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Natural phenolic acids from wheat bran inhibit *Fusarium culmorum* trichothecene biosynthesis *in vitro* by repressing *Tri* gene expression

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Abstract The effect of natural phenolic acids from wheat bran on type B trichothecene biosynthesis by *Fusarium culmorum* was investigated *in vitro*. Durum wheat bran contained various monomeric forms of phenolic acids, with ferulic acid being the most abundant. In addition, various oligomeric forms of ferulic acid and mainly dimeric forms were also detected. When liquid cultures of *F. culmorum* were supplemented with a natural wheat bran extract, trichothecene production was fully inhibited. The exact mechanism by which toxin synthesis is repressed remains to be clarified but we showed that the phenolic acid treatment resulted in a drastic reduction in the expression level of structural trichothecene biosynthetic genes. The inhibitory efficiency of the natural phenolic acid extract was significantly higher than that of a reconstituted mixture containing similar concentrations of monomeric forms. Thus, to elucidate the full repression of type B trichothecene production induced by the natural phenolic acid extract from wheat bran, two hypotheses can be raised: (i) a synergistic impact of monomeric and dimeric forms of phenolic acids, (ii) the occurrence of an unidentified oligomeric form able to efficiently repress toxin yield. As a first attempt to investigate the effect of oligomeric forms, one of the most abundant dimer of ferulic acid, the 8-5′-benzofuran dimer, has been synthesized *in vitro* and was shown to inhibit trichothecene biosynthesis to the same extent than the monomer of ferulic acid.

Keywords Diferulic acid · *Fusarium* · Mycotoxin · Phenolic acid · *Tri* gene · Type B trichothecene

Abbreviations

3-ADON	3-acetyl-4-deoxynivalenol
15-ADON	15-acetyl-4-deoxynivalenol
DON	deoxynivalenol
FX	fusarenone X
NIV	nivalenol
DiFA	dimer of ferulic acid
Fpps	farnesyl pyrophosphate synthetase

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Introduction

Wheat, one of the most important agricultural crops, is subject to *Fusarium* Head Blight (FHB) leading to significant yield reduction. This yield reduction is very frequently accompanied by mycotoxin contamination of the harvested grains. *Fusarium graminearum* Schwabe and *Fusarium culmorum* (W.G. Smith) Saccardo are the main species inducing FHB and leading to mycotoxin contamination of cereals (Bottalico and Perrone 2002). Both species produce type B trichothecenes, including deoxynivalenol (DON), acetyldeoxynivalenol (ADON), nivalenol (NIV) and fusarenone X (FX). Maximum contamination levels acceptable for cereals and maize-based food were set by the European Community for DON, the most frequently observed mycotoxin, in June 2005 (EC No. 856/2005) and revised in July 2007 (EC No. 1126/2007). Grains or derived products exceeding the established limits will not be authorised for human consumption. Type B trichothecenes are heat-stable molecules and are not fully eliminated by current food manufacturing processes. The toxicity of type B trichothecenes to animals and humans is now well documented and it is of great concern to reduce trichothecene occurrence in cereals and derived products.

Presently, the most efficient way to prevent trichothecene occurrence in grains is to limit their biosynthesis by the fungus during cultivation of the crop by using cultivars resistant to either *Fusarium* development (Champeil et al. 2004) or mycotoxin accumulation (Boutigny et al. 2008). We previously raised the hypothesis that the resistance to trichothecene accumulation in some durum wheat cultivars contaminated by *F. culmorum* could be ascribed to a particular biochemical composition of the kernel, rich in specific endogenous compounds able to reduce trichothecene biosynthesis (Boutigny et al. 2008). Phenolic acids which are relatively abundant in bran (Kim et al. 2006) could be candidates for these inhibitory compounds. Several *in vitro* experiments have described an inhibitory effect of various individual phenolic acids on mycotoxin production, including aflatoxin production by *Aspergillus parasiticus* (Sinha and Premlata 1981), fumonisin B1 production by *Fusarium verticillioides* (Beekrum et al. 2003) and type B trichothecene production by *F. culmorum* (Boutigny et al. 2009). However, various phenolic acids are present simultaneously and at different amounts in wheat bran, among which the most abundant is the *trans*-ferulic acid. Phenolic acids are present as soluble forms or cell-wall bound forms (Kim et al. 2006). In plant cell walls, ferulic acid is mainly linked to various polysaccharides and to lignin through ester and ether bonds. Ferulic acid dehydrodimerize through an oxidative mechanism to form mainly 8-5′-, 8-5′-benzofuran form, 8-O-4′-, 5-5′- and 8-8′-coupled dimers (often simply referred to DiFA). Such dimers are involved in the cross-linking of polysaccharide chains (Ralph et al. 1994). It could be worthwhile to investigate the effect of the co-occurrence of different monomeric and dimeric phenolic acids forms contained in bran on type B trichothecene biosynthesis by *Fusarium* species.

