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Possible Role of Plant Phenolics in the Production of Trichothecenes by *Fusarium graminearum* Strains on Different Fractions of Maize Kernels

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Four trichothecene-producing strains of *Fusarium graminearum* were grown on three maize grain fractions, whole grain, degermed grain, and the germ, to determine the effect of natural substrates on mycotoxin production. Monitoring the ergosterol content after 25 days of incubation indicated that fungal growth on all grain fractions was comparable. Trichothecene (TCT) production was highest on degermed grain, less on whole grain, and very low or nondetectable on the germ; similar results were found with four different strains. It was concluded that inhibitor(s) of TCT biosynthesis were present in maize germ. The presence of phenolic compounds was investigated in the different fractions. The hydroxamate 4-acetylbenzoxazolin-2-one (4-ABOA), a known inhibitor of mycotoxin production, was found in the degermed and whole grain fractions but not in the germ. Therefore, the TCT inhibition observed on the maize germ fraction used in our study is clearly not linked to 4-ABOA. Other soluble phenolic compounds were found at a much higher concentration in the germ than in the two other fractions. The inhibition property of the soluble ester-bound extracts was tested in liquid culture. A possible role for these compounds is discussed.

KEYWORDS: ***Fusarium*; mycotoxins; trichothecenes; phenolic compounds; maize**

INTRODUCTION

Fusarium graminearum is a common contaminant of cereals and is also known for its ability to produce different mycotoxins such as type B trichothecenes (TCTs) including deoxynivalenol (DON), acetyldeoxynivalenol (ADON), and nivalenol (NIV) and its acetylated form fusarenone X (FX). The toxic effects of mycotoxins on animals and humans are well documented (*1*, *2*). These compounds have been reported worldwide as contaminants of cereals (*3*−*6*). It has been demonstrated that TCTs are heat stable (*7*, *8*) and that their transmission through processed food is a potential problem (*9*, *10*). Moreover, decontamination methods have not been found to be fully satisfactory (*11*). It is therefore necessary to better understand the mechanisms affecting mycotoxin production.

While in vitro trichothecene production on different natural solid substrates is well documented (*12*−*14*), the tissue-specific production of TCT in grain has not been well studied (*15*).

The effects of plant secondary metabolites on mycotoxin biosynthesis have been previously studied. Screening of naturally occurring flavonoids and furanocoumarins demonstrated that several compounds inhibit mycotoxin production by *Fusarium sporotrichioides* in liquid culture (*16*). A benzoxazolinone, 4-acetylbenzoxazolin-2-one (4-ABOA), extracted from a *Fusarium*-resistant North American maize genotype is also a potent mycotoxin inhibitor in vitro (*17*). However, it is not obvious that compounds exhibiting an effect in artificial media will behave similarly on natural solid substrates. Our aim was to investigate trichothecene biosynthesis on different maize grain fractions and then to characterize plant secondary metabolites in the various fractions to explore a possible relationship to mycotoxin production.

In the present paper, four toxinogenic *F. graminearum* strains were grown on sterilized maize grain, degermed maize grain, and maize germ. TCT biosynthesis and growth, estimated by the ergosterol concentrations, were measured on these three substrates. The implication of the difference in the phenolic content in species-specific tissue is discussed.

MATERIALS AND METHODS

F. graminearum Strains. Five *F. graminearum* strains were used in this study. Strains 18532 and 31673 came from the CBS Baarn collection; strain 6131 was provided by the MUCL collection. Strain FgDAA was isolated from infected wheat ears in the laboratory and identified according to the key of Nelson et al (*18*). Taxonomic

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determination was also confirmed by means of *F. graminearum*-specific PCR primers designed by Ouellet and Seifert (*19*). The strain DAOM 180376 was obtained from the Canadian Collection of Fungus Cultures, Ottawa, and was previously isolated from infected maize ear. *Fusarium* strains were stored on potato dextrose agar (PDA) at 4 °C.

Inoculation and Growth Conditions. The same batch of maize grains, provided by La CANA (Treilliere, France) and exempt of TCTs, was fully moistened by addition of sterile water and homogenized for 5 days at 4 °C until the water activity (A_w) was maximal. A part of the moistened maize grains was manually degermed as described by Pelschenke and Lindemann (*20*). Samples of 110 g each of the whole grains, degermed grains, and germ were distributed into 500 mL Erlenmeyer flasks, and sterilized twice for 25 min at 110 °C. A_w was checked and adjusted to $a_w = 1$ before the inoculations (*21*, *22*). All flasks were inoculated by a suspension of 10^5 conidia. Incubations of *Fusarium* strains were conducted in triplicate for each strain. The flasks were incubated at 26 °C for 25 days with daily manual shaking for the first 5 days of inoculation to uniformly distribute the inoculum.

