PCR-Based Identification of Toxinogenic *Fusarium* Species

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Abstract: Aim of this study was to develop sensitive PCR assay for mycotoxin producing *Fusarium* species. Strains *Fusarium oxysporum* 4199 and *Fusarium culmorum* 4044 were used as representatives of this group. Primers JB chosen to demonstrate the affiliation to genus *Fusarium* were derived from ITS region of rDNA. Gene from trichothecene pathway *Tri4* was employed to design primers for toxin biosynthesis. Specificity of PCR based on JB primers was tested on DNA isolated from *F. culmorum* 4044, *F. oxysporum* 4199, *Aspergillus oryzae* 4002 and *Mucor circinelloides* 4018, *Trichoderma* sp. Both *Fusarium* species gave positive reaction, while the later ones did not react. Primers based on *Tri4* highly specific sequences were giving positive reaction only with DNA from *F. culmorum* 4044 and *F. oxysporum* 4199. DNA isolated from six samples of contaminated wheat grains gave positive result on the presence of genus *Fusarium* and mycotoxines by optimised PCR protocol using JB and *Tri4* primers. The results corresponded to LC/MS analysis that was established quantitatively in all samples to ascertain the amount and type of fusarious mycotoxines.

Keywords: toxinogenic *Fusarium*; trichothecenes; PCR; JB primers; *Tri4* primers

The genus *Fusarium* was introduced by Link in 1809, and is now reaching its third century as a genus that contains many plant-pathogenic fungi. The members of this genus beside many diseases in plants, humans and domesticated animals produce an intriguing array of secondary metabolites named mycotoxines. The need to identify strains and to recognise their toxinogenic potential is in *Fusarium* stronger than in any fungal genus. Studies based on the morphology of macroconidia are relieable but time demanding.

Amplification of target DNA through PCR with sequence specific primers is potentially more sensitive and rapid than microbiologic techniques, as a number of constraints are removed. Unlike culture, PCR does not require the presence of viable organisms for success and may be performed even when sample volumes are small. The nuclear-encoded ribosomal RNA genes (rDNA) of fungi exist as a multiple-copy gene family comprised of highly similar DNA sequences (typically from 8–12 kb each) arranged in a head-to-toe manner. The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the detection of pathogens at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbett 1992). They also occur in multiple copies with up to 200 copies per haploid genome (Bruns *et al*. 1991). Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan *et al*. 1995). Therefore we focused on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *Fusarium* spp. Taxon-selective ITS amplification has already been used for detection of the fungal pathogens such as *Fusarium* (O'Donnell 1992) and *Verticillium* spp. (Nazar *et al*. 1991).

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Trichothecenes comprise different types of mycotoxines produced at least by 24 diverse *Fusarium* species. Trichothecenes chemical structure is rather varied. They are formed mostly by a mixture of tricyclic sesquiterpens (hydrocarbons with fifteen carbon atoms) with six-membered circle containing double bond between C-9 and C-10 and epoxygroup in position C-12 and C-13 (Velíšek 2002). In the last twenty years trichothecenes biosynthesis was elucidated using precursors experiments and mutants. Certain genes of trichothecenes biosynthesis could be characterised by their heterologous expression in the yeast *Saccharomyces cerevisiae* (McCormick *et al*. 2006; Makoto *et al*. 2007). Using the pathway proposed by Makoto *et al*. (2007), *Tri4* gene coding multifunctional oxygenase responsible for trichodiene (TDN) transformation to isotrichotriol was chosen as label of mycotoxin biosynthesis. According to Hohn *et al*. (1995), *Tri4* encodes a protein of 520 residues (M(r) = 59 056) that shows significant homology with members of the superfamily of cytochromes P450. It appears most similar to CYP3A subfamily (24.6% amino acid identity). Because it contains less than 40% homology with other cytochromes P450, the *Tri4* gene has been placed in a new cytochrome P450 gene family designated CYP58.

The objective of our investigation was to develop a reliable and sensitive PCR assay for the selective detection of pathogenic *Fusarium* species in food and feed samples.

MATERIALS AND METHODS

Fungal strains. *Fusarium culmorum* DBM 4044, *Fusarium oxysporum* DBM 4199, *Aspergillus oryzae* DBM 4002, *Mucor circinelloides* DBM 4018, *Trichoderma* sp. 4092. Origin of all strains was in the Czech Republic.