The exact mechanism by which phenolic acids are able to repress type B trichothecene biosynthesis remains unknown. Today, the effect on transcription of the main genes involved in the type B trichothecene biosynthesis pathway (Desjardins 2006) can be analyzed. Expression of the genes encoding first steps of the type B trichothecene biosynthesis pathway i.e. the *Tri5* gene, the *Tri4* gene and the *Tri11* gene can be representative of the initial flux of the biosynthetic pathway. The non-cluster *Tri101* gene (Kimura et al. 1998) and the *Tri12* gene (Alexander et al. 1999) are playing a role in self protection of the fungus against trichothecenes and can be limiting steps in the biosynthetic pathway. The two positive regulators *Tri6* (Hohn et al. 1999) and *Tri10* (Tag et al. 2001; Peplow et al. 2003) were shown to control expression of most *Tri* genes in *F. sporotrichioides*. Recently, in *F. graminearum, Tri6* has also been shown to regulate genes from the isoprenoid biosynthetic pathway including the farnesyl pyrophosphate synthetase gene (*Fpps*) that generates the farnesyl pyrophosphate (FPP), the immediate precursor of trichothecene biosynthesis pathway (Seong et al. 2009). Specific effect of modulators of trichothecene biosynthesis on expression of all these genes can be evaluated (Ponts et al. 2007; Boutigny et al. 2009).

This study aimed at evaluating *in vitro* the inhibitory effect of the pool of natural phenolic acids extracted from wheat bran on type B trichothecene accumulation by *F. culmorum* and on expression of various *Tri* genes involved in the toxin biosynthesis pathway. Then, the inhibitory efficiency of the natural phenolic acid extract was compared to that of a reconstituted mixture containing the corresponding monomeric forms. Finally, the effect on toxin biosyn-

thesis of one of the most abundant dimers of ferulic acid occurring in wheat bran, the 8-5′-BenDiFA, was assessed.

Materials and methods

Wheat samples

Orjaune used in this study is a winter durum wheat (*Triticum turgidum* L. subsp. *durum*) variety bred by Sudwestdeutsche Saatzucht and registered in France on the Catalogue National Liste A on 3rd January 1996. Bran fractions were provided by the "Grande Semoulerie de l'Ouest" (GSO, Angoulême, France).

Fusarium culmorum strains

The INRA 117 strain is the Fc21 strain from Quirico Migheli (University of Sassari, Italy). The INRA 319 strain is from the INRA-MycSA laboratory collection. In liquid cultures, the INRA 117 strain predominantly produces 3-ADON, and to a lesser extent DON (DON/3-ADON chemotype) while the INRA 319 strain predominantly produces FX and to a lesser extent NIV (NIV/FX chemotype). Stock cultures were maintained at 4°C on Potato Dextrose Agar (PDA) slants under mineral oil. When inoculum was required, the *F. culmorum* strains were grown on PDA slants at 25°C in the dark for 8 days and spore suspensions prepared by adding 6 ml of sterile distilled water to the PDA slant with gentle shaking.

Medium and culture conditions

Liquid-culture experiments were performed in a Mycotoxin Synthetic medium (MS medium) (KH_2PO_4, 0.5 gl^{-1}; K_2HPO_4, 0.6 gl^{-1}; $MgSO_4$, 0.017 gl^{-1}; $(NH_4)_2SO_4$, 1 gl^{-1}; glucose, 20 gl^{-1}; biotine, 0.1 mg l^{-1} and 0.1 ml l^{-1} of Vogel trace element solution (Vogel 1956)). Sterile Petri dishes (55 mm in diameter) containing 8 ml of MS medium supplemented or not with phenolic acid extracts were inoculated with spore suspensions to achieve a final concentration of 10^4 spores ml^{-1}. Fungal liquid cultures (static) were incubated in the dark at 25°C. Cultures were done in triplicate. Following incubation, the culture medium was removed by centrifugation and stored at −20°C until analysis for type B trichothecene content while the mycelium was stored at −80°C. Mycelia were used either for RNA extractions or were lyophilized in order to quantify the fungal biomass production.

Culture supplementation with phenolic acids

A mixture of monomeric forms of phenolic acids was reconstituted by pooling commercial preparations of phenolic acids to get the same qualitative and quantitative composition in phenolic acid monomers than that of the natural extract isolated from wheat bran. The correct composition of this reconstituted mixture was checked by HPLC-DAD analysis. Liquid cultures of *F. culmorum* (INRA 117 and INRA 319 strains) were supplemented, or not, before fungal inoculation with either the natural extract of phenolic acids (monomers and oligomers) or with the reconstituted mixture (monomers only). Ferulic acid content served as reference to adjust the two mixtures at the same final concentration for ferulic acid. Both mixtures were assayed at two different concentrations corresponding to either 0.1 mM or 0.5 mM of ferulic acid. Effect on type B trichothecene accumulation was analyzed in 10-day-old cultures. It had previously been checked that initial pH of the culture medium was not affected by supplementation with the phenolic acid extracts compared to the control.

In a different experiment, liquid cultures of *F. culmorum* (INRA 117 strain) were supplemented, or not, before fungal inoculation either with ferulic acid or with 8-5′-BenDiFA (purity >86%) at two different concentrations 0.25 mM and 0.5 mM. Effect on type B trichothecene accumulation was analyzed in 10-day-old cultures. It had previously been checked that initial pH of the culture medium was not affected by supplementation with these compounds compared to the control.

