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# Cinnamic-Derived Acids Significantly Affect *Fusarium graminearum* Growth and In Vitro Synthesis of Type B Trichothecenes

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## ABSTRACT

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The impact of five phenolic acids (ferulic, coumaric, caffeic, syringic, and *p*-hydroxybenzoic acids) on fungal growth and type B trichothecene production by four strains of *Fusarium graminearum* was investigated. All five phenolic acids inhibited growth but the degree of inhibition varied between strains. Our results suggested that the more lipophilic phenolic acids are, the higher is the effect they have on growth. Toxin accumulation in phenolic acid-supplemented liquid glucose, yeast extract,

and peptone cultures was enhanced in the presence of ferulic and coumaric acids but was reduced in the presence of *p*-hydroxybenzoic acid. This modulation was shown to correlate with a regulation of *TRI5* transcription. In this study, addition of phenolic acids with greater antioxidant properties resulted in a higher toxin accumulation, indicating that the modulation of toxin accumulation may be linked to the antioxidant properties of the phenolic acids. These data suggest that, in planta, different compositions in phenolic acids of kernels from various cultivars may reflect different degrees of sensitivity to “mycotoxinogenesis.”

*Additional keywords:* ferulic acid.

*Fusarium graminearum* is a prevalent phytopathogen on cereals. In planta, it induces Fusarium head blight, also known as Fusarium scab, of small grain cereals and maize ear rot, two diseases with a huge economic impact in many cereal-growing areas around the world. Furthermore, some *F. graminearum* strains can produce deoxynivalenol (DON) and its 3- and 15-acetylated forms (3ADON and 15ADON, respectively) that belong to the type B trichothecenes (TCTB) family of mycotoxins. DON toxicity has been well documented (12) and represents a real public health problem.

TCTB are highly stable. Because these toxins are resistant to biodegradation and food processing and there is no suitable detoxification procedure available at present, the occurrence of TCTB in kernels cannot be reduced by post-harvest treatments. In June 2005, maximum DON contamination levels in cereals and corn food and feed were set in Europe (EC No856/2005). These levels were revised in July 2007 (EC No1126/2007).

Implementing strategies to provide an efficient management of the mycotoxic risk requires a better understanding of events leading to TCTB accumulation in kernels. A number of strategies for reducing TCTB concentrations in wheat and maize ears are currently under investigation (1) and among the most promising is the identification of naturally occurring plant compounds able to inhibit TCTB biosynthesis.

The TCTB biosynthesis pathway is fairly well understood (24) as a result of the enhanced effort at identifying biosynthetic steps and genes involved in mycotoxin production developed during the last 10 years. Nearly all the genes implicated in the TCTB biosynthesis pathway, commonly named *TRI* genes, have been

identified. Most of them are localized in one genomic locus which is referred as the “Fg*TRI5* cluster”. Among the clustered genes, *TRI5* plays a crucial role in TCTB production because it encodes the trichodiene synthase that catalyzes the first step of the TCTB biosynthesis pathway (24). One of the key challenges that research on *F. graminearum* now has to answer is the elucidation of the effects of environmental factors on initiation or repression of toxin biosynthesis. Knowledge concerning the factors that could control TCTB production has been recently updated (10). The main consistent data published recently concern the effects of carbon and nitrogen sources (20) and the identification of specific amines such as agmatin and putrescine as potent inducers of trichothecenes B biosynthesis (13). A previous work has also demonstrated that pro-oxidant compounds significantly modulated DON and 15ADON biosynthesis by *F. graminearum*. In particular, H<sub>2</sub>O<sub>2</sub> was shown to have a long-term activating effect on TCTB accumulation while the kinetics of fungal biomass accumulation was not affected (32). This event was indicated to be linked to a general upregulation of some *TRI* genes involved in the DON and 15ADON biosynthesis pathway (27,31). Because plant metabolites with pro-oxidant properties have a strong effect on mycotoxin biosynthesis, we could reasonably suppose that plant secondary metabolites with antioxidant properties may also interfere with mycotoxin biosynthesis. Phenolic acids are typical cereal and corn compounds described as potent antioxidants (7,21,36). In corn kernels, monomers and dimers of ferulic acid are largely predominant (5). Phenolic compounds have been extensively studied with regard to Fusarium head blight and have been shown to be involved in the disease resistance (7,17). Moreover, the content in phenolic compounds was shown to be higher in resistant maize silks infected with *F. graminearum* than in sensitive *Fusarium* spp.-infected maize silks (33). Furthermore, some phenolic compounds have been reported as fungistatic and inhibitors of toxin production. For example, caffeic acid and

