JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Maize Kernel Antioxidants and Their Potential Involvement in Fusarium Ear Rot Resistance

Adeline Picot,[†,‡,#] Vessela Atanasova-Pénichon,[‡] Sebastien Pons,[‡] Gisèle Marchegay,[‡] Christian Barreau,[§] Laëtitia Pinson-Gadais,[‡] Joël Roucolle,[∥] Florie Daveau,[⊥] Daniel Caron,[†,▽] and Florence Richard-Forget*[,‡]

[†]ARVALIS—Institut du végétal, 6 Chemin de la Côte Vieille, F-31 450 Baziège, France

[‡]INRA, UR1264 MycSA, 71 Avenue Edouard Bourlaux, F-33 883 Villenave d'Ornon, France

[§]CNRS, UR1264 MycSA, 71 Avenue Edouard Bourlaux, F-33 883 Villenave d'Ornon, France

[∥]Monsanto SAS Peyrehorade, Croix de Pardies, F-40 300 Peyrehorade, France

[⊥]Euralis Semences, 117 Avenue de Vendôme, F-41 000 Blois, France

ABSTRACT: The potential involvement of antioxidants (α-tocopherol, lutein, zeaxanthin, β-carotene, and ferulic acid) in the resistance of maize varieties to Fusarium ear rot was the focus of this study. These antioxidants were present in all maize kernel stages, indicating that the fumonisin-producing fungi (mainly *Fusarium verticillioides* and *Fusarium proliferatum*) are likely to face them during ear colonization. The effect of these compounds on fumonisin biosynthesis was studied in *F. verticillioides* liquid cultures. In carotenoid-treated cultures, no inhibitory effect of fumonisin accumulation was observed while a potent inhibitory activity was obtained for sublethal doses of α-tocopherol (0.1 mM) and ferulic acid (1 mM). Using a set of genotypes with moderate to high susceptibility to Fusarium ear rot, ferulic acid was significantly lower in immature kernels of the very susceptible group. Such a relation was nonexistent for tocopherols and carotenoids. Also, ferulic acid in immature kernels ranged from 3 to 8.5 mg/g, i.e., at levels consistent with the in vitro inhibitory concentration. Overall, our data support the fact that ferulic acid may contribute to resistance to Fusarium ear rot and/or fumonisin accumulation.

KEYWORDS: *tocopherols, carotenoids, ferulic acid, antioxidants, maize, Fusarium verticillioides, fumonisin, inhibition*

INTRODUCTION

Among *Fusarium* mycotoxins, fumonisins, a family of polyketide-derived mycotoxins, are common contaminants of maize crops. These mycotoxins are mainly produced by *Fusarium* species belonging to the *Gibberella fujikuroi* complex, including *Fusarium verticillioides* and *Fusarium proliferatum*. Fumonisin B_1 (FB_1), B_2 (FB_2), and B_3 (FB_3) are predominantly encountered on maize kernels, with FB_1 occurring at the highest level. The levels of these toxins have been recently subjected to European regulations, limiting the FB_1 + FB_2 content in unprocessed maize for human consumption to a maximum level of 4 mg/kg.[1]

Fumonisins are heat-stable molecules and are only partially eliminated during food processing.[2] Currently, the best way to reduce or prevent fumonisin contamination is to limit their biosynthesis during cultivation of maize plants. Several approaches are under investigation, and among them, the identification of naturally occurring mechanisms in plants that lead to reduced mycotoxin accumulation seems particularly relevant. First, it is known that the plants have the ability to detoxify toxins through the formation of glucose conjugates, as reported for some resistant wheat lines in which the mycotoxin deoxynivalenol (DON) can be converted to DON-3-*O*-glucoside.[3,4] Second, toxin biosynthesis can also be inhibited through the action of plant endogenous compounds, both constitutive and induced in response to pathogen infection.[5] Multiple pieces of evidence indicate that oxidative stress is an enhancing factor for the biosynthesis of several mycotoxins, including trichothecene B, fumonisin, ochratoxin, aflatoxin, and patulin.[6,7] Therefore, the kernel content in antioxidant compounds may represent a common selectable trait for the control of these mycotoxins in the field. Among plant natural antioxidants, phenolic compounds have been widely studied for their in vitro ability to inhibit mycotoxin production such as aflatoxin,[8−11] trichothecenes B,[12,13] and fumonisins[14−17] and for their involvement in resistance to pests and diseases.[18,19] In addition to phenolic compounds, tocopherols and carotenoids are potent maize antioxidant compounds but their inhibitory activity against mycotoxins is poorly known. To our knowledge, no studies have yet investigated the effect of tocopherols and carotenoids on fumonisin biosynthesis. A few studies investigated their impact on other mycotoxin production and lead to opposite results, depending on the mycotoxin targeted. Thus, although capsanthin (a major carotenoid in paprika) has been shown to inhibit aflatoxin yield,[20] more recent results demonstrated its lack of inhibitory effect on ochratoxin production.[21]

Tocopherols and carotenoids have been reported to naturally occur at varying levels in mature maize kernels,[22−25] while ferulic acid is one of the predominant phenolic compounds.[26,27] Apart from their inhibitory activity, an additional prerequisite for the potential involvement of these compounds in interfering with fumonisin accumulation is that their presence is required

Received: February 6, 2013
Revised: March 12, 2013
Accepted: March 13, 2013
Published: March 13, 2013

© 2013 American Chemical Society

at the time fumonisin production occurs in developing maize kernels. In a previous field study, fumonisin was found to be initially produced on immature maize kernels from the dough to the dent stages.[28] The evolution of carotenoid[25] and ferulic acid[26] levels during maize kernel ripening has been recently investigated, showing that these compounds were actually present at all maturation stages. To our knowledge, there is currently no data available concerning the evolution of the tocopherol content over the course of maize kernel development.

The goal of this study was to clarify the potential involvement of maize antioxidants, namely ferulic acid, tocopherols, and carotenoids, in resistance to Fusarium ear rot and fumonisin accumulation. Three complementary approaches were conducted: (i) quantitation of the tocopherol and carotenoid content at various maturity stages, (ii) investigation of their inhibitory effect on fumonisin biosynthesis in *F. verticillioides* liquid cultures, and (iii) investigation of a relationship between maize resistance levels to Fusarium ear rot and the antioxidant composition in early stages of kernel development. This work is a complement to a field study whose objective was to monitor fungal growth and fumonisin accumulation in developing maize kernels after silk inoculation with *F. verticillioides*.[28] The carotenoid and tocopherol contents were quantified on the same samples as those collected during this field study.