HPLC-DAD analysis of type B trichothecenes

A 4 ml sample of culture medium was extracted with 8 ml of ethyl acetate. Six ml of the organic phase were evaporated to dryness at 70°C under a nitrogen flux. Dried samples were dissolved in 200 µl of methanol/water (1/1, v/v) before analysis using high performance liquid chromatography (HPLC) as previously described (Bily et al. 2004). HPLC analysis was carried out using an Agilent Technologies 1100 series liquid chromato-

graph equipped with an auto sampler system, an Agilent photodiode array detector (DAD) and the ChemStation chromatography manager software (Agilent, France). Quantification was performed by using external calibration with NIV, DON, FX, 15-ADON and 3-ADON standard solutions prepared from commercial pure powders purchased from Sigma-Aldrich (France).

Type B trichothecene yields (sum of DON+3-ADON for the INRA 117 strain and sum of NIV+FX for the INRA 319 strain) are expressed as μg g^{-1} of dry fungal biomass. Data are reported as mean values ±SD of three biological replications. The data were normalized against a given yield obtained from the control sample. Values were compared at the 5% significance level using a standard Student's *t*-test (control vs. treated).

Extraction of phenolic acids

Phenolic acids were released from cell walls by alkaline hydrolysis. Milled bran samples (15 g) were shaken for 4 h in 600 ml of sodium hydroxide (2 M). Filtrates were acidified to pH 2 with hydrochloric acid (12 M). Samples were extracted with 600 ml of ethyl acetate. After centrifugation (4500×g), the ethyl acetate fraction was collected and evaporated to dryness using a rotavapor (BÜCHI Rotavapor R-200). Finally, dried samples were dissolved in 400 μl of methanol before HPLC analysis.

HPLC-DAD analysis of phenolic acids

HPLC separation was performed according to a previously described procedure (Kim et al. 2006) with some modifications. Separation of phenolic acids was achieved on a ZORBAX SB-C18 column (5 μm; 250 mm×4.6 mm) (Agilent, France) maintained at 30°C. The mobile phase consisted of 2% formic acid in water (v/v) (solvent A) and acetonitrile (solvent B). Phenolic acids were separated by a gradient elution as follows: 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 min post-run reconditioning. The injection volume was 5 μl. The flow rate was kept at 1 ml min^{-1} for a total run time of 75 min. The UV-VIS spectra were recorded from 200 to 550 nm and peak areas were measured at 260 nm, 280 nm and 320 nm according to the studied phenolic acid. Quantification was performed by using external calibrations with phenolic acid standard solutions prepared from commercial pure powders (gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *trans*-ferulic acid, *p*-coumaric acid, caffeic acid and sinapic acid) purchased from Sigma-Aldrich (France). The dimers of ferulic acid were quantified using an external 8-5′-BenDiFA standard (chemically synthesized in our laboratory, purity >86%). Peak areas at 320 nm were converted to mg l^{-1} equivalent 8-5′-BenDiFA using a standard curve prepared with different concentrations of 8-5′-BenDiFA in methanol/water (50/50, v/v).

HPLC-MS analyses of phenolic acids

HPLC-MS analyses were performed using a QTrap 2000 LC/MS/MS system (Applied Biosystems, France) equipped with a TurboIonSpray ESI source and a 1100 Series HPLC system (Agilent, France). Chromatographic separation was achieved as previously described with some modifications. The mobile phase consisted of 0.1% formic acid in water (v/v) (solvent A) and 0.1% formic acid in acetonitrile (v/v) (solvent B). The flow rate was kept at 1 ml min^{-1} and was split after UV detection so that 0.25 ml min^{-1} went to the electrospray source. The injection volume was 10 μl. Detection was monitored at 280 nm. The electrospray interface was used in the negative ion mode at 400°C with the following settings: curtain gas, 25 p.s.i.; nebulizer gas, 35 p.s.i.; auxiliary gas, 65 p.s.i.; ion spray voltage, −4200 V; declustering potential, −30 V; entrance potential, −10 V; collision energy, −30 eV; collision-activated dissociation gas, high.

Synthesis and purification of a dimer of ferulic acid

Dimerisation products of ferulic acid methyl ester were synthesized following the method of Rakotondramanana et al. (2007) that yields a mixture of two diastereoisomers. Alkaline hydrolysis (NaOH 2 M, 1 h) of this mixture produced ferulic acid (FA), 8-5′-BenDiFA (*trans*-5-[(E)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxy-phenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid), 8-O-4′-DiFA ((Z-β-(4-[(E)-2-carboxyvinyl]-2-methoxyphenoxy)-4-hydroxy-3-methoxycinnamic acid) and an intermediate product (methyl ferulic acid dimer). The hydrolyzed mixture was acidified to

pH 2.0 with HCl and extracted with ethyl acetate. The diferulic acids (DiFAs) were purified using a Toyopearl gel HW-40 S column (300 mm×16 mm, 0.5 ml min^{-1}) on a Biosciences Amerscham LC system. Equilibration and elution were performed with ethanol/water/trifluoroacetic acid (55/45/0.05, v/v/v). Elution was monitored by absorbance at 280 nm. Two fractions were collected. The first fraction contained ferulic acid and a methyl ferulic acid dimer and the second fraction contained mainly 8-5′-BenDiFA (86%) and traces of 8-O-4′-DiFA (2%). The two last compounds were characterized by UV-VIS and MS spectroscopy. 8-5′-BenDiFA was as follows: UV-VIS λ_{max} 325 nm; ion-spray MS, *m/z* relative intensity, 385 (10), 341 (100), 326 (33), 297 (20), 282 (13), 267 (10). 8-O-4′-DiFA was as follows: UV-VIS λ_{max} 325 nm and a shoulder at 290 nm; ion-spray MS, *m/z* relative intensity, 385 (5), 298 (5), 193 (100), 178 (10), 149 (14), 134 (16). Spectral characteristics of the synthesized DiFAs were in complete agreement with the same dimers detected in the phenolic extract from wheat bran.

