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Distribution of toxigenic *Fusarium spp.* and mycotoxins production in milling fractions of *Durum* wheat

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Distribution of toxigenic *Fusarium spp.* and mycotoxins production in milling fractions of Durum wheat

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Abstract

A reliable and sensitive PCR assays to specifically detect trichothecene-producing *Fusarium spp.* in milling fractions and kernel tissues of naturally infected durum wheat is reported. The assays were based on a combination of primers derived from the trichodiene synthase and the β -tubulin genes. The occurrence of toxigenic *Fusarium spp.* in semolina and wheat tissues (grain ends, crease, pericarp, aleurone layer, germ and albumen) was detected, even for a weakly contaminated wheat sample. We therefore demonstrated the penetration of toxigenic *Fusarium spp.* into the interior of durum wheat kernel for the Nefer variety and indicated that none of the tissue structures within the wheat kernel acted as an effective barrier to fungal invasion. Moreover, after inoculation by toxigenic *Fusarium* strains, semolina was shown to allow high yields of trichothecenes while bran was demonstrated to contain biochemical inhibitors able to significantly reduce trichothecene production. These results will be useful for improving breeding strategies to control trichothecene contamination of durum wheat kernels.

Keywords: *Fusarium spp.*, trichothecenes, deoxynivalenol, nivalenol, durum wheat, PCR detection

Introduction

Fusarium head blight (FHB) also known as scab, belongs to the most damaging disease of small grains cereals (particularly wheat and barley). FHB may be caused by a complex of ear blight pathogens including *Fusarium* species from the discolor section (mainly *F. culmorum*, *F. graminearum*, *F. avenaceum* in Europe) and *Microdochium nivale* (Bottalico and Perrone 2002). FHB does not only result in reduced yield, but also in reduced milling and malting quality of grains. Moreover, *Fusarium* species can produce highly toxic metabolites, such as trichothecenes mycotoxins and zearalenone. *F. graminearum* and *F. culmorum* have been described as the most toxigenic species in Europe (Logrieco et al. 2002). Of the mycotoxins produced, the most abundant belong to the type B trichothecenes family (Larsen et al. 2004). Those are deoxynivalenol (DON) and its 3- and 15-acetylated derivatives (3 and 15-ADON), nivalenol (NIV) and fusarenone X (FX). Because they are resistant to food processing and do not readily degrade at high temperature, type B trichothecenes enter the animal and human food supply. Type B trichothecenes are detrimental to livestock and possibly affect human health (Schlatter 2004). Studies on laboratory animals have revealed a complex spectrum of toxic effects induced by a chronic exposure to type B trichothecenes: anorexia, reduced weight gain, diminished nutritional efficiency, neuroendocrine changes, and immune modulation (Rotter et al. 1996).

Surveys on the occurrence of DON in cereal grains showed it is a common contaminant of wheat worldwide (Pittet 1998). In Europe, a recent data collection on the occurrence of *Fusarium* toxins in food was performed within a SCOOP project (Gareis et al. 2001). This study demonstrated an incidence of DON positive samples (above a limit of 2 µg kg⁻¹) of around 55% of some 6000 samples of both common and durum wheat. DON contamination of