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ferulic acid can reduce fumonisin B<sub>1</sub> production by *F. verticillioides* (6). Aflatoxin production by *Aspergillus* spp. is reduced by ferulic acid, syringaldehyde and sinapinic acid (18), and caffeic acid (22). Finally, previous studies have also suggested that caffeic, ferulic, *p*-coumaric, and 3-hydroxybenzoic acids were able to act on 15ADON production by *F. graminearum* (5,9). However, these reports required to be clarified, and the mechanisms by which phenolic acids could affect trichothecenes production remained to be elucidated.

Thus, the main objective of this investigation was to clarify the effect of phenolic acids on TCTB accumulation. Five phenolic acids (ferulic, *p*-coumaric, caffeic, syringic, and *p*-hydroxybenzoic acids) were considered. These phenolic acids were selected because of their relative abundance in cereals and their well-known antioxidant properties. Their effects on *F. graminearum* growth and on DON and 15ADON accumulation were investigated and attempts to establish structure-modulating efficiency relationships were achieved.

## MATERIALS AND METHODS

***F. graminearum* strains.** Four strains of *F. graminearum* were used: *F. graminearum* strain CBS185.32 (Centraal Bureau voor Schimmelkulturen, The Netherlands), strain 245AP4 (Hort-Research and AgResearch, New Zealand), and strains INRA 159 and INRA 202 (collection of INRA MycSA, France). They all produce predominantly 15ADON and a lower amount of DON. Usually, INRA 159 produces very low amounts of toxins (<1 µg of toxin per gram of dry fungal biomass after 15 days of incubation in glucose, yeast extract, and peptone (GYEP) medium at 25°C) (26) while CBS185.32 and INRA 202 are strong toxin-producing strains (>30 µg of toxin per gram of dry fungal biomass after 15 days of incubation in GYEP medium at 25°C); the strain 245AP4 produces average amounts of toxins (close to 10 µg of toxin per gram of dry fungal biomass after 15 days of incubation in GYEP medium at 25°C). Strains were maintained on potato dextrose agar (PDA) (Sigma-Aldrich, St. Louis) slants at 4°C. When inocula were required, spore suspensions were prepared by adding sterile distilled water to the slants followed by gentle shaking. Spores concentrations were evaluated using a Thoma cell.

**Effect of phenolic acids on the growth of *F. graminearum* CBS185.32, INRA 159, INRA 202, and 245AP4.** Growth inhibition tests were performed on PDA medium in petri dishes (diameter = 9 cm) inoculated at the centre with 5 µl of spore suspension at 10<sup>3</sup> spores ml<sup>-1</sup>. PDA media were supplemented with ferulic, *p*-coumaric, caffeic, syringic, or *p*-hydroxybenzoic acid (Sigma-Aldrich) at 2, 3, 4, 5, or 6 mmol liter<sup>-1</sup>. For each condition, plates (in triplicate) were incubated at 25°C for 5 days; that is to say, until 80% of the petri dish area was colonized in the control dishes. In each plate, the colony diameter value was given as the average of two perpendicular diameter length measurements (in centimeters). Growth inhibition values (GIVs) were expressed as the average percentage of the petri dish covered by mycelium in the control dishes minus the percentage overgrown by the fungus in the treated dishes. For each phenolic acid, the relation  $GIV = f([\text{phenolic acid}])$  was plotted and concentration that inhibits 50% of growth (IC<sub>50</sub>) was determined by linear regression for  $GIV = 50\%$ .

**Estimation of the relative lipophilicities of the tested phenolic acids.** Samples were prepared and analyzed by reverse-phase high-performance liquid chromatography (HPLC) using the procedure of Ponts et al. (32). HPLC analyses were monitored at 280 nm. The retention times of the phenolic acids were compared and classified in order to evaluate their relative degrees of lipophilicity.