Before analysis, each fraction of maize grains was dried at 45 °C in a ventilated oven for 6 h, ground, and homogenized. For each homogenized sample, 15 g was used for ergosterol analysis, 25 g was used for TCT analysis, and 1.5 g was for phenolics analysis.

Extraction and Assay of Ergosterol. Ergosterol analysis was carried out as described by Cahagnier et al. (*23*). Ergosterol was extracted after saponification in a mixture of methanol, ethanol, and potassium hydroxide and purified on an Extrelut 20 R solid-phase extraction column (Merck, Darmstadt, Germany). Quantification was performed using an external standard (Merck). The results were expressed in milligrams of ergosterol per kilogram of dry matter or parts per million, and ergosterol concentrations were compared by the Student's *t* test.

Trichothecene Analysis. Maize grain fractions (25 g) were analyzed by GC−ECD as previously described (*24*). Briefly, samples were extracted with acetonitrile/water (84:16, v/v) and purified on a charcoal and aluminum oxide column. Trimethylsilyl derivatives were quantified by gas chromatography using electron capture detection. A standard solution (1 μg of DON, NIV, FX, 3-ADON, 15-ADON, all from Sigma) was also analyzed. The results were expressed in milligrams of TCT per kilogram of dry matter or parts per million, and TCT concentrations on the different substrates were compared by the Student's *t* test. Detection limits were 0.01 ppm for DON, 3-ADON, 15-ADON, and FX and 0.02 ppm for NIV.

Extraction of Phenolic Compounds. Four different kinds of phenolic compounds were investigated: insoluble cell-wall-bound phenolics, free phenolics, soluble ester-bound phenolics, and soluble glycoside-bound phenolics. Extraction methods were adapted from Weidner et al. (*25*) and Sosulski et al. (*26*) with the following modifications: freeze-dried powders of maize grain fractions (1.5 g) were extracted for 1 h with 30 mL of 80% methanol in water after 20 s of homogenization with a Polytron mixer (Brinkman Instruments, Westbury, NY). The extracts were centrifuged for 10 min at 1000*g*. Supernatants were separated from pellets, and the pellets were re-extracted twice. The pellets contain insoluble cell-wall-bound phenolics, and the supernatants contain soluble phenolics.

The insoluble cell-wall-bound phenolics were extracted with the method described in ref *27*.

The pooled supernatants, containing soluble phenolics, were evaporated under vacuum to 20 mL of aqueous solution. These aqueous solutions were adjusted to pH 2 and filtered through Whatman paper no. 1. Free phenolics were extracted using liquid−liquid extraction with 20 mL of ethyl acetate. The remaining aqueous solution was divided into two parts.

The NaOH digested was used to release ester-conjugated soluble phenolics, and the HCl was used to release glycoside-conjugated phenolics. A 10 mL sample of 2 N NaOH was added to the first aqueous solution, and digested for 3 h under darkness and a nitrogen atmosphere. A 10 mL sample of 2 N HCl was added to the second aqueous solution, and digested for 1 h at 4 °C. After both digestions, the pH of the solutions was adjusted to 2, and the phenolics were extracted with 20 mL of ethyl acetate. The ethyl acetate phase was dried using a Savant Speed Vac (Bio101, Vista, CA) and dissolved in 3 mL of methanol, HPLC grade. The final extracts were stored at −18 °C prior to analysis.

The 4-ABOA was also extracted according to Miller et al. (*17*) in selected samples.

HPLC Analysis of Phenolics. Standards of the most common phenolics (caffeic acid, ferulic acid, *p*-coumaric acid, salicylic acid, 4-hydroxybenzoic acid) were purchased from Sigma (St. Louis). The 4-ABOA was kindly provided by D. Fielder (CeaPro Inc., Edmonton, Alberta, Canada). The 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) were synthesized in house. All standards and samples were filtered through a 2 μm PTFE filter and injected into the HPLC column in a 1 μL volume.