■ MATERIALS AND METHODS

Fusarium Isolates. INRA 63, INRA 64, and INRA 444 were previously isolated from naturally infected maize grains and are maintained in the MycSA collection.[29]

Preparation of Fungal Inoculum. In the field experiment, the *F. verticillioides* isolate INRA 63 was inoculated into the silk channel of two maize varieties. The spore suspension was prepared and inoculated as previously described by Picot et al.[28]

The effect of carotenoids (lutein, zeaxanthin, and β-carotene) and α-tocopherol was tested on *F. verticillioides* liquid cultures, using the three isolates INRA 63, INRA 64, and INRA 444. Spore suspensions were prepared by adding sterile distilled water to 7−10-day-old culture grown on potato dextrose agar (PDA) slant tubes.

Field Experiments. The experimental protocol was previously described by Picot et al.[28] Briefly, two field maize varieties, PR38H20 and CRAZI (here referred to as V1 and V2, respectively), were sown in 2008 (3 May) and in 2009 (7 May) in one location (Montesquieu-Lauragais) in the southwest of France. V1 is a hybrid of the dent type, while V2 is a hybrid of the flint to flint-dent type. A spore suspension (0.5 mL) containing 4×10^6 spores/mL prepared from *F. verticillioides* INRA 63 isolate was injected into the silk channel with a self-refilling syringe equipped with an obtuse needle. Inoculations were performed the same day for both varieties, at days four and seven after silking, for V1 and V2, respectively. Silking was assigned when silk emergence could be observed in 50% of the maize ears. At each sampling date, 10 inoculated ears were randomly hand-picked and kernels from the first third of each ear were quickly removed (within less than 5 min) using a scalpel and placed in a container maintained in dry ice. Once collected, the container was immediately placed in a CX 500 Cryo Express dry shipper (Taylor-Wharton Cryogenics, Theodore, AL) before final storage at −80 °C in a freezer. Kernels were then ground to a fine powder in liquid nitrogen, lyophilized, and stored at −20 °C before carotenoid and tocopherol quantitation. Measurements of carotenoid and tocopherol contents were performed on five sampling dates, 10, 15, 22, and 42 days postinoculation and at harvest maturity. Each sampling date corresponds to the blister, milk, dough, dent, and harvest maturity stage of maize kernels.

To determine the relationship between resistance levels to Fusarium ear rot and kernel composition in antioxidants, healthy kernels from a set of 10 maize genotypes were analyzed for their content in tocopherols, carotenoids, and ferulic acid in a trial set up in 2008 located in the southwest of France. The 10 genotypes tested discriminate in three groups according to their levels of resistance to Fusarium ear rot: very susceptible, susceptible, and moderately susceptible. This classification is based on symptom scores over several years of field experiments in France. The noninfected kernels were hand collected 20 days after silking, corresponding to the milk stage.

Carotenoid and Tocopherol Extraction and Measurement. The extraction and quantitation procedures were adapted from Kurilich and Juvik.[22] Carotenoids and tocopherols were extracted from 1 g of dried maize kernels by adding 5 mL of methanol that contained 0.1% of butylated hydroxytoluene (BHT, w/v), followed by vortexing for 20 s and shaking for 10 min at 50 rpm. Then, 3 mL of 12% (w/v) KOH were added to the supernatant in order to saponify potentially interfering oils. Samples were vortexed for 30 s and agitated for 30 min. Following saponification, an internal standard, namely β-apo-8′-carotenal (50 μL, 100 μg/mL), was added. Carotenoids and tocopherols were then extracted three times with hexane layers (3 mL) after centrifugation for 10 min at 3000*g*. Combined organic layers were washed with 3 mL of deionized water. The organic layer was then evaporated to dryness under a nitrogen stream. Dried samples were reconstituted in 200 μL of methanol, and 30 μL aliquots were used for HPLC analysis. An Agilent 1100 series HPLC system consisting of a 250 mm × 4.6 mm i.d., 5 μm, Zorbax SB C18 column, with a 4.6 mm × 7.5 mm i.d. guard column of the same material (Agilent Technologies, Santa Clara, CA), maintained at 30 °C, a quaternary HPLC pump, an autosampler, and a photodiode array detector, was used. The mobile phase consisted of acetonitrile/methanol/methylene chloride (75/20/5, v/v/v). Isocratic elution was performed with the following flow conditions: 16 min at 0.5 mL/min, 1 min of progressive flow from 0.5 to 1.8 mL/min, 23 min at 1.8 mL/min, 1 min of progressive flow from 1.8 to 0.5 mL/min, and 10 min at 0.5 mL/min. Chromatograms were generated at 290 and 450 nm for tocopherols (δ-, α-, and γ-tocopherols) and carotenoids (lutein, zeaxanthin, internal standard, and β-carotene), respectively. Identification of these compounds was achieved by comparison of their retention times and absorption spectra with those of standards.

Standards of lutein and zeaxanthin were purchased from Indofine Chemicals Company (Hillsborough, NJ), while α-, δ-, γ-tocopherols, β-carotene, and internal standard were purchased from Sigma-Aldrich (France). Standards of carotenoids and tocopherols were dissolved in tetrahydrofuran (THF) at concentrations of 1 mg/mL for β-carotene, 2.5 mg/mL for lutein, zeaxanthin, and γ-tocopherol, and 20 mg/mL for δ-, and α-tocopherols. Stock solutions were stored at −20 °C. For the preparation of HPLC standards, stock solutions were evaporated to dryness and dissolved in methanol at the required concentrations. Linear relations between peak area and the concentration values were obtained over the following intervals: 0.1−25 μg/mL for lutein, 0.1−50 μg/mL for zeaxanthin, and 0.5−10 μg/mL for β-carotene, respectively. Linearity domain for α-, δ- and γ-tocopherols ranged from 1 to 100 μg/mL. Concentrations of carotenoids and tocopherols in maize were expressed in μg/g of dry maize powder after correction with the extraction efficiency that comprised between 0.95 and 1.27.

Ferulic Acid Extraction and Measurement. Phenolic acids were released from cell walls by alkaline hydrolysis. First, 100 mg of lyophilized powders of maize kernels were shaken for 2 h in 4 mL of 2 N NaOH in the dark in a nitrogen atmosphere. Then the hydrolysis was stopped with 12 N HCl until the pH reached 2.0. After centrifugation (5 min at 3000*g*), supernatants were extracted twice with 5 mL of ethyl acetate. The organic phases were evaporated to dryness under a nitrogen stream at 40 °C. The final precipitates were dissolved in 100 μL of methanol/water (50:50, v/v) before analysis.

