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Role of Maize Hybrids and Their Chemical Composition in *Fusarium* Infection and Fumonisin Production

Chiara Dall'Asta,[†] Claudia Falavigna,[†] Gianni Galaverna,[†] and Paola Battilani*[,‡]

[†]Department of Organic and Industrial Chemistry, University of Parma, Viale Usberti 17/A, 43124 Parma, Italy

[‡]Institute of Entomology and Plant Pathology, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122, Piacenza, Italy

Supporting Information

ABSTRACT: This study was designed to investigate the role of hybrids in maize *Fusarium* section *Liseola* interaction and fumonisin production, with particular emphasis on the occurrence and accumulation of hidden fumonisins in maize (masking phenomenon). In this 2 year study, naturally infected field crops were chosen with 10 maize hybrids, six of them grown in both years. Maize samples collected in 2010 showed a higher incidence of fungal infection as well as higher fumonisin contamination than those obtained in 2009 but a very similar incidence of *F.* section *Liseola*. Fumonisin masking was confirmed in raw maize, with a lower amount of hidden forms as compared to free fumonisins detected in the year with higher contamination. The chemical composition of the different hybrids was determined and correlated with the contamination data: the results obtained highlight the main role of fatty acids, with a higher fumonisin contamination in hybrids showing a higher linoleic acid content and a higher masking action in hybrids with higher oleic to linoleic ratio. These results represent a good basis to explain maize hybrid susceptibility to *F.* section *Liseola* infection, fumonisin contamination, and masking not related to a specific commercial hybrid but extendable to all hybrids.

KEYWORDS: *mycotoxin, masking, fatty acids, hidden fumonisins, oleic acid, linoleic acid*

INTRODUCTION

Fusarium verticillioides Sacc. (Nirenberg), belonging to *F.* section *Liseola*, is the most common toxigenic fungus in maize worldwide, causing Fusarium ear rot, a very important disease affecting maize production.[1] The main toxins produced by *F. verticillioides* in grains are fumonisins; while approximately 60 fumonisin analogues have been identified, fumonisin B_1 (FB_1) and, to a lesser extent, fumonisins B_2 (FB_2) and B_3 (FB_3) are the predominant forms found in maize kernels.[2] Fumonisin-contaminated maize products are a major food concern on account of their toxic effects in humans and animals;[3] indeed, FB_1 has been declared as a class 2B carcinogen by the International Agency for Research on Cancer. Recently, the European Union has enforced the new legislation for fumonisins in food (EU Commission Regulation No. 1126/2007), and recommendations have also been made regarding FB_1 + FB_2 content in animal feeds (EU Commission Regulation No. 576/2006). Besides fumonisins usually detected with common analytical methods, several studies reported the presence in food of fumonisins potentially bound or strongly associated with proteins or other food components, which escape conventional analysis and can be determined only in an indirect way through the application of a hydrolysis step.[4,5] In particular, it has been observed that, performing alkaline hydrolysis of contaminated corn products (especially extruded products such as corn flakes), the amount of released hydrolyzed fumonisins is often higher than that stoichiometrically derived by the conversion of the fumonisins detectable by routine analytical methods. The presence of hidden fumonisins is of concern for food safety, since they are potentially able to contribute to overall toxicity after release upon gastrointestinal digestion.[6] In this context, Dall'Asta et al.[5] reported for the first time the occurrence of hidden fumonisins in raw maize, proving that these hidden forms do not originate from processing. The same authors, moreover, reported sound evidence supporting a noncovalent nature of these masked forms,[5] originating via association to macromolecules such as starch or proteins through supramolecular complexation. This mechanism is particularly relevant from a toxicological point of view, since the complexed fumonisins are released completely upon gastrointestinal digestion.[6]

This intriguing problem is probably related to the production and accumulation of fumonisins during maize growth, which is a very complex process governed by the interaction of the fungal pathogen with the host plant and ecological factors, all being involved in the modulation of fungal secondary metabolite production. Fumonisin biosynthesis is regulated by the FUM gene cluster[7] through a mechanism that has not yet been totally clarified; however, it is known that a number of environmental factors, including water activity (a_w) and temperature,[8] can be pointed out as key factors regulating fumonisin production and possibly its partitioning between FB_3 and FB_4, respectively, precursors of FB_1 and FB_2.

The role of hybrids in fumonisin contamination is stated as important by many authors, but the reasons for host resistance to *Fusaria* are not explained[9−11] and have not been attributed to specific genetic traits.[12] Indeed, genetic resistance has been studied,[13−16] but experimental data do not support definitive conclusions as no genetically resistant hybrids are available on

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the market. Grain hardness has been suggested as relevant for fumonisin contamination in maize, with soft grains being more susceptible than hard.[17] Moreover, the role of the season length of hybrids has been supposed but only partially confirmed by experimental results.[10,17,18] The strong influence of a_w dynamic during ripening has recently been described by Battilani et al.[19]

Fumonisin production seems to be influenced also by physicochemical and nutritional factors such as pH, C:N ratio, and amylopectin content in kernels,[20−22] but specific studies to investigate a possible correlation between maize composition, in terms of other macroconstituents, and fumonisin accumulation have not been performed yet.

The aim of this research was to study, in commercial fields, the role of maize hybrids in fumonisin production by *F.* section *Liseola* and the eventual effect on masking phenomena, by investigating a possible correlation between fumonisin accumulation and hybrid chemical composition in terms of macroconstituents. Special attention was devoted to the fatty acid composition, on account of the peculiar role played by these compounds in the host−pathogen interaction. In more detail, hybrids were collected in a small geographic area in the first year, to focus on the role of commonly seeded hybrids, and a wider area was sampled in the second year to evaluate the possible interaction of the hybrids with the growing area.