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durum wheat kernels has frequently been described (Dexter et al. 1997; Pascale et al. 2002). If mean contamination level in positive samples rarely exceeds 1 mg kg⁻¹, levels as high as 6 mg kg⁻¹ have been reported (Pascale et al. 2002). European legislation (Regulation EC, No 856/2005), dealing with maximum levels of DON in cereals and cereals products, will be applied in July 2006. Concerning durum wheat, the proposed limits are as follows: 1750 µg kg⁻¹ for unprocessed durum wheat, 750 µg kg⁻¹ for wholemeal wheat flour, bran and pasta (dry), 500 µg kg⁻¹ for cleaned cereals intended for human consumption and all derived products. Several studies have been carried out on durum wheat to determine the stability and partitioning of DON during semolina milling (Nowicki et al. 1988, Dexter et al. 1997, Visconti et al. 2004). According to these studies, levels of DON in the bran were two or more times greater than in the wheat, indicating a concentration of toxin in the outer parts of the kernel. Moreover, the average amount of DON retained in the semolina ranged between 30 and 50 % of the amount found in conditioned wheat. Retention level in semolina was shown to depend from the DON contamination levels of the unprocessed wheat (Visconti et al. 2004) and/or the variety of *durum* wheat (Nowicki et al. 1988). Occurrence of toxins in the endosperm tissues of kernel may be ascribed to two types of mechanisms, a translocation from the kernel surface to the interior or/and a fungal production inside the endosperm tissues. The occurrence of a significant diffusion of type B trichothecenes in all the tissues of seeds was demonstrated by Kang and Buchenauer (1999). The previous study has reported that toxins diffused inside the kernel before the pathogen invaded into the host tissues. However, according to Seitz and Bechtel (1985) and Nowicki et al. (1988), type B trichothecenes are localized at the site of their production rather than being transported inside the kernel, as indicated by the high correlation they obtained between ergosterol and DON levels in the milling fractions. These contradictory conclusions certainly result from the fact that most of the investigations dealing with *Fusarium* colonization of wheat kernels were performed on

artificially inoculated spikelets and therefore with high levels of contamination (Kang and Buchenauer 1999 and 2002; Jackowiack et al. 2005). Few data concerning naturally contaminated heads have been published and the detection methods used in these studies (microscopy examinations or ergosterol analysis) were rarely specific for *Fusarium* strains able to produce type B trichothecenes (Seitz and Bechtel 1985 ; Bechtel et al. 1985).

As another step to establish the penetration degree of *Fusarium* inside naturally contaminated durum wheat kernels, we report here the development and optimization of molecular tools specific for potential type B trichothecenes producing strains. We then used the defined tools to analyze milled fractions and wheat tissues obtained from lightly and heavily naturally DON contaminated kernels. In addition, to investigate the occurrence of an *in situ* type B trichothecenes production, we compared the amounts of toxins synthesized by two *Fusarium culmorum* strains on different milling fractions.

Materials and Methods

Wheat samples, milling fractions and kernel tissues

The three durum wheat samples were of Nefer elite line and from the 2002 French crop. Milling fractions were provided by the UMR Agropolymer Engineering and Emerging Technologies (INRA, Montpellier, France). They were obtained from a conventional milling process performed in a semi-industrial semolina mill (150 kg h⁻¹) as described by Chaurand et al. (1999). DON content of the 3 wheat samples and associated milling fractions was HPLC-UV determined after clean up with immunoaffinity column, by a commercial analytical laboratory (INZO, Château-Thierry, France). Sample 1 originally contained a high DON level close to 8000 µg kg⁻¹. Sample 2 was less contaminated with a DON content of about 750 µg

kg⁻¹. The amounts of DON assayed in semolina were 3500 µg kg⁻¹ for sample 1 and close to 500 µg kg⁻¹ for sample 2. No toxins were detected in sample 3.

Wheat tissues were hand-isolated from 20 kernels of sample 1 and 2, according to Peyron et al. (2002) and provided by the UMR Agropolymer Engineering and Emerging Technologies (INRA, Montpellier, France). For each wheat sample, available amounts (dry matter) of tissues were as follow: 850 mg of albumen, 80 mg of aleurone layer, 35 mg of pericarp, 30 mg of germ, 50 mg of grain ends and 30 mg of crease.

Mycological screening

Each milling fraction (1 g) was plated directly onto different culture media (malt extract agar, potato dextrose agar and osmophilic medium) in triplicate. Culture media were prepared according to Cahagnier (1998). Plates were incubated in constant daylight at 25 °C for 7 days. Fungal colonies were identified in accordance with guidelines published for each genus (Pitt and Hocking 1999).

