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Phenylpropanoids from maize pericarp: resistance factors to kernel infection and fumonisin accumulation by *Fusarium verticillioides*

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Abstract The aim of this work was to investigate the role of pericarp phenylpropanoids as resistance factors to *F. verticillioides* in eleven maize genotypes. Disease severity and kernel fumonisin accumulation were measured after inoculation with *F. verticillioides* and related to contents of pericarp phenylpropanoids in field trials conducted during 2 years. Grain fumonisin concentrations were highly dependant on disease severity of the genotypes (r=0.88). A detailed analysis of pericarp phenylpropanoids indicated the presence of *trans*-ferulic acid (tFA), *cis*-ferulic acid (cFA), p-coumaric acid (pCA), and five diferulates (DFAs). The most prominent diferulates were 8,5′-diferulic acid benzofuram form (8,5′-DFAbz), followed by 8,5′-DFA and 8,8′-DFA. Except for cFA, the most resistant genotypes exhibited high levels of phenylpropanoids which were related to low levels of disease severity and grain fumonisin concentration ($-0.61 > r > -0.90$). A stepwise regression analysis revealed that total diferulates was the best explanatory parameter for variability of disease severity (r^2=0.71). Grain fumonisin concentration was well depicted by contents of total diferulates, 8,5′DFAbz and pCA (r^2=0.82). Our findings suggest that high level of phenylpropanoids in the kernel pericarp is a trait associated to less disease severity and fumonisin accumulation caused by *F. verticillioides*. Further research is in progress to map quantitative trait loci for these cell wall components in bi-parental populations derived by crossing resistant and susceptible genotypes included in this study.

Keywords Fusarium ear rot · Mycotoxins · Plant breeding · Diferulates

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Introduction

Fusarium verticillioides (Saccardo) Nirenberg [synonym, *Fusarium moniliforme* (Sheldon)], teleomorph *Gibberella moniliformis* (Wineland) is the most important ear and kernel rotting pathogen of maize (*Zea mays* L.) in tropical and temperate regions of Argentina (Chulze et al. 1996). It not only reduces yield but

also contaminates the infected kernels with mycotoxins, mainly fumonisins (FB1, FB2, and FB3). Fumonisins are of greatest concern because of their noxious effects on humans and animals. Dietary exposure to fumonisin has been associated with leucoencephalomalacia in equines (Marasas et al. 2004), pulmonary edema in swine (Harrison et al. 1990), and liver cancer in rats (Norred et al. 1992). Epidemiological studies have shown connection between intake of fumonisins and oesophageal cancer in human populations with high maize consumption in South Africa, Brazil, China, and Italy (Marasas et al. 2004). Due to this problem, several official agencies such as the FAO/WHO Expert Committee on Food Additives, U.S. Food and Drug Administration and the European Union, have established for human consumption the fumonisin threshold of 4 ppm in non-processed maize (Maiorano et al. 2009). In Argentina, this concentration can be surpassed whenever outbreaks occur reducing the margin of maize available for export. Although these mycotoxins are unavoidable natural contaminants in maize based foods and feeds, no definitive control strategies are currently available to prevent their accumulation in kernels.

The development of resistant hybrids is a feasible strategy to prevent infection by *F. verticillioides* and grain fumonisin accumulation. In Argentina, a search for disease resistance after silk inoculation with this fungus led to the identification of a set of eleven genotypes varying not only in disease resistance but also in grain fumonisin accumulation. Previous studies indicated that the pericarp seems to have a role on fumonisin accumulation of these genotypes (Sampietro et al. 2009). A working theory has proposed that suceptibility of maize to pests and diseases depends on pericarp contents of phenylpropanoids at kernel level (Santiago and Malvar 2010). The aim of this work was to determine the role of pericarp phenylpropanoids as factors of disease resistance to *F. verticillioides* in maize. Our objectives were: 1) to determine the contents of simple phenolic acids and diferulic acids (DFAs) in the pericarp of a set of eleven maize genotypes, which had previously showed pericarp related differences in susceptibility to *F. verticilliodes*; and 2) to assess the effect of pericarp phenylpropanoid contents on disease severity and fumonisin accumulation in field conditions.