An LC separation procedure was developed to provide baseline separation of the major phenolic compounds found in our samples. A Chemstation Agilent HP 1100 (Agilent, Palo Alto, CA) was used, and the separation was achieved using a 2 × 100 mm YMC ODS-AM column (Waters Corp., Milford, MA) at 50 °C with a binary gradient with acetonitrile (A) and trifluoroacetic acid (0.05%, pH 2.4) (B) at the following conditions: initial 8% A, then 35% A in 12 min, 100% in 2 min, isocratic for 30 s, and then return to the initial conditions in 3 min. The eluent was monitored at 325, 280, and 240 nm.

Confirmation of the identities of the phenolic compounds was achieved using retention time and UV spectrum comparisons with solution freshly prepared from commercial standards. For 4-ABOA, the identity was also confirmed by monitoring the eluent with an atmospheric pressure chemical ionization (APCI) Agilent VL mass detector coupled to the LC systems. The MS conditions were positive ionization mode, a drying gas flow of 7 L/min of nitrogen at 220 °C, vaporizer at 450 °C, a nebulizer pressure of 60 psig, a fragmentor voltage of 85 V, a capillary voltage of 3000 V, and a corona current of 4.0 μA. The MS data were collected in scan mode.

Test of Phenolic Extract Effect on Liquid Culture.

To investigate the effect of some soluble ester-bound phenolic extracts (**Table 1**) of maize germ (G) or degermed maize grain (D) on trichothecene production by *F. graminearum* in liquid culture, aliquots of 1 mL of the final phenolic extract were reduced to dryness under vacuum. The extracts were then redissolved in 1 mL of ethanol in water (1:1, v/v). A two-step liquid culture protocol previously described by Miller and Blackwell (*28*) was used with the following modifications: the second step was performed in 250 mL flasks containing 50 mL of autoclaved medium. The extracts dissolved in ethanol were then added in the second medium 3 days after transfer from the first to the second medium. A 1 mL sample of ethanol (50%) was added in the control culture (C). Twelve days after the addition of phenolic extract, liquid cultures were filtered using Whatman paper GFA. The mycelium was washed with 15 mL of distilled water. The remaining mycelium was dried and weighed, and the culture surnageant was extracted twice with 50 mL of ethyl acetate. The organic fractions were pooled and reduced to dryness before being redissolved in methanol and stored at −18 °C until analysis.

RESULTS AND DISCUSSION

Analysis of ergosterol, a fungus-specific membrane compound, which is commonly used to estimate the fungal biomass in natural solid substrates such as grains (*29*, *30*), was found to be a sensitive indicator of growth on grain fractions. The experimental conditions ($a_w = 1$, 26 °C) used in this study allowed a substantial mycelial growth on all grain fractions as indicated by a high ergosterol grain content (**Figure 1**). These concentrations of ergosterol were comparable to published values in which *Fusarium* strains had grown on moistened and sterilized maize grain (*31*, *32*). For three strains of *F. graminearum*, ergosterol levels were significantly ($p < 0.01$) higher on whole grain. No significantly different biomass was measured on the three substrates for *F. graminearum* CBS 18532.

F. graminearum CBS 18532, FgDAA, and CBS 31673 produced DON and ADON, whereas MUCL 6131 produced NIV and FX. In all cases, DON or NIV and their related

Table 1. Contents (μg/g[a] of DW) of the Major Phenolic Compounds Detected in the Different Grain Fractions[b]

	soluble phenolics						cell-wall-bound phenolics	
	free		released by NaOH		released by HCl		released by NaOH	
compd	G	D	G	D	G	D	G	D
caffeic acid	0.43 ± 0.15	1.04 ± 0.17	0.26 ± 0.09					
3-hb acid			0.21 ± 0.04					
p-coumaric acid	5.66 ± 0.62	1.57 ± 0.56	15.22 ± 1.22	0.99 ± 0.11	0.43 ± 0.05	0.09 ± 0.00	43.95 ± 5.52	60.35 ± 7.54
(*Z*)-ferulic acid	6.62 ± 0.84	2.94 ± 0.74	149.80 ± 15.6	5.75 ± 1.09		0.18 ± 0.04	567.04 ± 32.5	614.58 ± 23.8
(*E*)-ferulic acid	0.78 ± 0.31	1.01 ± 0.10	46.00 ± 5.99	3.28 ± 0.54			90.35 ± 6.95	95.98 ± 9.74
4-ABOA				23.66 ± 2.14				
salicylic acid			2.47 ± 0.67	1.11 ± 0.32	1.59 ± 0.37	1.27 ± 0.14		
DFA 8-5′							92.92 ± 7.82	96.25 ± 8.95
DFA 5-5′							67.71 ± 5.36	72.58 ± 7.52
DFA 8-*O*- 4′							57.78 ± 4.32	63.14 ± 3.25
DFA 8-5′b							21.47 ± 2.99	32.58 ± 4.69

[a] Values are means ± SD of three replicates. [b] Identical to the ones used for fermentations. G = germ fraction. D = degermed grain. DW = dry weight. 3-hb = 3-hydroxybenzoic.