MATERIALS AND METHODS

Maize Samples Collection. In 2009, an agreement was established with a consortium of farmers settled in Parma (Emilia Romagna Region, North Italy) with the aim of sampling at harvest around 30 maize fields seeded with the prevalent hybrids commercialized in the area, with several replicated fields. One hundred subsamples (around 100 g each) were collected from the flux of the kernels during the harvest combine machine discharge in each field; the final sample, around 10 kg, was sent to the laboratory for mycological analysis.

In 2010, seven hybrids were selected, based on results from 2009 and taking into account the three main commercial brands of maize seeds in the Italian market. An agreement was established with three farming consortia based in Emilia Romagna to sample at harvest around 60 fields with the selected hybrids replicated and grown in five districts in the region (Piacenza, Parma, Modena, Bologna, and Ferrara); the same protocol previously described was followed.

Data on the cropping system applied were collected for each field, and hourly data on air temperature, relative humidity, and rain were downloaded from a meteorological station situated close to the sampled maize growing area.

In both years, the whole production of each selected field was delivered to a store house where maize humidity was reduced to under 14% by forced ventilation (air heated at 100 °C) in around 6 h; the grain temperature during drying was around 35−40 °C. Sampling was performed at the end of each drying turn, during the grain discharge, with subsamples taken from the kernels flux, similarly to the approach followed at harvest, previously described. Dried samples were finely ground to 0.25 mm particle size by means of a IKA MF 10 laboratory mill (OptpLab, Modena, Italy) and stored at −18 °C until analyses for determination of fumonisin content, and chemical compositions were carried out.

Incidence of Fungal-Infected Kernels. Fifty kernels of raw maize were randomly selected from each 10 kg sample, collected from the harvesting combine after an accurate mixing. They were surface disinfected with a solution of 1% sodium hypochlorite and 90% ethyl alcohol for 2 min and washed with sterile distilled water. Kernels were plated in Petri dishes (9 cm diameter), with potato dextrose agar (PDA) (Oxoid Ltd., Basingstoke, Hampshire, England) added with 0.1% streptomycin (Sigma-Aldrich) as the medium, and incubated at 25 °C for 7 days. White mold colonies, looking like *Fusaria*, were transferred on Petri dishes with PDA and identified at section level according to Summerell et al.[23] Black and green colonies, looking like *Aspergilli* or *Penicilli*, were observed for their macro- and microscopic characters and identified at section (Raper and Fennell[24]) or genus levels, respectively. Results were expressed as incidence of infected kernels (%).

Chemicals. Fumonisin B_1, B_2, and B_3 mixed standard solutions, 50 μg/mL each, in acetonitrile/water, 1:1 v/v, and FB_1 in powder, 5 mg, were purchased from RomerLabs (Tulln, Austria). Hydrolyzed fumonisins were produced as already reported.[6]

All solvents used were of LC grade. Methanol and diethyl ether were obtained from Carlo Erba (Milan, Italy), and acetonitrile and 96% ethanol were from J. T. Baker (Mallinckrodt Baker, Phillipsburg, NJ); bidistilled water was produced in our laboratory utilizing an Alpha-Q system (Millipore, Marlborough, MA). Potassium hydroxide was purchased from Carlo Erba (Milan, Italy). Fatty acid standards were purchased by Sigma-Aldrich (St. Louis, MO).

Sample Preparation for the Analysis of Free and Total Fumonisins. For free fumonisin analysis, 5 g of ground maize sample was blended in an Ultraturrax T25 high-speed blender (IKA, Stauffen, Germany) with 40 mL of water/methanol, 30:70 v/v, for 3 min at 6000 rpm and then filtered. After filtration on 0.45 μm nylon filters, 1 mL of extract was analyzed by LC-ESI-MS/MS.

For analysis of total fumonisins, aliquots (2.5 g) of the ground maize sample were blended in an Ultraturrax T25 high-speed blender (IKA, Stauffen, Germany) with 50 mL of 2 M KOH for 10 min at 6000 rpm and then stirred for 50 min (magnetic stirring). Then, 50 mL of acetonitrile was added, and after they were stirred for 10 min, two layers were formed, which were separated by centrifugation at 3500 rpm for 15 min. A 2 mL portion of the acetonitrile-rich upper layer was evaporated to dryness under a stream of nitrogen, and the residue was redissolved in water/methanol, 30:70 v/v, filtered through a 0.45 μm nylon filter and analyzed by LC-MS/MS. Fumonisins obtained after sample hydrolysis were measured as the sum of hydrolyzed FB_1, FB_2, and FB_3. Results are expressed as the sum of FB_1, FB_2, and FB_3 equivalents, considering a correction factor due to the different molecular weight of native and hydrolyzed compounds and referred to as "total fumonisins after hydrolysis". All of the results have been related to dry matter since maize kernels showed different moisture contents at harvesting.

Analysis of Free and Total Fumonisins by LC-MS/MS. The LC-MS/MS analysis for fumonisins and hydrolyzed fumonisins was performed as already reported by Dall'Asta et al.[6] For each sample, the entire procedure was performed in duplicate (n = 2). Validation experiments (matrix-matched calibration, recovery, repeatability, and limit of detection) were based on the analysis of spiked corn samples already measured as a blank for both free and hidden fumonisins. The spiking experiments were performed at six concentration levels in the range 25−5000 μg/kg. For the total fumonisin determination, the spiked samples were previously submitted to hydrolysis, and the hydrolyzed forms were then determined. Recovery was found to be 93% for FB_1 and FB_2, 89% for FB_3, 91% for HFB_1, and 88% for HFB_2 and HFB_3. Repeatability (six determinations at three spiking levels) was found to be in the range of 6−9% for fumonisins and 8−11% for hydrolyzed fumonisins. Matrix-matched calibration curves (calibration range, 25−5000 μg/kg) were used for the quantification of extractable fumonisins and hydrolyzed fumonisins. The limit of quantification (LOQ) was 25 μg/kg for both fumonisins and hydrolyzed fumonisins. Limits of detection (LOD) were found to be lower than 10 μg/kg for all of the considered analytes. All of the results were corrected for recovery. Samples showing contamination levels higher than the highest calibration level (5000 μg/kg) were diluted to match the proper calibration range.