**Effect of phenolic acids on DON and 15ADON accumulation in liquid cultures of *F. graminearum* CBS185.32, INRA 159, INRA 202, and 245AP4.** The liquid medium GYEP (26)

was used throughout the study. The medium was supplemented before inoculation with ferulic, *p*-coumaric, caffeic, syringic, or *p*-hydroxybenzoic acid. The final concentration of each phenolic acid was 0.5 mmol liter<sup>-1</sup>. Then, 100 ml of GYEP in 500-ml Erlenmeyer flasks was inoculated with 10<sup>6</sup> spores and incubated at 25°C and 150 rpm into darkness in a Multitron incubator shaker (INFORS AG, Switzerland). Cultures were stopped by filtering under vacuum through Whatman paper number 4. The filtrates were stored at -20°C. TCTB levels were determined in culture filtrates, and fungal biomass production was determined by weighting the mycelia after 48 h of freeze drying. Each culture condition was repeated in triplicate.

TCTB were analyzed according to the protocol described by Ponts et al. (32). Briefly, 15 ml of filtrates was extracted with 30 ml of ethyl acetate; 20 ml of the organic phase was evaporated to dryness at 70°C under a nitrogen stream. Dried samples were resuspended in 200 µl of methanol/water (50%, vol/vol) before analysis by HPLC-DAD. Quantification was performed by using external calibration with DON and 15ADON standard solutions prepared from commercial pure powders (Sigma-Aldrich). Toxin levels were expressed in micrograms of toxin per gram of dry fungal biomass divided by the toxin yield measurement after 15 days of control culture. Because effects of phenolic acids on DON and 15ADON accumulation were consistent for all tested strains, DON and 15ADON yields were summed without distinguishing both chemical species (noted as DON+15ADON). Data are expressed as arithmetic mean values ± standard deviation. Values were compared by computing standard Student's *t* test. The *P* value 0.05 was chosen as the point of statistical significance.

**Extraction of total RNA and preparation of cDNA.** Fresh mycelium (200 mg) of 4-day-old cultures was grounded in 1 ml of TRIzol reagent (Invitrogen Life Technologies) using the TissueLyser System (Qiagen). Total RNA was extracted using the TRIzol reagent manufacturer's instructions. The quality of the RNA was verified by agarose gel electrophoresis. Total RNA (3 µg) was reverse transcribed using the Superscript II First-Strand Synthesis System (Invitrogen Life Technologies).

**Real-time polymerase chain reaction analysis.** Abundance of the transcripts of the gene *TRI5* was evaluated using 3 µl of each cDNA solution diluted 10 times by real-time polymerase chain reaction (PCR) using LightCycler FastStart DNA Master SYBR Green I and a LightCycler real-time detector (Roche Applied Science). Expression of  $\beta$ -*tubulin* was used as an endogenous reference. Analyses were performed in triplicate. Primers used to amplify these genes were Tri5-F GACCCTAAGCGACTACAG and Tri5-R GTGCTACGGATAAGGTTTC (35) for *TRI5* amplification, and  $\beta$ 2a GGTAACCAAATCGGTGCTGCTTTC (15) and  $\beta$ tub-R536 ( $\beta$ 2) GATTGACCGAAAACGAAGTTG (30) for  $\beta$ -*tubulin* amplification. They were used at a final concentration of 500 nM. MgCl<sub>2</sub> was added to the PCR mix to achieve a final concentration of 4 mM. Amplification was performed in triplicate for each of the three biological replications.

cDNA sample solutions (10 µl each) were mixed together to prepare a standard mixture to be used to determine PCR efficiencies (*E*) by external calibration, according to a previously described protocol (31). *E* was calculated using the LightCycler Software (version 4.05; Roche Applied Science). PCR efficiencies and standard errors obtained for reference and *TRI5* genes were 1.93 ± 0.03 and 1.97 ± 0.02, respectively. All cDNA samples were tested for residual genomic DNA (31). The absence of genomic DNA was verified by running both melting curves and agarose gels analyses on the final PCR products. The same procedure was followed to check the absence of nonspecific PCR amplification or primer dimers formation.

**Real-time PCR data analysis.** Data were analyzed according to the procedure of Ponts et al. (31). The crossing point (C<sub>p</sub>) values obtained experimentally (mean value of three replicates) for  $\beta$ -

*tubulin* were compared in order to verify the stability of  $\beta$ -*tubulin* expression in the presence of phenolic acids. When the cultures were treated with either ferulic or *p*-hydroxybenzoic acid, data were analyzed with the REST software. The expression levels of *TR15*, normalized to reference gene expression, are expressed as a regulation factor in the treated condition relative to the control, with the ratio =  $(ETR15)^{ACP TR15 (control-treated)} / (Eref)^{ACP ref (control-treated)}$ .  $P = 0.001$  was chosen as the statistical point throughout.