Materials and methods

Field assay

Field assays were conducted in 2009/2010 and 2010/2011 in Pergamino, Province of Buenos Aires, Argentina, at 33°54′south latitude and 60°35′ west longitude. Eleven maize genotypes from temperate and subtropical regions of Argentina were included (Table 1).

Resistance of the genotypes was tested after inoculation with *F. verticillioides* strain P364, a high fumonisin producer. P364 was isolated from maize ears collected in Pergamino (province of Buenos Aires, Argentina). A non-inoculated treatment for each genotype was also included for grain sampling purposes. Treatments were randomized in a complete block design with two blocks. Each experimental unit consisted of two 5-m rows separated 0.7 m from each other and sown at a density of 5 seeds/m. P364 was grown in a liquid medium following Reid et al. (1996). After 2 weeks, the cultures were filtered through cheesecloth to remove mycelium and conidial concentrations were adjusted to 1×10^6 conidia ml^{-1} with sterile water. Suspensions were stored at 4 °C for a maximum of 3 days prior to inoculation. In inoculated treatments, all plants were inoculated by injecting 2 ml of conidial suspensions into the silk channel 4 days after silking (Reid et al. 1996). When reaching about 18–20 % grain moisture, all ears were harvested and percentage of area visibly affected by mould (disease severity) was visually assessed in all plants and plot means were used for statistical analysis. Percentages of disease severity were arcsin 1/2 transformed to normalize errors.

After disease severity assessment, ears were allowed to dry at room temperature and shelled. Grain was thoroughly mixed and a sample of 1 kg was taken from each experimental unit for mycotoxin analyses.

Extraction and analysis of cell-wall phenylpropanoids

Ears with no visibly diseased kernels harvested from non-inoculated treatments were dried at room temperature until they reached a moisture content of 12 % and shelled. Equal weight subsamples of kernels from each ear were mixed and the composite samples were stored at 4 °C before processing. All samples were free of fungicides, insecticides, and dyes.

Table 1 Pedigree, origin, colour and kernel type of eleven Argentinian maize genotypes

Genotype	Pedigree	Resistance to *F. verticillioides*	Colour and kernel type	Origin	
				Germplasm	Region
Leales 25	Open pollinated variety	Moderately resistant	Yellow semident	Broad-based composite	Subtropical
Leales Pob D	Breeding population	Moderately resistant	Yellow semident	Broad-based composite	Subtropical
L4637	Inbred	Moderately resistant	Yellow Flint	(LP561 × LP611)	Temperate
L1186 × L1196	Hybrid	Susceptible	Yellow semident	(LP915 × L3125)	Temperate
L4671 × L4674	Hybrid	Susceptible	Yellow Flint	(LP561 × LP611) × R4973 Synthetic	Temperate
L4674	Inbred	Susceptible	Yellow semident		Temperate
L6856 × L4674	Hybrid	Susceptible	Yellow semident	AX888	Temperate
L943 × L944	Hybrid	Moderately resistant	Yellow semident	LP2541 × LP915	Temperate
ARZM03018	Landrace	Moderately esistant	White flint (perlita race)	Unknown	Temperate
ARZM04014	Landrace	Resistant	Red pericarp flint (perlita race)	Unknown	Subtropical
ARZM05040	Landrace	Resistant	White dent	Unknown	Subtropical

Kernel samples from the 11 maize genotypes were freeze-dried and then soaked for 4 h at 4 °C. Then, the hydrated kernels were placed for 10 s in an electric willey mill (Thomas Co., Philadelphia, PA). This treatment allowed us to obtain pericarp pieces, which were dissected from germ and endosperm residues using a scalpel. Then, the pericarp fraction was milled to a powder and freeze-dried. Pericarp samples (5 g) were digested with 60 ml of 2N NaOH for 3 h in darkness, with mixing at half-hour intervals. Then, samples were acidified to pH 2.0 with 16 ml of concentrated 12N HCl, vigorously mixed, and extracted twice with 100 ml of ethyl acetate. Collected organic fractions were combined and solvent was evaporated under reduced pressure until dryness. The final extract was dissolved in 3 ml of high-performance liquid chromatography (HPLC) grade methanol and stored at 18 °C until HPLC analysis.