Proximate Composition of Maize Samples. The chemical composition of maize samples (moisture, starch, fat, and protein percentages) was determined by means of NIR spectroscopy. Dispersive near-infrared reflectance (NIR/VIS) data (including the visual region) were collected using a 5000 spectrophotometer model from FOSS NIR Systems, Inc. (Silver Spring, MD, model 20910-2441). The spectrophotometer uses a split detector system with a Silicon (Si) detector between 1100 and 2500 nm and a tungsten

Table 1. Free and Total Fumonisin Contamination (Mean, *Range* in μg/kg) Determined in Maize Samples Collected in 2009 and 2010 in Emilia Romagna District (North Italy)[a]

	2009			2010		
toxins	free	total	rate F/T	free	total	rate F/T
FB_1	1266	1983	0.64	5203	7591	0.68
	70−5782	*135−6996*		*LOQ−30075*	*LOQ−44274*	
FB_2	386	634	0.61	1643	2486	0.66
	LOQ−1459	*75−1841*		*LOQ−9342*	*LOQ−16471*	
FB_3	212	391	0.54	580	1180	0.50
	LOQ−779	*60−1138*		*LOQ−2598*	*LOQ−7947*	
FB_2/FB_1	0.31	0.36		0.32	0.28	
	0.12−0.55	*0.23−0.54*		*0.01−0.71*	*0.01−0.59*	
FB_3/FB_1	0.14	0.26		0.11	0.13	
	0.01−0.33	*0.05−0.54*		*0.02−0.24*	*0.02−0.77*	

[a]LOQ, limit of quantification (25 μg/kg).

halogen lamp and has an internal ceramic standard. All spectral data were recorded in duplicate as log R^{-1}, where R is the reflectance, in the wavelength range 1100−2500 nm every 2 nm, to give a total of 346 data points per sample. Four g of ground maize was sampled for each entry. The software for scanning, mathematical processing, and statistical analysis was supplied with the spectrophotometer by Infrasoft International (ISI Port Matilda, PA).

Sample Preparation for Fatty Acid Analysis. Five grams of ground maize sample was extracted with 60 mL of diethyl ether through Soxhlet extraction. At the end of the process, the fatty residue was weighed, added with 10 μL as internal standard (caproic acid), and redissolved using 9 mL of hexane and 3 mL of KOH solution, 5% in methanol. After 1 min of stirring, 1 μL of the upper organic phase was injected in gas chromatography−mass spectrometry (GC-MS).

Fatty Acids Profile by GC-MS Analysis. GC-MS analysis was performed by a Hewlett-Packard 5890 separation system (GMI Inc., Minneapolis, MN), equipped with a Hewlett-Packard 5971 single quadrupole mass spectrometer with an electronic impact source (GMI Inc.). The chromatographic conditions were as follows: the column was a Carbowax 250 mm × 2.5 mm i.d., 250 nm f.t. (Supelco, Bellefonte, PA); the injection volume was 1 μL; gradient elution was performed using helium as the carrier gas: initial conditions at 80 °C, 0−3 min isothermal step at 80 °C, 3−16 min linear gradient to 210 °C, 16−21 min isothermal step at 210 °C (total analysis time, 21 min); injector temperature, 220 °C; and source block temperature, 230 °C. MS detection was performed using a full scan mode from 50 to 500 m/z. Peak identification was obtained by both database matching (Wiley275, NBS75K) and comparison with standard retention time.

Statistical Analyses. Statistical analyses were performed using SPSS v.19.0 (SPSS Italia, Bologna, Italy). Arcsin and logarithm transformation were applied, respectively, to data on the percentage of infected kernels and on fumonisin contamination, before using analysis of variance (ANOVA). Hybrid, hybrid and sampling location, and hybrid and year were, respectively, considered as factors in 2009, 2010, and in the joint data analysis. Mean data were statistically compared by a posthoc Tukey test (α = 0.05). Data correlation was evaluated by Spearman's correlation test (α = 0.05). Contamination data were compared by a Student's t test (α = 0.05). Regression parameters were statistically evaluated by linear regression model.

RESULTS AND DISCUSSION

Fungal Infection and Fumonisin Contamination.

Field Data and Kernels Contamination in 2009. Nine maize hybrids, marketed by different brands, were sampled in 2009 from 27 fields. One hybrid (code: H2) was sampled in 10 fields (10 replicates), three (H4, H7, and H5) in three, three (H1, H8, and H9) in two, and two (H3 and H6) in one field, respectively; the last two hybrids were not considered in the data analysis. Most of the hybrids were medium season (two and six hybrids of FAO class 500 and 600, respectively); only hybrid H1 was a short season (FAO class 300). The selected fields were representative of hybrids prevalent in the growing area (Parma, Emilia Romagna Region). All of the maize fields were seeded between early and mid-April and harvested between late August and mid-September; silk emergence was observed between early and mid-July. The preceding crops were variable, mainly cereals or arable crops; all of the fields were plowed, at around 30 cm depth, during winter and regularly fertilized. Sixty-five percent of maize crops were irrigated, and 18% were sprayed with pesticides for European Corn Borer (ECB; *Ostrinia nubilalis* Hübner) control. Kernel humidity at harvest ranged between 12 and 20%.

