Changes in the Phenolic Acid Content and Antioxidant Activity During Kernel Development of Corn (*Zea mays* L.) and Relationship with Mycotoxin Contamination

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ABSTRACT

Corn grain production could be affected by several fungal pathogens responsible for the production of mycotoxins. The aims of this study were to determine the evolution of phenolic acids and total antioxidant activity (TAA) during kernel development and to evaluate their potential protective role in minimizing mycotoxin contamination in six corn genotypes (four open-pollinated varieties and two hybrids) characterized by a wide array of kernel traits. TAA and free and cell wall-bound phenolics showed significant differences among corn genotypes at different stages of development, with the highest values found at the beginning of kernel development. Ferulic, *p*-coumaric, and caffeic acids were the main cell wall-bound phenolic acids during kernel development, whereas chlorogenic acid was the main free phenolic acid. A significant negative correlation was observed between deoxynivalenol contamination at harvest maturity and free phenolic acids and TAA at the beginning of kernel development, whereas no significant correlation was observed with fumonisin contamination. In conclusion, free phenolic acids are evidently involved in the resistance mechanism toward deoxynivalenol contamination, whereas their role toward fumonisin contamination was not elucidated under field conditions, implying that components other than phenolic acids may be responsible for this latter type of resistance.

Corn (*Zea mays* L.) is one of the most cultivated and consumed cereals in the world. Corn red and pink ear rots are two of the major fungal diseases affecting corn production worldwide. *Fusarium* ear rot results usually in white to salmon-pink discoloration of kernels, and a white to pinkish mycelium covers the kernels, especially on the tip of the ear. The predominant species responsible for corn red ear rot in Europe are *Fusarium graminearum* Schwabe and *Fusarium culmorum* (Wm. G. Sm.) Sacc., whereas pink ear rot is caused by *Fusarium verticillioides* (Sacc.) Nirenberg and *Fusarium proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg. All these species could be responsible for mycotoxin production. *F. graminearum* and *F. culmorum* are two well-known type B trichothecene producers, and deoxynivalenol (DON) is the most diffuse trichothecene produced in temperate areas, whereas *F. verticillioides* and *F. proliferatum* are responsible for production of fumonisins (FBs) (Logrieco et al. 2002). Mycotoxins are secondary metabolites that are harmful to humans and livestock; thus, their occurrence in food and feed is a significant problem throughout the world (Charmley et al. 1995; CAST 2003). Mycotoxins are generally heat-stable molecules that cannot be fully eliminated during the processes currently used in cereal-based manufacturing (Hazel and Patel 2004). Prevention of contamination by mycotoxins during crop cultivation is the best way to reduce their accumulation. The environmental conditions, agricultural practices, and susceptibility of the genotype are the main factors playing a significant role in the fungal development and, consequently, in the mycotoxin accumulation (Blandino et al. 2009). The identification of naturally occurring mechanisms that reduce mycotoxin accumulation in plants has gained a lot of interest, and there is increased effort from the corn seed industry to identify the genetic source of *Fusarium* ear rot resistance.

The role of phenylpropanoids in disease resistance has been analyzed extensively in the last few years. One such role ascribed to phenylpropanoids in plant defense mechanism results from their antioxidant properties (Boutigny et al. 2008). Moreover, they could interfere directly with pathogens and reinforce plant structural components acting as a protective mechanical barrier (Siranidou et al. 2002). In corn grains, phenolic acids represent the most common form of phenolic compounds that exist as soluble (free and conjugated) or cell wall-bound forms. These phenolic acids could play a role in the inhibition of mycotoxigenic *Fusarium* species, as suggested by their ability to reduce in vitro fungal growth and mycotoxin accumulation (Boutigny et al. 2009). To the best of our knowledge, little information is available on the possible relationship between resistance to *Fusarium* spp. and corn phenolic acid content under field conditions. It was observed that in the earliest phase of kernel development of two yellow corn hybrids, chlorogenic acid may play a role in corn resistance to red ear rot and DON accumulation (Atanasova-Penichon et al. 2012). In addition, a possible role of cell wall-bound ferulic acid in corn resistance to *F. verticillioides* was suggested (Picot et al. 2013). To date no report has analyzed the evolution of phenolic acids at different stages of kernel development and their role in the resistance to mycotoxin accumulation in corn genotypes characterized by a wide array of kernel traits. Therefore, the aims of this study were 1) to determine phenolic acids and total antioxidant activity (TAA) evolution during kernel development of six corn genotypes (four open-pollinated varieties and two representative hybrids) characterized by a wide array of kernel traits including kernel color, size, and hardness; and 2) to evaluate the potential protective effects of phenolic acids toward *Fusarium* ear rot and consequently to DON and FB accumulation.

MATERIALS AND METHODS

Experimental Design. Four open-pollinated varieties of corn and two representative hybrids, characterized by a wide array of kernel traits, were sown in 2014 in northwestern Italy (Chivasso, Piedmont; 45°12′42″N, 7°55′96″E) in a completely randomized block design with four replications. The plot size was 6 × 3 m, and each plot consisted of four rows of 36 plants (open-pollinated varieties) or 45 plants (hybrids). The corn genotypes were chosen for their different kernel traits and in particular for their color. However, their selection was such that the genotypes would have similar maturity lengths and morphological traits, such as plant height, to reduce the influence of these factors on the fungal infection and mycotoxin contamination. The main information about the open-pollinated varieties of corn, characterized by a maturity duration of 120 relative days, and about the hybrids, belonging to FAO maturity class 600 (132 relative days to maturity), are reported in Table I.

Conventional agronomic techniques were adopted for field experiments. Briefly, the previous crop was corn, and the mechanical

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sowing was carried out on April 7, following an autumn plowing (30 cm) and disk harrowing to prepare a proper seedbed. All the plots received the same amount of nutrients: 300, 100, and 100 kg/ha of N, P_2O_5, and K_2O, respectively. Irrigation was carried out by employing the furrow method, according to the conventional farm management system in force in the experimental area, to avoid any drought stress until the end of the dough stage (growth stage [GS] 85). All the plots were sprayed at GS 75 with pyrethroid lambda-cyhalothrin insecticide (Karate Zeon, Syngenta Crop Protection, Milan, Italy) at 0.019 kg of active ingredient/ha. The application was performed on July 22, to minimize the ear injuries caused by the activity of the European Corn Borer (ECB) (*Ostrinia nubilalis*, Hübner), which could favor a late infection of *Fusarium* spp.

Daily temperatures and precipitations were measured by using a meteorological station located near the experimental area (Table II). After flowering, corn kernel GSs were identified according to the BBCH scale (Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie) (Meier 2001) and to the thermal time after silking (Table II).