Each methanolic sample was filtered through a 0.2-μm pore polytetrafluoroethylene filter (Microclar, Argentina) before analysis. All the analyses were performed on a Gilson HPLC system coupled to a Diode Array Detector, using a Smartgrace C18 (25 cm× 4.7 mm, 5 μm particle size) at a flow of 1 ml/min. The mobile phase was a binary gradient with solvent A (2 % formic acid in water) and solvent B (2 % formic acid in acetonitrile) in the ratio of 0 % B for 0.38 min, increasing to 20 % B in 14.62 min, increasing to 75 % B in 15 min, and mantaining at 75 % B for 5 min. Diferulates (DFAs) and phenylpropanoid phenolic acids were identified by comparison with standards and UV–vis spectra. Additional confirmation of identities was obtained by injecting standards and selected samples in a liquid chromatography-mass spectrometry system (Agilent 1100 LC system, Agilent Technologies Inc, USA) equipped with a binary pump and coupled with a MSD VL quadrupole (Agilent Technologies, USA) with an electrospray ionization (ESI) interface (Sgariglia et al. 2010).

Fumonisin analysis

Fumonisin concentration was assessed in maize samples from inoculated field plots by using the Ridascreen® Fast Fumonisin assay (R-Biopharm AG, Darmstadt, Germany). The procedure was described by Presello et al. (2008). This competitive enzyme immunoassay is based on monoclonal antibodies raised against fumonisin B1. The antibodies cross-react with fumonisin B2 (40 %) and B3 (100 %) resulting in an average overestimate of 20 % in FB1 readings (Murphy et al. 1993). The detection limit of the assay is 0.22 μg/g of maize kernel. Fumonisins were extracted by blending each 5-g subsample of

maize kernels in 25 ml of 70 % methanol. The mix was shaken for 2 min in a vortex, filtered through filter paper Whatman No. 1 and diluted 1:14 with sterile distilled water. Diluted kernel extracts and five standards of the test kit (0, 0.222, 0.666, 2 and 6 ppm of fumonisins), were subjected to ELISA. Absorbance was measured at 450 nm with a microplate reader BioRad 680 (BioRad, USA). Concentration of total fumonisins in the samples was estimated on the basis of a logit–log function between fumonisin concentration and relative absorbance of the four positive standards using RIDA® SOFT Win software (R-Biopharm AG, Darmstadt, Germany).

Statistical analysis

Disease severity and grain fumonisin accumulation from inoculated plots and, pericarp contents of phenylpropanoid monomers and dimers from non-inoculated plots of the same experiments were subjected to analysis of variance. Differences among means were compared by the Fisher's LSD test at a probability level of $P=0.05$. Pearson correlations coefficients among traits were computed and tested at the same probability level. A principal component analysis (PCA) was conducted using data of disease severity, fumonisin accumulation, and contents of phenylpropanoids from each year as input traits. Stepwise regression was used to develop linear models estimating severity and grain fumonisin concentration on the basis of pericarp concentration of phenylpropanoids. A probability level of 0.15 was used for entering and rejecting a new trait to the model. Infostat 2007 software (Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina) was used for statistical analyses.

Results

Disease severity and field fumonisin accumulation

Differences of disease severity among genotypes were observed after inoculation with *F. verticillioides* in both years. Although genotype by environment interactions (G × E) were observed for fumonisin accumulation and disease severity, estimates of variance components showed that environment (E) contributed less than 10 % of the observed phenotypic variation (data not shown). Moderately resistant genotypes (Table 1) exhibited lower disease severity and less grain fumonisin accumulation compared with the suceptible ones (Table 2). Grain fumonisin concentration was correlated to disease severity ($r=0.88$).