Concerning the fungal infection data, the collected samples had a mean percentage of kernels infected by fungi of around 50%, ranging between 16 and 92%; most fungal isolates were identified as *F.* section *Liseola*, whose more frequent species in South Europe is *F. verticillioides*, as confirmed by morphological identification in this study (42 and 74% of kernels being mean and maximum, respectively). The incidence of all of the other potential mycotoxin producers, *Aspergillus* or *Penicillium* spp., was below 1% of infected kernels each.

As far as the occurrence of fumonisins is concerned, all of the considered samples were found to be contaminated, as reported in Table 1. Free fumonisins, expressed as the sum of FB_1, FB_2, and FB_3, were in a concentration range from 70 to 8020 μg/kg dry matter (d.m.) Total fumonisins, obtained after alkaline hydrolysis and expressed as the equivalent sum of FB_1, FB_2, and FB_3, were in the range 270−9975 μg/kg d.m., being significantly higher than free fumonisins in 22 out of 28 samples (Student's t test, α = 0.05). Hidden forms, considered as the difference between total and free fumonisins, were found in all of the considered samples, ranging between 11 and 96% of free fumonisins. The FB_2 to FB_1 and FB_3 to FB_1 ratios were also calculated for both the free and the total forms: the values were not significantly different, thus indicating that all of the target compounds were involved in the masking mechanism to the same extent. Although the calculation applied for hidden FB determination is actually based on results obtained from two independent analytical procedures, validation data reported in this study showed a good reliability for both methods, thus proving that the occurrence of hidden forms cannot be ascribed to analytical bias.

Field Data and Kernels Contamination in 2010. Sixty-nine maize samples were collected from five Emilia-Romagna districts; seven maize hybrids, marketed by the three main

Table 2. Chemical Composition (Means ± SEs) of the Maize Hybrids Sampled in 2009 and 2010

		g/100 g d.m.					
hybrid	FAO class	starch	fat	proteins	C16:0	C18:1	C18:2
				2009			
H1	300	71.21 ± 1.55	2.80 ± 0.33	7.95 ± 0.57	0.43 ± 0.08	0.78 ± 0.07	1.58 ± 0.08
H2	600	69.51 ± 0.13	3.89 ± 0.08	8.16 ± 0.06	0.47 ± 0.04	1.09 ± 0.05	2.45 ± 0.07
H3[a]	600	68.45	3.67	9.08	0.47	1.02	2.34
H4	500	70.40 ± 0.39	3.63 ± 0.15	7.78 ± 0.12	0.49 ± 0.02	1.16 ± 0.02	1.58 ± 0.03
H5	600	70.20 ± 0.77	3.74 ± 0.18	7.81 ± 0.39	0.49 ± 0.03	1.23 ± 0.02	2.28 ± 0.02
H6[a]	600	69.10	3.94	8.32	0.45	1.17	2.17
H7	500	70.90 ± 0.59	3.33 ± 0.21	7.53 ± 0.22	0.49 ± 0.02	1.10 ± 0.04	2.11 ± 0.02
H8	600	69.62 ± 0.77	3.63 ± 0.15	7.98 ± 0.65	0.45 ± 0.01	1.20 ± 0.01	2.43 ± 0.01
H9	500	69.24 ± 0.45	3.93 ± 0.23	8.05 ± 0.03	0.45 ± 0.01	1.00 ± 0.04	2.02 ± 0.04
H10[b]	500						
				2010			
H1	300	72.03 ± 0.35	3.00 ± 0.37	7.23 ± 0.37	0.46 ± 0.03	0.97 ± 0.02	1.49 ± 0.02
H2	600	71.24 ± 0.59	3.18 ± 0.12	8.29 ± 0.22	0.43 ± 0.07	0.93 ± 0.05	1.74 ± 0.04
H3	600	71.04 ± 0.74	3.01 ± 0.18	7.96 ± 0.15	0.43 ± 0.03	0.92 ± 0.09	1.60 ± 0.05
H4	500	71.39 ± 0.66	2.98 ± 0.16	7.60 ± 0.14	0.42 ± 0.03	0.96 ± 0.11	1.39 ± 0.11
H5	600	71.04 ± 0.44	3.27 ± 0.12	7.43 ± 0.09	0.44 ± 0.03	0.99 ± 0.03	1.79 ± 0.03
H6	600	72.59 ± 0.36	2.58 ± 0.08	7.36 ± 0.15	0.37 ± 0.02	0.71 ± 0.06	1.45 ± 0.06
H7[b]	500						
H8[b]	600						
H9[b]	500						
H10	500	71.89 ± 0.67	2.84 ± 0.14	6.81 ± 0.19	0.43 ± 0.02	0.92 ± 0.07	1.96 ± 0.04

[a]Mean is reported when only one sample of the hybrid was collected. [b]Hybrid H10 in 2009 and hybrids H7, H8, and H9 in 2010 were not sampled.

maize seed brands, were included. Most of the hybrids were medium season (four and six hybrids were of FAO class 500 and 600, respectively); only one was a short season (FAO class 300). The hybrids were sampled as follows: H1 in 2 (two replicates), H2 in 11, H3 in 12, H4 in 9, H5 in 13, H6 in 16, and H10 in 6 fields, respectively. All of the maize fields were seeded between late March and April and harvested in September, with few exceptions; silk emergence was observed between early and mid-July. The preceding crops were variable, mainly cereals or arable crops, and all of the fields were tilled and regularly fertilized. Forty percent of maize crops were irrigated, and 46% were sprayed for ECB control. Kernels humidity at harvest ranged between 11 and 36%. The distribution of samples from the districts was as follows: 2 from Piacenza, 19 from Parma, 9 from Modena, 21 from Bologna, and 16 from Ferrara. The number of samples of each hybrid collected in each district varied between one and five.