For each corn genotype, 10 corn ears were randomly handpicked at four stages of kernel development: end of the silking stage (GS 69, about 5 days after silking [DAS]), blister stage (GS 71, about 7 DAS), dough stage (GS 85, about 32 DAS), and harvest maturity (GS 99, about 75 DAS). Silking was assigned when silk emergence was observed in 50% of corn ears. Samples were immediately frozen. The ears were then manually shelled, and kernels were dipped in liquid nitrogen, freeze-dried, ground to a fine powder (particle size < 250 μm) with a Cyclotec 1093 sample mill (Foss, Padova, Italy), and stored at –25°C until analyses (phenolic acid content and TAA) were performed.

At harvest maturity, ears were collected by hand from 2.25 m^2 of each plot to quantify grain yield and to obtain a representative grain sample for analyses of mycotoxins. Ears were shelled with an electric sheller, and kernels from each plot were mixed thoroughly to obtain a random distribution. Samples (4 kg) were then taken, dried at 60°C for three days, and ground to a powder (particle size < 1 mm) to analyze the DON and FB content.

Mycological Measurements. Fungal ear rot incidence and severity were calculated on 20 ears randomly sampled from each plot at harvest maturity (GS 99). The fungal ear rot incidence was calculated within each plot as the percentage of ears displaying symptoms, and its severity was calculated as the percentage of symptomatic kernels per ear. A scale from 1 to 7 was used to quantify the percentage interval of surfaces exhibiting visible symptoms of the disease: 1 = 0%, 2 = 1–3%, 3 = 4–10%, 4 = 11–25%, 5 = 26–50%, 6 = 51–75%, and 7 = >75% (Reid et al. 1999). The ear rot severity scores were then converted to percentages of the ear exhibiting symptoms, and each score was replaced with the midpoint of the interval (Campbell and Madden 1990).

Reagents. Methanol (LC-MS Ultra Chromasolv, ≥99.9%), acetonitrile (LC-MS Chromasolv, ≥99.9%), ethyl acetate (Chromasolv for HPLC, ≥99.7%), acetic acid (puriss. p.a., ACS reagent, ≥99.8%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%), and phenolic acid standards (vanillic acid, ≥97%; caffeic acid, ≥98%; syringic acid, ≥95%; chlorogenic acid, ≥95%; *p*-coumaric acid, ≥98%; *trans*-ferulic acid, ≥99%; and sinapic acid, ≥98%) were purchased from Sigma-Aldrich (Saint Louis, MO, U.S.A.).

Extraction of Phenolics. The extraction of phenolics in two separated forms (free and cell wall-bound) was performed on all the biological replicates ($n = 4$) of each corn genotype collected at four stages of kernel development. The extraction was performed according to the procedure proposed by Urias-Peraldí et al. (2013) with some modifications (Atanasova-Penichon et al. 2012). Each sample (100 mg) was mixed with 1 mL of 80:20 (v/v) methanol–water solvent. The mixtures were sonicated for 10 min in an ultrasonic bath (35 kHz, Sonorex Super RK 156 BH, Bandelin Electronic, Berlin, Germany) while maintaining the temperature at 4°C. Samples were then mixed for 10 min and centrifuged at 10,600 × *g* for 5 min at 4°C. The supernatant was transferred into a new microcentrifuge tube, and a second extraction was carried out. The supernatants collected contained free phenolics, whereas the pellets contained cell wall-bound phenolics.

Extraction of Free Phenolics. The supernatants obtained in the first extraction step were combined, and free phenolics were concentrated under a nitrogen stream, prior to the addition of 500 μL of water. The aqueous solutions were acidified to pH 2 with 1N HCl (20 μL) before liquid-liquid extraction with 500 μL of ethyl acetate. Samples were shaken for 5 min with magnetic stirring bars (8 × 3 mm) and then centrifuged at 10,600 × *g* for 5 min at 4°C. The supernatant was transferred into a new microcentrifuge tube, and the extraction was repeated another two times. The combined supernatants were reduced to dryness under a nitrogen stream and then reconstituted in 200 μL of 80:20 (v/v) methanol–water solvent.

TABLE I
Corn Genotypes Compared in the Study and Their Kernel Characteristics[z]

Code	Name	Corn Type	Kernel Color	Kernel Size	Kernel Hardness
V1	Rostrato vinato	Open-pollinated variety	Dark red	Small	High
V2	Pignoletto rosso	Open-pollinated variety	Light red	Small	High
V3	Pignoletto giallo	Open-pollinated variety	Yellow	Small	High
V4	Ostenga	Open-pollinated variety	White	Large	Medium
I1	SNH48.02	Hybrid	Yellow	Medium	Medium
I2	PR32B10	Hybrid	White	Medium	Medium

[z] V1, V2, V3, and V4 corn genotypes were provided by CREA-MAC (Bergamo, Italy); I1 corn genotype was provided by Planta Research and Seeds (Vicenza, Italy); and I2 corn genotype was provided by Pioneer (Cremona, Italy).

TABLE II
Sowing, Silking, and Sampling Dates of the Corn Genotypes Compared[z]

	Open-Pollinated Varieties				Hybrids					
Phase	Date	DAS	GDD	Rainfall	Date	DAS	GDD	Rainfall	GS	Stage
Sowing	April 7	…	…		April 7	…	…		…	…
Silking	July 4	0	0	0	July 11	0	0	0	65	Silking
Sampling	July 9	5	52	60	July 15	4	48	8	69	End of silking
	July 11	7	74	60	July 18	7	93	8	71	Blister
	August 6	33	396	216	August 11	31	392	160	85	Dough
	September 15	73	841	283	September 25	76	849	243	99	Harvest maturity

[z] DAS = days after silking; GDD = growing degree days (°C) from silking; Rainfall = cumulative rainfall (mm of rain) from silking; and GS = growth stage identified according to the BBCH scale.

Extraction of Cell Wall-Bound Phenolics. The pellet remaining after the first extraction step was hydrolyzed with 400 µL of 6M NaOH in a hot water bath set at 80°C for 30 min. The hydrolysates were then acidified to pH 2 with 12N HCl (260 µL), and 800 µL of hexane was added to remove lipids. After discarding the supernatant, 300 µL of ethyl acetate was added to each sample, followed by mixing and centrifugation at 10,600 × *g* for 5 min at 4°C. The supernatant was transferred into a new microcentrifuge tube, and the extraction was repeated another three times. The combined supernatants were reduced to dryness under a nitrogen stream and then reconstituted in 200 µL of 80:20 (v/v) methanol–water solvent.

Determination of the Free (FP) and Cell Wall-Bound (CWBP) Phenolic Contents. The FP and CWBP contents were determined with the Folin–Ciocalteu colorimetric method (Blandino et al. 2013). The results were expressed as ferulic acid equivalents (FAE) per gram of sample dry weight (DW) through a calibration curve (linearity range: 0.03–1 mg/mL; $r^2 = 0.999$).