DNA isolation

For fungal DNA extraction, isolates of each identified genus were grown initially in 5 ml malt extract agar for 5 days at 25 °C. Spore suspensions were prepared by adding 6 ml of sterile diluting solution (tryptone 1 % w/v, NaCl 8,5 % w/v, tween 80 0,3 %) to the malt slants with gentle shaking. 0.5 ml of spore suspensions was added to 100 ml liquid malt extract (30 % w/v) medium and incubated for 5 additional days at 25 °C. Mycelia were vacuum-filtered on Whatman No.1 filter paper, washed three times with sterile water, lyophilised and stored at – 20°C until use. DNA was extracted from the mycelia and purified according to the method described by Bakan *et al.* (2002). The DNA pellets were re-dissolved in 300 µl of TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). DNA was extracted from naturally-infected

grains or milling fractions with the Dneasy® Plant Mini Kit from Qiagen following the manufacturer's instructions. Before extraction, samples were ground to a fine powder under liquid nitrogen.

PCR amplification

The primers used in this study are listed in Table I. All PCR amplification were carried out in volumes of 50 µl containing 1 µl of DNA template. Reactions mixtures contained 25 pmol of each primers pair for tox5-1/tox5-2 and $\beta t1/\beta t2$, 5 pmol for ITS3/P3, 2.5 U of DNA polymerase (Invitrogen, Cergy, France), 400 µM of deoxynucleotide triphosphate. Reactions were performed using the following PCR conditions: denaturation at 95°C for 1.5 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 40 s and extension at 72°C for 30s; final extension at 72°C for 5 min, followed by cooling at 4°C until recovery of the samples. Amplification products were checked on 1% agarose gel stained with ethidium bromide.

[Insert Table I about here]

Sequencing of PCR products

The PCR products were purified with a GFX gel band Kit (Pharmacia Amersham Biotech) as indicated by the manufacturer. All DNA sequencing of purified PCR products was done by a commercial sequencing laboratory (Genome Express, Grenoble, France).

Fusarium isolates, inoculation and incubation conditions

The two *F. culmorum* strains used in this study, INRA 305 and INRA 337, were single spore purified from diseased wheat spikes and characterized for toxin production according to the procedure described by Bakan *et al.* (2002). INRA 305 was a DON and 15-ADON producer,

INRA 337 a NIV and FX one. Stock cultures were maintained on Potato Dextrose Agar (PDA) slants at 4 °C.

Kernels, bran and semolina from *Durum* wheat sample 3 (the uncontaminated one) were moistened by addition of sterile water and homogenized for 5 days at 4°C until a_w level was maximal. Grains or milling fractions were weighed (50 g) into 250-ml Erlenmeyer flasks and sterilized twice for 25 min at 110 °C. a_w was checked before inoculations. Each flask was inoculated with a suspension of 10^4 fungal conidia. Incubations were conducted at 25 °C for 21 days, in triplicate for each wheat substrate and strain. Before analysis, flask contents were dried at 50°C in a ventilated oven for 72 h, ground and homogenized.

Ergosterol and type B trichothecenes analysis

Ergosterol was analysed according to the protocol optimised by Cahagnier et al. (1993). Ergosterol was extracted after saponification in a mixture of methanol, ethanol, and potassium hydroxide and purified on a solid-phase extraction column (Extrelut 20 R, Merck, Darmstadt, Germany). The organic phase (n-hexane) eluted from the column was evaporated to dryness and the residue resuspended in the HPLC mobile phase (dichloromethane). Ergosterol was assayed by HPLC on a silica column and detected at 282 nm. Quantification was achieved using an external standard (Merck), results were expressed as mg kg⁻¹ of ergosterol on a dry weight basis.