Concerning fungal infection, the collected samples had a mean incidence of kernels infected by fungi of around 95%, ranging between 48 and 100%; many fungal isolates were identified as *F.* section *Liseola* (mean and maximum 46 and 94%, respectively); *F. verticillioides* (identification based on morphological characters) was largely dominant, similarly to what was found in 2009. Furthermore, the mean incidence of kernels infected by *A.* section *Flavi* was around 5% with a maximum of 80%, by *A.* section *Nigri* around 1% with a maximum of 30% and by *Penicillium* spp. around 2%, with a maximum of 44% (the maximum was detected in only 1 sample for each group).

As far as fumonisin contamination is concerned, also in 2010, all of the considered samples were found positive to fumonisins (Table 1) at a level higher than that recorded for the first year of sampling.

The free fumonisin concentration ranged from LOQ to 42015 μg/kg d.m., while the total fumonisin level after alkaline hydrolysis was found to be in the range LOQ−68692 μg/kg d.m., being significantly higher than free fumonisins in 60 out of 67 samples (Student's t test, $\alpha = 0.05$). Hidden fumonisins ranged between 388 and 26677 μg/kg d.m. Also for this data set, the calculated FB_2/FB_1 and FB_3/FB_1 ratios were not significantly different for both free and total fumonisins.

The collected data showed a significant difference in fungal incidence as well as in fumonisin contamination over the 2 years. In particular, samples collected in 2010 showed a higher fungal incidence, as well as a higher fumonisin level, than those obtained for maize sampled in 2009. These results are supported by the meteorological data collected for 2009 and 2010 in Emilia-Romagna. Indeed, the year 2010 was colder as compared to 2009, except in July, the flowering period of maize. Relative humidity was generally higher in 2010, as was rainfall; the amount of rain in May, June, and July almost tripled in 2010 as compared to 2009, and it remained abundant also in August and September. These conditions resulted in a longer in field maturation period, supporting a longer period of favorable conditions for fungal growth and fumonisin accumulation in kernels.[19,25]

Chemical Composition of Kernels. *Kernel Composition in 2009.* Mean data (±standard error) for the chemical composition of the considered hybrids are reported in Table 2. The compositional data are in agreement with those usually reported for raw maize: the mean starch content in kernels was about 70 g/100 g d.m. with a maximum of 73 g/100 g d.m., while fat and protein mean percentages were 3.7 g/100 g d.m. with a maximum of 4.7 g/100 g d.m. and 8.0 g/100 g d.m. with a maximum of 9.1 g/100 g d.m.. The main fatty acids were found to be palmitic acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2), with traces of stearic (C18:0) and linolenic (C18:3) acids. The oleic to linoleic ratio was calculated in the range 0.37−0.67, with a mean value of 0.50.

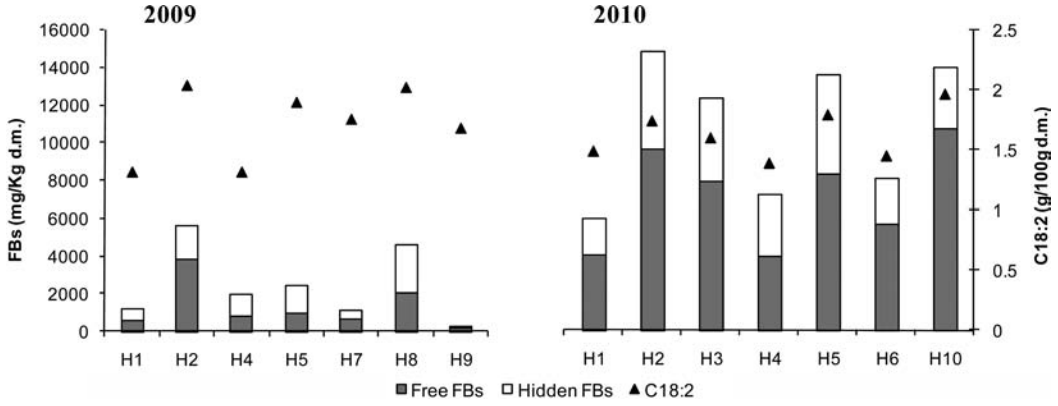

Figure 1. Free and total fumonisins levels and linoleic acid content in the considered maize hybrids.

