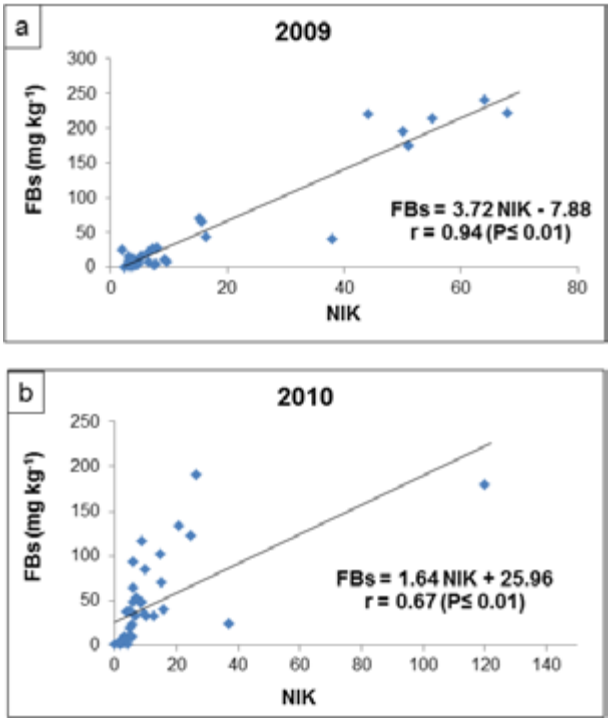

Figure 4. Scatter plots associating fumonisin concentration (FBs mg kg^{-1}) *vs* Number of Infected Kernels (NIK) for the 40 inbred maize lines inoculated with *F. verticillioides* at CRA-MAC in 2009 (a) and 2010 (b).

Table 3. Simple correlation coefficients (r) among FBs content, number of infected kernels (NIK) and internal kernel infection (IKI) in maize inbred lines inoculated with *F. verticillioides* in 2009 and 2010 (n= 66).

	NIK	IKI
FBs	0.81**	0.37**
NIK		0.29*

* Significant at $P \leq 0.05$, ** significant at $P \leq 0.01$

Discussion

Influence of climate, fumonisin accumulation and artificial inoculation

The most influential risk factors with regard to Fusarium ear rot and FBs accumulation are temperature, drought stress, insect damage, other fungal diseases, and maize genotype (Miller, 2001). Genotype by environment interactions are likely to be very important in determining mycotoxin contamination. Abbas *et al*. (2005) reported that some years favoured aflatoxin production while other years appeared to favour FB production. Logistic regression modelling of cropping systems to predict FB contamination in maize, based on 438 maize samples collected in five

regions of Northern Italy in a six year period (2002–2007), explained around 69% of variability with major roles for longitude, maturity class, and growing weeks (Battilani *et al*., 2008).

Many plant breeders rely on natural infection to create sufficient levels of disease severity for the selection of disease resistant genotypes. However, there are few locations where natural infection is sufficiently uniform to make this selection efficient and successful (Mesterházy *et al*., 2012). In addition, year-to-year variation in the degree of fungal attack, and very likely, mycotoxin contamination, is highly dependent on weather conditions, although response to weather varies among fungal species (Pan and May, 2009). To overcome this drawback, artificial infection is a suitable strategy for testing host genotypes forwards resistance to mycotoxin production (Reid *et al*., 1996a; Miedaner *et al*., 2010). The interaction between *F. verticillioides* and pests that attack maize kernels has been widely reported. The European corn borer [ECB: *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae)], is a serious pest in maize growing areas, including Northern Italy, where it has been shown that mitigation of FB_1 contamination is correlated with ECB control (Alma *et al*., 2005; Blandino *et al*., 2008; Mazzoni *et al*., 2011). Therefore, considering that in Northern Italy fungal infection through wounded kernels is predominant, kernel inoculation with a spore suspensions obtained from a mix of two toxigenic *F. verticillioides* strains, was chosen as a first step in our research. Our results indicate that the kernel inoculation technique used to infect ears was effective for inducing *F. verticilliodes* infections and FB accumulation. During the two years of research, NIK increased four- to seven-fold in the *Fusarium* inoculated material compared to the experimental controls inoculated with sterile water. Similarly, FB concentration increased five- to ten-fold in the grain from inoculated plants.