Extraction of total RNA and preparation of cDNA

Total RNA was extracted from 3, 4 and 5-day-old mycelium from *F. culmorum* INRA 117 grown in medium supplemented or not with the natural extract of phenolic acids. Two biological replications were prepared for each condition. Frozen mycelium (50 mg) was ground in 1 ml of TRIzol® Reagent (Invitrogen, France) using the TissueLyser System® (Qiagen, France). Total RNA was extracted following the TRIzol® Reagent manufacturer's instructions. Quality of the prepared RNA was assessed by agarose gel electrophoresis. RNA was quantified by absorbance measurements at 260 nm. Total RNA (5 μg) was reverse-transcribed using the SuperScript™ II First-Strand Synthesis System for RT-PCR (Invitrogen). Reverse transcription was performed with an iCycler™ (Bio-Rad, France) programmed according to the thermal cycling conditions recommended by the manufacturer (Invitrogen). cDNA samples were stored at −20°C until used for PCR analyses.

RT-PCR analyses

Classic RT-PCR analyses were performed using the iCycler™ thermal cycler (Bio-Rad). PCR experiments were conducted in 25 μl reaction mixtures containing 1 μl of each diluted cDNA sample (corresponding to 10 ng of total RNA), 2.5 U GoTaq® DNA polymerase (Promega, France), 3.5 mM $MgCl_2$, 0.5 mM of each dNTP, and 0.5 μM of each primer. The primer pairs used and annealing temperatures are described in Boutigny et al. (2009). PCR amplification was realized using 28 cycles. Amplification products were separated by electrophoresis through 1.5% agarose gel.

Real time RT-PCR analyses were performed using a LightCycler® 2.0 system and the LightCycler Software 3.5.3 (Roche, France). Abundance of the transcripts of the genes *Ef2, Tri5*, *Tri4*, *Tri11*, *Tri101*, *Tri6*, *Tri10* and *Fpps* was evaluated in 2 μl of each diluted cDNA (corresponding to 10 ng of total RNA) in a final reaction volume of 10 μl using the QuantiTect™ SYBR® Green PCR Kit (Qiagen). $MgCl_2$ was supplemented to the PCR mix to achieve a final concentration of 4 mM. Primers were used at the final concentration of 0.5 μM each. Expression of the *Ef2* elongation factor was used as an endogenous reference. Real time PCR amplification was realized using 45 cycles according to the thermal cycling conditions recommended by the kit manufacturer (Qiagen). Amplifications were performed in triplicate for each of the two biological replications. In order to determine PCR efficiencies, a standard cDNA mixture was prepared by mixing a fraction of each cDNA sample. A range of four log dilutions was prepared from the standard mixture. For each gene, PCR efficiency (*E*) was determined with the serial dilutions as described previously (Ponts et al. 2007). The PCR efficiencies obtained were 1.95 for *Ef2*, 1.93 for *Tri5*, 1.96 for *Tri4*, 1.87 for *Tri11*, 1.93 for *Tri101*, 2.02 for *Tri6*, 1.82 for *Tri10*, 2.02 for *Fpps*. All the cDNA samples were tested for residual genomic DNA (Ponts et al. 2007). Absence of non-specific PCR amplification products or primer dimer formation was checked by running both melting curves and agarose gel analyses on the final PCR products.

Real-time RT-PCR data analyses

Crossing point values (*Cp*) experimentally measured (mean value of three replicates) for *Ef2* were compared in order to verify the stability of *Ef2* expression under the treatment. The *Cp* values experimentally measured obtained for our target *Tri* genes and *Fpps* gene were

compared in control and treated conditions and normalized relative to the *Cp* values obtained for the reference gene *Ef2* using the REST© software REST-384 (Relative Expression Software Tool). The mathematical model used takes into account differences for efficiencies between the reference gene and the target gene and the mean crossing point deviation between the control and the treated condition (Pfaffl et al. 2002). Expression levels of the target genes, normalized by the reference gene expression, were expressed as regulation factor in the treated condition relative to the control condition, with the ratio $= \left(E_{\text{target}}\right)^{\Delta CP\ \text{target(control}-\text{treated)}} / \left(E_{ref}\right)^{\Delta CP\ \text{ref(control}-\text{treated)}}$. Values obtained for expression ratio were tested for significance by running Pair Wise Reallocation Randomisation Test© with a p value of 0.001 using the REST© software REST-384 (Pfaffl et al. 2002).