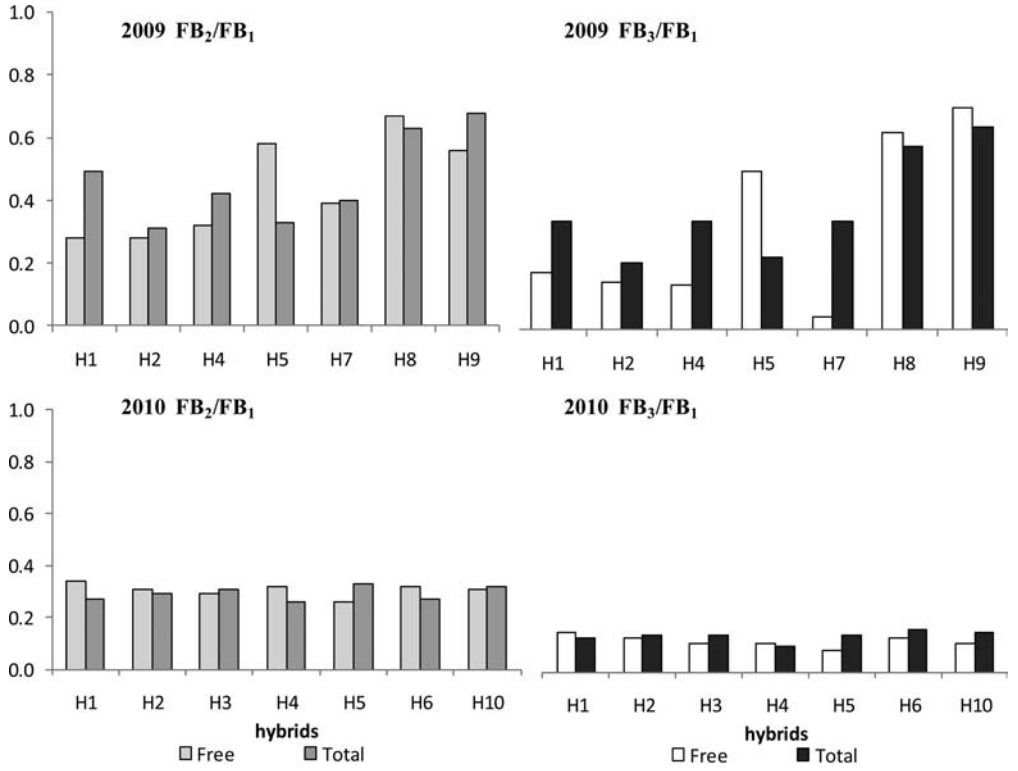

Figure 2. Free and total FB_2/FB_1 (left) and FB_3/FB_1 (right) ratios in the hybrids sampled in 2009 and 2010.

Kernel Composition in 2010. Also, for maize samples in 2010, mean data (±standard error) for the chemical composition of the considered hybrids are reported in Table 2. The mean starch content was about 71.5 g/100 g d.m. (range, 66.4−75.2 g/100 g d.m.), while the fat and the protein mean percentages were around 3 g/100 g d.m. (range, 2.1−4.2 g/100 g d.m.) and 7.6 g/100 g d.m. (range, 6.1−9.6 g/100 g d.m.), respectively. A slightly different distribution in fatty acids content was observed: in particular, the oleic to linoleic ratio was calculated in the range of 0.42 and 0.76, with a mean of 0.6, slightly higher than the values obtained in 2009.

Role of Hybrids, Growing Area, and Years. To highlight the possible role played by the genotype in fungal infection and fumonisin accumulation in field, all of the chemical and infection data collected in this study were subjected to ANOVA. No relations were found between the applied cropping system and the incidence of infected kernels or their fumonisin content, also considering each hybrid separately. Data analysis did not show a significant effect of the considered hybrids on the incidence of kernels infected by *F.* section *Liseola* over the 2 years; the same result was obtained for starch content.

Year 2009. The incidence of kernels infected by *F.* section *Liseola*, *A.* section *Flavi*, *A.* section *Nigri*, and *Penicillium* spp., considered separately, was not significantly influenced by hybrids, while significant differences between hybrids were found for the incidence of kernels infected by fungi and for both total and free fumonisin levels. Considering the FB_2/FB_1 and FB_3/FB_1 ratios, the collected data showed a high variability between hybrids, as reported in Figure 2. As far as the hybrid chemical composition is concerned, the lipid fraction related variables were found to be a key parameter, since the main significant differences between hybrids were observed in fat content and fatty acid profiles, as reported in Table 2.

Year 2010. Concerning data obtained for samples collected in 2010, the statistical analysis was performed considering as

factors both the maize genotype and the harvesting district. The main source of variation between samples was found to be the genotype; the harvesting district never resulted as significant nor did the interaction of both factors.

The incidence of kernels infected by fungi was significantly influenced by hybrids, with H1 being less contaminated. The incidence of kernels infected by *F.* section *Liseola*, *A.* section *Flavi*, *A.* section *Nigri*, and *Penicillium* spp., although higher than in the 2009 season, did not show significant differences between hybrids. Similarly, also, the contamination levels for both free and hidden fumonisins were not significantly different between genotypes (Figure 1) but higher than those determined in 2009. An interesting trend was observed for the FB_2/FB_1 and FB_3/FB_1 ratios, these values being more similar between hybrids than those observed in the 2009 season, as shown in Figure 2.

In the harvest year 2010, significant differences between hybrids were observed for protein content. Moreover, as already observed for the 2009 data set, the lipid fraction-related variables showed higher variability between hybrids. Accordingly, also, changes in fatty acid profile were found to be significant, as reported in Figure 2

Years 2009 and 2010: Common Hybrids. To better understand the role played by the maize hybrids considered toward *Fusarium* infection and fumonisin contamination, the data collected in 2009 and in 2010 harvest seasons were merged and statistically analyzed taking into account the six common hybrids; this approach is justified by the fact that the sampling place did not play a significant role.

Because the main aim of this research was to study the role of hybrids, the contamination levels were normalized on the maximum recorded amount for each year to obtain comparable data. Then, the ANOVA was rerun, considering as factors both the hybrid (H1−H6) and the year (2009 and 2010). Both hybrid and year showed a statistical significance, whereas the interaction between the two factors was found to be negligible. Significant differences over the 2 years of observation were found for the total infection level, for the fat and the protein content. As far as differences between hybrids are concerned, significant changes were found for the same variables. On the other hand, no significant difference was found between hybrids in relation to the incidence of kernels infected by *F.* section *Liseola* (fungal incidence 95 vs 49 and *F.* section *Liseola* 41 vs 46%, respectively in 2009 and 2010). The free and masked fumonisin levels, as well as the linoleic acid content, are reported in Figure 3: for these variables, significant differences were found between hybrids when the data obtained over 2 years were combined. Observation of the 2009 and 2010 data sets indicates that the highest variability between hybrids was due to the lipid fraction related variables, as shown in Table 2.