Type B trichothecenes content of inoculated kernels and wheat milling fractions was determined by GC-ECD as described by Bakan et al. (2001). Samples were extracted with acetonitrile/water (84:16, v/v) and purified using one-step cleanup column (Trichothecenes P[®] Columns, R-Biopharm, Glasgow, Scotland). Trichothecenes derivatization was achieved

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3 using N-trimethylsilylimidazole according to the protocol described by Seibel et al. (1993).
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5 Trimethylsilyl derivatives were quantified by gas chromatography using electron capture
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7 detection. External calibration was performed with standards solution purchased from Sigma
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9 (St Louis, MO, USA). Results were expressed in milligrams of type B trichothecenes per
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11 kilogram of dry matter. Detection limits were 0.01 mg kg⁻¹ for DON and 0.02 mg kg⁻¹ for
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20 **Results and Discussion**

21 *Mycological screening*

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23 Mycological analyses performed on the durum wheat samples considered in this study
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25 (kernels and associated bran and semolina) revealed the occurrence of eight fungal genera in
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27 addition to *Fusarium*. The recovered genera, summarized in Table II, are common cereals
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29 contaminants and 3 of them, namely *Aspergillus*, *Penicillium* and *Fusarium*, have been the
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31 focus of numerous reviews (Schuster et al. 2002; Xu and Berrie 2005). Several species of
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33 these genera are able to produce the major mycotoxins subject to regulation by most
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35 countries, *i.e.* aflatoxin, ochratoxin and deoxynivalenol. The nine genera were detected in all
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37 milled fractions and were particularly abundant in both kernels and bran. Specificity analysis
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39 of the detection tests defined in this study will have to include these fungal genera.
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45 [Insert Table II about here]
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50 *Specificity and sensitivity of the defined diagnostic PCR system*

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52 The first and main objective of our investigations was to establish the penetration degree of
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54 mycotoxigenic *Fusarium spp.* inside harvested kernels. To achieve this objective, we decided
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56 to develop a reliable and sensitive PCR assay for selective detection of potential
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58 trichothecenes-producing *Fusarium* species. We therefore checked if the Tox 5 PCR assay
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reported by Niessen and Vogel (1998) could fit in with the specificity and sensitivity requirements of our study. Tox 5 PCR assay is based on primers derived from the trichodiene synthase gene (*Tri5*). This enzyme catalyses the first step of type B trichothecenes biosynthetic pathway (Hohn and Desjardins 1992), *i.e.* the isomerization and cyclization of farnesyl pyrophosphate to yield trichodiene. Purified DNA, isolated from mycelial cultures of a *Fusarium culmorum* strain (INRA 16), was subjected to duplex PCR analysis using primer pairs Tox5-1/Tox5-2 and ITS1/P3. Amplification resulted in the production of two fragments of about 650 bp and 1200 bp, according to the size markers (Figure 1). The 650-bp size was in accordance with the predicted size (658 bp) of the *Tri5* fragment (Niessen and Vogel 1998). The 1200 bp product was associated with the amplified portion from the ITS region of *Fusarium culmorum*. Identity of the two products was checked after sequencing. Specificity of the Tox 5 PCR assay designed to detect trichothecenes-producing *Fusarium*-species was largely documented by Niessen and Vogel (1998); more than 64 species and varieties of *Fusarium* were considered in this study. However, the number of fungal genera analysed by Niessen and Vogel (1998) was lower than 10 and no data concerning *Alternaria*, *Aspergillus*, *Botrytis*, *Epicoccum*, *Rhizopus* were available. Because of the occurrence of these genera in the *Durum* wheat samples involved in our study (Table II), it was essential to test Tox 5 primers cross-reactivity with DNA from all fungal isolates we previously identified. Results of PCR assays obtained with DNA extracted from mycelial culture of each isolate are given in Figure 1. None of the non-*Fusarium* isolate produces the *Tri5* fragment (theoretical size : 658 bp). Besides, occurrence of an ITS amplicon (corresponding to the expected size) for each isolate illustrated the quality of purified DNA and the success of the PCR reaction. The negative Tox 5 PCR reaction obtained with *Alternaria*, *Aspergillus*, *Botrytis*, *Epicoccum*, *Mucor*, *Penicillium* and *Rhizopus* was well correlated with the fact that none of these genera has been reported as trichothecenes producers in the reviewed literature. The negative reaction