Results

Phenolic acid composition of the natural extract from bran of durum wheat

The phenolic acid composition of a wheat bran extract from the Orjaune variety is illustrated in Fig. 1. Monomeric forms of phenolic acids were identified and quantified using HPLC-DAD on the basis of their retention time and their UV-VIS spectra. The cinnamic acid derived monomers, i.e. ferulic acid (1007 μg g^{-1} of bran), sinapic acid (73 μg g^{-1} of bran) and *p*-coumaric acid (34 μg g^{-1} of bran) were the most abundant. Two benzoic acid derived, vanillic acid (13 μg g^{-1} of bran) and *p*-hydroxybenzoic acid (7 μg g^{-1} of bran), were also detected, but in lower amounts. Ferulic acid was by far the most abundant phenolic acid (89% of the total monomeric phenolic content), in accordance with previously published studies (Onyeneho and Hettiarachchy 1992; Kim et al. 2006). HPLC-MS analysis revealed the presence of both *trans*-ferulic acid and *cis*-ferulic acid, with the *trans* form being predominant. In addition to the monomeric forms of phenolic acids, several additional peaks eluting at the end of the chromatogram were observed. Based on the UV-VIS and mass spectra obtained after LC/DAD-MS analysis (negative ion mode), the main peaks were assigned to ferulic acid oligomers (Waldron et al. 1996; Bunzel et al. 2005). Dimers (*m/z* 385 or *m/z* 357), trimers (*m/z* 577) and tetramers (*m/z* 769) of ferulic acid could be detected. The dimers of ferulic acid were the most abundant with the occurrence of various forms previously reported in wheat bran (Garcia-Conesa et al. 1997), the most important of which were 8-O-4′-DiFA (151 μg g^{-1} eq. 8-5′-BenDiFA of bran), 5-5′-DiFA (125 μg g^{-1} eq. 8-5′-BenDiFA of bran) and 8-5′-BenDiFA

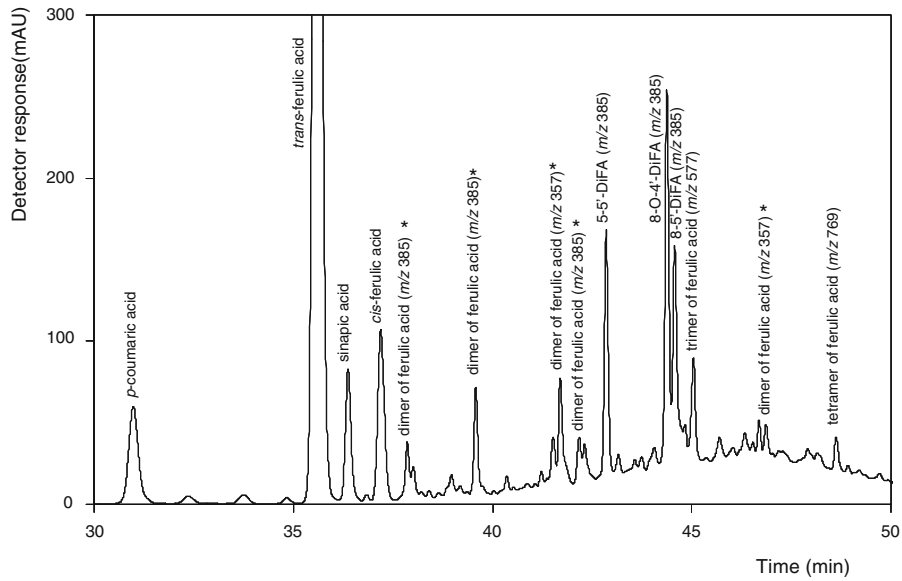

Fig. 1 HPLC elution profile of the natural extract of phenolic acids released from cell walls of wheat bran from the Orjaune variety. Peaks were detected at 320 nm. *Peaks marked with an asterisk remain to be unambiguously identified

(80 μg g^{-1} eq. 8-5′-BenDiFA of bran). The bran phenolic extract contains also other compounds characterized on the basis of UV-VIS spectra and molecular weight (MW 386 and 358) as similar to dimers of ferulic acids (Fig. 1, peaks marked with *). These compounds remain to be unambiguously identified. The concentration of ferulic acid dimers, including all identified and unidentified compounds, was estimated to 570 μg g^{-1} eq. 8-5′-BenDiFA of bran.

The natural extract of phenolic acids is a potent inhibitor of type B trichothecene biosynthesis by *F. culmorum in vitro*

When the natural extract of phenolic acids from bran was supplied to liquid cultures inoculated with *F. culmorum* strains INRA 117 and INRA 319, a significant inhibitory effect on trichothecene biosynthesis was observed. Two different concentrations were used. Content in ferulic acid monomers of the natural extract served as a reference to obtain final concentrations in the culture medium corresponding to either 0.1 mM ferulic acid (lower concentration) or 0.5 mM ferulic acid (higher concentration). Supplementation of liquid culture at the two concentrations had no effect on fungal biomass production compared to control cultures (data not shown). At 0.1 mM ferulic acid, the natural extract reduced type B trichothecene yield by 68% for the INRA 117 strain and by 84% for the INRA 319 strain (Table 1). At 0.5 mM ferulic acid, type B trichothecene production of both strains was fully inhibited by the natural extract. The full inhibition of toxin production with the natural extract of the Orjaune variety was successfully observed for three biological replications with the INRA 117 strain and a similar effect was also observed with an extract issued from another variety of durum wheat (data not shown).

