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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  $\beta$ -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<sup>-1</sup>) of around 55% of some 6000 samples of both common and durum wheat. DON contamination of

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3 durum wheat kernels has frequently been described (Dexter et al. 1997; Pascale et al. 2002). If  
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5 mean contamination level in positive samples rarely exceeds  $1 \text{ mg kg}^{-1}$ , levels as high as  $6 \text{ mg}$   
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7  $\text{kg}^{-1}$  have been reported (Pascale et al. 2002). European legislation (Regulation EC, No  
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9 856/2005), dealing with maximum levels of DON in cereals and cereals products, will be  
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11 applied in July 2006. Concerning durum wheat, the proposed limits are as follows:  $1750 \text{ }\mu\text{g}$   
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13  $\text{kg}^{-1}$  for unprocessed durum wheat,  $750 \text{ }\mu\text{g kg}^{-1}$  for wholemeal wheat flour, bran and pasta  
14  
15 (dry),  $500 \text{ }\mu\text{g kg}^{-1}$  for cleaned cereals intended for human consumption and all derived  
16  
17 products. Several studies have been carried out on durum wheat to determine the stability and  
18  
19 partitioning of DON during semolina milling (Nowicki et al. 1988, Dexter et al. 1997,  
20  
21 Visconti et al. 2004). According to these studies, levels of DON in the bran were two or more  
22  
23 times greater than in the wheat, indicating a concentration of toxin in the outer parts of the  
24  
25 kernel. Moreover, the average amount of DON retained in the semolina ranged between 30  
26  
27 and 50 % of the amount found in conditioned wheat. Retention level in semolina was shown  
28  
29 to depend from the DON contamination levels of the unprocessed wheat (Visconti et al. 2004)  
30  
31 and/or the variety of *durum* wheat (Nowicki et al. 1988). Occurrence of toxins in the  
32  
33 endosperm tissues of kernel may be ascribed to two types of mechanisms, a translocation  
34  
35 from the kernel surface to the interior or/and a fungal production inside the endosperm tissues.  
36  
37 The occurrence of a significant diffusion of type B trichothecenes in all the tissues of seeds  
38  
39 was demonstrated by Kang and Buchenauer (1999). The previous study has reported that  
40  
41 toxins diffused inside the kernel before the pathogen invaded into the host tissues. However,  
42  
43 according to Seitz and Bechtel (1985) and Nowicki et al. (1988), type B trichothecenes are  
44  
45 localized at the site of their production rather than being transported inside the kernel, as  
46  
47 indicated by the high correlation they obtained between ergosterol and DON levels in the  
48  
49 milling fractions. These contradictory conclusions certainly result from the fact that most of  
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51 the investigations dealing with *Fusarium* colonization of wheat kernels were performed on  
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3 artificially inoculated spikelets and therefore with high levels of contamination (Kang and  
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5 Buchenauer 1999 and 2002; Jackowiack et al. 2005). Few data concerning naturally  
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7 contaminated heads have been published and the detection methods used in these studies  
8  
9 (microscopy examinations or ergosterol analysis) were rarely specific for *Fusarium* strains  
10  
11 able to produce type B trichothecenes (Seitz and Bechtel 1985 ; Bechtel et al. 1985).  
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17 As another step to establish the penetration degree of *Fusarium* inside naturally contaminated  
18  
19 durum wheat kernels, we report here the development and optimization of molecular tools  
20  
21 specific for potential type B trichothecenes producing strains. We then used the defined tools  
22  
23 to analyze milled fractions and wheat tissues obtained from lightly and heavily naturally DON  
24  
25 contaminated kernels. In addition, to investigate the occurrence of an *in situ* type B  
26  
27 trichothecenes production, we compared the amounts of toxins synthesized by two *Fusarium*  
28  
29 *culmorum* strains on different milling fractions.  
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## 34 35 36 **Materials and Methods**