Ferulic acid was quantified by HPLC-DAD using an Agilent Technologies 1100 series liquid chromatograph with the same column and guard column as previously described for carotenoid and tocopherol analyses. The mobile phase consisted of 2% formic acid in water (v/v) (solvent A) and acetonitrile (solvent B) according to the following gradient: 5−15% B in 30 min, 15−50% B in 20 min, 50−90% B in 8 min, 90% B for 5 min, 90−5% B in 2 min, and 5% B for 10

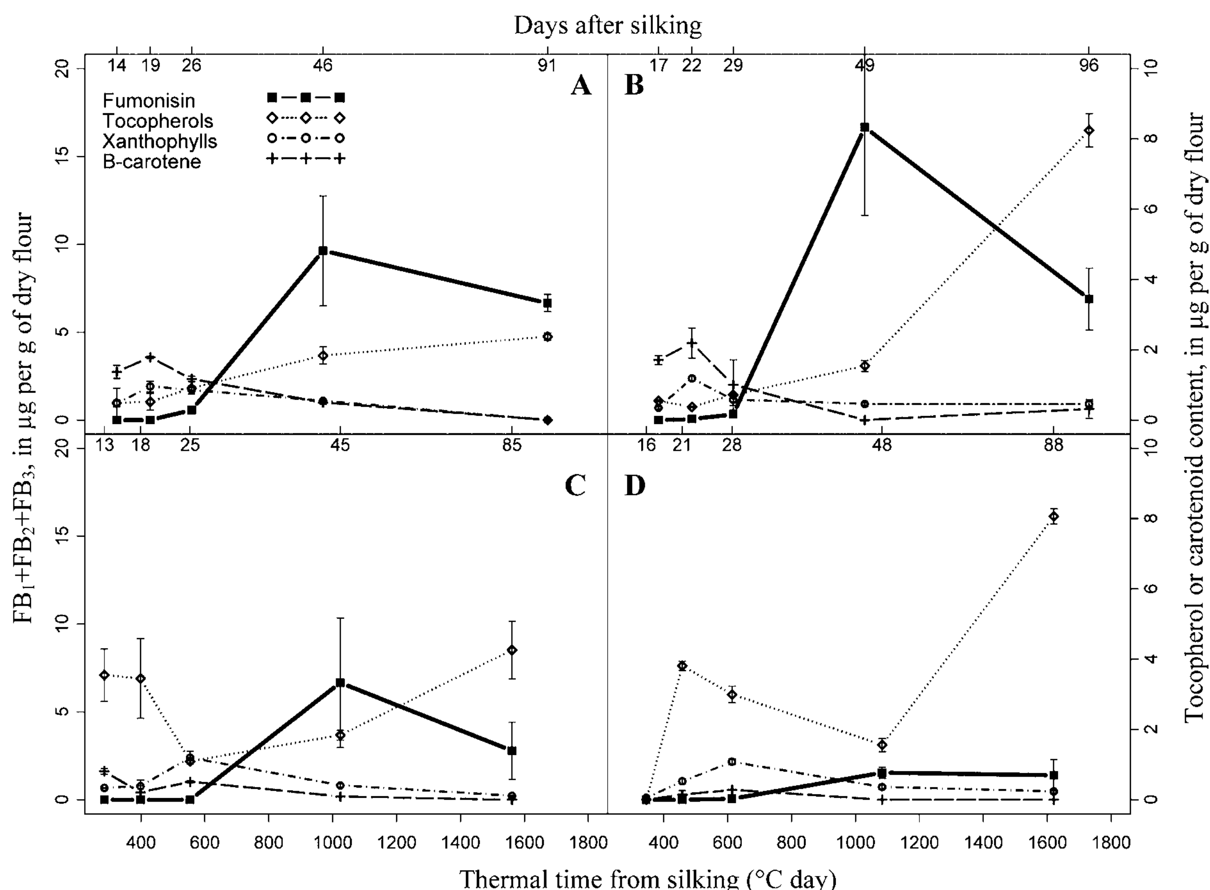

Figure 1. Time-course of fumonisin and antioxidant accumulation in infected maize kernels. (A) V1, 2008; (B) V2, 2008; (C) V1, 2009; (D)V2, 2009. Vertical bars show standard deviation. ■, Fumonisins; ◇, α- + γ-tocopherol; ○, xanthophylls (lutein + zeaxanthin); +, β-carotene. Bottom *X*-axis, the thermal time from silking; top *X*-axis, days after silking for each sampling.

min postrun reconditioning. The sample injection volume was 5 μL. The flow rate was kept at 1 mL/min for a total run time of 75 min. The UV/vis spectra were recorded from 200 to 550 nm. Quantitation of ferulic acid was performed by using external calibration with standard solutions prepared from commercial powder purchased from Sigma-Aldrich (France). Final results were converted into μg/g of dry maize powder.

Liquid Culture Supplementation. The inhibitory effect of lutein, zeaxanthin, β-carotene, α-tocopherol, and ferulic acid on fumonisin accumulation was tested in liquid cultures of three *F. verticillioides* isolates. Cultures were performed in GAYEP medium (glucose, amylopectin, yeast extract, and peptone) in 25 mL Erlenmeyer flasks. This medium was obtained by mixing 900 mL of a solution containing 40 g/L glucose and 10 g/L amylopectin (autoclaved for 30 min at 105 °C) and 100 mL of a solution containing 1 g/L neopeptone and 1 g/L yeast extract, autoclaved for 20 min at 121 °C. For each strain, a 10 mL aliquot of GAYEP, supplemented with lutein, zeaxanthin, β-carotene, α-tocopherol, or ferulic acid dissolved in methanol was inoculated with 2×10^4 spores/mL. It was verified that the percentage of solvent used (0.1% of the volume of the initial medium) did not affect fungal growth. The effect on fumonisin biosynthesis was evaluated at concentrations ranging from 0 to 1 mM for ferulic acid, 0 to 0.1 mM for α-tocopherol, at 0.7 μM for lutein and zeaxanthin, and at 0.4 μM for β-carotene. Flasks were then incubated in darkness at 25 °C and with shaking in a Multitron incubator (Infors AG, Bottmingen, Switzerland) for 10 days. Following incubation, cultures were centrifuged at 3000*g* for 10 min. Supernatants were stored at −20 °C before quantitation of fumonisin. *F. verticillioides* biomass was measured by weighing the mycelial pellet after 48 h of freeze-drying. Cultures were done in triplicate. It was checked that the initial pH of the culture medium was not affected by the supplementation and that final pH conditions did not vary between treatments.