## RESULTS

**Effect of phenolic acids on the growth of *F. graminearum* CBS185.32, INRA 159, INRA 202, and 245AP4.** When considering the CBS185.32 strain, phenolic acid  $IC_{50}$  values ranged from 1.7 mmol liter<sup>-1</sup> to >15 mmol liter<sup>-1</sup> (Table 1). The lower the  $IC_{50}$  value, the higher the fungicidal properties of the phenolic acid under consideration. Thus, ferulic acid can be considered to be the most toxic compound and *p*-hydroxybenzoic acid the least toxic one (little growth inhibition). Similar experiments were performed on *F. graminearum* INRA 159, *F. graminearum* INRA 202, and *F. graminearum* 245AP4; results are reported in Table 1. It clearly appears that ranking of the fungicidal activity of the phenolic acids is independent of the *Fusarium* strain tested. Ferulic acid is the most toxic compound, followed by *p*-coumaric acid, syringic acid, caffeic acid, and *p*-hydroxybenzoic acid.

**Lipophilic characteristics of phenolic acids and their effects on growth of *F. graminearum*.** Because previous studies indicated that fungitoxic properties of phenolic compounds may primarily depend on their lipophilic properties (16), it was investigated whether our experimental  $IC_{50}$  values of phenolic acids

TABLE 1. Experimental concentration that inhibits 50% of growth ( $IC_{50}$ ) values of five phenolic acids measured on the growth of *Fusarium graminearum* CBS185.32, INRA 202, 245AP4, and INRA 159

Phenolic acid	Experimental $IC_{50}$ (mmol liter <sup>-1</sup> )			
	CBS 185.32	INRA 202	245AP4	INRA159
Ferulic acid	1.7	1.8	2.2	0.7
<i>p</i> -Coumaric acid	4.1	4.2	4.1	1.0
Syringic acid	5.2	5.7	6.2	3.5
Caffeic acid	6.6	6.4	7.1	4.0
<i>p</i> -Hydroxybenzoic acid	>15.0 <sup>a</sup>	10.3	8.4	6.6

<sup>a</sup> Little growth inhibition.

TABLE 2. Retention times (in minutes) measured when separation of phenolic acids is performed by reverse-phase high-performance liquid chromatography following four different methods<sup>a</sup>

Phenolic acid	Jandera			TCTB analysis method <sup>b</sup>
	Bolarinwa and Linseisen (8)	et al. (19)	Antolovich et al. (3)	
Ferulic acid	51.0	53.3	14.0	11.0
<i>p</i> -Coumaric acid	42.3	37.2	13.8	10.0
Syringic acid	36.5	25.0	12.3	8.1
Caffeic acid	30.9	21.3	12.2	7.6
<i>p</i> -Hydroxybenzoic acid	ND	ND	ND	6.7

<sup>a</sup> ND = not determined.

<sup>b</sup> TCTB = type B trichothecenes; described in Materials and Methods.

could be linked to their degrees of lipophilicity. Lipophilicities of phenolic acids were scored using their retention times when separated by the reverse-phase HPLC method (see Materials and Methods). Our results were compared with retention times obtained with other reverse-phase HPLC methods (3,8,19). All data are consistent (Table 2), and a classification of phenolic acids according to their decreasing degrees of lipophilicity can be established: phenolic acids with higher retention times are more lipophilic. Ferulic acid is the most lipophilic compound, followed by *p*-coumaric acid, syringic acid, caffeic acid, and *p*-hydroxybenzoic acid. For each *F. graminearum* strain and each reverse-phase HPLC method, Pearson's correlation coefficients were calculated between  $IC_{50}$  values of phenolic acids and their retention times (Table 3). Depending on the HPLC method that was used and the *F. graminearum* strain considered, correlation coefficients ranged from -0.8849, when retention times were determined with the method from Antolovich et al. (3) and for strain CBS185.32, up to -0.9988, when retention times were determined with the method from Jandera et al. (19) for isolate INRA 202. These high values support the initial hypothesis that more lipophilic phenolic acids have greater fungistatic efficiency.