### 37 38 *Wheat samples, milling fractions and kernel tissues*

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40 The three durum wheat samples were of Nefer elite line and from the 2002 French crop.  
41  
42 Milling fractions were provided by the UMR Agropolymer Engineering and Emerging  
43  
44 Technologies (INRA, Montpellier, France). They were obtained from a conventional milling  
45  
46 process performed in a semi-industrial semolina mill (150 kg h<sup>-1</sup>) as described by Chaurand et  
47  
48 al. (1999). DON content of the 3 wheat samples and associated milling fractions was HPLC-  
49  
50 UV determined after clean up with immunoaffinity column, by a commercial analytical  
51  
52 laboratory (INZO, Château-Thierry, France). Sample 1 originally contained a high DON level  
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54 close to 8000 µg kg<sup>-1</sup>. Sample 2 was less contaminated with a DON content of about 750 µg  
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3 kg<sup>-1</sup>. The amounts of DON assayed in semolina were 3500 µg kg<sup>-1</sup> for sample 1 and close to  
4  
5 500 µg kg<sup>-1</sup> for sample 2. No toxins were detected in sample 3.  
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10 Wheat tissues were hand-isolated from 20 kernels of sample 1 and 2, according to Peyron et  
11 al. (2002) and provided by the UMR Agropolymer Engineering and Emerging Technologies  
12 (INRA, Montpellier, France). For each wheat sample, available amounts (dry matter) of  
13 tissues were as follow: 850 mg of albumen, 80 mg of aleurone layer, 35 mg of pericarp, 30  
14 mg of germ, 50 mg of grain ends and 30 mg of crease.  
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#### 24 *Mycological screening*

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

42 For fungal DNA extraction, isolates of each identified genus were grown initially in 5 ml malt  
43 extract agar for 5 days at 25 °C. Spore suspensions were prepared by adding 6 ml of sterile  
44 diluting solution (tryptone 1 % w/v, NaCl 8,5 % w/v, tween 80 0,3 %) to the malt slants with  
45 gentle shaking. 0.5 ml of spore suspensions was added to 100 ml liquid malt extract (30 %  
46 w/v) medium and incubated for 5 additional days at 25 °C. Mycelia were vacuum-filtered on  
47 Whatman No.1 filter paper, washed three times with sterile water, lyophilised and stored at –  
48 20°C until use. DNA was extracted from the mycelia and purified according to the method  
49 described by Bakan *et al.* (2002). The DNA pellets were re-dissolved in 300 µl of TE buffer  
50 (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA ). DNA was extracted from naturally-infected  
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3 grains or milling fractions with the Dneasy® Plant Mini Kit from Qiagen following the  
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5 manufacturer's instructions. Before extraction, samples were ground to a fine powder under  
6  
7 liquid nitrogen.  
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9

### 10 11 12 *PCR amplification*

13  
14 The primers used in this study are listed in Table I. All PCR amplification were carried out in  
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16 volumes of 50 µl containing 1 µl of DNA template. Reactions mixtures contained 25 pmol of  
17  
18 each primers pair for tox5-1/tox5-2 and βt1/βt2, 5 pmol for ITS3/P3, 2.5 U of DNA  
19  
20 polymerase (Invitrogen, Cergy, France), 400 µM of deoxynucleotide triphosphate. Reactions  
21  
22 were performed using the following PCR conditions: denaturation at 95°C for 1.5 min; 40  
23  
24 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 40 s and extension at 72°C for  
25  
26 30s; final extension at 72°C for 5 min, followed by cooling at 4°C until recovery of the  
27  
28 samples. Amplification products were checked on 1% agarose gel stained with ethidium  
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30 bromide.  
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36 [Insert Table I about here]  
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### 41 42 *Sequencing of PCR products*

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44 The PCR products were purified with a GFX gel band Kit (Pharmacia Amersham Biotech) as  
45  
46 indicated by the manufacturer. All DNA sequencing of purified PCR products was done by a  
47  
48 commercial sequencing laboratory (Genome Express, Grenoble, France).  
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### 51 52 53 *Fusarium isolates, inoculation and incubation conditions*

54  
55 The two *F. culmorum* strains used in this study, INRA 305 and INRA 337, were single spore  
56  
57 purified from diseased wheat spikes and characterized for toxin production according to the  
58  
59 procedure described by Bakan *et al.* (2002). INRA 305 was a DON and 15-ADON producer,  
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3 INRA 337 a NIV and FX one. Stock cultures were maintained on Potato Dextrose Agar  
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5 (PDA) slants at 4 °C.  
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11 Kernels, bran and semolina from *Durum* wheat sample 3 (the uncontaminated one) were  
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13 moistened by addition of sterile water and homogenized for 5 days at 4°C until  $a_w$  level was  
14  
15 maximal. Grains or milling fractions were weighed (50 g) into 250-ml Erlenmeyer flasks and  
16  
17 sterilized twice for 25 min at 110 °C.  $a_w$  was checked before inoculations. Each flask was  
18  
19 inoculated with a suspension of  $10^4$  fungal conidia. Incubations were conducted at 25 °C for  
20  
21 21 days, in triplicate for each wheat substrate and strain. Before analysis, flask contents were  
22  
23 dried at 50°C in a ventilated oven for 72 h, ground and homogenized.  
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#### 29 *Ergosterol and type B trichothecenes analysis*