Cultivation. Each fungal culture was started from slants of Potato Dextrose Agar (Oxoid, GB). Liquid cultures used 50 ml aliquotes of Malt Extract Broth (Oxoid, GB) in 250 ml Erlenmayer flasks were incubated for one week at 25°C on a rotary shaker 200 rpm.

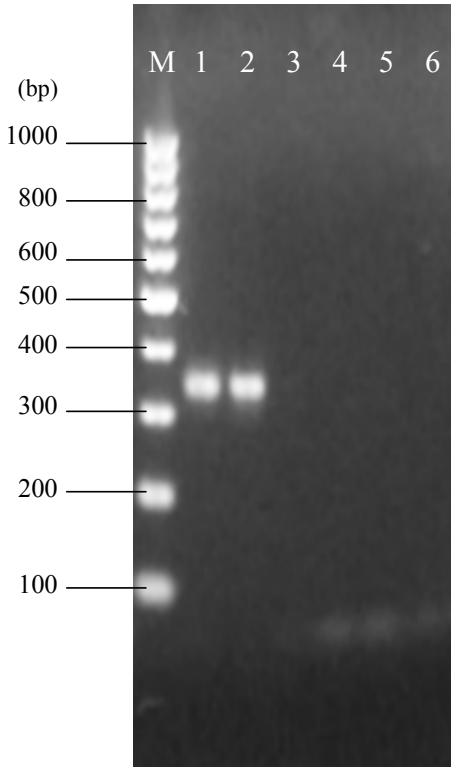
DNA isolation. Mycelium was collected by centrifugation (5000 rpm, 5 min). Cell wall was disrupted by liquid nitrogen in a grinding mortar. DNA was extracted by Dneasy Plant Mini Kit (Cat. No. 69106, Qiagen, Germany) according instruction provided by manufacturer. The wheat grains were grinded before the extraction. The purity and concentration of extracted DNA was checked spectroscopically at 260 nm (Spectrophotometer ND-100, Bio-Rad, USA). DNA was resuspended in deionised water and stored at –20°C.

PCR. JB primers (566 and 572) were borrowed from Glynn *et al*. (2006) and were producing 389 bp product. Primers Tri 1433 and Tri 1434, McCormick *et al*. (2006) were used for verification of the ability to synthetise trichothecenes. Reaction was done in 25 µl format. Primers provided by Generi Biotech, CR were as follow: JB566 (5'-GTT TTT AGT GGA ACT TCT GAG T-3'), JB572 (5'-AAG TTG GGG TTT AAC GGC-3'), Tri 1433 Reverse (5'-AGG GCG AGG TAC ATC TCA GC-3'), Tri 1434 Forward (5'-GCT GGT ACT GAG ACT ACT TC-3'). DyNAzyme™ II DNA Polymerase (2 U/µl) was Finnzymes, USA, and nucleotides Promega, USA. Both reactions were done in 25 µl format with undermentioned composition: dNTP mix (dATP, dTTP, dCTP, dGTP) 10mM, 0.5 µl; primers, 0.02nM/µl, 0.25 µl; *Taq* polymerase 2 U/µl, 0.25 µl; template DNA 2.5 µl; sterile distilled water 18.75 µl. Temperature program for JB primers was started by 3 min initial denaturation at 94°C, followed by 35 cycles with annealing temperature 60°C for 15 s, elongation temperature 72°C for 45 s, template denaturation 95°C for 15 sec with 7 min final extension at 72°C. Temperature program for *Tri4* primers used 5 min for initial denaturation at 94°C, with 45 cycles with annealing temperature 60°C for 45 s, elongation temperature 72°C for 1 min, template denaturation 95°C for 1 min with 10 min final extension at 72°C. Optimal concentration of template DNA was 20 ng/µl.

Analysis of mycotoxines. *Fusarium* mycotoxins in cereals were quntitatively analysed by usage of acredited LC-MS method, which was described in detail in Lancova *et al*. (2008).