**Effect of phenolic acids on DON and 15ADON accumulation in liquid cultures of *F. graminearum* CBS185.32, INRA 159, INRA 202, and 245AP4.** Effects of ferulic, *p*-coumaric, syringic, caffeic, and *p*-hydroxybenzoic acids on DON and 15ADON accumulation were first studied at 0.5 mmol liter<sup>-1</sup> in liquid cultures of *F. graminearum* CBS185.32. Cultures were grown for 4, 11, and 15 days. Whatever the considered phenolic acid, a 0.5 mmol liter<sup>-1</sup> concentration is much lower than the  $IC_{50}$  of the compounds, and fungal growth after 11 and 15 days in GYEP culture was not affected (data not shown). As a reference, the effect of butylhydroxytoluene (BHT) at 0.5 mmol liter<sup>-1</sup> after 11 days of culture was also determined because of its efficient antioxidant properties. BHT at 0.5 mmol liter<sup>-1</sup> did not affect biomass accumulation in GYEP cultures of *F. graminearum* CBS 185.32 (data not shown). After 11 days of culture, DON+15ADON accumulation was 24 times higher in the presence of BHT (Fig. 1). Ferulic acid and *p*-coumaric acid both activated DON+15ADON accumulation (11 and 20 times, respectively). Caffeic acid seemed also to enhance toxin accumulation but high variance of toxin yields in caffeic acid-supplemented cultures strongly lowers the significance of this observation (biological variation). Syringic acid supplementation had no effect on DON+15ADON accumulation. Only *p*-hydroxybenzoic acid had a significant inhibitory effect, with three times less toxin accumulating in supplemented cultures than in control cultures ( $P = 0.02$ ). These trends remain observable after 15 days of culture but with weaker intensities (Fig. 1). The impact of ferulic acid at 0.5 mmol liter<sup>-1</sup> or *p*-hydroxybenzoic acid at 0.5 mmol liter<sup>-1</sup> on the levels of *TR15* gene expression was evaluated in 4-day-old cultures of *F. graminearum* (CBS 185.32). Four-day-old cultures were chosen for investigation because it has been previously demonstrated that transcripts of the *TR15* gene reach their highest level between 3 and 4 days of culture in GYEP media to then attain a low residual level (13,31). Analysis of the  $\beta$ -*tubulin* Cp values measured in control and phenolic-acid-supplemented cultures showed that levels of  $\beta$ -*tubulin* mRNA in

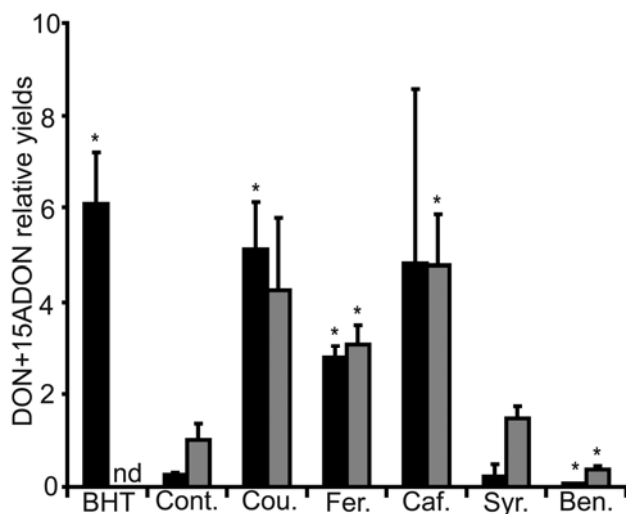
TABLE 3. Pearson's correlation coefficients computed between concentration that inhibits 50% of growth ( $IC_{50}$ ) values of phenolic acids measured for four *Fusarium graminearum* strains (CBS 185.32, INRA 202, 245AP4, and INRA 159) and retention times of phenolic acids given in Table 2

HPLC method <sup>a</sup>	CBS185.32	INRA202	245AP4	INRA159
Bolarinwa and Linseisen (8)	-0.9976	-0.9898	-0.9920	-0.9241
Jandera et al. (19)	-0.9840	-0.9988	-0.9921	-0.9209
Antolovich et al. (3)	-0.8849	-0.9083	-0.9535	-0.9968
TCTB analysis method	-0.9580	-0.9431	-0.9961	-0.9620