30  
31 Ergosterol was analysed according to the protocol optimised by Cahagnier et al. (1993).  
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33 Ergosterol was extracted after saponification in a mixture of methanol, ethanol, and potassium  
34  
35 hydroxide and purified on a solid-phase extraction column (Extrelut 20 R, Merck, Darmstadt,  
36  
37 Germany). The organic phase (n-hexane) eluted from the column was evaporated to dryness  
38  
39 and the residue resuspended in the HPLC mobile phase (dichloromethane). Ergosterol was  
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41 assayed by HPLC on a silica column and detected at 282 nm. Quantification was achieved  
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43 using an external standard (Merck), results were expressed as mg kg<sup>-1</sup> of ergosterol on a dry  
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45 weight basis.  
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53 Type B trichothecenes content of inoculated kernels and wheat milling fractions was  
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55 determined by GC-ECD as described by Bakan et al. (2001). Samples were extracted with  
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57 acetonitrile/water (84:16, v/v) and purified using one-step cleanup column (Trichothecenes P<sup>®</sup>  
58  
59 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  
6  
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<sup>-1</sup> for DON and 0.02 mg kg<sup>-1</sup> for  
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13 NIV.  
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## 20 **Results and Discussion**

### 21 *Mycological screening*

22  
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  
26  
27 addition to *Fusarium*. The recovered genera, summarized in Table II, are common cereals  
28  
29 contaminants and 3 of them, namely *Aspergillus*, *Penicillium* and *Fusarium*, have been the  
30  
31 focus of numerous reviews (Schuster et al. 2002; Xu and Berrie 2005). Several species of  
32  
33 these genera are able to produce the major mycotoxins subject to regulation by most  
34  
35 countries, *i.e.* aflatoxin, ochratoxin and deoxynivalenol. The nine genera were detected in all  
36  
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*