Pericarp concentration of phenylpropanoids

The extracts of the pericarp layer obtained from kernels of the 11 maize genotypes revealed a similar pattern of peaks. Three major simple phenolic acids and five DFA isomers were identified. A representative chromatogram of the maize pericarp extract is shown in Fig. 1. The tFA was the most abundant cell wall-bound phenylpropanoid detected in all genotypes and environments (Table 3). It represented on average 78 % of total phenolic compounds, while pCA and total DFAs participated with 4 % and 17 %, respectively. The cFA was found in low levels (0.3–1 %). The 8,5′DFAbz contributed to 56 % of total DFAs, followed by 8,5′DFA (14 %), 8,8′DFA (14 %), 8O4DFA (12 %) and 5,5′DFA (3 %). The UV spectra of the peaks corresponded to those of the standard

Table 2 Ear rot severity and fumonisin concentration in kernels of eleven maize genotypes subjected to artificial inoculation with conidial suspensions of *Fusarium verticillioides.* Means are presented as combined data from experiments conducted in 2010 and 2011

Genotype	Grain fumonisin concentration ppm)[1]	Visibly diseased ear area (%)[2]
Leales 25	64.65a	4.0a
Leales D	25.65a	5.0a
L4637	190.37a	2.4a
L1186 × L1196	443.35c	32.2b
L4671 × L4674	677.25d	63.0d
L4674	618.19d	47.7b
L6856 × L4674	623.40d	42.6b
L943 × L944	232.95b	8.4a
ARZM03018	196.65a	28.6b
ARZM04014	89.95a	5.0a
ARZM05040	23.40a	6.2a

[1] Means followed by the same letter are not different at a probability level of $P<0.05$ (t test) based on data transformed into ln (grain fumonisin concentration)

[2] Means followed by the same letter are not different at a probability level of $P=0.05$ (t test) based on data transformed into arc sin (disease severity)$^{1/2}$

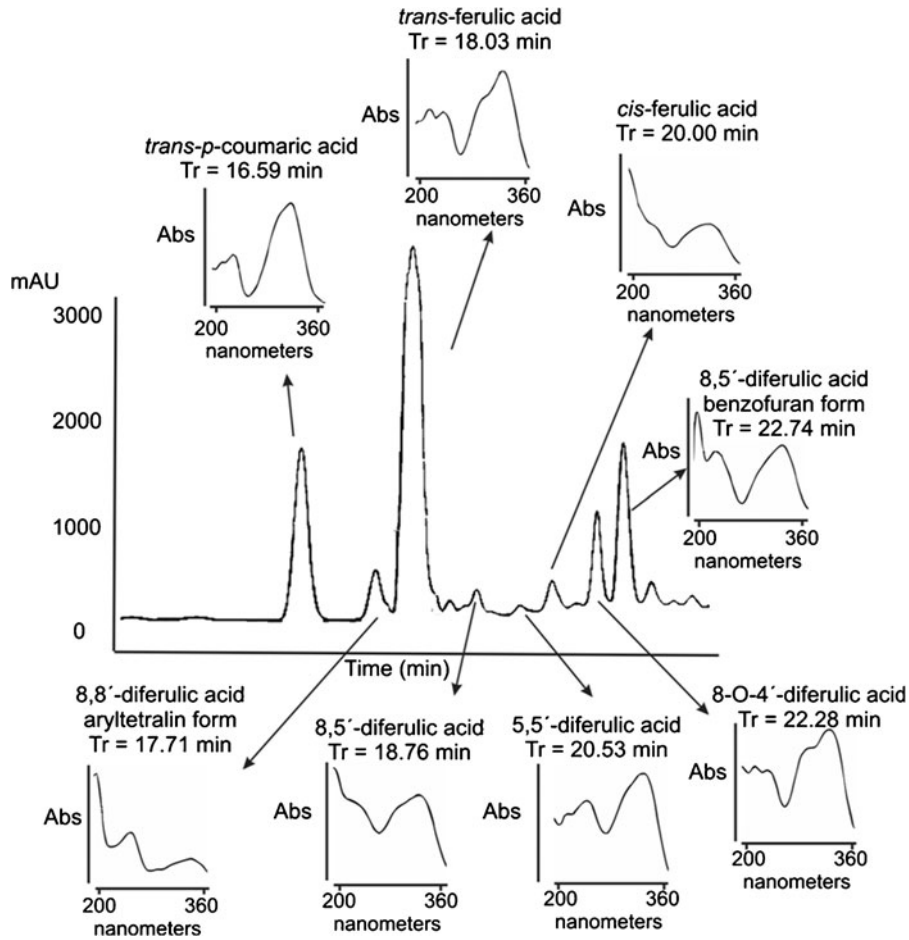

Fig. 1 HPLC chromatogram of a typical pericarp sample and UV–vis spectra of main phenylpropanoid phenolic acids and diferulates detected. Tr = retention times

compounds and identity of the phenylpropanoids was confirmed by HPLC-MS (high performance liquid chromatography–mass spectrometry) of the pericarp extracts.