LC-MS/MS Analysis of Phenolic Acids. The phenolic extracts were filtered through a 0.2 µm filter and then analyzed with a high-performance liquid chromatograph (Alliance 2695, Waters, Milford, MA, U.S.A.) coupled with a Waters 2996 photodiode array detector. Separations were carried out with a 150 × 4.6 mm, 5 µm, Gemini RP-18 column (Phenomenex, Torrance, CA, U.S.A.). The temperatures of the sample holder and of the column were set at 15 and 35°C, respectively. The operating linear gradient was used with a mobile phase consisting of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in methanol (solvent B) and was programmed as follows (Liu et al. 2010): 0–7 min, 15 to 20% B; 7–8 min, 20 to 15% B; 8–20 min, 15% B; 20–21 min, 15 to 24% B; 21–33 min, 24% B; 33–34 min, 24 to 13% B; 34–36 min, 13% B; 36–37 min, 13 to 20% B; 37–45 min, 20% B; 45–46 min, 20 to 42% B; 46–62 min, 42% B; and finally bringing the mobile phase to 100% B in 1 min followed by 7 min of equilibration. The flow rate of the mobile phase was 0.9 mL/min, and the injection volume was 10 µL. The quantification of phenolic acids was based on the area of the peak at a wavelength of 280 nm by using external calibration curves. Phenolic acids were identified by comparing retention time and UV-VIS spectra with standards and were confirmed by MS/MS analyses. The quadrupole time-of-flight mass analyzer was calibrated with sodium iodide standard in negative mode, and a resolution of 5,000 was achieved. Full mass spectra were recorded in the range of *m/z* 100–1,000 in negative mode by using a capillary voltage of 900 V and a sample cone voltage of 35 V. The flow rates of desolvation and cone gas were 900 and 50 L/h, respectively. The desolvation gas and the ion source temperatures were set at 250 and 120°C, respectively. The tandem mass spectra were acquired by using the collision energy of 20 V for monomeric phenolic acids and 35 V for dimeric forms.

Determination of the TAA. The TAA analyses were performed on all the biological replicates ($n = 4$) of each corn genotype collected at four stages of kernel development. TAA was determined by adapting the classical DPPH radical scavenging method (Locatelli et al. 2009) to the QUENCHER approach (Gökmen et al. 2009). Briefly, 0.5–3 mg of ground kernels (particle size < 250 µm) were weighed, and 700 µL of distilled water and 700 µL of a DPPH methanolic solution (100 µM) were added. The samples were vortex mixed, and the reaction was then carried out in the dark at 20°C while stirring at 1,000 rpm (PCMT Thermoshaker, Grant Instruments, Cambridge, U.K.) for 25 min. The samples were promptly centrifuged for 1 min at 20,800 × *g*, and the absorbance was measured after exactly 30 min of reaction at 515 nm. A control solution was tested under the same conditions to calculate the DPPH inhibition percentage of the sample. The final results were expressed as millimoles of Trolox equivalents (TE) per kilogram of sample (DW) through a calibration curve (linearity range, 2–24 µM; $r^2 = 0.994$).

DON and FB Extraction and LC-MS/MS Analysis. DON and FB analyses were performed on all the biological replicates ($n = 4$) of each corn genotype collected at harvest maturity. The DON extraction was performed by mixing samples (50 g) with 200 mL of water in a blender at high speed for 30 min. The entire extract was then filtered and cleaned up with antibody-based immunoaffinity columns (DONtest WB columns, VICAM, Milford, MA, U.S.A.). The FB extraction was performed by mixing samples (50 g) with 100 mL of 80:20 (v/v) methanol–water solvent in a blender at high speed for 20 min. The entire extract was then filtered, and 10 mL of filtered extract was diluted to 50 mL with phosphate-buffered saline, pH 7.8 (Sigma-Aldrich, Saint Louis, MO, U.S.A.). Antibody-based immunoaffinity columns (FUMtest WB columns, VICAM) were used to clean up the diluted sample extracts.

DON and FB (FB_1 and FB_2) contents were quantified with a LC-MS/MS system consisting of a Varian 212-LC chromatography pump and 310-MS TQ mass spectrometer. The analyses of DON were carried out with a 100 × 2.0 mm, 3 µm, Polaris C18-A column (Agilent Technologies, Santa Clara, CA, U.S.A.). The operating linear gradient consisting of a mobile phase of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in methanol (solvent B) was programmed as follows: 0–3 min, 10 to 90% B; 3–5 min, 90% B; 5–10 min, 90 to 10% B; and 10–13 min, 10% B. The analyses of FBs were carried out with a 50 × 2.1 mm, 5 µm, Pursuit C18 column (Agilent Technologies). The operating linear gradient consisting of a mobile phase of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in acetonitrile (solvent B) was programmed as follows: 0–2 min, 20% B; 2–8 min, 20 to 80% B; 8–13 min, 80 to 20% B; and 13–17 min, 20% B. In both cases the flow rate of the mobile phase was 0.2 mL/min and the injection volume was 10 µL. DON mass spectrometric analyses were performed in the negative ion mode. The nebulizing gas was N_2 (20 psi); the drying gas was at 300°C (25 psi); and needle, shield, capillary, and collision energy voltages were set to –3,000, –600, –60, and 16 V, respectively. The deprotonated molecules ($[M-H]^-$ *m/z* 295) were fragmented to their product ions (*m/z* 264) and used for quantification purposes. The quantification of DON was performed by using external calibration curves.

The FB analyses were performed in the positive ion mode, and needle, shield, capillary, and collision energy voltages were set to 5,000, 600, 67, and –38 V, respectively. The protonated molecules ($[M+H]^+$ *m/z* 722 and 706) were fragmented to their product ions (*m/z* 352 and 336) and used for quantification purposes. The quantification of FB_1 and FB_2 was performed by using external calibration curves.

Because ear size was higher for the hybrids than the varieties, the average ear weight was calculated and then multiplied for mycotoxin concentration to obtain mean mycotoxin contamination per ear.

Statistical Analyses. One-way analysis of variance (ANOVA) was applied to compare phenolic acid content and TAA among different GSs within each corn genotype and among different corn genotypes within each GS. Rank transformation (Conover and Iman 1981) of the data was performed because the ANOVA assumptions were not always verified. The REGW-Q test was performed for multiple comparisons. A 0.05 threshold was used for the rejection of the null hypothesis. The same statistical analyses were performed to compare grain yield, mycotoxin content, and fungal ear rot severity and incidence among corn genotypes at harvest maturity.

To further investigate the linkage between phenolic acids and TAA, correlation coefficients were determined for each corn genotype by two-tailed Spearman's correlation. A similar approach was used to analyze the linkage between DON or FB contamination at harvest maturity and phenolic acid content during kernel development.

Statistical analyses were carried out with SPSS for Windows statistical package, version 22.0 (SPSS, Chicago, IL, U.S.A.).

RESULTS AND DISCUSSION

Meteorological Data and Mycotoxin Contamination. The growing season was characterized by frequent rainfall from flowering to harvesting time. In particular, the highest cumulative rainfall level (201 mm), which occurred in July, was three times as

high the average values reported in the experimental area in the period 2000–2013. Moreover, the average temperature observed in July was about 1°C lower than usual. These conditions observed during the growing season, and in particular close to corn flowering, were favorable to infection by *Fusarium* species, causing corn red ear rot associated with DON production.