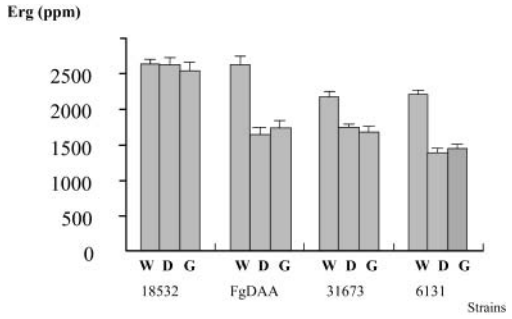

Figure 1. Growth of *F. graminearum* strains on different maize grain fractions: whole maize grain (W), degermed maize grains (D), and maize germ (G). Erg = ergosterol. The error bars represent the standard deviation of three replicates.

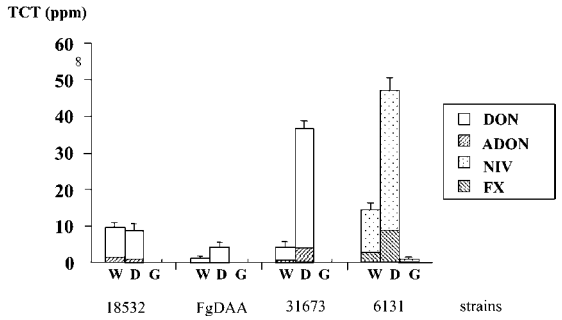

Figure 2. Trichothecene production by *F. graminearum* strains on different maize grain fractions: whole maize grain (W), degermed maize grains (D), and maize germ (G). The error bars represent the standard deviation of three replicates.

acetylated forms were produced in the same proportion on the different substrates. Trichothecene production by the *F. graminearum* strains is presented in **Figure 2**. TCT production was equivalent (strain CBS 18532) or significantly ($p < 0.01$) higher on degermed maize grain than on whole maize grain (strains FgDAA, CBS 31673, and MUCL 6131). Of note is the TCT inhibition which was observed on maize germ even for strains CBS 18532 and MUCL 6131, which are high toxin producers. The result was confirmed in three separate fermentations, and the combined results are shown in **Figure 2**.

The type of substrate significantly affected toxin production ($p < 0.01$) and growth as well ($p < 0.01$). There was no direct correlation between growth variation and toxin production on the different maize grain fractions. TCT production by *F. graminearum* is clearly not homogeneous on the different maize grain fractions.

Our results on maize germ also indicated that trichothecene biosynthesis is not a mandatory phenomenon. As a matter of fact, very low (0.08 μg/g for MUCL 6131) or nondetectable (for strains FgDAA, CBS 31673, and CBS 18532) trichothecene amounts were produced on maize germ, whereas a high ergosterol concentration was quantified for all strains.

Added to the weak or nondetectable TCT production on maize germ, higher TCT amounts were measured on degermed maize grains than on the whole grain. As a consequence, we hypothesized that some TCT inhibitors were present in the sterilized maize germ. To our knowledge, this is the first observation of TCT inhibition on a natural solid substrate.

Several studies have indicated an inhibitory effect of plant secondary metabolites on mycotoxin production.

In the case of aflatoxin production by *Aspergillus flavus*, it has been shown that some corn seed proteins were able to inhibit toxin formation with little effect on fungal growth (*33*). In our study, the different fractions used as a substrate were autoclaved, so it is highly probable that heat-labile compounds such as protein were denaturated and therefore had lost their biological activity.