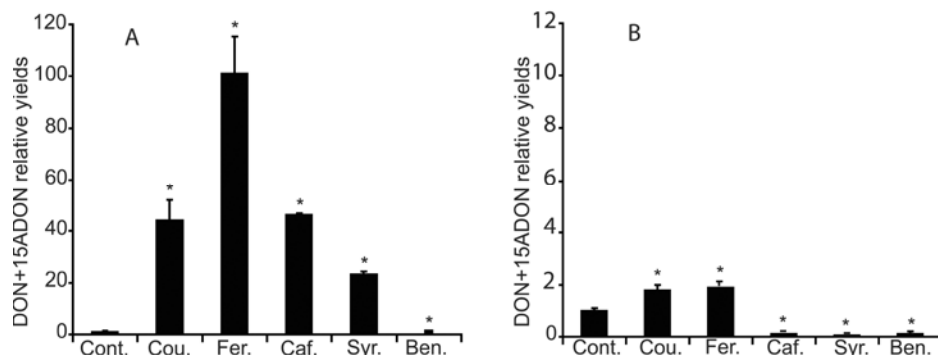
<sup>a</sup> HPLC = high-performance liquid chromatography; TCTB = type B trichothecenes; described in Materials and Methods.

total RNA were not significantly affected by the treatment, supporting the use of  $\beta$ -tubulin as a reference gene. The expression values of *TRI5* relative to the reference gene were expressed as a up- or downregulation factor in the treated conditions relative to the control. The ferulic acid treatment was shown to induce a significant upregulation of *TRI5* with a regulation factor of  $4.3 \pm 1.3$  ( $C_{pTRI5} = 26.9 \pm 0.25$  in control cultures and  $25.2 \pm 0.18$  in ferulic-acid-treated cultures) in accordance with the enhancement of the DON+15ADON accumulation that was reported above. A weak downregulation of *TRI5* (regulation factor of  $1.82 \pm 0.25$ ) that was, however, not significant was observed in *p*-hydroxybenzoic-acid-supplemented cultures. These data indicated that the effect of phenolic acids on TCTB accumulation could be linked to a regulation of the expression of the *TRI5* gene involved in the trichothecenes biosynthetic pathway.

Impact of phenolic acids on TCTB production was investigated in 15-day-old cultures of *F. graminearum* INRA 159, INRA 202, and 245AP4. Whatever the considered strain and phenolic acid,



**Fig. 1.** Effects of five phenolic acids or butylhydroxytoluene (BHT) (0.5 mmol liter<sup>-1</sup>) on deoxynivalenol (DON) plus 15-acetylated DON (DON+15ADON) accumulation in 11-day-old (black bars) and 15-day-old (gray bars) glucose, yeast extract, and peptone cultures of *Fusarium graminearum* CBS185.32. Cont. = control, Cou. = *p*-coumaric acid, Fer. = ferulic acid, Caf. = caffeic acid, Syr. = syringic acid, Ben. = *p*-hydroxybenzoic acid, nd = not determined. Data obtained from three biological replications were normalized relative to the average DON+15ADON yield in 15-day-old control cultures (=50  $\mu$ g of DON and 15ADON per gram of dry biomass). Asterisk (\*) indicates a significant difference when compared with the corresponding control ( $P \leq 0.05$ ).



**Fig. 2.** Effects of five phenolic acids (0.5 mmol liter<sup>-1</sup>) on deoxynivalenol (DON) plus 15-acetylated DON (DON+15ADON) accumulation in 15-day-old glucose, yeast extract, and peptone cultures of *Fusarium graminearum* **A**, 245AP4 and **B**, INRA 202. Cont. = control, Cou. = *p*-coumaric acid, Fer. = ferulic acid, Caf. = caffeic acid, Syr. = syringic acid, Ben. = *p*-hydroxybenzoic acid. For each strain, data obtained from three biological replications were normalized relative to the average DON+15ADON yield in corresponding control cultures (INRA 202: 70  $\mu$ g of DON+15ADON per gram of dry fungal biomass; 245AP4: 8  $\mu$ g of DON+15ADON per gram of dry fungal biomass). Asterisk (\*) indicates a significant difference when compared with the corresponding control ( $P \leq 0.05$ ).

fungal biomass production was not affected by the 0.5 mmol liter<sup>-1</sup> concentration used (data not shown). One of the tested strains, INRA 159, usually produces very low amounts of toxins (<1  $\mu$ g of toxin per gram of dry fungal biomass). In the presence of ferulic acid, toxin production by this *F. graminearum* strain seems to be slightly enhanced whereas, in the presence of *p*-hydroxybenzoic acid, it seems to be slightly reduced. However, none of the tested phenolic acid had a quantifiable effect (data not shown). With regard to *F. graminearum* strains 245AP4 (Fig. 2A) and INRA 202 (Fig. 2B), DON+15ADON accumulation was significantly enhanced in the presence of *p*-coumaric acid (2 times in the case of INRA 202 and 44 times in the case of 245AP4) and ferulic acid (2 times in the case of INRA 202 and 100 times in the case of 245AP4). *p*-Hydroxybenzoic acid significantly inhibited DON+15ADON accumulation for both strains. Finally, opposite effects of caffeic acid and syringic acid were observed as far as these two *F. graminearum* strains are concerned: caffeic acid and syringic acid enhanced toxin production by *F. graminearum* strain 245AP4 but inhibited it with regard to strain 202.