Fumonisin Analysis. Fumonisins were extracted as described by Shephard et al.[30] with some modifications. Briefly, culture filtrates were adjusted at pH 6.5, and fumonisins were purified using Bond Elut Strong Anion Exchange (SAX) cartridges (Varian, Palo Alto, CA). Fumonisins were eluted with 10 mL of methanol/acetic acid (99/1, v/v) and evaporated to dryness under a nitrogen stream. Dried samples were dissolved in 200 μL of methanol. Fumonisin concentration was determined using a high-performance liquid chromatograph Agilent 1100 series (Agilent Technologies, Santa Clara, CA) equipped with a 150 mm × 4.6 mm i.d., 5 μm, Equisorb ODS2 column, maintained at 25 °C, and a fluorescence detector (λ of excitation, 335 nm; λ of emission, 440 nm). Then 10 μL of samples were derivatized with 90 μL of *o*-phthaldialdehyde reagent, and 20 μL of this solution were injected into the HPLC system within 1 min after derivatization. Quantitation was performed using external calibration with FB_1, FB_2, and FB_3 standard solutions ranging from 1 to 100 μg/mL. The sum of FB_1, FB_2, and FB_3 was used to quantitate the amount of fumonisins, and results were converted in μg/mg of dry biomass. Note that this protocol based on fluorescence detection may underestimate the total levels of fumonisins because forms such as hydrolyzed fumonisins or minor fumonisins cannot be detected under these conditions.[31]

Fumonisins were extracted from maize kernels and quantified according to a similar protocol, described in Picot et al.[28]

Statistical Analysis. Statistical analysis was performed using Splus software (TIBCO Sofware Inc., USA) using ANOVA. Differences between treatments were determined with multiple comparisons with the Sidak method. Level of significance was set at α = 0.05.

Table 1. Effect of (A) Lutein, Zeaxanthin, and β-Carotene, (B) α-Tocopherol, and (C) Ferulic Acid on Fungal Biomass and Fumonisin Accumulation by Three Isolates of *F. verticillioides*

A

compound	$FB_1 + FB_2 + FB_3$ (μg/mg of dry biomass) INRA 63	INRA 64	INRA 444	dry biomass (mg) INRA 63	INRA 64	INRA 444
control	1.34 ± 0.51	2.39 ± 0.39	0.63 ± 0.85	88.8 ± 5.8	83.8 ± 1.9	92.0 ± 4.2
lutein, 0.7 μM	1.31 ± 0.26	1.25 ± 0.10	0.78 ± 0.52	95.6 ± 2.0	90.9 ± 0.9	114.9 ± 8.3
zeaxanthin, 0.7 μM	2.12 ± 0.19	2.86 ± 1.12	0.62 ± 0.49	82.0 ± 3.4	82.2 ± 4.3	112.1 ± 9.5
β-carotene, 0.4 μM	1.13 ± 0.26	2.70 ± 0.31	0.62 ± 0.22	98.5 ± 26.9	104.2 ± 2.0	136.7 ± 8.4

B

α-tocopherol (mM)	$FB_1 + FB_2 + FB_3$ (μg/mg of dry biomass) INRA 63	INRA 64	INRA 444	dry biomass (mg) INRA 63	INRA 64	INRA 444
0	2.08 ± 0.72	2.64 ± 0.52	1.21 ± 0.24	84.9 ± 12.8	88.2 ± 9.4	104.6 ± 14.2
0.001	1.74 ± 0.60	2.22 ± 0.78	0.98 ± 0.44	77.5 ± 11.2	84.4 ± 12.2	95.1 ± 13.0
0.01	1.67 ± 0.42	2.02 ± 0.91	1.46 ± 0.55	77.4 ± 11.7	81.6 ± 15.1	100.9 ± 17.4
0.1	1.44 ± 0.13[a]	1.48 ± 0.60[a]	0.29 ± 0.08[a]	78.9 ± 4.5	82.1 ± 9.1	100.3 ± 14.8

C

ferulic acid (mM)	$FB_1 + FB_2 + FB_3$ (μg/mg of dry biomass) INRA 63	INRA 64	INRA 444	dry biomass (mg) INRA 63	INRA 64	INRA 444
0	2.52 ± 0.31	3.13 ± 0.29	NT	89.93 ± 3.05	78.6 ± 5.21	NT
0.5	1.67 ± 0.74	3.07 ± 0.24	NT	82.57 ± 3.35	81.47 ± 3.04	NT
1	0.36 ± 0.12[a]	1.48 ± 0.66[a]	NT	81.47 ± 5.88	1.67 ± 0.42	NT

[a]Significant differences with control treatment ($\alpha = 0.05$). NT: not tested. Data are reported as mean values ± standard deviation, based on three biological replications. Experiments were repeated twice for α-tocopherol (B).

RESULTS AND DISCUSSION

Kernel Antioxidant Composition during Maize Ear Ripening. As previously reported, the main carotenoid and tocopherol compounds found in our maize samples included lutein and/or zeaxanthin, β-carotene, and α- and γ-tocopherols while δ-tocopherol was never detected. The levels of xanthophylls (i.e., the sum of lutein and zeaxanthin), β-carotene, and tocopherols, measured on the V1 and V2 maize varieties, over two years of experiment (2008 and 2009) and at different maturation stages, are summarized in Figure 1. The range of values for α- + γ-tocopherols, xanthophylls, and β-carotene were from not detected (ND) to 8.24 μg/g, ND to 1.2 μg/g, and ND to 2.19 μg/g, respectively. In addition, note that quantitation of carotenoids and tocopherols was also performed on 10 noninoculated control samples. Results showed no significant differences between the inoculated samples and their respective control ones (data not shown).

Analysis of variance (ANOVA) exhibited significant differences among the maturation stages ($P < 0.00001$ for all studied compounds), the years of experiments ($P = 0.036$, $P = 0.0056$, and $P < 0.00001$ for α- + γ-tocopherols, xanthophylls, and β-carotene, respectively) and the two-way interactions (variety by sampling date and year by sampling date interactions). The levels of lutein and zeaxanthin generally reached their maximum content at the dough (19−22 days after silking) or milk stage (26−29 days after silking), depending on varieties and years and then decreased at dent (46−49 days after silking) and final harvest stages (Figure 1). The levels of tocopherols were not significantly different among immature kernels, from blister to dent stages, and reached their maximum levels at final harvest maturity (Figure 1). Overall, our results show that tocopherols and carotenoids are present in the maize kernels from blister to the final mature stages, i.e., when fumonisin is initially produced and when it accumulates in the kernels (Figure 1). The pattern of evolution described here was similar to that recently reported in yellow maize for lutein and zeaxanthin by Xu et al.[25] In the latter study, total carotenoids first decreased from blister to milk stage, then increased to dent stage, and finally decreased to its lowest levels at the maturity stage.