The yield, incidence, and severity of fungal ear rot and mycotoxin contamination of corn genotypes analyzed at harvest maturity are reported in Table III and Figure 1. As expected, the yields of corn hybrids were higher than the yields of varieties. The lowest fungal ear rot incidence and severity were observed in the I1 corn genotype, whereas the V4 genotype had the highest. On average, DON contamination was higher compared with FBs. Significant differences were observed for DON and FB contamination among the different corn genotypes. The V3 and V4 corn genotypes showed the highest DON and FB contamination levels, respectively.

TABLE III
Yield and Fungal Ear Rot Incidence and Severity for Each Corn Genotype Analyzed[z]

		Fungal Ear Rot	
Genotype	**Yield (t/ha)**	**Incidence (%)**	**Severity (%)**
V1	7.6b	30.0bc	4.8ab
V2	9.0ab	47.5ab	7.0ab
V3	6.6b	40.0abc	18.6a
V4	9.2ab	52.5a	18.5a
I1	15.8a	25.0c	3.1b
I2	14.6ab	50.0a	8.7ab

[z] Mean values within a column with different letters are significantly different ($P < 0.05$).

Free Phenolics in Developing Kernels. The levels of free phenolics in developing kernels were monitored from the end of the silking stage to harvest maturity (Table IV). FPs ranged from 988.5 to 5,826.1 µg of FAE/g DW at the end of the silking stage, from 804.0 to 6,499.4 µg/g at the blister stage, from 323.8 to 1,454.2 µg/g at the dough stage, and from 251.3 to 415.8 µg/g at harvest maturity.

On average, the V1 and V2 corn genotypes showed the highest FP content at all stages of kernel development. Little information is available about phenolic evolution in other cereals. Previous studies performed on colored waxy corn (Hu and Xu 2011) showed that black corn was characterized by a higher free phenolic content than white and yellow corn. Similar results were obtained for colored rice (Shao et al. 2014), for which the red rice was characterized by a higher free phenolic content than the black and white rice during kernel development.

The highest FP levels were observed in the first and second sampling dates, corresponding to the end of the silking and the blister stages. FPs increased significantly from the end of the silking stage to the blister stage only in the V2 corn genotype. No

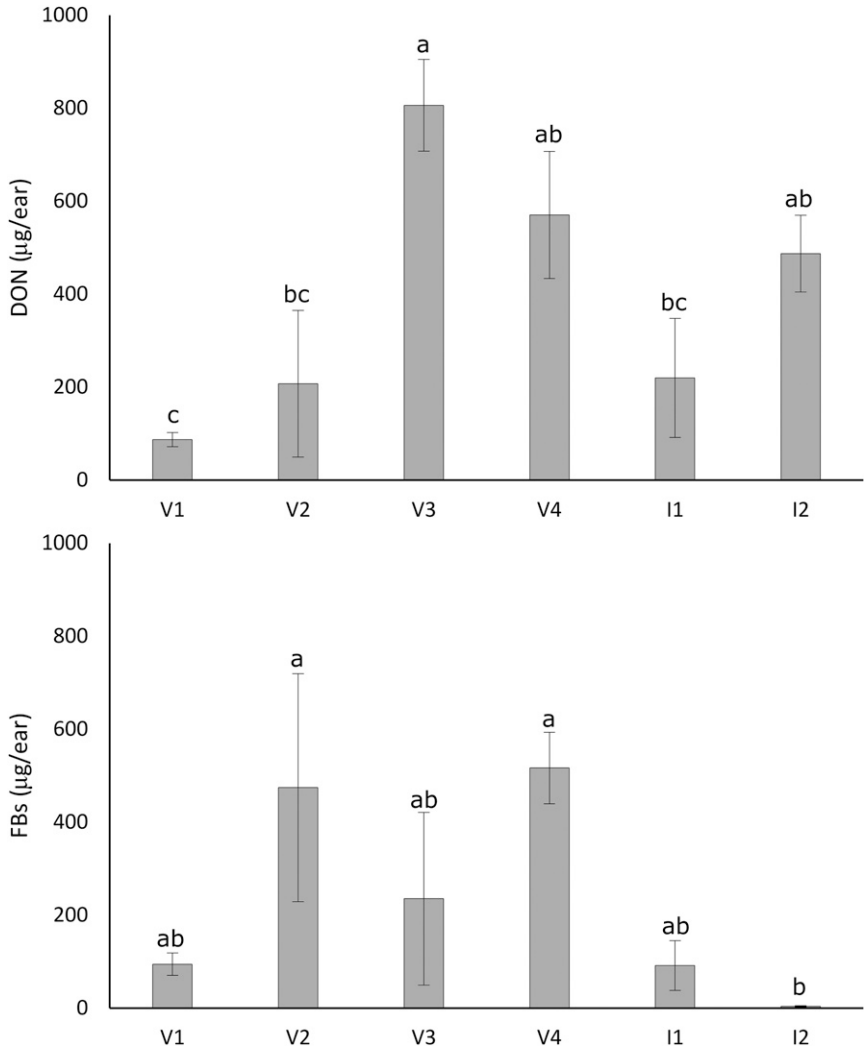

Fig. 1. Deoxynivalenol (DON) and fumonisin (FB) concentrations detected at harvest maturity in the six corn genotypes analyzed. The error bars represent the standard deviations of the four replicates. Values with different letters differ significantly ($P < 0.05$).

significant changes were found when these two stages were compared in the V1 and V4 corn genotypes, whereas a significant decrease was observed in the V3, I1, and I2 corn genotypes. On average, a significant decrease in FPs was observed at dough stage and at harvest maturity. The lowest concentration was observed for all corn genotypes at harvest maturity, with the exception of V3, for which an increase was observed from the dough stage to harvest maturity.

At harvest maturity the FP content detected in all corn genotypes was higher than the sum of free phenolic acid reported for common and durum wheat, spelt, einkorn, and emmer (Li et al. 2008) but was similar to the values of free phenolics observed in colored rice (Shao et al. 2014). Moreover, FP content observed in this study during kernel development was higher than values reported in other studies performed on yellow corn (Xu et al. 2010). The detected differences in the concentration were probably owing not only to the different genotypes analyzed but also to the different methods employed for free phenolic extraction. In fact, this study used an ultrasound-assisted extraction method that, as previously demonstrated, could increase the yield of extraction of phenolics (Chooklin 2013; Ghasemzadeh et al. 2015).