obtained with *Trichoderma* was more surprising since *Trichoderma* such as 3 other genera (*Stachybotrys*, *Myrothecium* and *Trichothecium*) have been reported to be able to produce macrocyclic trichothecenes (Wilkins et al. 2003). *Tri5* sequences were published for *Myrothecium roridum* (Trapp et al. 1998) and *Stachybotrytis chartarum* (Straus et al. 1999) and a putative trichodiene synthase was reported in database for *Trichoderma Harzanium* (accession number AJ784992.1). However, the lack of Tox 5 signal observed with our *Trichoderma* sample was in accordance with the negative reaction described by Niessen and Vogel (1998) for the other macrocyclic trichothecenes producers and was consistent with the conclusion of Fekete et al. (1997), *i.e.* the occurrence of evolutionary variants of the *Tri5* gene in these fungi. In fact, comparison of the *Stachybotrytis* and *Myrothecium* available *Tri5* sequences (accession number AF053926 and AF009416.1) revealed significant divergences with *Tri5* sequences present in *Fusaria spp.* (*Fusarium sporotrichoides*, accession number M27246), especially in the Tox5 primers binding region. These data demonstrated that the PCR assay previously defined can be used for the specific detection of trichothecenes-producing *Fusarium*-species inside wheat kernels.

The diagnostic assay developed in the current study was then applied to bran and semolina but also to wheat tissues, with less than 50 mg of material available for several samples such as pericarp, germ, crease and grain ends. When *Tri5* amplification were used in the literature to detect and/or quantify trichothecenes-producing *Fusarium spp.* in cereals, more than 1 g of ground grains were usually used to extract the DNA (Doohan et al. 1998, Niessen and Vogel 1998, Edwards et al. 2001; Schnerr et al. 2002). To determine the minimum amount of wheat material that could be successfully analyzed by our established Tox5 PCR assay, reactions were set up with increasing quantities ranging from 10 mg to 500 mg of the highest contaminated semolina fraction (DON close to 3500 µg/kg). Reliable amplification of specific

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Tox5 PCR fragment was obtained with only more than 100 mg of semolina (data not shown). Therefore, a new pair of primers specific for *Fusarium* species was designed to be applied on samples below 100 mg and to screen wheat tissues. These primers, called β t1 and β t2 (described in Table I), were designed to amplify a 296 bp portion of β -tubulin gene. Specificity of the primers was tested against 20 strains of *Fusarium culmorum* and *graminearum* and all the fungal isolates reported in Table II. Whatever the considered *Fusarium* strain, an amplicon of correct size (296 bp) was obtained (data not shown). These results attesting for the high homology among β -tubulin sequences of *F. graminearum* and *culmorum* were in accordance with the work of Gagkaeva and Yli-Mattila (2004). Data obtained with the other fungal genera are given on Figure 2. If no cross-reaction occurred with the isolates of *Alternaria*, *Epicoccum*, *Mucor* and *Trichoderma*, isolates of *Aspergillus*, *Botrytis*, *Penicillium* and *Rhizopus* showed amplification with the β t primers pair. However, the amplified fragments were higher than 296 bp (370 bp for *Aspergillus*, 410 bp for *Penicillium*, 425 bp for *Botrytis* and 470 bp for *Rhizopus*) and can therefore be easily distinguished from the specific *Fusarium* PCR-product. Purified PCR products were sequenced and compared to the published corresponding β -tubulin gene sequences, which accession numbers are reported in Table III. The *Botrytis* and *Penicillium* fragments were identified as β -tubulin amplicons. In opposite, *Aspergillus* and *Rhizopus* ones were not β -tubulin. [Insert table III about here] With a view to use the β -tubulin PCR assay on wheat tissues and to avoid false positive results from cross-reaction with plant DNA, the primers were tested with total genomic DNA extracted from healthy wheat. No amplification was obtained with healthy materials. A minimum amount of 30 mg of contaminated semolina (the highest contaminated one, DON close to 3500 μ g kg⁻¹) was sufficient for a reliable amplification of *Fusarium* β -tubulin that was detectable in the gel (data not shown). Sensitivity of the β -tubulin PCR assay was therefore significantly greater than that of Tox5