On the other hand, several studies have indicated an inhibitory effect of phenolic compounds on mycotoxin production (*16*, *34*, *35*). Phenolic compounds are widespread metabolites. In addition, these compounds are heat stable (*36*, *37*). In the cereals, these compounds are major components (structure shown in **Figure 3**), mainly bound to the cell wall polysaccharides by ester linkages (*38*). Soluble phenolics can also be found free or conjugated in the extracellular medium or released during the degradation of tissues by fungal or plant hydrolases (*39*). In the present study, four different kinds of phenolic compounds were investigated: insoluble cell-wall-bound phenolics, free phenolics, soluble NaOH-extracted phenolics (including soluble ester-bound phenolics), and soluble HCL-extracted phenolics (including glycoside-bound phenolics). Phenolic concentrations were compared in the maize germ, where TCT production is inhibited, and in the degermed maize grain fractions, which allowed the highest biosynthesis of TCT. Results are presented in **Table 1**. Unsurprisingly, the highest levels of phenolic compounds were released from alkaline treatment of the cell-wall-bound phenolic fractions (CWB). Nevertheless, weak variations of insoluble cell-wall-bound phenolics were observed in the different maize grain fractions. The CWB phenolics, mainly the hydroxycinnamic acids, are known to be major

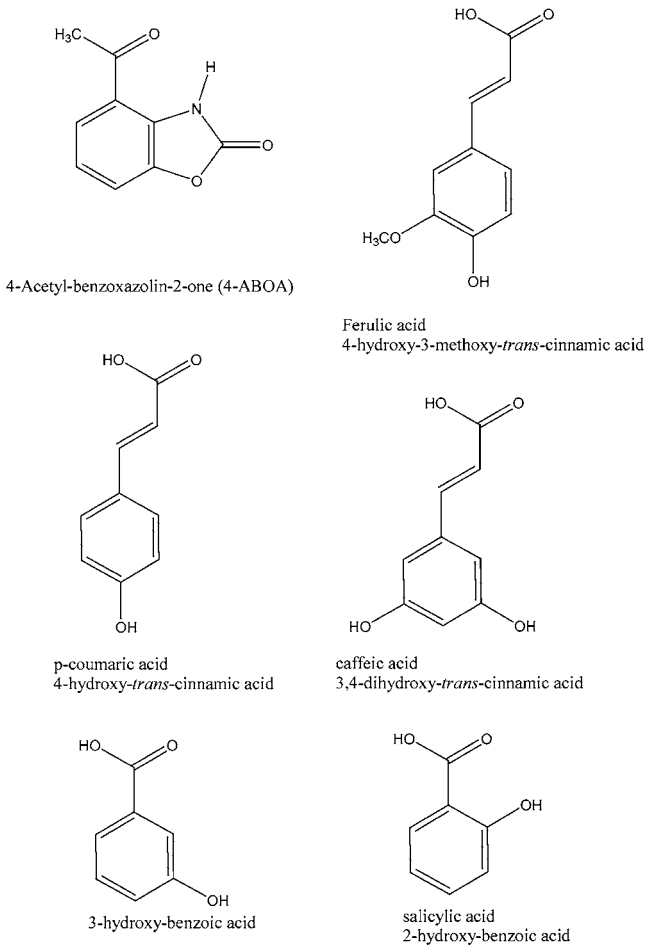

Figure 3. Structures of the phenolic compounds found in the different grain fractions.

components of the primary cell wall of cereals. These compounds are ester bound to the C5 hydroxyl of the arabinosyl side chains of cell wall arabinoxylan chains (*40*). The feruloyl residues, predominant species, can also be dimerized under an oxidative coupling mediated by peroxidases, form cross-links, or dehydrodimers of ferulic acid (DFA), and then lead to a reinforcement of the primary cell wall (*40*). It is possible that, for accessibility issues, the levels of CWB contribute only very little to the pool of phenolics released during the fungal growth and tissue invasion compared to soluble phenolics.

Levels of free *p*-coumaric and ferulic acids measured in the degermed grain were similar to values found in maize (*26*) and in other cereal grains, such as Triticale or oats (*41*). Levels of free *p*-coumaric and ferulic acids were higher in the germ fraction than in the degermed fraction (by 3- and 2-fold, respectively).

HCl extraction yielded only weak levels of phenolic compounds whose concentrations were not significantly different in the maize grain fractions.