Regardless of the corn genotype considered, chlorogenic ($[M-H]^-$ *m/z* 353), ferulic ($[M-H]^-$ *m/z* 193), and vanillic ($[M-H]^-$ *m/z* 167) acids were the main phenolic acids detected (Table V). A smooth decrease in chlorogenic and ferulic acid concentrations occurred from the silking stage to maturity, with the exception of the V2 corn genotype, for which chlorogenic acid showed a peak at the blister stage. Chlorogenic and ferulic acids, respectively, represented on average 18.3 and 7.8% of FPs at the end of the silking stage, whereas they represented only 1.6 and 0.6% at harvest maturity. On average, vanillic acid was detected only at the beginning of kernel development (end of the silking and blister stages), representing about 2.2% of FPs. Only the V1 corn genotype showed a low concentration of vanillic acid at harvest maturity.

The highest concentration of sinapic acid (data not reported in table) was detected at the beginning of kernel development with an average of 13.8 and 14.8 μg/g DW at the end of the silking stage and at the blister stage. At the dough stage and at harvest maturity its concentration was lower than 5 μg/g DW. Sinapic acid was not detected in the V1 and V4 corn genotypes regardless of the stage of kernel development. *p*-Coumaric acid concentration (data not reported in table) was lower than 10 μg/g for all corn genotypes regardless of the stage of development, with the highest concentration observed at the beginning of kernel development with an average of 4.6 μg/g DW at the end of the silking stage and 4.1 μg/g DW at

TABLE V
Mean Concentration (μg/g DW) of Different Free Phenolic Acids During Kernel Development[z]

Genotype	DAS	Chlorogenic Acid	Ferulic Acid	Vanillic Acid
V1	5	990.6aA	275.0aAB	151.0aA
	7	827.3abA	226.9aA	135.9aA
	33	574.7bcA	nd	23.9bA
	73	5.8cAB	2.5bA	0.8c
V2	5	499.3aAB	168.9aB	63.6aAB
	7	805.3aA	154.5aA	68.2aAB
	33	21.4bA	2.4bA	1.1bB
	73	11.6bA	2.1bAB	nd
V3	5	125.1aC	42.5aD	18.3aD
	7	105.1aB	36.5aB	14.8aC
	33	6.5bD	1.3bB	nd
	73	3.4cBC	1.4bAB	nd
V4	5	121.0aC	80.4aC	24.1aBC
	7	122.3aB	33.8aB	22.5aB
	33	4.1bE	1.6bAB	nd
	73	4.6bABC	2.2bAB	nd
I1	4	447.3aB	346.8aA	23.1aC
	7	218.0bA	151.3bA	14.7bC
	31	10.3cB	0.9dC	nd
	76	2.7dC	1.4cB	nd
I2	4	181.1aC	97.3aC	15.6aD
	7	60.8bB	41.1bB	8.9bD
	31	9.2cC	1.3dB	nd
	76	3.7dBC	2.4cA	nd

[z] Mean values within a column with different lowercase letters for the same corn genotype at different stages of development are significantly different ($P < 0.05$). Mean values within a column with different uppercase letters for different corn genotypes at the same stage of development are significantly different ($P < 0.05$). DAS = days after silking; DW = dry weight; and nd = not detected.

TABLE IV
Mean Concentrations of FPs, CWBPs, and TAA During Kernel Development[z]

Genotype	DAS	FPs (μg of FAE/g DW)	CWBPs (μg of FAE/g DW)	TAA (mmol of TE/kg DW)
V1	5	5,826.1aA	11,344.9aA	224.4aA
	7	6,499.4aA	12,422.1aA	248.3aA
	33	1,454.2bA	6,759.5bA	149.4bA
	73	415.8cA	5,448.9cA	31.4cA
V2	5	2,283.1bAB	9,355.0bAB	57.3bB
	7	2,729.2aB	11,339.9aAB	107.3aB
	33	559.0cAB	5,013.4cAB	22.6cB
	73	395.7dA	4,782.7cAB	12.3dAB
V3	5	1,127.8aC	9,046.2aB	31.0aD
	7	925.1bD	10,138.4aB	27.2bE
	33	323.8cE	4,723.4bBC	12.5cE
	73	398.7cA	4,124.9bCD	9.1dCD
V4	5	1,134.5aC	6,939.3aCD	27.6aE
	7	1,191.7aC	7,703.2aC	27.5aE
	33	503.5bBC	5,043.6bAB	13.2bD
	73	251.3cB	4,261.9cBC	10.1cBC
I1	4	1,579.0aB	5,899.3bD	41.8bC
	7	1,218.6bC	7,531.0aC	45.2aC
	31	456.2cCD	4,261.1cC	15.5cC
	76	254.9dB	3,931.0cCD	9.4dCD
I2	4	988.5aC	7,961.4aBC	31.8aD
	7	804.0bE	7,764.8aC	30.3aD
	31	405.7cDE	4,203.2bC	11.1bF
	76	321.8cB	3,830.0cD	8.6cD

[z] Mean values within a column with different lowercase letters for the same corn genotype at different stages of development are significantly different ($P < 0.05$). Mean values within a column with different uppercase letters for different corn genotypes at the same stage of development are significantly different ($P < 0.05$). DAS = days after silking; FPs = free phenolics; CWBPs = cell wall-bound phenolics; TAA = total antioxidant activity; FAE = ferulic acid equivalents; TE = Trolox equivalents; and DW = dry weight.

the blister stage. The only exception was the V1 corn genotype, in which it was detected only at harvest maturity (1.2 µg/g DW).

In addition to these monomeric forms, phenylalanine ([M-H]$^-$ *m/z* 164) was identified at the beginning of kernel development (i.e., end of the silking and blister stages). Moreover, two peaks eluted toward the end of the chromatogram for samples collected at the dough stage and at harvest maturity. According to the UV-VIS spectra and MS/MS fragmentation patterns, these compounds were identified as *p*-coumaroyl-feruloylputrescine ([M-H]$^-$ *m/z* 409) and diferuloylputrescine ([M-H]$^-$ *m/z* 439). In agreement with previous studies (Atanasova-Penichon et al. 2012), for each corn genotype, these two hydroxycinnamic polyamines were the main peaks in chromatograms of mature kernels.

Cell Wall-Bound Phenolics in Developing Kernels. The levels of cell wall-bound phenolics in developing kernels were monitored from the end of the silking stage to harvest maturity (Table IV). CWBPs were significantly higher than values of FPs, in agreement with previous results on corn (Xu et al. 2010; Hu and Xu 2011; Atanasova-Penichon et al. 2012) and other cereals (Adom and Liu 2002). CWBPs ranged from 10 to 17 times higher than FPs at harvest maturity, depending on the corn genotype. CWBPs ranged from 5,899.3 to 11,344.9 µg of FAE/g DW at the end of the silking stage, from 7,531.0 to 12,422.1 µg/g at the blister stage, from 4,203.2 to 6,759.5 µg/g at the dough stage, and from 3,830.0 to 5,448.9 µg/g at harvest maturity. The V1 corn genotype showed the highest CWBPs at all stages of kernel development. As for free phenolics, similar results were obtained for colored rice (Shao et al. 2014), with black and red rice characterized by a higher cell wall-bound phenolic content than white rice during kernel development.