51  
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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3 reported by Niessen and Vogel (1998) could fit in with the specificity and sensitivity  
4 requirements of our study. Tox 5 PCR assay is based on primers derived from the trichodiene  
5 synthase gene (*Tri5*). This enzyme catalyses the first step of type B trichothecenes  
6 biosynthetic pathway (Hohn and Desjardins 1992), *i.e.* the isomerization and cyclization of  
7 farnesyl pyrophosphate to yield trichodiene. Purified DNA, isolated from mycelial cultures of  
8 a *Fusarium culmorum* strain (INRA 16), was subjected to duplex PCR analysis using primer  
9 pairs Tox5-1/Tox5-2 and ITS1/P3. Amplification resulted in the production of two fragments  
10 of about 650 bp and 1200 bp, according to the size markers (Figure 1). The 650-bp size was in  
11 accordance with the predicted size (658 bp) of the *Tri5* fragment (Niessen and Vogel 1998).  
12 The 1200 bp product was associated with the amplified portion from the ITS region of  
13 *Fusarium culmorum*. Identity of the two products was checked after sequencing. Specificity  
14 of the Tox 5 PCR assay designed to detect trichothecenes-producing *Fusarium*-species was  
15 largely documented by Niessen and Vogel (1998); more than 64 species and varieties of  
16 *Fusarium* were considered in this study. However, the number of fungal genera analysed by  
17 Niessen and Vogel (1998) was lower than 10 and no data concerning *Alternaria*, *Aspergillus*,  
18 *Botrytis*, *Epicoccum*, *Rhizopus* were available. Because of the occurrence of these genera in  
19 the *Durum* wheat samples involved in our study (Table II), it was essential to test Tox 5  
20 primers cross-reactivity with DNA from all fungal isolates we previously identified. Results  
21 of PCR assays obtained with DNA extracted from mycelial culture of each isolate are given in  
22 Figure 1. None of the non-*Fusarium* isolate produces the *Tri5* fragment (theoretical size : 658  
23 bp). Besides, occurrence of an ITS amplicon (corresponding to the expected size) for each  
24 isolate illustrated the quality of purified DNA and the success of the PCR reaction. The  
25 negative Tox 5 PCR reaction obtained with *Alternaria*, *Aspergillus*, *Botrytis*, *Epicoccum*,  
26 *Mucor*, *Penicillium* and *Rhizopus* was well correlated with the fact that none of these genera  
27 has been reported as trichothecenes producers in the reviewed literature. The negative reaction  
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3 obtained with *Trichoderma* was more surprising since *Trichoderma* such as 3 other genera  
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5 (*Stachybotrys*, *Myrothecium* and *Trichothecium*) have been reported to be able to produce  
6  
7 macrocyclic trichothecenes (Wilkins et al. 2003). *Tri5* sequences were published for  
8  
9 *Myrothecium roridum* (Trapp et al. 1998) and *Stachybotrytis chartarum* (Straus et al. 1999)  
10  
11 and a putative trichodiene synthase was reported in database for *Trichoderma Harzanium*  
12  
13 (accession number AJ784992.1). However, the lack of Tox 5 signal observed with our  
14  
15 *Trichoderma* sample was in accordance with the negative reaction described by Niessen and  
16  
17 Vogel (1998) for the other macrocyclic trichothecenes producers and was consistent with the  
18  
19 conclusion of Fekete et al. (1997), *i.e.* the occurrence of evolutionary variants of the *Tri5* gene  
20  
21 in these fungi. In fact, comparison of the *Stachybotrytis* and *Myrothecium* available *Tri5*  
22  
23 sequences (accession number AF053926 and AF009416.1) revealed significant divergences  
24  
25 with *Tri5* sequences present in *Fusaria spp.* (*Fusarium sporotrichoides*, accession number  
26  
27 M27246), especially in the Tox5 primers binding region. These data demonstrated that the  
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29 PCR assay previously defined can be used for the specific detection of trichothecenes-  
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31 producing *Fusarium*-species inside wheat kernels.  
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41 The diagnostic assay developed in the current study was then applied to bran and semolina but  
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43 also to wheat tissues, with less than 50 mg of material available for several samples such as  
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45 pericarp, germ, crease and grain ends. When *Tri5* amplification were used in the literature to  
46  
47 detect and/or quantify trichothecenes-producing *Fusarium spp.* in cereals, more than 1 g of  
48  
49 ground grains were usually used to extract the DNA (Doohan et al. 1998, Niessen and Vogel  
50  
51 1998, Edwards et al. 2001; Schnerr et al. 2002). To determine the minimum amount of wheat  
52  
53 material that could be successfully analyzed by our established Tox5 PCR assay, reactions  
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55 were set up with increasing quantities ranging from 10 mg to 500 mg of the highest  
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57 contaminated semolina fraction (DON close to 3500 µg/kg). Reliable amplification of specific  
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3 Tox5 PCR fragment was obtained with only more than 100 mg of semolina (data not shown).  
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6 Therefore, a new pair of primers specific for *Fusarium* species was designed to be applied on  
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8 samples below 100 mg and to screen wheat tissues. These primers, called  $\beta$ t1 and  $\beta$ t2  
9  
10 (described in Table I), were designed to amplify a 296 bp portion of  $\beta$ -tubulin gene.  
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12 Specificity of the primers was tested against 20 strains of *Fusarium culmorum* and  
13  
14 *graminearum* and all the fungal isolates reported in Table II. Whatever the considered  
15  
16 *Fusarium* strain, an amplicon of correct size (296 bp) was obtained (data not shown). These  
17  
18 results attesting for the high homology among  $\beta$ -tubulin sequences of *F. graminearum* and  
19  
20 *culmorum* were in accordance with the work of Gagkaeva and Yli-Mattila (2004). Data  
21  
22 obtained with the other fungal genera are given on Figure 2. If no cross-reaction occurred  
23  
24 with the isolates of *Alternaria*, *Epicoccum*, *Mucor* and *Trichoderma*, isolates of *Aspergillus*,  
25  
26 *Botrytis*, *Penicillium* and *Rhizopus* showed amplification with the  $\beta$ t primers pair. However,  
27  
28 the amplified fragments were higher than 296 bp (370 bp for *Aspergillus*, 410 bp for  
29  
30 *Penicillium*, 425 bp for *Botrytis* and 470 bp for *Rhizopus*) and can therefore be easily  
31  
32 distinguished from the specific *Fusarium* PCR-product. Purified PCR products were  
33  
34 sequenced and compared to the published corresponding  $\beta$ -tubulin gene sequences, which  
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36 accession numbers are reported in Table III. The *Botrytis* and *Penicillium* fragments were  
37  
38 identified as  $\beta$ -tubulin amplicons. In opposite, *Aspergillus* and *Rhizopus* ones were not  $\beta$ -  
39  
40 tubulin. [Insert table III about here] With a view to use the  $\beta$ -tubulin PCR assay on wheat  
41  
42 tissues and to avoid false positive results from cross-reaction with plant DNA, the primers  
43  
44 were tested with total genomic DNA extracted from healthy wheat. No amplification was  
45  
46 obtained with healthy materials. A minimum amount of 30 mg of contaminated semolina (the  
47  
48 highest contaminated one, DON close to 3500  $\mu\text{g kg}^{-1}$ ) was sufficient for a reliable  
49  
50 amplification of *Fusarium*  $\beta$ -tubulin that was detectable in the gel (data not shown).  
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60 Sensitivity of the  $\beta$ -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  $\beta$ -tubulin fragment (296 bp), will now allow the screening of wheat tissues for contamination  
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8 by *Fusarium spp.* mycelium.  
9