Conversely, NaOH extraction yielded high levels of phenolic compounds, and much greater variation was observed between the different grain fractions. The 4-ABOA, which was first isolated from kernels of a resistant hybrid, Funks 4106 (*42*), was only found in the degermed fraction of the maize studied. There was no evidence of 4-ABOA presence in the germ. Miller et al. (*17*) established that the addition of 4 μM 4-ABOA, isolated from a resistant maize variety, could inhibit by 50% ADON production by *Fusarium culmorum* grown in liquid culture, without a significant effect on mycelium weight. Our

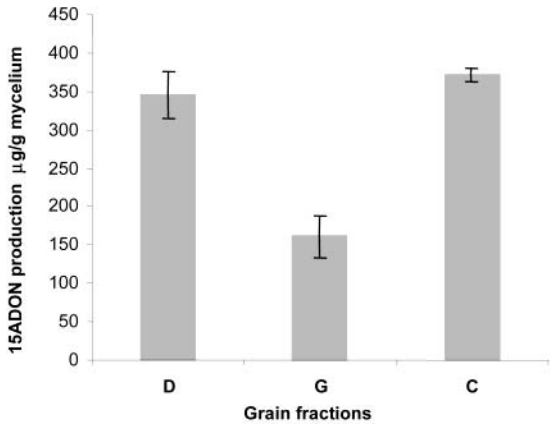

Figure 4. Trichothecene production by *F. graminearum* DAOM 180376 in liquid culture after treatment with different extracts of "soluble phenolics released by NaOH" (see **Table 1**) of degermed maize grain (D), maize germ material (G), and a control culture (C) containing 1 mL of ethanol in water (1:1, v/v). The 15-ADON production is expressed in micrograms per gram of mycelium. The error bars represent the standard deviation of two replicates.

results indicate that the distribution of 4-ABOA is not homogeneous in the different parts of the grains. To our knowledge, there has been no previous report of specific localization of this compound in grain tissues. Added to that, the TCT inhibition observed on the maize germ fraction used in our study is clearly not linked to 4-ABOA.

No DIMBOA or DIBOA aglycons or conjugates were found in any grain fractions used in this study. This is in agreement with other reports where no 1,4-benzoxazin-3-one was found in dry maize (*43*) or wheat (*44*) seeds. These hydroxamates are known to appear after germination and be present in seedling tissues (*44*).

Among NaOH-liberated phenolics, ferulate ester levels were found to be 20 times higher in germ tissue than in degermed grain. This result is in accordance with a previous study that showed, by microspectrofluorometry, that the maize embryo contains the highest level of phenolic compound (*45*). Soluble ferulic ester-conjugated forms in maize are represented by ferulic acid esterified to carbohydrates, ferulic arabinose, and ferulic arabinoxylose complexes (*38*). Ferulic ester has also been reported to be conjugated to sterols (*46*) and possibly to proteins (*40*, *47*). During growth on the host tissues, *Fusarium* expresses a different type of hydrolases, such as esterases, that can release free forms of ferulic ester. Once released, free ferulate may influence the ability of *Fusarium* to produce mycotoxins. As a consequence, low molecular weight ferulic ester conjugates in the germ could be implicated in the inhibition of TCT observed on the natural substrate. Added to that, in the model *Aspergillus parasiticus*, 200 ppm ferulic acid reduced aflatoxin production by 90% in liquid culture, while reduction of fungal growth was only 8.2% (*48*). The influence of other germ compounds on TCT production cannot be totally ruled out. Nevertheless, our results suggest a role of ferulate and/or some conjugated form in TCT production by *F. graminearum*.

To verify the inhibiting properties of the soluble phenolic extract from germs, the soluble phenolic extract released by NaOH resulting from the extraction of 500 mg of germ tissues (extract G) or degermed tissues (extract D) was added to a liquid culture of *F. graminearum.* After 12 days of fermentation, the 15-ADON production was not significantly different for the control culture and the culture treated with the D extract (**Figure 4**). On the contrary, the 15 A-DON amount produced in the

culture treated with the G extract was significantly ($p < 0.01$) lower than in the control culture. This inhibiting effect of the G extract, observed in vitro, was not accompanied by any significant reduction of fungal growth (1.2% of the control). This result confirms our hypothesis that soluble phenolic compounds in the germ are playing a significant role in the inhibition of mycotoxin production observed in vitro.

Further studies are in progress to pinpoint and isolate the most active inhibitors in the soluble phenolic extracts from the germ tissues, and the effects of these different phenolics on TCT biosynthesis by *F. graminearum*. A thorough investigation of toxin inhibitors present in specific plant tissues should add valuable information on mycotoxin production.

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