**Antioxidant characteristics of phenolic acids and their effect on DON and 15ADON accumulation in liquid cultures of *F. graminearum*.** Because previous studies have suggested that DON and 15ADON accumulation could be significantly affected by the oxidative status of the medium (32), it was investigated whether DON+15ADON yields in phenolic-acid-supplemented cultures could be linked to the antioxidant properties of the compounds. To assess antioxidant capacities of phenolic compounds from literature data is difficult, because results largely depend on the experimental protocol that is used. Nevertheless, authors' classifications of phenolic acids according to their decreasing antioxidant capacities are consistent (21,34). The highest antioxidant capacity is measured for *p*-coumaric acid, followed by ferulic acid, caffeic acid, syringic acid, and then *p*-hydroxybenzoic acid. The antioxidant capacities of these five phenolic acids, valued using the vitamin C equivalent antioxidant capacity (VCEAC) method (21), are presented in Table 4. Pearson's correlation coefficients were computed between the VCEAC values of phenolic acids and DON+15ADON yields in 11- and 15-day-old cultures of *F. graminearum* CBS185.32. For the 11-day-old cultures, correlation coefficient was 0.74. It reached 0.76 when the 15-day-old cultures were considered. Correlation coefficients were close to 0.70 and 0.86 with regard to 15-day-old cultures of the strains INRA 202 and 245AP4, respectively. These correlation coefficient values support the hypothesis that effect of phenolic acids on DON and 15ADON accumulation may be tightly linked with their antioxidant capacities: the more antioxidant the supplemented phenolic acid is, the more DON and 15ADON accumulate in the culture.

## DISCUSSION

According to our results, all the tested phenolic acids inhibited 50% of *F. graminearum* radial growth at concentrations ranging from 0.7 mmol liter<sup>-1</sup> (ferulic acid) to >15 mmol liter<sup>-1</sup> (*p*-hydroxybenzoic acid). Although these concentrations were dependent on the *F. graminearum* strain that was used, the classification of phenolic acids according to their increasing IC<sub>50</sub> values was consistent for all strains. The most toxic compound was ferulic acid, then *p*-coumaric acid, syringic acid, caffeic acid, and *p*-hydroxybenzoic acid. Ferulic and *p*-coumaric acid are both derived from cinnamic acid, while *p*-hydroxybenzoic and syringic acid are derived from benzoic acid. Therefore, our data suggest that cinnamic-derived phenolic acids are roughly more toxic than benzoic acid-derived ones. These data are consistent with those of previous tests on *Aspergillus* spp. growth (11) and on *F. verticillioides* and *F. proliferatum* growth (6). When IC<sub>50</sub> values of phenolic acids were plotted against their lipophilicity scores, high correlation coefficients were obtained. This result suggests a strong link between toxicity of phenolic acids and lipophilic properties: the best fungistatic effect seems to be obtained for the compounds that are highly lipophilic. These data are in accordance with previous results (23,28) that supported the hypothesis that lipophilic properties of phenol compounds are primary factors for their antimicrobial action.

As far as the effect of phenolic acids on TCTB accumulation is concerned, two previous reports indicated that ferulic acid supplementation led to a reduction of 15ADON production by *F. graminearum* (7,9). This is apparently in contrast to our results that suggest that, in GYEP liquid cultures of *F. graminearum*, cinnamic-derived acids, including ferulic acid, are enhancers of DON+15ADON accumulation while benzoic-derived phenolic acids are more likely inhibitors. Two main hypotheses can be raised to explain these apparently contradictory results. The first one concerns the culture medium. In our experiments, contrary to the medium used by Bily (7) and Boutigny et al. (9), the GYEP medium was a very rich medium that also contains yeast extract, the organic compounds of which could interfere with the effect of phenolic acids. The high glucose concentration (50 g liter<sup>-1</sup>) may also influence phenolic acids activity, as reported previously (4). The second hypothesis concerns the time of ferulic acid addition. In our experiments, ferulic acid was supplemented at the same time point as the seeding of the culture and was present during the germination stage; in Bily's experiments (7), ferulic acid was added during the stationary phase of fungal growth. Previous data have reported that the *F. graminearum* toxin-producing potential was closely linked to the biochemical environment surrounding its spores during germination (32).