The analysis of variance revealed that contents of phenylpropanoids were not affected by (E) and G × E (data not shown). Except for cFA, all phenylpropanoids showed significant variation among genotypes (Table 3). In the PCA (Fig. 2), the first two principal components explained 86 % of the joint variation of disease severity, fumonisin accumulation and contents of phenylpropanoids. Vectors for each phenylpropanoid exhibited accuted angles between years confirming the scarce influence of E and G × E effects. Principal component 1 (73 %) was mainly associated to the total concentration of phenylpropanoids in pericarp and clearly separated the moderately resistant genotypes from the susceptible ones. Contents of phenylpropanoids, except those of cFA, were negatively correlated to fumonisin accumulation and disease severity ($-0.61 > r > -0.90$). This is also indicated in the PCA by the wide obtuse angles formed between each phenylpropanoid vector and that of either disease severity or fumonisin concentration. Principal component 2 (14 %) separated genotypes from the moderately resistant group according to their phenylpropanoid profile, with L4637 and Leales 25 exhibiting the maximum levels of divergence (Fig. 2). On the basis of the stepwise linear regression model, variability of disease severity was best explained by total DFAs while that of field fumonisin accumulation was best explained by 8,5DFAbz, total DFAs and pCA (Table 4).

Discussion

All genotypes developed symptoms after inoculation with *Fusarium verticillioides*. The most resistant

Table 3 Content of total diferulates (DFAs) and total phenolics in the kernel pericarp of eleven maize genotypes subjected to artificial inoculations with conidial suspensions of *Fusarium verticillioides*. Means are presented as combined data of two consecutive harvests (2010 and 2011)

Genotype	Diferulic acids (DFAs)					Total DFAs	Simple phenolic acids (SPhA)			Total Phenolics (SPhA + DFAs)
	8,5′DFAbz	8O4DFA	5,5′DFA	8,5′DFA	8,8′DFA		tFA	cFA	pCA	
mg g^{-1} dry pericarp										
Leales 25	2.57a	0.54a	0.10b	0.58a	0.74a	4.48a	15.65a	0.06a	1.12a	21.31a
Leales D	1.83d	0.46a	0.09b	0.46c	0.69a	3.52c	15.79a	0.15a	1.14a	20.61a
L4637	2.53a	0.40a	0.18b	0.56a	0.56b	4.23a	14.82a	0.28b	1.01a	20.34a
L1186 × L1196	0.87b	0.25b	0.05a	0.30c	0.25c	1.72b	11.98b	0.19a	0.26b	13.38b
L4671 × L4674	0.84b	0.20b	0.04a	0.21c	0.20	1.49b	11.21b	0.16a	0.34b	13.97b
L4674	0.84b	0.21b	0.03a	0.20c	0.19c	1.50b	10.73b	0.14a	0.24b	12.61b
L6856 × L4674	0.88a	0.15b	0.05a	0.33c	0.29c	1.73b	11.71b	0.12a	0.27b	13.33b
L943×L944	2.20a	0.48a	0.12b	0.66b	0.66a	4.11a	15.81a	0.21a	0.96a	21.09a
ARZM03018	2.35a	0.55a	0.13b	0.65b	0.67a	4.33a	14.82a	0.16a	0.96a	20.27a
ARZM04014	3.10c	0.59a	0.19b	0.64b	0.41b	4.86a	19.92c	0.28b	1.56d	26.58c
ARZM05040	2.53a	0.62a	0.15b	0.51[a]	0.54b	4.39a	15.19a	0.21b	1.08a	20.87a

[1] Contents expressed in mg per gram of dry pericarp. Values are expressed as mean ± .standard deviation

[2] Means in each column followed by the same letter are not significantly different according to LSD test (P=0.05)