The fact that water-inoculated ears develop fewer NIK and lower FB content compared with the *Fusarium* inoculated ears indicated that, in the area where the trials were carried out, the inoculation method *per se* did not allow natural infection to proceed through wounds at levels similar to that observed after inoculation.

In our research, the same *F. verticillioides* inoculum strains were used in the two years, avoiding a possible difference in complex FB-producer fungus communities (Miller, 1994; Bottalico, 1998). This indicates that the differing environmental factors in the two years did not influence host susceptibility. In particular, reduced rainfall in 2009 compared with 2010, especially during late July and August, the period after silking, did not cause increased FB accumulation. This could have been expected, considering that dry conditions after pollination imply an increased risk of FB contamination, as reported by Battilani *et al*. (2008) and Parsons and Munkvold (2012). In other studies, FB levels were negatively correlated with season-long rainfall or with rainfall in June (Munkvold, 2003a).

Response of inbred lines to kernel inoculation

Maize germplasm was shown to possess wide genetic variability for the main components of grain (protein, lipid, starch and carotenoid contents) as reported by Berardo *et al*. (2009). Italian cultivated maize germplasm presents diversity, as a consequence of several processes of differentiation due to environmental pressure. This had caused this species to adapt to various microclimates and develop many ecotypes (Brandolini and Brandolini, 2005). The risk of losing this diversity suggested that conserving the genotypes *ex situ* was important. Therefore, in the 1950s, a large number of local maize populations were collected across Italy and organised in a germplasm collection that is still maintained at CRA-MAC, and which was used for the present research. Genotype differences are normally much wider under artificial inoculation conditions than in natural ones (Mesterházy *et al*., 2012). The kernel inoculation technique applied in the present study was effective in discriminating inbred lines with regard to their response to kernel infection by *F. verticillioides* and to FB accumulation. In susceptible genotypes, infection spreads rapidly to unwounded, healthy kernels, as white to pink fungal mycelium, whereas in resistant genotypes, the spread is very limited or does not occur at all (Reid *et al*., 1996a). In our research, only in Lo577 in 2010 infected kernels were absent at the inoculation point; in the same year Lo249 showed the greatest spread infection after inoculation.

A high proportion (58%) of the inbred lines investigated here showed similar NIK responses to artificial kernel inoculation in 2009 and 2010. Of the remaining genotypes, 30% showed higher NIK in 2009 than 2010, and 12.5% showed the opposite trend. This suggests that the interaction between genotype

and environmental factors could be important in determining Fusarium ear rot symptoms (Parsons and Munkvold, 2012).

The experimental data presented in this paper also indicated that 25% of the inbred lines under study, including nine Italian lines, responded to *F. verticillioides* inoculation by producing low levels of FBs (from 0 to 10 mg kg^{-1}), in both 2009 and 2010, similar to the non-inoculated or water-inoculated controls. Four of these lines (Lo18, Lo21, Lo577, and Lo578), belong to the "Nostrano" group; lines Lo387 and Lo435 are early "Cinquantino" types. Lo446 derives from a cross between the Scagliolo and Nostrano types. Lines Lo295 and Lo309 are dent lines. These genotypes could be considered as Italian germplasm sources of resistance to *Fusarium*, suitable for introduction into advanced breeding programs.

On the contrary, two of the inbred lines (Lo186 and B73) showed high fumonisin concentrations (from 101 to 250 mg kg^{-1}) after inoculation, in both years, reaching FB concentrations 10- to 20-fold greater than the uninoculated controls and the other inoculated genotypes. Our research confirmed that public line B73, as previously reported, is susceptible to Fusarium ear rot (Troyer 1999; Clements *et al*., 2004). Line Lo186, together with resistant Italian inbred lines highlighted in this study, could be useful tools to address research about differential gene expression in maize kernels with contrasting levels of FB accumulation. Recent studies have shown that pathogenesis-related genes were differentially activated after *F. verticillioides* infection, in Canadian maize genotypes with differential resistance to *F. graminearum* (Lanubile *et al*., 2010, 2012).

