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Original article

Detection of *Fusarium tricinctum* from cereal grain using PCR assay

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Abstract. Contamination of cereals with mycotoxins produced by *Fusarium* is a worldwide problem requiring rapid and sensitive detection methods. This paper describes the development of a PCR protocol facilitating the detection of *F. tricinctum*, which belongs to the FHB (*Fusarium* Head Blight) complex responsible for contamination of cereal grains with enniatins and moniliformin. Sequence alignment of partial IGS rDNA revealed a single nucleotide polymorphism, which was used to design primers differentiating *F. tricinctum* from other members of the FHB complex. The specificity of the assay was tested on 68 isolates belonging to 21 *Fusarium* species originating from different parts of the world and hosts/substrates. Positive PCR results were obtained from all 12 *F. tricinctum* isolates tested; however, unexpected amplicons were amplified from the templates of *F. acuminatum* (CBS 618.87) and *F. nurragi* (CBS 393.96). No cross reactivity was found with any other *Fusarium* species tested.

The PCR assay was tested on 24 asymptomatic wheat seed samples originating from Northern Poland and resulted in 13 positive samples, of which 11 samples were contaminated with moniliformin and/or antibiotic Y.

Keywords: *F. tricinctum*, PCR, IGS rDNA, detection.

Introduction

Phytopathogenic fungi of the genus *Fusarium* cause a number of diseases in a diversity of host plants worldwide (Leslie and Summerell 2006). In cereals, infection with *Fusarium* spp. reduces the yield and quality of seeds and results in the accumulation of various mycotoxins, such as beauvericin (BEA), enniatins (ENs), fumonisins, moniliformin (MON), trichothecenes and zearalenol (ZEN). These compounds have been found to cause a variety of toxic effects on humans and livestock (Desjardins 2006). Contamination of grain with certain groups of mycotoxins depends on many factors; however, *Fusarium* species composition and toxigenic potential of the complex present in plant material are crucial.

In order to detect the presence of the pathogens directly from different types of material, PCR diagnostic protocols that rely on the use of species-specific primers have been developed (Yoder and Christianson 1997; Nicholson et al. 2004; Kulik et al. 2004; Jurado et al. 2005). In these cases, species-specific primers were designed based on the existing taxonomy of the genus *Fusarium*; however, subsequent studies demonstrated cross-reactivity of the primers with other closely related species or unexpected negative results with isolates belonging to the target species (Tan and Niessen 2003; Yli-Mattila et al. 2004; Demeke et al. 2005). The difficulties in generating fungal specific sets of diagnostic primers result from high and unpredictable genetic variability in fungi. Species differing in morphology can be closely related genetically. Additionally, the incomplete species concept within the genus *Fusarium,* problems with the taxonomic status of particular strains studied (Leslie and Summerell 2006), the existence of hybrids (O'Donnell et al. 2000) and horizontal gene transfer (Rosewich and Kistler 2000) also complicate efforts to develop reliable diagnostic protocols for *Fusarium*.

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From the toxicological point of view, the use of PCR assays corresponding to the toxigenic potential of fungi is the most useful. Such PCR protocols can predict potential contamination of the material with certain mycotoxins. This is achieved by the use of primers targeting genes responsible for toxin synthesis (Lee et al. 2001, 2002; Chandler et al. 2003; Gonzáles-Jaén et al. 2004; Kulik et al. 2007). Moreover, the ability to produce certain mycotoxins could be determined by phylogenetic analysis of genes not involved in toxin synthesis, such as rDNA (Mule et al. 1997; Llorens et al. 2006) or the ef-1α gene (Kristensen et al. 2005). Among the numerous genes/DNA regions used to design diagnostic primers for the *Fusarium* complex, the IGS rDNA region is of particular interest. IGS rDNA is organized in units repeated many times in the genome, thus this region is especially considered as the most sensitive target for fungal diagnostic purposes.

This paper outlines the development of a PCR assay for the detection of *F. tricinctum*, a common FHB agent in Europe. To date, no PCR diagnostic protocol has been described for the detection of this species. The paper also presents results from a study in which the assay was applied to wheat seed samples naturally contaminated with the complex of *Fusarium* originating from different sites in Northern Poland.

Materials and methods

Fungal isolates tested for primers specificity

The fungal isolates used for primer specificity are listed in Table 1 and are held in the CBS (The Centraalbureau voor Schimmelcultures, The Netherlands) fungal collection. The local (Polish) isolates tested in this study are held in the fungal collection of the DDPP (Department of Diagnostics and Plant Pathophysiology, University of Warmia and Mazury in Olsztyn, Poland). The *Fusarium* isolates were maintained on PDA (potato dextrose agar) at 25°C.