Concentrations in ferulic acid were not determined in this time course. A recent study accurately describes the phenolic acid accumulation during maize ear ripening.[26] The V1 variety was included in the set of considered genotypes. They showed a gradual decrease in free ferulic acid from silking to harvest maturity while cell-wall-bound ferulic acid (which represents 90% of the total ferulic acid content), reached a maximum content at blister and milk stages (up to 8500 μg/g) before drastically decreasing to the final stage. Overall, ferulic acid was the major cell-wall-bound phenolic acid in kernels at all stages, representing between 75% and 95% of the total cell-wall-bound monomeric phenolic content.

It has been previously demonstrated that infection can occur from the blister stage, while fumonisin production is initiated during the dough stage.[28] Therefore, *F. verticillioides*, during its colonization of the maize kernel, is likely to encounter tocopherol, carotenoid, and more abundantly, ferulic acid. The exact localization and distribution of these antioxidants in maize kernels has yet to be clarified in order to precisely determine whether these compounds can directly interact with the fungal cells during the infection process. In kernels, phenolic acids are present in both soluble (free) and insoluble (bound to cell wall polysaccharides) forms. Because of the presence of hydroxy groups at both ends of the molecule, lutein and zeaxanthin can span the membranes while β-carotene remains entirely in the inner part of the membrane.[32] Tocopherols are anchored in membrane by their polyprenyl chain, but the presence of an hydroxyl group at the chromanol ring places them in the lipid−water interfacial region of the cell membrane.[33]

In Vitro Inhibitory Activity of Antioxidants. The effect of lutein (0.7 μM or 0.4 μg/mL), zeaxanthin (0.7 μM or 0.4 μg/mL), β-carotene (0.4 μM or 0.2 μg/mL), α-tocopherol (concentrations ranging from 0 to 0.1 mM or 40 μg/mL), and ferulic acid (concentrations ranging from 0 to 1 mM or 194 μg/mL) on fungal development and fumonisin biosynthesis was investigated in GAYEP liquid cultures using three isolates of *F. verticillioides* (Table 1). Fungal biomass was never affected by the supplementation of antioxidant compounds (Table 1). Using the carotenoid concentrations reported above, consistent with the physiological concentrations we determined in maize kernels, a reduction (52%) in fumonisin production was observed for the *F. verticillioides* INRA 64 strain in lutein-supplemented liquid cultures, but the difference was not significant compared to control treatments (Table 1A). The use of alternative solvents such as chloroform or THF may have allowed including higher concentrations of carotenoids.[34] However, on the basis of previous studies performed in our laboratory, these solvents were shown to impair fungal growth and consequently may have masked the antifumonisin activity of carotenoids. The use of methanol at 0.1% was therefore preferred.

Regarding α-tocopherol, a 0.1 mM concentration was shown to induce a significant reduction of fumonisin biosynthesis for 10-day-old cultures whatever the strain tested. At this concentration, fumonisin accumulation was decreased by 31%, 44%, and 76%, for *F. verticillioides* INRA 63, INRA 64, and INRA 444, respectively (Table 1B). A similar or higher degree of inhibition was reached with 1 mM ferulic acid treated cultures, with 86% and 53% of fumonisin inhibition, for *F. verticillioides* INRA 63 and 64 strains, respectively (Table 1C).

In our study, 0.1 mM α-tocopherol induced significant reduction of fumonisin accumulation for three *F. verticillioides* strains. This concentration (equaling to 40 μg/mL of liquid medium) is approximately 20 times higher than the highest levels of tocopherols found in our maize varieties. Ferulic acid treated cultures needed to be 10 times more concentrated to reach a similar degree of fumonisin inhibition. Given that ferulic acid is very abundant in kernels, this in vitro inhibitory concentration remains largely consistent with the physiological levels found in early kernel stages. Norton[35] also investigated the effect of α- and γ-tocopherols on aflatoxin B1 biosynthesis by one strain of *Aspergillus flavus*. In contrast to our results, aflatoxin and fungal growth were never inhibited by supplementation with α- and γ-tocopherols, at concentrations ranging from 20 to 2000 μg/mL (0.05−5 mM). The discrepancy in these results might be due to the fact that the inhibitory effect of tocopherols may be species specific or depends on the secondary metabolite considered. Ferulic acid has also been previously described as a potent repressor of fumonisin biosynthesis.[14] It has been reported as an efficient inhibitor of type B trichothecene biosynthesis in *Fusarium culmorum* and *Fusarium graminearum* liquid cultures at concentration as low as 0.5 mM.[13] In the latter study, percentages of inhibition ranged from 17 to 90%, depending on the species and strains tested. Other studies also showed that ferulic acid, at 1 mM, reduced aflatoxin B_1 and G_1, with an inhibition factor that could reach as much as 75%.[8,10]

The mechanisms by which α-tocopherol and ferulic acid are able to reduce mycotoxin biosynthesis under in vitro conditions remain to be elucidated. One hypothesis is that these antioxidant compounds may directly affect gene transcription or enzymatic activity involved in the fumonisin biosynthetic pathway. This pathway includes many oxidation steps that require high oxygen levels.[36] In *Aspergillus parasiticus*, it has been demonstrated that oxygen requirements progressively increase during the different steps of aflatoxin biosynthesis and are lowest in the nontoxigenic strain and highest in the aflatoxin producing strain.[37] Therefore, it can be hypothesized that antioxidants may inhibit redox enzymes such as P450 cytochrome monooxygenases, notably encoded by the FUM6 gene. The associated product of this gene, which catalyzes the oxidation of the backbone at carbons 14 and 15, plays a crucial role in the fumonisin biosynthetic pathway given that disruption of FUM6 gene blocked fumonisin production.[38]

Antioxidant compounds may also modulate the environmental and physiological factors affecting fumonisin biosynthesis and/or inhibit signaling circuit upstream of the fumonisin pathway. For instance, antioxidant compounds, by reducing the levels of oxidant species, may modify the redox equilibrium in the medium surrounding *F. verticillioides*, inducing therefore a regulation cascade upstream of the fumonisin biosynthetic pathway that will in turn inhibit or reduce fumonisin production. Notably, it has been shown that supplementation with caffeic acid in *A. flavus* liquid cultures induced an increased expression of genes encoding alkyl hydroperoxide reductases that detoxify organic peroxides. The induction of such genes leads to an alleviation of the oxidative stress and to reduced levels of aflatoxin without affecting fungal growth.[39] In addition, acetyl-CoA molecules, originated from β-oxidation of short-chain fatty acids inside mitochondria, are required in the early steps of fumonisin biosynthesis. As reported in the study of Kim et al. for *A. flavus*,[40] antioxidant compounds may disrupt the mitochondrial respiration chain and therefore affect the amount of acetyl-CoA available for fumonisin biosynthesis.