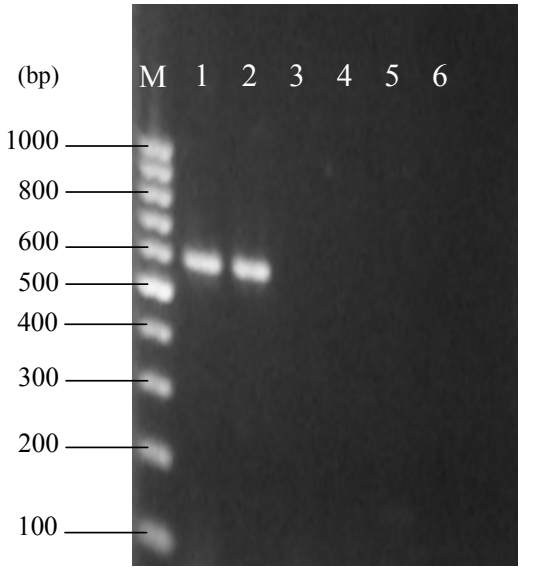
RESULTS AND DISCUSSION

PCR methods for identification of fungi have increased enormously over the past 15 years. After completing a number of optimalisations (Lacmanova 2009) we amplified extracted DNA of *Fusarium culmorum*, *Fusarium oxysporum*, *Aspergillus oryzae*, *Mucor circinelloides*, *Trichoderma* sp. with JB primers and products approximately



Lane M – size markers (100, 200, 300, 400, 500, 600, 800, 1000 bp); lane 1 – *Fusarium oxysporum* 4199; lane 2 – *Fusarium culmorum* 4044; lane 3 – *Aspergillus oryzae* 4002; lane 4 – *Trichoderma* sp. 4092; lane 5 – *Mucor circinelloides* 4018; lane 6 – no template control (H_2O)

Figure 1. Specifity of JB primers for PCR using collection strains



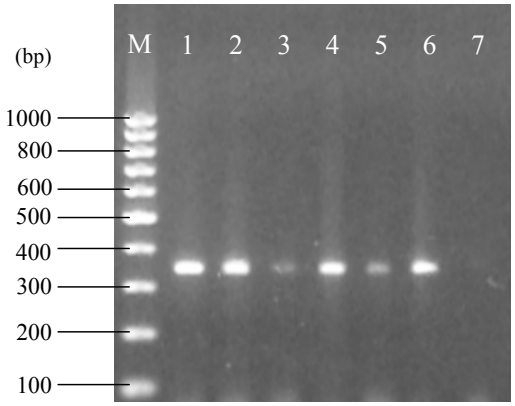
Lane M – size markers (100, 200, 300, 400, 500, 600, 800, 1000 bp); lane 1 – *Fusarium oxysporum* 4199; lane 2 – *Fusarium culmorum* 4044; lane 3 – *Aspergillus oryzae* 4002; lane 4 – *Trichoderma* sp. 4092; lane 5 – *Mucor circinelloides* 4018; lane 6 – no template control (H_2O)

Figure 2. Verification of *Tri4* primers specifity for PCR using collection strains

346 bp large were formed only by both *Fusarium* species. The time course of the PCR was optimised (Lacmanova 2009) for obtaining clear product (Figure 1). Using the DNA from the same collection strains, the second PCR for confirmation of ability to form trichothecenes was performed using *Tri4* primers (Figure 2). On the basis of *Tri4* sequences the product of approximately 600 bp was amplified by DNA from *F. culmorum* and *F. oxysporum.* The temperature of annealing was experimentally determined as 60°C for the both PCRs. All reactions were repeated three times. For *Tri4* primers the number of cycles was layed down at 45. Sensitivity of the PCR reactions was evaluated during the investigation. Decreasing amounts of the genomic DNA of *F. oxysporum* 4199 were used in order to determine the minimum amount of input DNA required to produce detectable product with primer set JB. Using 10 replicate samples per DNA concentration, amplification products of the primer set JB were visible on gels from 30 ng/µl, 20 ng/µl, 10 ng/µl, and 1 ng/µl, of *F. oxysporum* 4199 DNA 10 pg/µl and 1 pg/µl. PCR detectable products were not consistently observed across the 10 replicate reactions per primer set (data not shown).

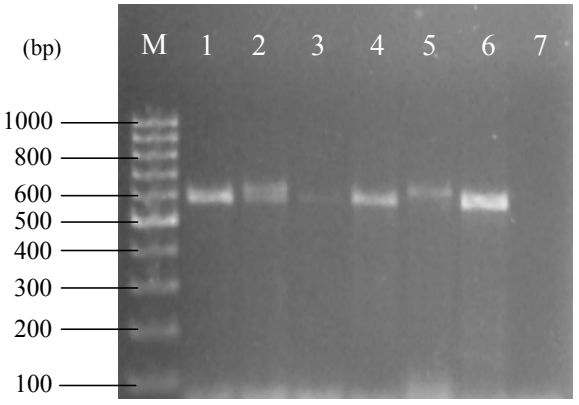
Several genes involved in the biosynthesis of trichothecenes have been described, most of them localised in a *Tri* gene cluster. The *Tri4* gene encodes the multifunctional oxygenase responsible for trichodiene (TDN) synthase, which catalyses the first step in the biosynthesis of trichothecenes.