#### 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  
16  
17 infected Nefer wheat kernels and associated milling fractions were analyzed. Six samples,  
18  
19 corresponding to two levels of DON contamination, were extracted as described in ‘*Material*  
20  
21 *and Methods*’ and subjected to Tox5 PCR. The results obtained with the bran and semolina  
22  
23 are given in Figure 3. Positive reactions were obtained for all samples, indicating the presence  
24  
25 of DNA from trichothecenes-producers in all milling fractions, even in the weakly  
26  
27 contaminated semolina. Consistent with these data, when  $\beta$ -tubulin PCR assay was applied to  
28  
29 wheat tissues (grain ends and crease, pericarp and aleurone layer, germ and albumen),  
30  
31 *Fusarium* DNA was detected in all samples (Figure 4). These observations clearly illustrated  
32  
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  
36  
37 demonstrated the penetration of toxigenic *Fusarium* strains into the interior of durum wheat  
38  
39 kernel at least for the Nefer variety used in this work. This suggests that none of the  
40  
41 considered wheat tissue act as an effective barrier to fungal invasion. The occurrence of  
42  
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.*

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3 invasion were performed on artificially inoculated spikelets with the risk of induced artifacts  
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5 (Kang and Buchenauer, 1999 and 2002, Jackowiack et al. 2005) and/or with analytical  
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7 methods that were not specific for type B trichothecenes producers (Seitz and Bechtel 1985,  
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9 Bechtel et al. 1985).  
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#### 12 13 14 *Trichothecenes production by F. culmorum on Durum wheat milling fractions*