The natural extract of wheat bran phenolic acids strongly down regulates *Tri* gene expression

In order to investigate the effect of the natural phenolic acid extract on type B trichothecene biosynthesis, the expression of *Ef2* gene, *Fpps* gene and of *Tri5*, *Tri4*, *Tri11*, *Tri12*, *Tri101*, *Tri6*, *Tri10* genes from the trichothecene biosynthesis pathway was analyzed. Experiments were first performed by classic reverse transcription PCR, using RNA extracted from 3, 4 and 5-day-old mycelium from cultures of *F. culmorum* INRA 117 strain supplemented or not with the natural extract of phenolic acids at the higher concentration, 0.5 mM ferulic acid. Results are reported on Fig. 2. It can be seen that expression of the *Ef2* reference gene was constant under the time and the conditions of treatment used. In control cultures, all target *Tri* genes were first detected at day three, when type B trichothecenes start to accumulate in the culture medium (data not shown) and the expression level of *Tri5*, *Tri4*, *Tri11*, *Tri101* and *Tri12* genes increased with time. In control cultures, *Tri6* seemed to be expressed at the same level at day three, day four and increased at day five while the expression of *Tri10* was greater at day three than at day four and five. In cultures treated with the natural extract of phenolic acids, expression of all considered *Tri* genes was strongly decreased compared to control cultures at any day of the experiment

Table 1 In vitro effect of natural extract and reconstituted mixture of phenolic acids on type B trichothecene (TCT B) production by *F. culmorum*

Condition	Ferulic acid (mM)[a]	INRA 117		INRA 319	
		Total TCT B (μg g^{-1})	% inhibition	Total TCT B (μg g^{-1})	% inhibition
Control culture	0	11210±465		9060±1194	
Natural extract	0.1	3536±133	68	1431±38	84
Reconstituted mixture	0.1	8837±175	21	7672±1260	15
Natural extract	0.5	nd	100	nd	100
Reconstituted mixture	0.5	2393±501	79	7820±647	14

[a] Each preparation was diluted in order to obtain the indicated final concentration for ferulic acid

nd: not detected

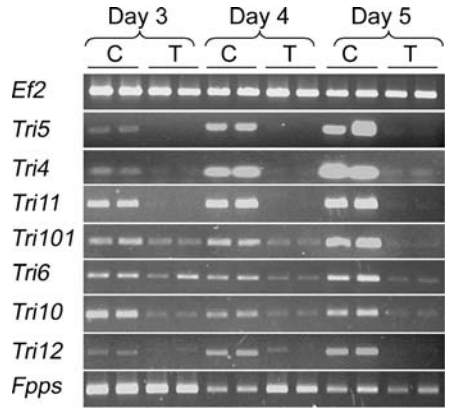

Fig. 2 Amplification products for *Ef2*, *Tri5*, *Tri4*, *Tri11*, *Tri101*, *Tri6*, *Tri10*, *Tri12* and *Fpps* genes obtained after RT-PCR using RNA extracted from *F. culmorum* grown in liquid cultures supplemented or not with the natural extract of phenolic acids. C: control cultures, T: treated cultures supplemented with the natural extract of phenolic acids adjusted at 0.5 mM ferulic acid. Two biological replicates were made for each condition. In control cultures, TCT B yields were 250±29 μg g^{-1} at day 3, 244±97 μg g^{-1} at day 4 and 1534±403 μg g^{-1} at day 5. In treated cultures, no production of TCT B was observed

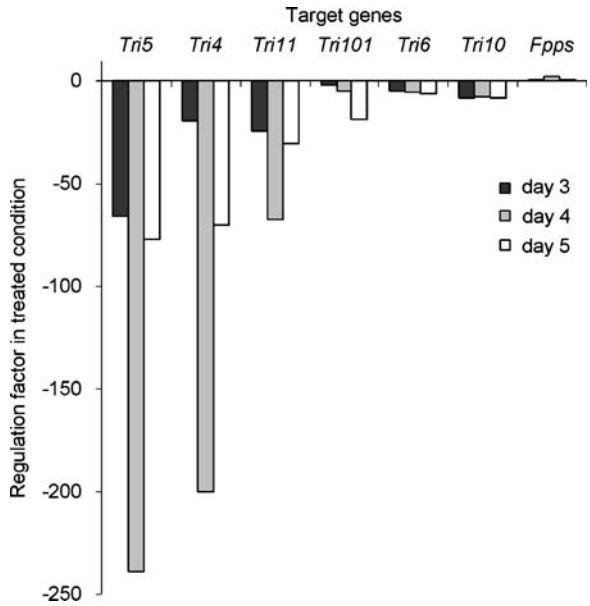

Fig. 3 Regulation factor obtained for structural *Tri* genes in culture supplemented with the natural extract relative to the control culture at day 3, day 4 and day 5

meanwhile the expression of *Ef2* and *Fpps* genes was not affected by the treatment. Inhibition of expression seems particularly strong for *Tri4, Tri5 and Tri11* as only residual amplification was observed under the conditions used.