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3 PCR assay. This high sensitivity, which probably results from the minor size of the amplified
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5 β -tubulin fragment (296 bp), will now allow the screening of wheat tissues for contamination
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8 by *Fusarium spp.* mycelium.
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10 11 12 *Detection of Fusarium in milling fraction and Durum wheat tissues*

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15 To establish the distribution of mycotoxinogenic *Fusarium* inside harvested kernels, naturally
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17 infected Nefer wheat kernels and associated milling fractions were analyzed. Six samples,
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19 corresponding to two levels of DON contamination, were extracted as described in '*Material*
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21 *and Methods*' and subjected to Tox5 PCR. The results obtained with the bran and semolina
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23 are given in Figure 3. Positive reactions were obtained for all samples, indicating the presence
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25 of DNA from trichothecenes-producers in all milling fractions, even in the weakly
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27 contaminated semolina. Consistent with these data, when β -tubulin PCR assay was applied to
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29 wheat tissues (grain ends and crease, pericarp and aleurone layer, germ and albumen),
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31 *Fusarium* DNA was detected in all samples (Figure 4). These observations clearly illustrated
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33 the usefulness of the developed PCR assays, which were capable of detecting specific fungal
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35 contaminants *in vivo*, even in weakly infected tissues. Moreover, our results undoubtedly
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37 demonstrated the penetration of toxigenic *Fusarium* strains into the interior of durum wheat
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39 kernel at least for the Nefer variety used in this work. This suggests that none of the
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41 considered wheat tissue act as an effective barrier to fungal invasion. The occurrence of
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43 barrier structures, able to restrain *F. gramineum* infection, were previously described by
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45 Voigt et al. (2005). These barriers were however not localized inside the kernel but at the
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47 transition zone between rachilla and rachis of the wheat spike.
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58 To our knowledge, this is the first observation of toxigenic *Fusarium spp* penetration inside
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60 naturally contaminated wheat kernel. The former published studies describing *Fusarium spp.*

invasion were performed on artificially inoculated spikelets with the risk of induced artifacts (Kang and Buchenauer, 1999 and 2002, Jackowiack et al. 2005) and/or with analytical methods that were not specific for type B trichothecenes producers (Seitz and Bechtel 1985, Bechtel et al. 1985).

*Trichothecenes production by *F. culmorum* on Durum wheat milling fractions*

Occurrence of toxins in endosperm tissues (Kang and Buchenauer 1999, Visconti et al. 2004) concomitant with the presence of toxigenic *Fusarium* species is consistent with an *in situ* type B trichothecenes production, as suggested by Seitz and Bechtel (1985) and Nowicki et al. (1988). According to Kang and Buchenauer (2002), *Fusarium* hyphae were able to yield toxins during penetration and growth on wheat spikelets at the earliest stages of infection. With the view to investigate the occurrence of a type B trichothecenes production on more mature tissues, two *F. culmorum* strains (a DON and 15-ADON producer, a NIV and FX producer) were inoculated on healthy kernels, bran and semolina from the Nefer durum wheat variety. Fungal biomass and type B trichothecenes yield were evaluated on each substrate 21 days after inoculation. Fungal biomass was estimated by quantification of ergosterol, a fungus-specific membrane compound. Results are given in Figure 5. A substantial fungal biomass was obtained whatever the considered strain and substrate, as attested by the high amounts of quantified ergosterol. If fungal biomass were not significantly different on the three substrates for INRA 305 strain, semolina and bran induced however a strong decrease for INRA 337. Our data are in accordance with the predicted chemotypes of the two considered strains: *F.culmorum* INRA 305 produced DON and 15-ADON whereas *F. culmorum* INRA 337 produced NIV and FX. For the two strains, high toxins yields (DON+15-ADON or NIV+FX), greater than 300 mg kg⁻¹, were obtained on whole kernels and semolina. However, on bran, weak amounts of type B trichothecenes were measured,