Statistic analysis of our data has led to high and positive correlation coefficients between antioxidant capacities of the tested phenolic acids and their effect on DON and 15ADON accumulation (0.70 to 0.85, depending on the *F. graminearum* strain under consideration). These observations suggest that the antioxidant capacities of the phenolic acids are tightly linked with their effect on DON+15ADON accumulation: the more antioxidant the supplemented phenolic acid is, the more DON and 15ADON accumulate in the culture. Because cinnamic acids were shown to inhibit the breakdown of hydrogen peroxide by fungi

(14), the enhancement of 15ADON accumulation observed in supplemented cultures was consistent with the activating effect ascribed to hydrogen peroxide by Ochiai et al. (27) and Ponts et al. (31). According to the publication of Amorati et al. (2), the radical scavenging efficiency of phenolic acids is only consistent for pH values higher than their pKa (usually close to 4.5). Therefore, we could reasonably assume that the opposite effect of ferulic acid observed in this study and in the one of Boutigny et al. (9) was linked to differences in pH values of the culture media. Actually, in the minimal medium used by Boutigny et al. (9), the pH value, initially close to 6.5, dramatically decreased as early as 24 h after fungal inoculation to attain a value close to two in 3-day-old cultures (25). In the GYEP cultures performed in this study, the pH value also decreases rapidly but its value remains >4.5 until the end of the cultures (data not shown). In a general manner, antioxidant compounds have already been involved in modulation of toxin production such as aflatoxins (29). According to Passone et al. (29), antioxidant compounds may act via a nonspecific mechanism, involving the perturbation of membrane function and leading to a modification of its permeability. In addition, antioxidant activity of phenolic acids was suggested to play an important part in their ability to regulate gene expression (37). Our results supported the previous conclusion. Accumulation of *TRI5* transcripts was modulated by phenolic acid supplementation, enhanced in the presence of a high antioxidant compound such as ferulic acid and repressed in the presence of a weak antioxidant phenolic acid such as *p*-hydroxybenzoic acid. These data support the previous conclusion of Boutigny et al. (9), indicating that modification of trichothecenes biosynthesis by ferulic acid could be regulated at the transcriptional level.

However, BHT-associated data, with regard to *F. graminearum* strain CBS185.32, do not fit into this correlation. The BHT VCEAC value is 77.4 (21). Although this VCEAC value is lower than the VCEAC values of ferulic acid or *p*-coumaric acid (Table 3), BHT activates DON+15ADON accumulation 24 times (compared with 11 and 20 times for ferulic acid and *p*-coumaric acid, respectively). These data indicate that, even if antioxidant capacities seem to be important properties of the phenolic compounds that influence their ability to interact with DON+15ADON accumulation, they are not sufficient to account for all the observed trends. Because BHT is very lipophilic (highly alkylated), it could be hypothesized that its transfer rate into cells may be high enough to compensate for the lower antioxidant capacity compared with ferulic acid.

This study showed that antioxidant properties of phenolic acids may, in part, explain important effects on DON and 15ADON production by *F. graminearum*. This effect may partially be ascribed to a transcriptional control of the *TRI5* gene. It should be noticed that the 0.5 mmol liter<sup>-1</sup> concentration of phenolic acids that was used throughout the study is compatible with levels of ferulic and *p*-coumaric acid that are commonly found in corn kernels (7). Thus, in planta, according to the in vitro modulation of TCTB biosynthesis associated with phenolic acids, different compositions in phenolic acids of kernels from various cultivars of cereals may reflect different degrees of sensitivity to mycotoxinogenesis. This hypothesis, which strongly fits in with the results of Hamzehzarghani et al. (17), is currently being investigated.

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TABLE 4. Vitamin C equivalent antioxidant capacity (VCEAC) value of five phenolic acids

Phenolic acid	VCEAC values (21)
Ferulic acid	170.5
<i>p</i> -Coumaric acid	186.0
Syringic acid	80.4
Caffeic acid	103.3
<i>p</i> -Hydroxybenzoic acid	4.8

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