To better define the changes in composition occurring over the 2 years, the mean values and the variation range were determined for the six common hybrids (Table 3). Only those factors that have been found as significant by statistical analysis were considered. Regarding fatty acids, the profile composition was given in terms of relative percentages. A wide overlapping can be noticed in fat and C18:1 content in the 2 years, while C18:2 was lower in 2010, with a minimum overlapping in the range of variation also noticed in the rate C18:1/C18:2 and FB_2/FB_1 (considering both free and total fumonisins, respectively). The range of variation was greater in 2009, with the exception of C18:1 with a very similar range in both years.

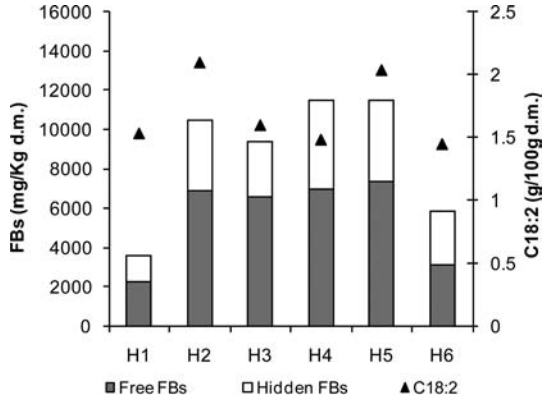

Figure 3. Free and masked fumonisins levels and linoleic acid content in the common maize hybrids for years 2009 and 2010.

Table 3. Mean Value and Variation Range (in Parentheses) of Selected Parameters Recorded for the Six Common Hybrids Considered in Both 2009 and 2010

	2009	2010
fat (g/100 g d.m.)	3.60 (2.80−3.90)	3.00 (2.60−3.30)
C18:1 (%)	27.70 (23.50−31.10)	30.20 (27.60−32.30)
C18:2 (%)	59.40 (56.60−64.20)	53.50 (49.70−56.30)
C18:1/C18:2	0.47 (0.37−0.55)	0.57 (0.49−0.65)
$(FB_2/FB_1)_{free}$	0.37 (0.28−0.67)	0.31 (0.26−0.32)
$(FB_2/FB_1)_{tot}$	0.39 (0.31−0.68)	0.29 (0.26−0.32)
$(FB_3/FB_1)_{free}$	0.24 (0.04−0.70)	0.12 (0.08−0.13)
$(FB_3/FB_1)_{tot}$	0.28 (0.21−0.64)	0.14 (0.10−0.16)
free FB/tot FB	0.58 (0. 40−0.91)	0.63 (0.55−0.70)

Considering fumonisin accumulation, another important point was related to the production of FB_2 and FB_3 in comparison to the main toxin FB_1. In the 2 years of observation, the mean values obtained for FB_2/FB_1 rate are comparable, while FB_3/FB_1 was lower in 2010; it can be argued that FB_1 could be more efficiently obtained from its precursor FB_3 in conducive conditions for fumonisin synthesis. Besides, when different hybrids are considered, significant differences can be highlighted (see Figure 2). In particular, the FB_2/FB_1 and FB_3/FB_1 ratios obtained for the 2009 data set showed a noticeable variability between hybrids, strongly reduced in 2010. As previously described for the masking rate, these data suggested that the role played by the genotype in fumonisin biosynthesis modulation is significant with low contamination, but it seems mitigated when high contamination occurs.

Moreover, when the masking rate between different maize genotypes is considered, the free-to-total FB_2/FB_1 and FB_3/FB_1 ratio values obtained for the samples collected in 2010 were narrower than in 2009. These data suggest that maize genotypes may support the masking phenomenon to a different extent, particularly with poorly conducive conditions for fumonisin accumulation.

Thus, on account of the data collected in this study, fumonisin accumulation in maize seems to be significantly related to the genotype. In particular, results obtained for the same maize hybrid grown in different areas during the same year showed a similar behavior, suggesting an infection response mainly due to the genotype characteristics.

Effect of Chemical Composition on Fumonisins. As already described, previous experimental studies considered several hybrid-related characteristics to define the factors able to

influence and/or mitigate fumonisin contamination in maize. However, none of these studies considered the composition of maize in terms of macroconstituents, mainly lipids and their fractions, as reported in this work. Starting from our data set, the starch amount resulted very similar between commercial hybrids, also over the 2 years, while protein content was found to change significantly over the 2 years but not between hybrids.

On the other hand, the lipid fraction was found to vary significantly between maize genotypes, irrespective of the growing year. The recorded trend in fatty acid profiles is, actually, very peculiar, since a decrease in C18:1 and an increase in C18:2 amount within all of the considered maize genotypes are recorded in 2010, when a stronger incidence of fungi is experienced in maize. In addition, the ranges of total fat percentage and of C18:1 and C18:2 amount within the six common hybrids were narrower in 2010 than in 2009, when the fumonisin contamination level was higher. These results are consistent with those obtained for the fumonisin accumulation and the masking rate and strongly support the hypothesis that different maize genotypes are able to differently react to fungal attack when the infection rate is low, whereas the hybrid-related response ability decreases when more conducive conditions for fumonisin production occur.