close to 40 mg kg⁻¹ of DON+15-ADON for the INRA 305 strain and 10 mg kg⁻¹ of NIV+FX for the INRA 337 strain. These data associated with the equivalent amounts of ergosterol determined on bran and semolina represent a further demonstration that growth and type B trichothecenes production may be independently regulated (Bakan et al. 2001, Reynoso et al. 2002). To explain the strong decrease in type B trichothecenes yield observed on bran, two hypotheses were raised: (i) lack of essential nutrients required for fungal toxinogenesis, (ii) occurrence of endogenous compounds acting as inhibitors of type B trichothecenes biosynthesis. To investigate the former hypotheses, the INRA 337 strain was inoculated in GYEP liquid medium supplemented with 0.05 g l⁻¹ and 0.1 g l⁻¹ of bran (Nefer variety). Results are reported on Figure 6. GYEP medium, a nutrient-rich laboratory substrate, was frequently shown to support great yields of type B trichothecenes (Miller and Greenhalgh 1985, Ponts et al. 2003, Covarelli et al. 2004). This high efficiency was confirmed by the significant levels of toxins, close to 25 µg ml⁻¹, obtained after 32 days of culture in control batches. If bran supplementation of GYEP did not significantly affect the amount of fungal biomass, it was shown to induce a strong decrease in NIV+FX production. The levels of produced toxins were more than twice reduced with 0.05 g l⁻¹ of bran and only traces were detected in media supplemented with 0.1g l⁻¹. These data strongly suggested the occurrence of endogenous biochemical compounds in bran of *durum* wheat that were able to significantly reduce the biosynthesis of type B trichothecenes. A similar conclusion was previously reported for maize germ fraction by Bakan et al. (2003). These authors have ascribed the observed inhibition to phenolic compounds and more precisely to the high content of ferulic acid occurring in maize germ. Bran of *durum* wheat is also characterized by significant concentrations of phenolic acids with mainly ferulic, *p*-coumaric and vanillic acids (Onyencho and Hettiarachchy 1992). Additional studies are in progress to ascertain the effects of these phenolic acids on the production of type B trichothecenes by *F. culmorum*. Moreover,

according to the literature data, other phenolic compounds such as flavonoïds and/or furanocoumarins (Desjardin et al. 1988) but also small peptides (Huang et al. 1997) could be implied in these inhibition mechanisms. Further studies will therefore been undertaken to isolate and identify the compounds in bran that may be responsible for toxin biosynthesis inhibition.

Conclusion

Two main conclusions can be drawn from the present study: (i) Toxigenic *Fusarium* strains are present in the core of durum wheat kernel infected under natural conditions, (ii) if semolina represents an excellent substrate for toxinogenesis, bran fractions appear to contain biochemical compounds that are able to significantly reduce type B trichothecenes yields. Since toxins have been shown to concentrate in the outer parts of the wheat kernel (Nowicki et al. 1988), identification of the inhibitor compounds occuring in bran may represent a significant improvement in the selection of durum wheat varieties less sensitive to DON contamination. Moreover the developed PCR assays, that can specifically detect *toxigenic Fusarium* strains *in vivo*, even in weakly infected tissues, may also be useful to select varieties more resistant to *Fusarium* penetration; use of these varieties in milling process may lead to less contaminated semolina.

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Figure Captions

Figure 1. Duplex PCR amplification patterns with primer pairs Tox 5-1/Tox 5-2 (lower band, close to 650 bp) and ITS1/P3 (upper band) from isolates of *F. culmorum* (lane 1) and fungal genera reported in Table II (lane 2: *Alternaria*, lane 3: *Aspergillus*, lane 4: *Botrytis*, lane 5: *Epicoccum*, lane 6: *Mucor*, lane 7: *Penicillium*, lane 8: *Rhizopus*, lane 9: *Trichoderma*). The molecular weight standard (lane M) is a 1-Kb ladder (Promega, Charbonnieres, France). Lane C⁻ represents the negative control.