Relationship between Resistance Levels and Antioxidant Content in Early Kernel Stages. A set of 10 maize genotypes was used to establish a relationship between resistance to Fusarium ear rot and maize kernel composition in antioxidants in immature and healthy kernels (around 20 days after silking, equivalent to the milk stages). Levels of tocopherols, xanthophylls, and β-carotene varied respectively between 0.46 and 1.70 μg/g, between 0.1 and 0.93 μg/g, and between ND and 0.3 μg/g. This range of concentrations is consistent with that found in our time-course reported in Figure 1. No relation was found between the resistance levels and the kernel content in tocopherols and carotenoids (data not shown). Regarding cell-wall-bound ferulic acid, concentrations ranged between 3 and 8.5 mg/g. Our data showed significant differences between the two extreme groups of genotypes: levels of ferulic acid were significantly higher in the moderately susceptible group and significantly lower in the very susceptible group (Figure 2).

On the basis of this study, α-tocopherol is a more potent inhibitor of fumonisin production than ferulic acid. Nevertheless, in planta, the latter is more abundant than the former, at any kernel stages, with concentrations up to 8.5 mg/g.[26] Also, our results indicated that the levels in carotenoids and tocopherols in early stages of kernel development were not related to the resistance levels to Fusarium ear rot while ferulic acid content was significantly higher in the least susceptible varieties and vice versa. This positive relation needs however to be confirmed using a wider set of genotypes and under various environments. A similar trend between *F. graminearum* disease and ferulic acid amounts in maize kernels was also suggested by the data of Assabgui et al.[41] However, the latter results were

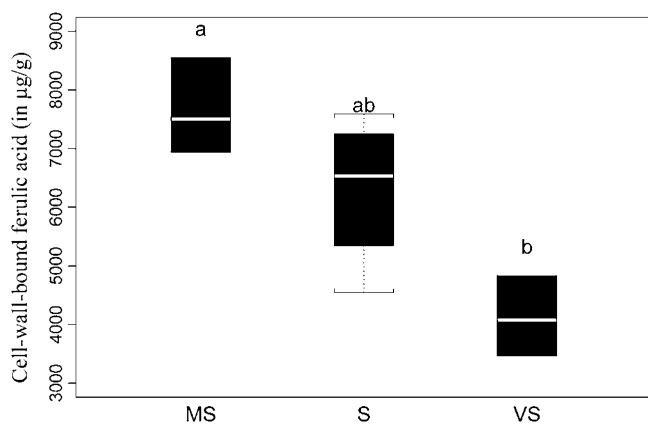

Figure 2. Cell-wall bound ferulic acid levels in milk kernel stages in three groups of varieties differing in their susceptibility to Fusarium ear rot: MS (moderately susceptible, $n = 3$), S (susceptible, $n = 4$), and VS (very susceptible, $n = 3$). Groups with different letters are significantly different (Tukey, $\alpha = 0.05$).

based on the phenolic acid content measured in mature maize kernels. Considering kernels from early stages should nevertheless be preferred because the greatest differences in phenolic composition among genotypes seems to be identified in immature kernels.[26,42] To our knowledge, this is the first study focusing on the occurrence of a correlation between resistance to Fusarium ear rot, fumonisin accumulation, and antioxidant content in early stages, i.e., at the starting kernel stage for fungal colonization and fumonisin production.

The in planta mode of action of ferulic acid on fungal growth and/or fumonisin accumulation has yet to be elucidated. It is widely acknowledged that, in response to pathogen infection, phenolic compounds are released from the cell wall or massively synthesized by the plant to accumulate rapidly at the site of infection.[43] Phenolic acids thus operate in defense response through direct interference with the fungus or through the reinforcement of plant structural components to act as a mechanical barrier against the pathogen.[44] Alternatively, the in vitro inhibitory activities toward mycotoxin accumulation indicate that they may also specifically inhibit fumonisin accumulation. Notably, it has been reported that the maize lipoperoxidative signaling pathway may positively regulate fumonisin biosynthesis.[45] In the latter study, fumonisin production was reduced when inoculating *F. verticillioides* in a maize mutant line which showed decreased levels of lipoperoxides resulting from the 9-LOX pathway. Because antioxidants are known to play a critical role in protecting the cell from lipid peroxidation and, in particular, ferulic acid has been demonstrated to inhibit LOX activity,[46] it provides a further explanation to the possible involvement of these compounds in the resistance of maize genotypes to fumonisin accumulation.

Overall, the present data provide insight into the potential involvement of ferulic acid in maize kernel resistance to Fusarium ear rot and fumonisin accumulation. They also indicate that, despite a higher in vitro inhibition activity toward fumonisin accumulation, α-tocopherol does not seem to be involved in resistance to Fusarium ear rot due to its low abundance and the absence of relation between resistance levels of maize and their tocopherol content in immature kernels. If the inhibitory effect of ferulic acid toward Fusarium ear rot and fumonisin accumulation can be validated for a larger set of genotypes and under a wider range of environments, then it may be a promising resistant biomarker.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +33 5 57 12 24 83. Fax: +33 5 57 12 25 00. E-mail: fforget@bordeaux.inra.fr.

Present Addresses

#(A. Picot) Department of Plant Pathology, University of California, Davis, Kearney Agricultural Center, Parlier, California 93648, United States.
▽(D. Caron) Diagnophyt, 7 Chemin de Saint-Sernin, F-31 290 Villenouvelle, France.

Funding

We are grateful to ARVALIS-Institut du végétal and the ANRT (National Agency for Research and Technology) for their financial support as part of a Ph.D. grant.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Commission Regulation (EC) No. 1126/2007. *Official Journal of the European Union*, 2007, 255/14, http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:255:0014:0017:EN:PDF (accessed February 24, 2013).

(2) Humpf, H. U.; Voss, K. A. Effects of thermal food processing on the chemical structure and toxicity of fumonisin mycotoxins. *Mol. Nutr. Food Res.* **2004**, *48*, 225−269.