Levels of CWBPs observed during kernel development were similar to values reported for other yellow corn genotypes (Atanasova-Penichon et al. 2012) but higher than the values reported for other yellow and blue corn genotypes (Xu et al. 2010; Urias-Peraldí et al. 2013). These discrepancies were likely related to the different genotypes analyzed, the extraction methods used, and in particular, the employment of different hydrolysis steps using NaOH.

TABLE VI
Mean Concentration (µg/g DW) of Different Cell Wall-Bound Phenolic Acids During Kernel Development[z]

Genotype	DAS	Ferulic Acid	*p*-Coumaric Acid	Caffeic Acid
V1	5	3,895.8aABC	751.5aAB	186.5aA
	7	4,407.5aBC	740.2aAB	182.4aA
	33	2,596.2bA	245.2bA	25.7bA
	73	1,712.8cC	314.7bA	24.0bA
V2	5	3,887.4bABC	761.1aAB	101.8aABC
	7	5,742.8aA	1,037.4aA	159.6aA
	33	3,200.1bA	160.6cA	16.8cA
	73	2,203.9cA	255.0bAB	25.2bA
V3	5	5,474.8aA	1,181.6aA	40.8aD
	7	4,810.4aAB	1,161.3aA	23.4aE
	33	2,915.2bA	156.2bA	nd
	73	1,894.8bBC	179.8bBC	nd
V4	5	3,746.3aBC	305.5aB	90.2aBC
	7	3,943.2aC	285.3aB	68.7aC
	33	3,959.6aA	172.8bA	9.6bB
	73	2,085.2bAB	131.6bC	9.0bB
I1	4	3,670.8bC	553.1aAB	103.2aAB
	7	4,783.1aB	711.2aAB	89.0aB
	31	2,925.4cA	130.7bA	nd
	76	1,857.6dC	142.1bC	nd
I2	4	4,751.4aAB	925.2aA	63.2aCD
	7	4,487.7aB	1,035.3aA	35.5bD
	31	3,038.5bA	144.3bA	nd
	76	2,132.5cA	159.5bCD	nd

[z] Mean values within a column with different lowercase letters for the same corn genotype at different stages of development are significantly different ($P < 0.05$). Mean values within a column with different uppercase letters for different corn genotypes at the same stage of development are significantly different ($P < 0.05$). DAS = days after silking; DW = dry weight; and nd = not detected.

The highest CWBP concentrations were observed at the end of the silking and at the blister stage. CWBP concentration increased significantly from the end of the silking stage to the blister stage in the V2 and I1 corn genotypes, whereas no significant change was observed in the other corn genotypes analyzed. On average, a significant decrease in CWBPs was found at the dough stage and at harvest maturity, at which the lowest concentration was observed for all corn genotypes.

Representative chromatograms of cell wall-bound phenolic acids at the end of the silking stage and at harvest maturity are shown in Figure 2. Cell wall-bound phenolic acids showed mainly quantitative than qualitative changes during kernel development (Table VI). Regardless of the corn genotype considered, ferulic ([M-H]$^-$ *m/z* 193), *p*-coumaric ([M-H]$^-$ *m/z* 163), and caffeic ([M-H]$^-$ *m/z* 179) acids were the main hydroxycinnamic acid-derived monomers observed during kernel development, but their relative proportions changed depending on the stage of development. Ferulic acid was the most abundant at all stages of development, as previously reported for corn (Atanasova-Penichon et al. 2012), representing 50.3 and 45.1% of CWBPs at the end of the silking stage and at harvest maturity, respectively. Ferulic acid concentration increased on average from the end of the silking stage to the blister stage. Subsequently, it decreased gradually until harvest maturity. The highest content of *p*-coumaric acid was observed at the beginning of kernel development, with a significant decrease at the dough stage for all corn genotypes. No significant difference was observed between *p*-coumaric acid levels measured at the dough stage and at harvest maturity, with the exception of the V2 corn genotype, which showed a significant increase at harvest maturity. Caffeic acid was

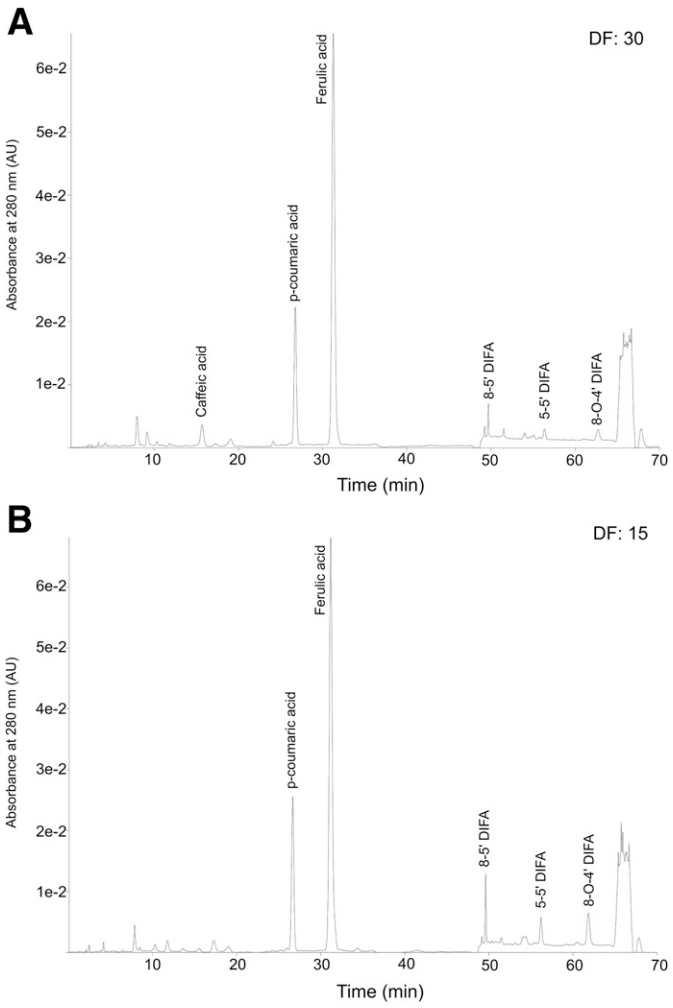

Fig. 2. Chromatogram at 280 nm of cell wall-bound phenolic acids in the V1 corn genotype at the end of the silking stage (**A**) and at harvest maturity (**B**). DF = dilution factor; and DIFA = ferulic acid dehydrodimer.

detected mainly at the beginning of kernel development. On average, its concentration significantly decreased during kernel development. At the dough stage and at harvest maturity, caffeic acid was detected only in the V1, V2, and V4 corn genotypes.

In addition to monomeric phenolic acids, three peaks eluted toward the end of the chromatogram, with a $[M-H]^-$ *m/z* signal equal to 385, which is a typical *m/z* value for ferulic acid dehydrodimer (DIFA). According to the MS/MS fragment ion pattern previously reported (Guo and Beta 2013), the three DIFAs were identified as 8-5′ DIFA, 5-5′ DIFA, and 8-*O*-4′ DIFA. At harvest maturity 8-*O*-4′ DIFA was the predominant DIFA form, followed by 8-5′ DIFA and 5-5′ DIFA.