Figure 2. PCR products generated with the β t1/ β t2 primer pair. A 296 bp fragment is exclusively amplified from *F. culmorum* (lane 1). Amplicons are detected for *Aspergillus* (lane 3), *Botrytis* (lane 4), *Penicillium* (lane 7) and *Rhizopus* (lane 8). No amplification are obtained with *Alternaria* (lane 2), *Epicoccum* (lane 5), *Mucor* (lane 6) and *Trichoderma* (lane 9). Lane M, the molecular weight standard is a 100-bp ladder (Promega, Charbonnieres, France).

Figure 3. Detection of toxigenic *Fusarium* in contaminated milling fractions. Lane C⁺: positive control showing the 658 bp *Tri5* fragment amplified from 10 ng of purified genomic DNA of *F. culmorum*. Lanes 1 to 4, milling fractions from two contaminated samples of *durum* wheat (DON content of sample 1 and 2 are close to 8000 μ g kg⁻¹ and 750 μ g kg⁻¹, respectively). Lane C⁻: negative control. Lane M: 100-bp ladder molecular weight Marker from Promega (Charbonnieres, france).

Figure 4. Detection of toxigenic *Fusarium* in contaminated wheat tissues. Lane C⁺: positive control showing the 296 bp β -tubulin fragment amplified from 10 ng of purified genomic

DNA of *F. culmorum*.. Lanes 1 to 12 : wheat tissues from two contaminated samples of *durum* wheat (DON content of sample 1 and 2 are close to 8000 $\mu\text{g kg}^{-1}$ and 750 $\mu\text{g kg}^{-1}$, respectively). Lane C: negative control. Lane M : 100-bp molecular weight Marker from Promega (Charbonnieres, france).

Figure 5. Fungal biomass (estimated by ergosterol content) and type B trichothecenes production (DON+15-ADON or NIV+FX) on *durum* wheat kernels, bran and semolina by two *F. culmorum* strains of different chemotypes (INRA 305, a DON + 15-ADON producer and INRA 337, a NIV + FX producer) after 21 days of incubation. Kernels, bran and semolina were obtained from the sample 3 (no toxin detected) of Nefer elite line. Bars represent mean $\pm$ standard error relevant to three flasks (with three extractions per flask).

Figure 6. Fungal biomass (estimated by ergosterol content) and NIV+FX yields after 26 and 32 days of incubation of the *F. culmorum* strain INRA 337 in GYEP liquid cultures supplemented or not with bran (0.05 g l^{-1} and 0.1 g l^{-1}). Bran was from the sample 3 (no toxin detected) of Nefer elite line. Bars represent mean $\pm$ standard error relevant to three flasks (with three extractions per flask).

Table I: Oligonucleotide primers used in the current study.

Primers	Nucleotide Sequences (5'to 3')	Target	Fragment length (bp)	Reference
tox5-1	GCTGCTCATCACTTTGCTCAG	<i>TRI5</i>	658	Niessen and Vogel 1998
tox5-2	CTGATCTGGTCACGCTCATC			
ITS1	TCCGTAGGTGAACCTGCGG	rDNA fragment including ITS1, 5,8S rDNA, ITS2 and 630 nucleotides of the 5' end of the 28S rDNA	1200	Edel et al 1996
P3	CCTTGGTCCGTGTTTCAAGACGG			
βt-1	GGTAACCAAATCGGTGCTGCTTTC	<i>βtubulin</i>	296	Doohan et al 1999 (βT2a)
βt-2	GATTGACCGAAAACG AAGTTG			This study

Table II: Fungal genera isolated from the *durum* wheat samples considered in the current study

Contaminants present on the <i>durum</i> wheat fractions
<i>Alternaria</i>
<i>Aspergillus</i>
<i>Botrytis</i>
<i>Epicoccum</i>
<i>Fusarium</i>
<i>Mucor</i>
<i>Penicillium</i>
<i>Rhizopus</i>
<i>Trichoderma</i>

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(NIV+FX) ($\mu\text{g/mL}$) [■ standard; ■ 0.05 g L⁻¹ of bran; ■ 0.1 g L⁻¹ of bran] Fungal biomass [—●—] (g)