In order to quantify the specific effect observed with *Tri* gene transcription, the expression level of *Tri* genes was determined by real time PCR using the same cDNA preparation as above. The elongation factor *Ef2* gene served as a reference gene. Analysis of the *Ef2* Cp values measured in control and supplemented cultures indicated that levels of *Ef2* transcripts were not significantly different ($p<0.05$) under the treatment at day three, day four and day five. The expression levels of the target genes in cultures supplemented with the natural extract were compared (Fig. 3) to the control condition. The expression values of the target *Tri* genes and the *Fpps* gene, normalized by the reference gene expression value, were reported as an up- or down-regulated factor in the treated condition relative to the control condition. Expression of all the studied *Tri* genes was significantly decreased ($p<0.001$) by the treatment at all days tested. Expression of *Tri5*, *Tri4* and *Tri11* structural genes, was considerably repressed in the treated condition. This is particularly evident at day four (between 50 to 250 times) and coincides with the time where the accumulation of toxin is maximum in control cultures (data not shown). Surprisingly, the repression of the regulators *Tri6* and *Tri10* genes was significantly lower than that observed for the three other genes of the trichothecene cluster. Expression of these genes was still clearly observed at day four and day five (see in Fig. 2), meanwhile expression of *Tri5*, *Tri4* and *Tri11* was barely detected. At day three and day four, expression of the non-cluster gene *Tri101* was only slightly decreased in treated cultures (Fig. 3). A stronger inhibitory effect on expression of *Tri101* was observable only at day five. By contrast, expression of *Fpps*, the gene encoding the farnesyl pyrophosphate synthase (the enzyme giving rise to farnesyl pyrophosphate, the precursor of the trichothecene pathway) was slightly but significantly activated at day four ($p<0.001$) and constant at day three and day five in treated cultures.

The inhibitory efficiency of wheat bran phenolic extract can not be totally ascribed to phenolic acid monomers

We previously showed that ferulic acid as well as various cinnamic derived phenolic acids inhibit trichothecene biosynthesis by *Fusarium in vitro* when they are individually tested (Boutigny 2007; Boutigny et al.

2009). However, these molecules have not yet been tested as a mixture. The inhibitory efficiency of the natural extract was compared with that of an artificial mixture reconstituted using commercial powders of phenolic acids adjusted at the same concentration as the corresponding monomers from the natural extract. This reconstituted mixture was tested *in vitro* for its effect on type B trichothecene production by the two INRA 117 and INRA 319 *F. culmorum* strains and the results compared to the effect of the natural extract (Table 1). Again, two different concentrations were used and content in ferulic acid of the reconstituted mixture served as a reference to obtain final concentrations of this monomer corresponding to either 0.1 mM or 0.5 mM. At the lower concentration, the reconstituted mixture reduced type B trichothecene yield by only 21% and 15% for the INRA 117 and the INRA 319 strains respectively. At the higher concentration, the reconstituted mixture reduced type B trichothecene yield by 79% for the INRA 117 strain but only by 14% for the INRA 319 strain. These results demonstrate that although the mixture reconstituted with monomeric forms of phenolic acids is an efficient inhibitor of trichothecene production, this cannot explain the whole inhibition induced by the natural extract. This result suggests the occurrence of additional compounds in the natural extract that significantly contribute to the strong inhibition.

Effect of 8-5′-BenDiFA on the biosynthesis of type B trichothecenes by *F. culmorum*

8-5′-BenDiFA, one of the most abundant diferulic acid in wheat bran phenolic extract, was successfully synthesized from ferulic acid methyl ester according to the procedure of Rakotondramanana et al. (2007). 8-5′-BenDiFA was tested *in vitro* for its effect on type B trichothecene production by the INRA 117 *F. culmorum* strain and the results compared to the effect of ferulic acid. Whatever the studied concentration, ferulic acid and 8-5′-BenDiFA supplementations did not affect fungal biomass production compared to control cultures (data not shown). Results show that only treatments with 0.5 mM of ferulic acid and 8-5′-BenDiFA lead to a significant decrease ($p<0.05$) in type B trichothecene accumulation by 29% and 23% respectively. The inhibitory effect of the diferulic acid was not significantly different to that of ferulic acid.

Discussion

Our results show a very strong *in vitro* inhibition of type B trichothecene biosynthesis by an extract of the phenolic acids naturally occurring in bran. This inhibition was observed for two different *F. culmorum* strains and with a natural extract from two different varieties of durum wheat. As the extract mainly contains ferulic acid, such an observation is consistent with a previous report describing a strong inhibitory effect of pure ferulic acid on type B trichothecene biosynthesis by *F. culmorum* (Boutigny et al. 2009). Our data are in agreement with a previous study describing the inhibitory effect of natural phenolic acids extracted from maize germ on *in vitro* type B trichothecene production by *F. graminearum* (Bakan et al. 2003). Natural phenolic acids extracted from the pellicle of walnut (Mahoney and Molyneux 2004) or from olive callus tissues (Paster et al. 1988) have also been shown to affect aflatoxin production by *Aspergillus* species.

Phenolic acids are known to have strong antioxidant properties (Rice-Evans et al. 1996). Wheat bran extract, containing a high concentration of phenolic acids, was characterized by a higher antioxidant activity than other fractions of wheat (Onyeneho and Hettiarachchy 1992). Trichothecenes are synthesized from trichodiene by a series of oxygenations (Desjardins 2006). Therefore, changes in the oxidative parameters of the medium could interfere with the secondary metabolism of the fungus and modulate the levels of trichothecene production. Recent *in vitro* experiments suggested that plant metabolites with pro-oxidant properties have a strong activating effect on type B trichothecene biosynthesis by *F. graminearum* and that suppressing the oxidant molecule from the medium strongly decreases synthesis of the toxin (Ponts et al. 2007). Moreover, other studies showed inhibitory effects of various antioxidant compounds on fumonisin production by *Fusarium verticillioides* and *Fusarium proliferatum* (Etcheverry et al. 2002; Torres et al. 2003) or on aflatoxin production by *Aspergillus* species (Passone et al. 2005; Kim et al. 2008). It has been suggested (Adams and Moss 1995) that antioxidants may act at the cell membrane, by eliminating the pH component of the proton active force and affecting energy transduction and substrate transport by alteration of cell membrane permeability. By perturbing the membrane function, antioxidant compounds could indirectly interfere with the sec-

ondary metabolism of the fungus and thus modulate mycotoxin production.