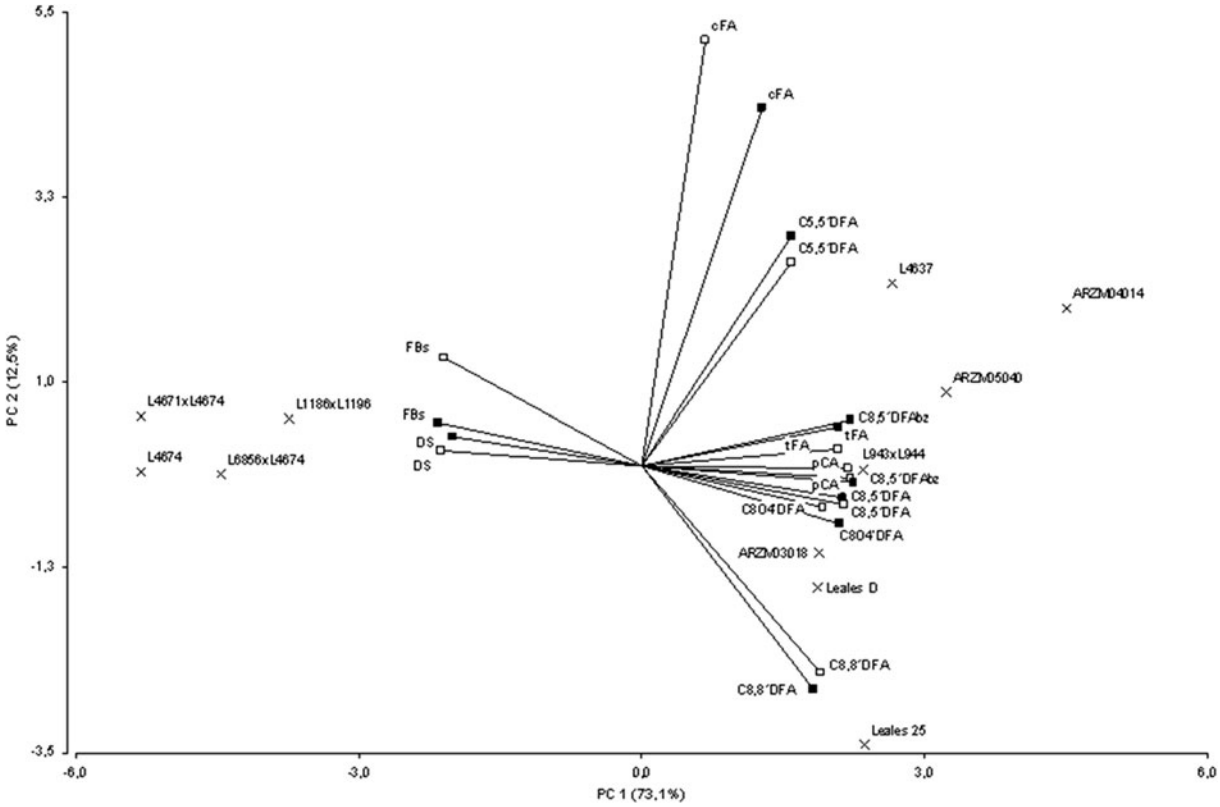

Fig. 2 Bi-plot of the two first principal components computed from means of disease severity (DS), grain fumonisin accumulation (FBs) and content of individual phenylpropanoid species of 11 maize genotypes obtained in 2 years (= 2010, = 2011; = maize genotype)

Table 4 Fixed regression equations for susceptibility parameters to Fusarium ear rot based on eleven maize genotypes

Trait	Equation	*p*	r^2
Field fumonisin accumulation	*A*=82.57–51 [DFAs] −63 [8,5′DFAbz] −865 [pCA]	0.001	0.82
Disease severity	*A*=65.57 –13.1 [DFAs]	0.001	0.71

TPhe Total phenolic compounds, *DFA* total diferulates, *8,5DFAbz* 8,5′-diferulate benzofuran form, *pCA* p-coumaric acid

genotypes showed a consistent low fumonisin accumulation which was highly associated to less kernel infection by *F. verticillioides* and elevated pericarp levels of both simple and dimeric phenylpropanoids. Relationship between disease severity and grain fumonisin concentration observed here was consistent with previous evidence indicating that most genotypic effects for fumonisin accumulation by *F. verticillioides* in kernels of maize are dependant on genotypic effects for disease symptoms (Presello et al. 2007; Presello et al. 2011).