As reported in Figure 1, when the free and total fumonisins and the C18:2 content in the considered maize hybrids are compared for the samples collected in 2009 and 2010, a similar trend is recorded. Furthermore, when average values for hybrids 1−6 are considered over 2 years (Figure 3), this trend is noticeably conserved, clearly pointing out a correlation between the fumonisin accumulation and the amount of fatty acids in maize.

When a Spearman's correlation test was applied to the data set, significant correlations were found between maize kernel chemical composition and fumonisin contamination ($\alpha = 0.05$). As expected, a positive correlation was found between free and total fumonisins for samples collected in 2009, as well as for those collected in 2010, and for the common hybrids analyzed together.

Significant positive correlations were obtained between the linoleic acid amount and the free and total fumonisin levels when common hybrids over 2 years are considered; therefore, the highest the fat and the linoleic acid amount in the considered maize genotypes, the highest the fumonisin contamination result, in agreement with results reported by Ebrahimi et al.[26] in peanuts infected by *A. flavus*. As far as separate data sets are concerned, free and total fumonisins were found to be positively correlated to the total fat amount as well as to the oleic and linoleic acid amounts in 2009. On the contrary, no significant correlations were found in 2010.

Unsaturated fatty acids have been frequently described as modulators of plant resistance pathway upon pathogen attack,[27] although no specific studies have been performed so far regarding their role in maize infected by *Fusaria*. The fatty acid profile is, indeed, influenced by the environmental conditions experienced by plants during the flowering/growing period; in particular, the temperature increase is related to higher oleic acid contents.[28] The collected data showed that the average oleic and linoleic acid relative contents were significantly different between years. This behavior may be partially influenced by the contribution of the different environmental conditions experienced by the sampled crops in particular after pollination (second half of July), this period being crucial for the accumulation of macroconstituents in kernels. Indeed, in 2010, a higher oleic acid relative percentage was found, in agreement with the climatic data indicating a slightly higher temperature recorded in July for that year.

Nevertheless, because the same statistical trend was found for free and total fumonisins, this result suggests that fatty acids may be implicated also in the masking phenomenon. Very recently, Bartok et al.[2] found several fatty acid esters of fumonisins in fungal mycelia. Although their occurrence has never been proven in food and/or in plants, these derivatives should be actually considered as masked fumonisins. Moreover, these compounds may be cleaved under alkaline conditions similar to those applied in this study, thus releasing the hydrolyzed forms. Although the formation of fumonisin derivatives esterified with fatty acids could offer a possible explanation of the masking phenomenon, according to Bartok et al.,[2] these derivatives are produced by fungi in very small amounts and are with difficultly excreted in the media, due to their low polarity. Because the masking rate reported in this paper is very high, it is very unlikely that hidden forms may be due to the formation of conjugation products between fatty acids and fumonisins. More likely, the masking phenomenon is ascribable to the supramolecular interactions already hypothesized by Dall'Asta et al,[5,6] while fatty acids may be actively involved in the plant−pathogen cross-talk regulating fumonisin accumulation and hidden fumonisin formation in planta. Indeed, a very good linearity was obtained, with a r^2 value of 0.89, when the masking rate and the oleic-to-linoleic ratio of the six hybrids is regressed. Moreover, if we exclude hybrid H1, which is the only short season hybrid considered in this study, a r^2 value of 0.99 was obtained. If this trend is confirmed by further analyses on a wider number of samples, it will open an important issue about fumonisin accumulation in maize also on account of the plant−pathogen interaction. The role played by fatty acids in fumonisin biosynthesis and bioactivity has already been addressed by several authors. In particular, fatty acids could be involved in the up- or down-regulation of fungal fumonisin biosynthesis.[29] In particular, the authors identified a number of fatty acid metabolism-related genes in the *F. verticillioides* FUM biosynthetic gene cluster and suggested a possible involvement of fatty acids as factors in regulating fungal differentiation. If proven also for the pathosystem *F. verticillioides* maize, this observation may suggest a fatty acid mediated cross-talk between the fungus and the host plant. On the other hand, several studies have reported the ability of FB_1 to significantly disrupt the fatty acid metabolism in animals. In particular, FB_1 is able to interfere with the fatty acid as well as the phospholipid biosynthetic pathways in rat liver, actually reporting a significant C18:2 accumulation upon short- and long-time feeding experiments.[30] The authors also suggested that alterations in n-3 and n-6 fatty acid biosynthetic pathways are likely to be important mediators for FB_1-induced cell proliferation. Thus, if the fatty acid trend recorded in this study is confirmed, a similar interfering action on the fatty acid biosynthetic pathway might be hypothesized also for fumonisins in maize.

In conclusion, data presented in this paper suggest a hybrid-dependent implication of fatty acids in the plant−pathogen cross-talk inducing a modulation of fumonisin-related mechanisms in maize. These results, if confirmed in further studies with a wider data set, may represent a basis to explain maize hybrid susceptibility to fungal infection, fumonisin contamination, and masking phenomenon not related to a specific

hybrid or commercial brand but extendable to all hybrids. This is of crucial importance, as maize hybrids are characterized by a very frequent turnover on the market,[25] and they can be very useful in breeding and hybrid selection.

■ ASSOCIATED CONTENT

Supporting Information

Figure of the linear correlation between the free-to-total fumonisin ratio and C18:1 to C18:2 ratio (short season hybrid is given in white); table of climatic data for 2009 and 2010; table of significance obtained by ANOVA tests for 2009 and 2010 data sets and for common hybrid data set; and table of significant Spearman's correlations found for 2009, 2010, and common hybrids data sets. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +39 0523 599254. Fax: +39 0523 599256. E-mail: paola.battilani@unicatt.it.

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Notes

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