15  
16 Occurrence of toxins in endosperm tissues (Kang and Buchenauer 1999, Visconti et al. 2004)  
17  
18 concomitant with the presence of toxigenic *Fusarium* species is consistent with an *in situ* type  
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20 B trichothecenes production, as suggested by Seitz and Bechtel (1985) and Nowicki et al.  
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22 (1988). According to Kang and Buchenauer (2002), *Fusarium* hyphae were able to yield  
23  
24 toxins during penetration and growth on wheat spikelets at the earliest stages of infection.  
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26 With the view to investigate the occurrence of a type B trichothecenes production on more  
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28 mature tissues, two *F. culmorum* strains (a DON and 15-ADON producer, a NIV and FX  
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30 producer) were inoculated on healthy kernels, bran and semolina from the Nefer *durum* wheat  
31  
32 variety. Fungal biomass and type B trichothecenes yield were evaluated on each substrate 21  
33  
34 days after inoculation. Fungal biomass was estimated by quantification of ergosterol, a  
35  
36 fungus-specific membrane compound. Results are given in Figure 5. A substantial fungal  
37  
38 biomass was obtained whatever the considered strain and substrate, as attested by the high  
39  
40 amounts of quantified ergosterol. If fungal biomass were not significantly different on the  
41  
42 three substrates for INRA 305 strain, semolina and bran induced however a strong decrease  
43  
44 for INRA 337. Our data are in accordance with the predicted chemotypes of the two  
45  
46 considered strains: *F.culmorum* INRA 305 produced DON and 15-ADON whereas *F.*  
47  
48 *culmorum* INRA 337 produced NIV and FX. For the two strains, high toxins yields  
49  
50 (DON+15-ADON or NIV+FX), greater than 300 mg kg<sup>-1</sup>, were obtained on whole kernels  
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52 and semolina. However, on bran, weak amounts of type B trichothecenes were measured,  
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3 close to 40 mg kg<sup>-1</sup> of DON+15-ADON for the INRA 305 strain and 10 mg kg<sup>-1</sup> of NIV+FX  
4  
5 for the INRA 337 strain. These data associated with the equivalent amounts of ergosterol  
6  
7 determined on bran and semolina represent a further demonstration that growth and type B  
8  
9 trichothecenes production may be independently regulated (Bakan et al. 2001, Reynoso et al.  
10  
11 2002). To explain the strong decrease in type B trichothecenes yield observed on bran, two  
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13 hypotheses were raised: (i) lack of essential nutrients required for fungal toxinogenesis, (ii)  
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15 occurrence of endogenous compounds acting as inhibitors of type B trichothecenes  
16  
17 biosynthesis. To investigate the former hypotheses, the INRA 337 strain was inoculated in  
18  
19 GYEP liquid medium supplemented with 0.05 g l<sup>-1</sup> and 0.1 g l<sup>-1</sup> of bran (Nefer variety).  
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21 Results are reported on Figure 6. GYEP medium, a nutrient-rich laboratory substrate, was  
22  
23 frequently shown to support great yields of type B trichothecenes (Miller and Greenhalgh  
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25 1985, Pons et al. 2003, Covarelli et al. 2004). This high efficiency was confirmed by the  
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27 significant levels of toxins, close to 25 µg ml<sup>-1</sup>, obtained after 32 days of culture in control  
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29 batches. If bran supplementation of GYEP did not significantly affect the amount of fungal  
30  
31 biomass, it was shown to induce a strong decrease in NIV+FX production. The levels of  
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33 produced toxins were more than twice reduced with 0.05 g l<sup>-1</sup> of bran and only traces were  
34  
35 detected in media supplemented with 0.1g l<sup>-1</sup>. These data strongly suggested the occurrence of  
36  
37 endogenous biochemical compounds in bran of *durum* wheat that were able to significantly  
38  
39 reduce the biosynthesis of type B trichothecenes. A similar conclusion was previously  
40  
41 reported for maize germ fraction by Bakan et al. (2003). These authors have ascribed the  
42  
43 observed inhibition to phenolic compounds and more precisely to the high content of ferulic  
44  
45 acid occurring in maize germ. Bran of *durum* wheat is also characterized by significant  
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47 concentrations of phenolic acids with mainly ferulic, *p*-coumaric and vanillic acids  
48  
49 (Onyeneho and Hettiarachchy 1992). Additional studies are in progress to ascertain the effects  
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51 of these phenolic acids on the production of type B trichothecenes by *F. culmorum*. Moreover,  
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3 according to the literature data, other phenolic compounds such as flavonoïds and/or  
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5 furanocoumarins (Desjardin et al. 1988) but also small peptides (Huang et al. 1997) could be  
6  
7 implied in these inhibition mechanisms. Further studies will therefore been undertaken to  
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9 isolate and identify the compounds in bran that may be responsible for toxin biosynthesis  
10  
11 inhibition.  
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## 14 15 16 17 **Conclusion**

18  
19 Two main conclusions can be drawn from the present study: (i) Toxigenic *Fusarium* strains  
20  
21 are present in the core of durum wheat kernel infected under natural conditions, (ii) if  
22  
23 semolina represents an excellent substrate for toxinogenesis, bran fractions appear to contain  
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25 biochemical compounds that are able to significantly reduce type B trichothecenes yields.  
26  
27 Since toxins have been shown to concentrate in the outer parts of the wheat kernel (Nowicki  
28  
29 et al. 1988), identification of the inhibitor compounds occurring in bran may represent a  
30  
31 significant improvement in the selection of durum wheat varieties less sensitive to DON  
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33 contamination. Moreover the developed PCR assays, that can specifically detect *toxigenic*  
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35 *Fusarium* strains *in vivo*, even in weakly infected tissues, may also be useful to select varieties  
36  
37 more resistant to *Fusarium* penetration; use of these varieties in milling process may lead to  
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39 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<sup>-</sup> represents the negative control.