The mechanisms by which the mixture of phenolic acids extracted from wheat bran inhibits the biosynthesis of the toxin remain unclear. However, our results demonstrate that the presence of wheat bran phenolic extract can strongly and specifically interfere with the transcription of the genes implicated in the type B trichothecene production by *F. culmorum*. The strong decrease in the level of expression of various *Tri* genes can fully explain the inhibition of toxin production. In treated cultures, the level of expression of the genes *Tri5*, *Tri4* and *Tri11* was drastically reduced. Expression of the non-cluster gene *Tri101* was also reduced but to a lower extent and the reduction occurred latter. In *Fusarium sporotrichioides, Tri6* and *Tri10* have been shown to regulate the expression of the other *Tri* genes in the schematic regulatory model of the biosynthesis pathway (Tag et al. 2001; Peplow et al. 2003). More recently it has been reported that in *F. graminearum* the main regulator of the biosynthesis pathway is *Tri6* (Seong et al, 2009). We observed that expression of these two genes was not so strongly reduced compared to the other *Tri* genes. Moreover, expression of the *Fpps* gene which has also been shown to be under the control of *Tri10* (Peplow et al. 2003) and *Tri6* (Seong et al. 2009) was not repressed by the treatment. These regulatory circuits have been described in *F. sporotrichioides* and *F. graminearum* and it cannot be excluded that they are different in *F. culmorum*. However, according to our observations, *Tri10* and *Tri6* are probably not the only transcription factors able to regulate structural *Tri* genes expression in *F. culmorum* cultures treated with natural phenolic acid extract and the strong and rapid repression observed suggests the action of a repressor. Regulation of aflatoxin and sterigmatocystin gene clusters in *Aspergillus* is under the control of specific transcription factors but is also modulated by more global regulatory factors that mediate environmental signals including carbon (CreA) and nitrogen (AreA) source, ambient temperature, light and pH (PacC) (Yu and Keller 2005). We hypothesize that our target *Tri* genes could also be under the control of more global regulators or of other yet unidentified transcription factors. We also cannot exclude at this stage that other mechanisms, including post transcriptional regulations, or more simply direct biochemical modulation of enzymatic activities, could also participate in the reduction of toxin synthesis by the phenolic acid extract.

As the natural extract and the reconstituted mixture of phenolic acids differentially reduce type B trichothecene production by *F. culmorum in vitro*, various interpretations can be proposed. The first simple interpretation could be that the total concentration of phenolics in the natural extract (sum of monomers plus oligomers) is greater than in the reconstituted mixture (monomers only). This could account for the greater inhibition caused by the natural extract. The sum of dimeric compounds has been estimated to be equivalent to 50% of the mass of monomers and then could increase the concentration by about 25%. However, this seems insufficient to explain the difference observed in the inhibitory effect induced by the natural extract and the reconstituted mixture. A second interpretation could be that the oligomeric forms or another unidentified compound occurring in the natural extract are far more potent inhibitors than the monomers of phenolic acids. Except for the monomers of phenolic acids, the most abundant molecules in the extract are oligomeric forms of phenolic acids (mainly DiFAs). Dimers of phenolic acids have already been suspected to act as a resistance factor to *F. graminearum* in maize (Bily et al. 2003). Here, as a first approach, we chemically synthesized the 8-5′-BenDiFA, one of the most abundant diferulic acid in the natural extract, and we showed that this compound inhibits type B trichothecene biosynthesis to the same extent as ferulic acid. However, the stronger inhibition may be due to one of the other dimers of ferulic acid. These are not as easy to synthesize and must be purified directly from the natural extract (Bunzel et al. 2004). It would be of particular interest to fractionate the pool of dimers from the whole natural extract and investigate if an inhibitory activity is associated with this fraction containing the ferulic acid oligomers. If yes, then we will try to isolate each major oligomeric components of the mixture in order to determine their potential as inhibitors. We also cannot exclude that something other than DiFAs in the natural extract enhances the inhibitory potential. However, because it would be present at a very low concentration in the extract, this unidentified compound should be biologically very active to cause such a high inhibition.

In bran, phenolic acids are present in free soluble forms but they are mainly insoluble and bound to cell wall polysaccharides by ester linkages, with ferulic

acid being the major phenolic acid (Kim et al. 2006). Content of free soluble forms is too low to account for any effect in the grain. However, during infection with *Fusarium*, some esterified forms of phenolic acids can be released from cell walls by esterase and then participate in the interaction (Faulds and Williamson 1995). It has been observed that incubation of wheat cell wall with ferulic acid esterase led to a release of diferulic acid (Bartolome et al. 1997). It cannot be excluded that such oligomeric forms of ferulic acid released from cell walls during the fungal attack could accumulate rapidly at the infection site and then interfere locally with trichothecene biosynthesis. We are now investigating if variations in ester-linked ferulic acid content in cell wall could explain, at least in part, the differences in the susceptibility of some durum wheat varieties to toxin accumulation. This would indicate the potential for the development of lines that inhibit toxin production.

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