Overall, a reduction in free and cell wall-bound phenolic acid contents during kernel development was observed in this study, as previously reported for both corn and other cereals (McKeehen et al. 1999; Xu et al. 2010; Atanasova-Penichon et al. 2012; Shao et al. 2014). Different hypotheses were proposed to explain phenolic acid accumulation kinetics (Atanasova-Penichon et al. 2012). Phenolic acids are mainly located in the outer layer of kernels (Ndolo and Beta 2014); during kernel development, the rate of endosperm development becomes higher than the rate of synthesis of the outer layers, causing a dilution of the phenolic compounds within the kernel. Moreover, phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase activities were shown to be maximal at the beginning of kernel development in common and durum wheat (McCallum and Walker 1990; Régnier and Macheix 1996). A decrease in oxidizable phenolic acids from the end of the milk stage was observed as a consequence of the contact with peroxidase enzymes induced by the breakdown of cellular structures in the pericarp (Régnier and Macheix 1996). Finally, during kernel development the formation of alkali-resistant bonds in cross-linked polymers in the cell wall could cause a decrease in the rate of extraction of phenolic acids (Iiyama et al. 1994).

TAA in Developing Kernels. The TAA of developing kernels from the end of the silking stage through harvest maturity is reported in Table IV. Significant differences were detected in TAA levels among corn genotypes at different stages of development. In general, the highest TAA was observed at the beginning of kernel development. The TAA at the dough stage was 2–5 times lower than at the blister stage. The lowest TAA was observed at harvest maturity for all corn genotypes. The highest TAA was observed in the V1 and V2 corn genotypes regardless of the stage of development.

Many studies reported that phenolic compounds could contribute to the antioxidant activity in some cereals or plants (Li et al. 2007; Hu and Xu 2011). During kernel development, a significant positive correlation between TAA and free and cell wall-bound phenolic contents was observed for all corn genotypes (Table VII). Moreover, a significant correlation was observed also between the main free phenolic acids (chlorogenic and ferulic) and cell wall-bound phenolic acids (ferulic and *p*-coumaric) detected during kernel development (Table VII). Some studies on cereals reported a moderate or high correlation between phenolic acids and antioxidant activity (Li et al. 2007; Xu et al. 2010; Hu and Xu 2011). On the other hand, other studies showed no correlation between total phenolics and DPPH scavenging capacities (Yu et al. 2002). These differences may be attributed to the samples and/or sample extracts analyzed, as well as to the method used for TAA determination. The direct QUENCHER procedure is a new approach for the measurement of TAA of solid samples. It does not require extraction and hydrolysis prior to the measurement of antioxidant activity. On the contrary, the TAA measured after extraction and hydrolysis procedures may be underestimated, because the procedure used to extract antioxidants is incomplete (Gökmen et al. 2009). The correlation observed between phenolic acids during kernel development and TAA determined by the QUENCHER method implied that phenolic acids are an important class of compounds responsible for antioxidant capacity in corn. Comparing TAA values observed at harvest maturity, the V1 and V2 corn genotypes showed higher TAA (31.4 and 12.3 mmol of TE/kg DW, respectively) than the other corn genotypes (9.3 mmol of TE/kg DW on average). Probably, in this case, higher TAA values observed in V1 and V2 corn genotypes could also be related to the pigments present in the pericarp tissue, as previously shown in other studies (Lopez-Martinez et al. 2009; Žilić et al. 2012).

Correlation Between Phenolic Acids and Mycotoxin Contamination. To the best of the authors' knowledge, this is the first study that analyzed the evolution of phenolic acids at different stages of kernel development and their correlation with DON and FB contamination at harvest maturity in corn genotypes characterized by a wide array of kernel traits. Although the phenolic acid composition was qualitatively similar in the corn genotypes analyzed within each stage of development, significant quantitative differences were observed. Negative correlations were detected between DON contamination at harvest maturity and FPs, the main free phenolic acids (chlorogenic, ferulic, and vanillic), cell wall-bound caffeic acid, and TAA at the beginning of kernel development (Table VIII);

TABLE VII
Spearman's Correlations (ρ) Between TAA and the Main Phenolic Acids for Each Corn Genotype During Kernel Development[z]

Phenolics	V1	V2	V3	V4	I1	I2
FPs	0.918**	0.941**	0.791**	0.803**	0.774**	0.885**
CWBPs	0.926**	0.897**	0.776**	0.906**	0.903**	0.841**
FFA	0.591*	0.811**	0.782**	0.637*	0.564*	0.732**
FCA	0.754**	0.934**	0.893**	0.657*	0.756**	0.941**
CWB*p*-CA	0.692**	0.697**	0.670**	0.764**	0.774**	0.682**
CWBFA	0.952**	0.879**	0.868**	0.575*	0.926**	0.882**

[z] FPs = free phenolics; CWBPs = cell wall-bound phenolics; FFA = free ferulic acid; FCA = free chlorogenic acid; CWB*p*-CA = cell wall-bound *p*-coumaric acid; and CWBFA = cell wall-bound ferulic acid. Correlation analyses were performed on 16 replicates (4 sampling dates × 4 replications) for each corn genotype. * and ** indicate significant at $P < 0.05$ and 0.01 (two-tailed test), respectively.

TABLE VIII
Spearman's Correlations (ρ) Between Deoxynivalenol (DON) and Fumonisin (FB) Contamination at Harvest Maturity and Phenolic Acids During Kernel Development[z]

Stage of Development	FPs	FCA	FFA	FVA	CWBPs	CWBFA	CWB*p*-CA	CWBCA	TAA
DON									
End of the silking stage	–0.853**	–0.895**	–0.855**	–0.891**	–0.252	0.371	–0.200	–0.832**	–0.797**
Blister stage	–0.706*	–0.818**	–0.800**	–0.664*	–0.399	–0.285	0.055	–0.937**	–0.874**
Dough stage	–0.860**	–0.758*	0.329	nc	–0.336	0.385	–0.161	nc	–0.762**
Harvest maturity	–0.126	–0.434	–0.636*	nc	–0.503	0.382	–0.427	nc	–0.580*
FB									
End of the silking stage	0.140	0.028	–0.042	0.300	–0.007	–0.042	–0.182	0.203	–0.231
Blister stage	0.469	0.455	0.091	0.400	0.035	0.055	–0.464	0.217	–0.140
Dough stage	0.112	–0.588	0.553	nc	0.350	0.615*	0.364	nc	0.224
Harvest maturity	0.140	0.322	–0.318	nc	0.406	0.182	–0.118	nc	0.476

[z] FPs = free phenolics; FCA = free chlorogenic acid; FFA = free ferulic acid; FVA = free vanillic acid; CWBPs = cell wall-bound phenolics; CWBFA = cell wall-bound ferulic acid; CWB*p*-CA = cell wall-bound *p*-coumaric acid; CWBCA = cell wall-bound caffeic acid; TAA = total antioxidant activity; and nc = not calculated. Correlation analyses were performed on 24 replicates (6 corn genotypes × 4 replications) for each sampling date. * and ** indicate significant at $P < 0.05$ and 0.01 (two-tailed test), respectively.

conversely, no significant correlations were observed with CWBPs and the main cell wall-bound phenolic acids (ferulic and *p*-coumaric). Phenolic acids and TAA were not significantly correlated with FB contamination at harvest maturity.