Figure 2. PCR products generated with the  $\beta t1/\beta t2$  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<sup>+</sup>: 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  $\mu\text{g kg}^{-1}$  and 750  $\mu\text{g kg}^{-1}$ , respectively). Lane C<sup>-</sup>: 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<sup>+</sup>: positive control showing the 296 bp  $\beta$ -tubulin fragment amplified from 10 ng of purified genomic

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3 DNA of *F. culmorum*. Lanes 1 to 12 : wheat tissues from two contaminated samples of  
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5 *durum* wheat (DON content of sample 1 and 2 are close to 8000  $\mu\text{g kg}^{-1}$  and 750  $\mu\text{g kg}^{-1}$ ,  
6  
7 respectively). Lane C: negative control. Lane M : 100-bp molecular weight Marker from  
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9 Promega (Charbonnieres, france).  
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15 Figure 5. Fungal biomass (estimated by ergosterol content) and type B trichothecenes  
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17 production (DON+15-ADON or NIV+FX) on *durum* wheat kernels, bran and semolina by  
18  
19 two *F. culmorum* strains of different chemotypes ( INRA 305, a DON + 15-ADON producer  
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21 and INRA 337, a NIV + FX producer) after 21 days of incubation. Kernels, bran and semolina  
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23 were obtained from the sample 3 (no toxin detected) of Nefer elite line. Bars represent mean  $\pm$   
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25 standard error relevant to three flasks (with three extractions per flask).  
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34 Figure 6. Fungal biomass (estimated by ergosterol content) and NIV+FX yields after 26 and  
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36 32 days of incubation of the *F. culmorum* strain INRA 337 in GYEP liquid cultures  
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38 supplemented or not with bran (0.05  $\text{g l}^{-1}$  and 0.1  $\text{g l}^{-1}$ ). Bran was from the sample 3 (no toxin  
39  
40 detected) of Nefer elite line. Bars represent mean  $\pm$  standard error relevant to three flasks  
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42 (with three extractions per flask).  
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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			
$\beta$ t-1	GGTAACCAAATCGGTGCTGCTTTC	<i><math>\beta</math>tubulin</i>	296	Doohan et al 1999 ( $\beta$ T2a)
$\beta$ t-2	GATTGACCGAAAACG AAGTTG			This study

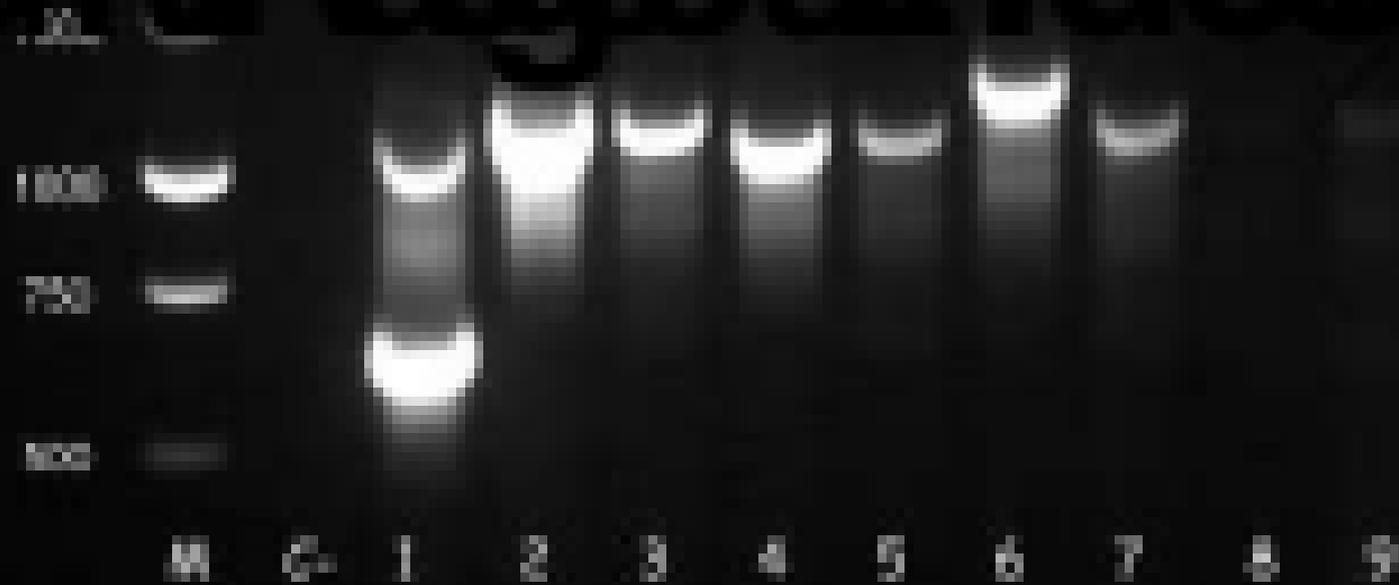
Table II: Fungal genera isolated from the *durum* wheat samples considered in the current study

<b>Contaminants present on the <i>durum</i> wheat fractions</b>
<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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02t

← ITS

← TRIS

# Old Rivers and Cont

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C- 1 M 2 3 4 5 6 7 8 9

How much money do you have?