Trichothecene biosynthesis may be regulated besides specific genes also by environmental conditions such as temperature, water activity, and substrate composition (Bakan *et al*. 2002). The choice of responsible genes may reveal the capacity of a certain strain to synthetise specific type of toxine. According the chemical analysis of contaminated wheat kernels as shown at Table 1, the composition of fusarious toxines contained all main types: DON deoxynivalonol (trichothecen typ B), D3G deoxynivalonol-3-glucoside (trichothecen typ B), ADONs 3- acetyldeoxynivalonol; 15-acetyldeoxynivalonol (trichothecen typ B), NIV



Lane M – DNA size markers (100, 200, 300, 400, 500, 600, 800, 1000 bp); lane 1 – wheat sample No. 5; lane 2 – wheat sample No. 8; lane 3 – wheat sample No. 9; lane 4 – wheat sample No. 40; lane 5 – wheat sample No. 51; lane 6 – wheat sample No. 64; lane 7 – no template control (H_2O)

Figure 3. Wheat grains PCR analysis on the confirmation of genus *Fusarium* with JB primers



Lane M – DNA size markers (100, 200, 300, 400, 500, 600, 800, 1000 bp) lane 1 – wheat sample No. 5; lane 2 – wheat sample No. 8; lane 3 – wheat sample No. 9; lane 4 – wheat sample No. 40; lane 5 – wheat sample No. 51; lane 6 – wheat sample No. 64; lane 7 – no template control (H_2O)

Figure 4. Wheat grains PCR analysis using *Tri4* primers on the ability to form trichothecenes

nivalenol (trichothecen typ B), FUS-X fusarenon-X (trichothecen typ B), HT-2 HT-2 toxine (trichothecen typ A), T-2 T-2 toxine (trichothecen typ A), ZON zearalenon. PCR of DNA isolated from these samples with JB primers confirmed the presence of genus *Fusarium* (Figure 3). Identically isolated DNA from wheat kernels was analysed by PCR using *Tri4* primers (Figure 4). All analysed samples produced expected amplicon, but while the amount of template DNA was 50 µg/ml for all reactions the intensity of product was not uniform. By comparing these results with analytical data in Table 1 it is evident that the lanes 1 and 6 presents the best amplified product, while the content of DON is the highest in sample 8 (304.3 g/kg) but the lane 2, corresponding to this sample is not distinctive. What is more satisfactory is the fact, that beside DON all analysed samples contained also other types of trichothecens. One of the biases in this study may be the rather limited set of samples which were studied.

Nevertheless, this study offers the necessary tools for a rapid detection of the presence of *Fusarium* species and their capacity to produce

Table 1. Amount of fusarious mycotoxines (µg/kg in wheat grains)

Sample number	Mycotoxines (µg/kg)							
	DON	D3G	ADONs	NIV	FUS-X	HT-2	T-2	ZON
5	144.8	17.3	< 5	< 5	< 1	< 1	< 0.3	17.9
8	304.3	31.4	< 5	181.6	< 1	< 1	< 0.3	9.7
9	102.3	55.2	< 5	< 5	< 1	< 1	< 0.3	8.7
40	194.6	50.9	< 5	< 5	< 1	< 1	< 0.3	6.2
51	113.9	< 5	< 5	< 5	< 1	< 1	< 0.3	< 5
64	210.6	< 1	< 5	< 5	< 1	< 1	< 0.3	37.3

Fusarious mycotoxines: DON – deoxynivalonol (trichothecen typ B); D3G – deoxynivalonol-3-glukoside (trichothecen typ B); ADONs – 3-acetyldeoxynivalonol; 15-acetyldeoxynivalonol (trichothecen typ B); NIV – nivalenol (trichothecen typ B); FUS-X – fusarenon-X (trichothecen typ B); HT-2 – HT-2 toxine (trichothecen typ A); T-2 – T-2 toxine (trichothecen typ A); ZON – zearalenon

trichothecenes. Further studies will be necessary to establish the relation of DNA content to trichothecens quantity and to more deep investigation of *Tri* genes.

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