(3) Lemmens, M.; Scholz, U.; Berthiller, F.; Dall'Asta, C.; Koutnik, A.; Schumacher, R.; Adam, G.; Buerstmayr, H.; Mesterházy, A.; Krska, R.; Ruckenbauer, P. The ability to detoxify the mycotoxin deoxynivalenol colocalizes with a major quantitative trait locus for Fusarium head blight resistance in wheat. *Mol. Plant−Microbe Interact.* **2005**, *23*, 977−986.

(4) Berthiller, F.; Lemmens, M.; Werner, U.; Krska, R.; Hauser, M. T.; Adam, G.; Schuhmacher, R.. Short review: metabolism of the *Fusarium* mycotoxins deoxynivalenol and zearalenone in plants. *Mycotoxin Res.* **2007**, *23*, 68−72.

(5) Boutigny, A. L.; Richard-Forget, F.; Barreau, C. Natural mechanisms for cereal resistance to the accumulation of *Fusarium* trichothecenes. *Eur. J. Plant Pathol.* **2008**, *121*, 411−423.

(6) Picot, A.; Barreau, C.; Pinson-Gadais, L.; Caron, D.; Lannou, C.; Richard-Forget, F. Factors of the *Fusarium verticillioides*−maize interaction modulating fumonisin production. *Crit. Rev. Microbiol.* **2010**, *36*, 221−231.

(7) Reverberi, M.; Ricelli, A.; Zjalic, S.; Fabbri, A. A.; Fanelli, A. L. Natural functions of mycotoxins and control of their biosynthesis in fungi. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 899−911.

(8) Chipley, J. R.; Uraih, N. Inhibition of *Aspergillus* growth and aflatoxin release by derivatives of benzoic acid. *Appl. Environ. Microbiol.* **1980**, *40*, 352−357.

(9) Sinha, K. K.; Premlata, S. Effect of some phenolics on aflatoxin production and growth of *Aspergillus parasiticus*. *Indian Phytopathol.* **1981**, *34*, 530−531.

(10) Fajardo, J. E.; Waniska, R. D.; Cuero, R. G. Phenolic compounds in peanut seeds: enhanced elicitation by chitosan and effects on growth and aflatoxin B1 production by *Aspergillus flavus*. *Food Biotechnol.* **1995**, *9*, 59−78.

(11) Norton, R. A. Inhibition of aflatoxin B1 biosynthesis in *Aspergillus flavus* by anthocyanidins and related flavonoids. *J. Agric. Food Chem.* **1999**, *47*, 1230−1235.

(12) Miller, J. D.; Fielder, D. A.; Dowd, P. F.; Norton, R. A.; Collins, F. W. Isolation of 4-acetyl-benzoxazolin-2-one (4-ABOA) and diferuloylputrescine from an extract of Gibberella ear rot-resistant corn that blocks mycotoxin biosynthesis, and the insect toxicity of 4-

ABOA and related compounds. *Biochem. Syst. Ecol.* **1996**, *24*, 647−658.

(13) Boutigny, A. L.; Barreau, C.; Atanasova-Penichon, V.; Verdal-Bonnin, M. N.; Pinson-Gadais, L.; Richard-Forget, F. Ferulic acid, an efficient inhibitor of type B trichothecene biosynthesis and *Tri* gene expression in *Fusarium* liquid cultures. *Mycol. Res.* **2009**, *113*, 746−753.

(14) Beekrum, S.; Govinden, R.; Padayachee, T.; Odhav, B. Naturally occurring phenols: a detoxification strategy for fumonisin B1. *Food Addit. Contam.* **2003**, *20*, 490−493.

(15) Samapundo, S.; De Meulenaer, B.; Osei-Nimoh, D.; Lamboni, Y.; Debevere, J.; Devlieghere, F. Can phenolic compounds be used for the protection of corn from fungal invasion and mycotoxin contamination during storage? *Food Microbiol.* **2007**, *24*, 465−473.

(16) Dambolena, J. S.; López, A. G.; Cánepa, M. C.; Theumer, M. G.; Zygadlo, J. A.; Rubinstein, H. R. Inhibitory effect of cyclic terpenes (limonene, menthol, menthone and thymol) on *Fusarium verticillioides* MRC 826 growth and fumonisin B1 biosynthesis. *Toxicon* **2008**, *51*, 37−44.

(17) Dambolena, J. S.; Zygadlo, J. A.; Rubinstein, H. R. Antifumonisin activity of natural phenolic compounds: a structure−property−activity relationship study. *Int. J. Food Microbiol.* **2011**, *145*, 140−146.

(18) Bily, A. C.; Reid, L. M.; Taylor, J. H. Dehydrodimers of ferulic acid in maize grain pericarp and aleurone: resistance factors to *Fusarium graminearum*. *Phytopathology* **2003**, *93*, 712−719.

(19) Santiago, R.; Malvar, R. Role of dehydrodiferulates in maize resistance to pests and diseases. *Int. J. Mol. Sci.* **2010**, *11*, 691−703.

(20) Masood, A.; Dogra, J. V. V.; Jha, A. K. The influence of colouring and pungent agents of red chilli (*Capsicum annum*) on growth and aflatoxin production by *Aspergillus flavus*. *Lett. Appl. Microbiol.* **1994**, *18*, 184−186.

(21) Santos, L.; Kasper, R.; Gil-Serna, J.; Marín, S.; Sanchis, V.; Ramos, A. J. Effect of *Capsicum* carotenoids on growth and ochratoxin A production by chilli and paprika *Aspergillus* spp. isolates. *Int. J. Food Microbiol.* **2010**, *142*, 354−359.

(22) Kurilich, A. C.; Juvik, J. A. Simultaneous quantitation of carotenoids and tocopherols in corn kernel extract by HPLC. *J. Liq. Chromatogr. Relat. Technol.* **1999**, *22*, 2925−2934.

(23) Chander, S.; Meng, Y.; Zhang, Y.; Yan, J.; Li, J. Comparison of nutritional traits variability in selected eighty-seven inbreds from Chinese maize (*Zea mays* L.) gemplasm. *J. Agric. Food Chem.* **2008**, *56*, 6506−6511.

(24) Berardo, N.; Mazzinelli, G.; Valoti, P.; Lagana, P.; Redaelli, R. Characterization of maize germplasm for the chemical composition of the grain. *J. Agric. Food Chem.* **2009**, *57*, 2378−2384.