In agreement with previous studies, the tFA was the predominant phenylpropanoid detected in the pericarp (Bily et al. 2003; García-Lara et al. 2004). On average, content of pCA and tFA was 4- and 2-fold higher in moderately resistant genotypes than in the susceptible ones, and were negatively correlated to fumonisin accumulation and disease severity. All together, these data suggest that these compounds participate in the variation of disease resistance to Fusarium ear rot observed on the evaluated genotypes. Phenylpropanoid monomers are often reported as factors involved in plant-pathogen interactions (Nicholson 1992). In their free unconjugated form, they showed to be fungistatic to *F. graminearum* and *F. culmorum* at EC_{50}s in the range 0.33–0.67 mg/g for tFA and 0.32–0.56 mg/g for pCA (Assabgui et al. 1993; McKeehen et al. 1999). The tFA also inhibited 90 % the in vitro fumonisin accumulation at 1 μg/ml, a concentration several times below to that needed for a significant inhibition of fungal growth (Beekrum et al. 2003). In the pericarp, however, tFA and pCA are mainly bound to cell wall polysaccharides by ester linkages and might act conferring mechanical resistance to infection by *F. verticillioides* (García Lara et al. 2004).

In these experiments, resistant genotypes contained about a 3-fold higher level of total DFAs than susceptible ones. The 8,5′DFAbz was the major DFA detected, followed by 8,5′DFA and 8,8′DFA. In contrast with these findings, Bily et al. (2003) and García Lara et al. (2004) found that 5,5′DFA and 8,8′DFA were the major DFAs detected in pericarp of maize hybrids from North America, a situation that might be due to genotype and/or environmental differences. Obtuse angles between each DFA vector and either disease severity or fumonisin concentration vectors, and the stepwise regression model suggest that the increase of all individual DFA species contributed to limit fumonisin accumulation and mycelial progress of *F. verticillioides*. The DFAs are formed during cell wall deposition and lignification by peroxidase-mediate coupling of ferulate monomers. They cross-link cell wall polysaccharides conferring pericarp hardness (Burr and Fry 2009). Hence, high contents of pericarp DFAs might act as a preformed structural barrier restricting fungal infection and mycelial progress from diseased to pericarp-intact neighbouring kernels. Cell wall DFAs also might have a direct inhibitory effect on mycotoxin production after released by fungal esterases and other extracellular enzymes during infection of *F. verticillioides*. The 8,5′-DFAbz, the major DFA detected in the pericarp of the genotypes, showed in vitro to be as effective as ferulic acid to inhibit the biosynthesis of trichothecenes by *F. graminearum* (Boutigny et al. 2010).

The contents of the phenylpropanoids detected in the pericarp of the maize genotypes suggest their contribution to kernel resistance to F. verticillioides. However, evidence indicating the exclusive importance of these molecules in the observed resistance is lacking. For example, fumonisin accumulation and fungal infection have been negatively correlated to pericarp thickness (Hoenish and Davis 1994), content of epicuticular kernel waxes (Sampietro et al. 2009), and the pericarp content of anthocyanins and phlobaphenes (Pilu et al. 2011). In addition, Flint maize showed higher ear rot resistance than dent maize (Czembor and Ochodzki 2009) which was attributed to higher amylase contents in kernels of the former one (Wit et al. 2011). However, the opposite situation was also reported (Loffler et al. 2010) and attributed to different silking dates. Other factors that have been also involved in resistance are silk age, husk coverage and husk coverage and tightness (Mesterhazy et al. 2012). Our current knowledge suggests that maize resistance to infection and fumonisin accumulation by *F. verticillioides* is a complex polygenic trait where several chemical and physical interacting factors are involved.

Conclusions

High levels of phenylpropanoids in the kernel pericarp are associated to less disease severity and fumonisin accumulation caused by *F. verticillioides*. Selection based on visual ratings of disease severity for Fusarium ear rot might be effective to develop genotypes accumulating low levels of fumonisins and high levels of pericarp phenylpropanoids. Results were confirmed in two independant years suggesting that plant production of phenylpropanoids is scarcely affected by environment, contributing to the stability of disease resistance. Further research is in progress to map quantitative trait loci for these cell wall components in bi-parental populations derived by crossing resistance and susceptible genotypes included in this study.

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