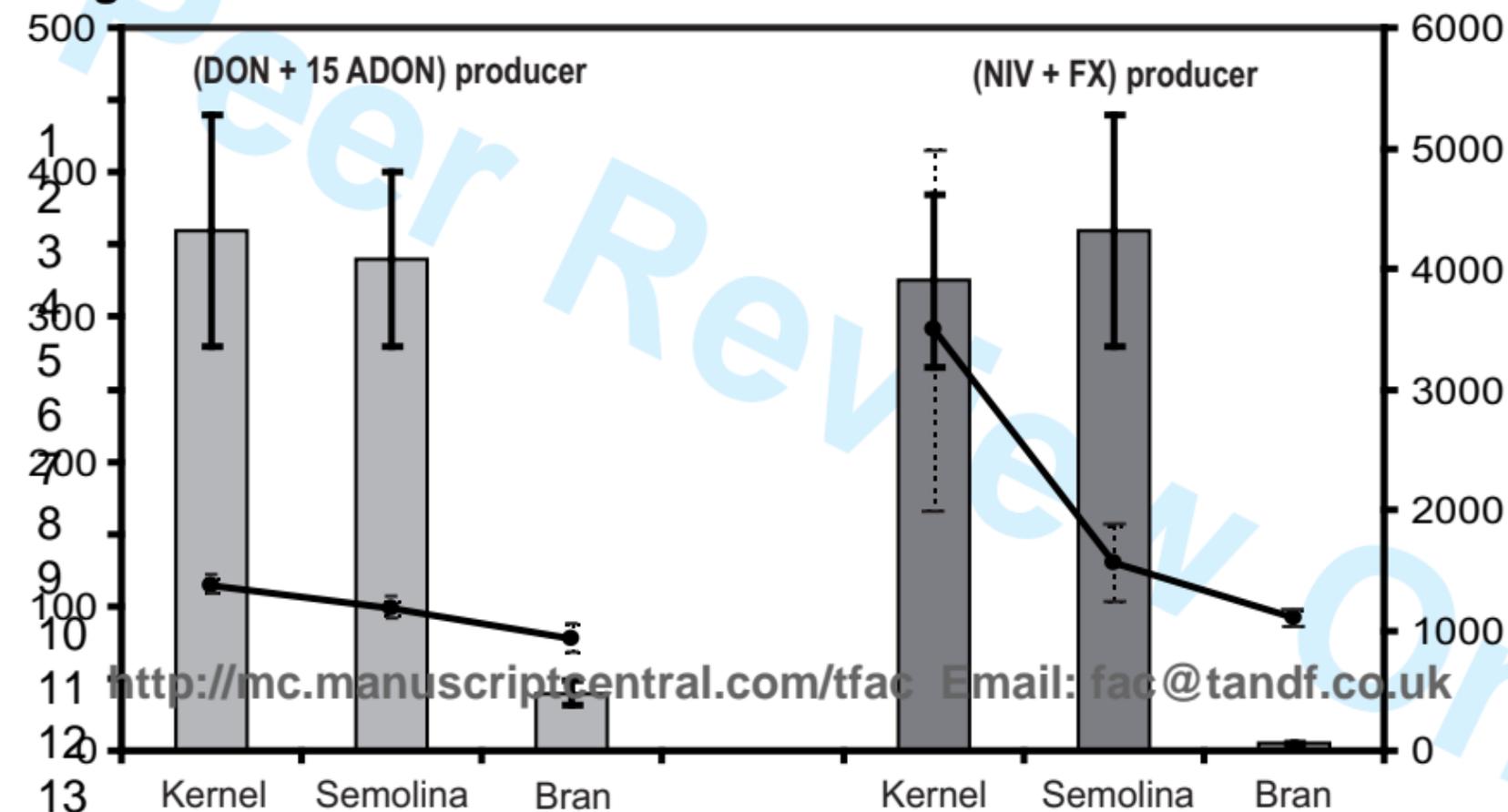
1 2 3 4

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