Correlations between fumonisin and phytopathological traits

Quantification of mycotoxin concentrations is expensive, laborious and time consuming. In contrast, determination of ear rot rating is more cost efficient, less laborious and more rapid. Therefore, indirect selection for reduced toxin concentrations using ear rot rating could gain greater selection efficiency. Successful indirect selection requires strong genetic associations between both traits. Studies have indicated a significant correlation between ear rot and mycotoxin levels in inbred lines and hybrids evaluated in field trials and inoculated with both *F. verticillioides* (Clements *et al*., 2003; Robertson-Hoyt *et al*., 2007; Löffler *et al*., 2010b; Parsons and Munkvold, 2012) and *A. flavus* (Walker and White, 2001; Henry *et al*., 2009). However, other studies have reported low or non-significant correlations between disease symptoms and FBs (Butron *et al*., 2006) or aflatoxin production (Hamblin and White, 2000; Walker and White, 2001). Balconi *et al*. (2010), recently showed that a non-wounding silk inoculation procedure for evaluating, in field experimental trials, maize hybrids for resistance to *A. flavus*, implied that secondary ear traits determined aflatoxin reduction, but no significant correlation between disease severity rating and mycotoxin content was found.

Our research, conducted on inbred lines where kernels were artificially inoculated in field trials, showed that the grain FB concentration was positively and significantly ($P \leq 0.01$) correlated with the number of kernels showing visible infection at the point of inoculation, both in 2009 and 2010. In areas such as Northern Italy, where pest incidence, such as ECB, is high, and consequently fungal infection can develop through kernel wounds from insect damage (Alma *et al*., 2005; Blandino *et al*., 2008; Mazzoni *et al*., 2011), visual evaluation could be a useful tool for selecting putative maize sources of resistance. Correlations between ear rot severity and mycotoxin concentrations reported from our study also agree with reports on Canadian maize for *Fusarium graminearum* (Reid *et al*., 1996b; Vigier *et al*., 2001) and United States maize for *F. verticillioides* (Kleinschmidt *et al*., 2005; Robertson *et al*., 2006).

Garcia *et al*. (2009) reported the prediction of mycotoxins in food, stating that not all fungal growth results in mycotoxin formation, and that the detection of mycotoxic fungi may or may not imply the presence of mycotoxins. Even if in many cases the extent of toxin contamination is proportional to the visual severity of infection, asymptomatic kernels may also be infected and may contain toxins (Desjardins *et al*., 1998; Munkvold *et al*., 1997). In our research the Internal Kernel Infection (IKI) trait was found to be positively and significantly ($P \leq 0.01$) correlated both with FB concentration and with the number of infected kernels at the point of inoculation (NIK). These results suggest that starting from the inoculation points, *F. verticillioides* develops visible cottony growth and spreads i) on the ear surfaces (determining NIK) and ii) internally (resulting in IKI); both modes of fungal growth resulted in FB accumulation. Nevertheless, depending on several

environmental factors, conditions favourable for fungal growth may not be conducive to mycotoxin production. It is particularly important, therefore, to consider the relationship between visual symptoms and the level of toxin contamination.

In conclusion, this research has shown that: i) there are some interesting Italian germplasm sources of resistance to Fusarium ear rot; ii) there is a strong association of visible symptoms of Fusarium ear rot with FB concentration, indicating that selection in maize for reduced visible moulds is likely to reduce the risk of mycotoxin contamination.

Resistance in maize to the two major modes of fungal penetration into the ears, via the silks through kernel wounds, is not clearly reported in literature for all genotypes. Therefore, it is important to test the inbred lines, for which we have shown that low FB accumulation results from kernel inoculation, also using silk inoculation methods.

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