The severity of fungal ear rot showed a significant and positive correlation with DON contamination at harvest maturity (Spearman's ρ: 0.713, $P < 0.01$), whereas a strong negative correlation was observed with free ferulic acid at the end of the silking stage and at the blister stage (Spearman's ρ: –0.952, $P < 0.01$; –0.755, $P < 0.01$, respectively) and with chlorogenic acid at the end of the silking stage (Spearman's ρ: –0.720, $P < 0.01$). A significant negative correlation was observed also with TAA at the end of the silking stage and at the blister stage (Spearman's ρ: –0.685, $P < 0.05$; –0.699, $P < 0.05$, respectively).

Phenolic acids display antifungal properties in several plant species and toward different fungal pathogens (Siranidou et al. 2002; de Ascensao and Dubery 2003; de Armas et al. 2007; Mandal et al. 2009; Santiago et al. 2009; Villarino et al. 2011). As far as the activity toward *Fusarium* ear rot in corn is concerned, previous studies reported that the antifungal properties of phenolic acids could vary depending on the plant tissue considered. Santiago et al. (2007) did not reveal a clear correlation between the content of phenolic acid monomers in pith tissue and the resistance to stalk infection by *F. graminearum*, but they suggested a possible role played by ferulic acid dehydrodimers. Similarly, Cao et al. (2011) observed that high concentrations in hydroxycinnamic acids of the silks were not related to a delayed progression of *F. graminearum*. Conversely, a study employed on kernels of two yellow corn hybrids collected at the beginning of their development showed that the highest free and cell wall-bound phenolic acid contents occurred in the corn hybrid characterized by a higher resistance to *F. graminearum* and DON contamination (Atanasova-Penichon et al. 2012). In addition to antifungal properties, several studies reported the in vitro ability of phenolic acids to inhibit the biosynthesis of various mycotoxins, including type B trichothecenes (Boutigny et al. 2009), fumonisins (Beekrum et al. 2003), aflatoxins (Nesci et al. 2007), and ochratoxin (Palumbo et al. 2007).

Correlation analyses suggested that at the beginning of kernel development phenolic acids may play a role in the inhibition of DON metabolism and, probably, of *Fusarium* spp. growth. In particular, chlorogenic acid detected at the end of the silking stage and at the blister stage was shown to be negatively correlated with DON contamination of the kernel at harvest maturity. Chlorogenic acid was shown to have toxicity toward *F. graminearum* and *F. culmorum* (Gauthier et al. 2016). Moreover, it was shown to be able to disrupt the structure of fungal cell membranes (Sung and Lee 2010) and to inhibit the production of melanin-like pigments (Villarino et al. 2011). The high concentration detected especially at the beginning of kernel development suggested that it may be a constitutive metabolite related to red ear rot and DON accumulation resistance.

Even if the concentration of CWBPs was higher than FPs at all stages of kernel development, no correlation was observed between CWBPs and DON contamination at harvest maturity. Similarly, Bakan et al. (2003) did not observe a relationship between cell wall-bound phenolic acids and the inhibition of trichothecene production by *F. graminearum* grown in different corn kernel fractions. The only cell wall-bound phenolic acid showing a significant negative correlation with DON contamination was caffeic acid, detected mainly at the end of the silking stage and at the blister stage, suggesting a possible role of this phenolic acid in cell wall fortification, especially at the beginning of kernel development (Siranidou et al. 2002; de Ascensao and Dubery 2003; Santiago et al. 2009).

Finally, DON contamination at harvest maturity was observed to be negatively correlated with TAA, which in turn was directly correlated with phenolic acids. Previous studies showed that the expression of toxin-producing potential by *F. graminearum* was closely linked to the biochemical environment surrounding its spores during germination and that the level of DON production was H_2O_2 mediated as early as the germination initiation step (Ponts et al. 2006). Consequently, the high TAA encountered by the fungal pathogen at the beginning of kernel development could be related to a negative modulation of DON biosynthesis.

Despite the negative correlation observed between phenolic acids and DON contamination at harvest maturity, pathogens responsible for FB production seem to be less affected by the content of phenolic acids at the beginning of kernel development than DON producers. Given the complexity of resistance mechanisms associated to a polygenic inheritance, components other than phenolic acids may contribute to the resistance mechanism toward pink ear rot and consequently to FB accumulation. Kernel age could influence mycotoxin production in corn. FBs were mainly produced at dent stage in kernels characterized by a moisture level of 40%, whereas lower contamination was observed in kernels collected at the blister and milk stages (Warfield and Gilchrist 1999). Conversely, the onset of fungal infection and DON contamination was observed already at the blister and milk stages (Atanasova-Penichon et al. 2012). Moreover, *Fusarium* species have shown different pathways in the infection of corn kernels. The primary pathway for infection of corn kernels by *F. graminearum* and *F. culmorum*, responsible for DON production, is via the silks, which are highly susceptible during the first six days after their emergence (Reid et al. 1996). In contrast, several infection pathways, including silk infection, insect injury, and systemic transmission from seeds or roots to kernels, have been identified for *F. verticillioides*, responsible for FB production (Munkvold et al. 1997; Sobek and Munkvold 1999). The relative importance of different pathways may vary among geographic areas. In the geographic area where the experimental activities were carried out, the ECB was identified as the main promoting agent of *F. verticillioides* infection. Although a pyrethroid insecticide was applied to minimize the fungal infection related to insect damage, ear injuries caused by the feeding activity of the ECB were, however, detected at harvest maturity (average ECB incidence and severity of 41.7 and 3.5%, respectively), eluding an antifungal effect that phenolic acids may carry out at the beginning of kernel development.

CONCLUSIONS

In conclusion, the results suggest that numerous changes in phenolic acid composition and TAA occur during kernel development. Comparing varieties and hybrids characterized by a wide array of kernel traits, the greatest difference in free and cell wall-bound phenolic acid contents and in TAA occurred especially at the early stages of kernel development. Correlation analyses showed that free phenolic acids could have a role in fungal red ear rot resistance and DON contamination, whereas no relationship was observed with pink ear rot resistance and FB contamination. Although in vitro experiments demonstrated that phenolic acids could inhibit FB biosynthesis, their role in the resistance to FB accumulation under field conditions was not elucidated, implying that components other than phenolic acids may be more important for the pink ear rot resistance mechanism.

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