(25) Xu, J. G.; Hiu, Q. P.; Wang, X. D.; Luo, J. Y.; Liu, Y.; Tian, C. R. Changes in the main nutrient, phytochemicals, and antioxidant activity in yellow corn grain during maturation. *J. Agric. Food Chem.* **2010**, *58*, 5751−5756.

(26) Atanasova-Penichon, V.; Pons, S.; Pinson-Gadais, L.; Picot, A.; Marchegay, G.; Bonnin-Verdal, M. N.; Ducos, C.; Barreau, C.; Roucolle, J.; Sehabiague, P.; Carolo, P.; Richard-Forget, F. Chlorogenic acid and maize ear rot resistance: a dynamic study investigating *Fusarium graminearum* development, deoxynivalenol production, and phenolic acid accumulation. *Mol. Plant−Microb. Interact.* **2012**, *12*, 1605−1616.

(27) Sampietro, D. A.; Fauguel, C. M.; Vattuone, M. A.; Presello, D. A.; Catalán, C. A. N. Phenylpropanoids from maize pericarp: resistance factors to kernel infection and fumonisin accumulation by *Fusarium verticillioides*. *Eur J. Plant Pathol.* **2013**, *135*, 105−113.

(28) Picot, A.; Barreau, C.; Pinson-Gadais, L.; Caron, D.; Lannou, C.; Richard-Forget, F. The dent stage of maize kernels is the most conducive for fumonisin biosynthesis under field conditions. *Appl. Environ. Microbiol.* **2011**, *77*, 8382−8390.

(29) *Toxigenic Fusarium Collection. A collection of Fusarium spp characterized for their genotypes and toxinogenic potential*; Mycology and Food Safety, INRA Bordeaux-Aquitaine: Villenave d'Ornon, 2012; http://www7.bordeaux-aquitaine.inra.fr/mycsa_eng/des_ressources_biologiques_uniques/la_collection_de_fusarium_toxinogenes (accessed February, 24th, 2013).

(30) Shephard, G. S.; Sydenham, E. W.; Thiel, P. G.; Gelderblom, W. C. Quantitative determination of fumonisins B1 and B2 by high-performance liquid chromatography with fluorescence detection. *J. Liq. Chromatogr.* **1990**, *13*, 2077−2080.

(31) Falavigna, C.; Cirlini, M.; Galaverna, G.; Sforza, S.; Dossena, A.; Dall'Asta, C. LC/ESI-MS/MS analysis outlines the different fumonisin patterns produced by *F. verticillioides* in culture media and in maize kernels. *J. Mass Spectrom.* **2012**, *47*, 1170−1176.

(32) Britton, G. Structure and properties of carotenoids in relation to function. *FASEB J.* **1995**, *9*, 1551−1558.

(33) Gómez-Fernández, J. C.; Villalían, J.; Aranda, F. J.; Ortiz, A.; Micol, V.; Coutinho, A.; Berberan-Santos, M. N.; Prieto, M. J. E. Localisation of α-tocopherol in membranes. *Ann. N. Y. Acad. Sci.* **1989**, *570*, 109−120.

(34) Craft, N. E.; Soares, J. H. Relative solubility, stability and absorptivity of lutein and β-carotene in organic solvents. *J. Agric. Food Chem.* **1992**, *40*, 431−434.

(35) Norton, R. A. Effect of carotenoids on aflatoxin B1 synthesis by *Aspergillus flavus*. *Phytopathology* **1997**, *87*, 814−821.

(36) Butchko, R. A. E.; Plattner, R. D.; Proctor, R. H. Deletion analysis of *FUM* genes involved in tricarballylic ester formation during fumonisin biosynthesis. *J. Agric. Food Chem.* **2006**, *54*, 9398−9404.

(37) Narasaiah, K. V.; Sashidhar, R. B.; Subramanyam, C. Biochemical analysis of oxidative stress in the production of aflatoxin and its precursor intermediates. *Mycopathologia* **2006**, *162*, 179−189.

(38) Seo, J. A.; Proctor, R. H.; Plattner, R. D. Characterization of four clustered and coregulated genes associated with fumonisin biosynthesis in *Fusarium verticillioides*. *Fungal Genet Biol.* **2001**, *34*, 155−165.

(39) Kim, J. H.; Mahoney, N.; Chan, K. L.; Molyneux, R. J.; Campbell, B. C. Controlling food-contaminating fungi by targeting antioxidant stress-response system with natural phenolic compounds. *Appl. Microbiol. Biotechnol.* **2006**, *70*, 735−739.

(40) Kim, J. H.; Yu, J.; Mahoney, N.; Chan, K. L.; Molyneux, R. J.; Varga, J.; Bhatnagar, D.; Cleveland, T. E.; Nierman, W. C.; Campbell, B. C. Elucidation of the functional genomics of antioxidant-based inhibition of aflatoxin biosynthesis. *Int. J. Food Microbiol.* **2007**, *122*, 49−60.

(41) Assabgui, R. A.; Reid, L. M.; Hamilton, R. I.; Arnason, J. T. Correlation of kernel (*E*)-ferulic acid content of maize with resistance to *Fusarium graminearum*. *Phytopathology* **1993**, *83*, 949−953.

(42) Santiago, R.; Reid, L. M.; Arnason, J. T.; Zhu, X.; Martinez, N.; Malvar, R. A. Phenolics in maize genotypes differing in susceptibility to Gibberella stalk rot (*Fusarium graminearum* Schwabe). *J. Agric. Food Chem.* **2007**, *55*, 5186−5193.

(43) Nicholson, R. L.; Hammerschidt, R. Phenolic compounds and their role in disease resistance. *Annu. Rev. Phytopathol.* **1992**, *30*, 369−389.

(44) Siranidou, E.; Kang, Z.; Buchenauer, H. Studies on symptom development, phenolic compounds and morphological defence responses in wheat cultivars differing in resistance to Fusarium head blight. *J. Phytopathol.* **2002**, *150*, 200−208.

(45) Gao, X.; Shim, W. B.; Göbel, C.; Kunze, S.; Feussner, I.; Meeley, R.; Balint-Kurti, P.; Kolomiets, M. Disruption of a maize 9-lipoxygenase results in increased resistance to fungal pathogens and reduced levels of contamination with mycotoxin fumonisin. *Mol. Plant−Microbe Interact.* **2007**, *20*, 922−933.

(46) Richard-Forget, F.; Gauillard, F.; Hugues, M.; Jean-Marc, T.; Boivin, P.; Nicolas, J. Inhibition of horse bean and germinated barley lipoxygenase by some phenolic compounds. *J. Food Sci.* **1995**, *60*, 1325−1329.