Grain samples

Wheat seed samples (24×1 kg) were collected from experimental fields of the Research Center for Cultivar Testing located in Northern Poland. The contamination of the seed samples with *Fusarium* species was determined by microbiological examination on PDA (100 asymptomatic seeds per sample tested) (Table 2). The contamination of the samples with moniliformin (MON) and antibiotic Y (ANT Y) were analyzed using the multimycotoxin method at the Chemistry and Toxicology Unit, Finnish Food Safety Authority (Evira), Mustialankatu 3, FIN-00790 Helsinki, FINLAND. DNA extraction from both the fungal cultures and grain samples followed the protocol described previously by Kulik et al. (2007).

Sequencing of partial IGS rDNA from *F. avenaceum, F. acuminatum, F. heterosporum* and *F. tricinctum* and the design of primer sets

The IGS rDNA region of *F. avenaceum* (CBS 115957, 408.86, 119834, 241.94)*, F. acuminatum* (CBS 334.75, 618.87)*, F. aywerte* (CBS 395.96), *F. heterosporum* (CBS 737.79), *F. nurragi* (CBS 393.96) and *F. tricinctum* (CBS 253.50, 393.93, 410.83, 261.51, 119842) was amplified with primers *igs15* cgg cca cag acc tcg cac ga and *igs16* taa cta tcg tgt gcc ggg gtt, designed on the basis of a conserved region of published sequence data available in the NCBI database. A part of the elongation factor-1 alpha gene of CBS 618.87 (*F. acuminatum*) was sequenced using primers *ef11* cat cgg cca cgt cga ctc tgg c and *ef12* gga agt acc agtgat cat gtt. The amplified products were sequenced in both directions using the above primers. Reactions were performed on an ABI PRISM 310 automated DNA sequencer with an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA). All sequences obtained in this study were deposited in the NCBI/GeneBank database under accession numbers EF670451, EF670452, EF670453, EF670454, EF670455, EF670456, EF670457, EF670458, EF670459, EF670460, EF670461, EF670462, EF670463, EF670464 and EU024695.

Based on the data obtained, primer pairs *tri1* cgt gtc cct ctg tac agc ttt ga and *tri2* gtg gtt acc tcc cga tac tct a were designed by aligning sequences of the partial IGS rDNA region for specific detection of *F. tricinctum*. All primers used in this study were synthesized by the Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland).

PCR protocol

PCR amplifications were performed in a total volume of 12 μL containing up to 10 pg of genomic DNA, 0.12 U FailSafe Polymerase, 6.5 μL PreMix E (Epicentre Biotechnologies, Madison, USA) and 2.5 pM concentration of each primer. Amplifications were carried out in a thermal

Table 1. A list of fungal isolates used to test the specificity of *tri1*/*tri2* primer set

Fungal species	CBS/DDPP code	Geographical origin, host or habitat of origin	PCR assay
1	2	3	4
F. tricinctum	CBS 261.51	Switzerland	+
	CBS 410.86	Denmark, mouldy grain of *Hordeum vulgare*	+
	CBS 253.50	Finland, grain of *Hordeum sativum*	+
	CBS 393.93	Germany, winter wheat cv. Diplomat, culm base	+
	CBS 119842	USA, Florida, leaf spots on *Hedera helix*, 1979	+
	DDPP 06501	Northern Poland, winter wheat kernel, 2006	+
	DDPP 079306	Northern Poland, winter wheat kernel, 2007	+
	DDPP 07323	Northern Poland, winter wheat kernel, 2007	+
	DDPP 07013	Northern Poland, winter wheat kernel, 2007	+
	DDPP 07010	Northern Poland, winter wheat kernel, 2007	+
	DDPP 07011	Northern Poland, winter wheat kernel, 2007	+
	DDPP 07606	Western Poland, winter wheat kernel, 2007	+
F. acuminatum	CBS 618.87	Denmark, soil from fruit plantation	+
	CBS 334.75	Turkey, *Musa sapientum*	–
F. armeniacum	CBS 485.94	Australia	–
F. arthosporioides	CBS 100485	Czech Republic, barley	–
	CBS 314.73	New Zealand, *Azalea* (Ericaceae)	–
F. avenaceum	CBS 119834	unknown	–
	CBS 409.86	USA, barley kernel, 1986	–
	CBS 241.94	Netherlands, *Dianthus caryophyllus*	–
	CBS 121.73	UK, root of *Dianthus caryophyllus*	–
	CBS 408.86	Denmark, mouldy barley kernel of *Hordeum vulgare*	–
	CBS 115957	Italy, *Fagus sylvatica* (Fagaceae), damaged roots, seedlings	–
	DDPP 06204	Poland, winter wheat kernel, 2006	–
	DDPP 07411	Northern Poland, winter wheat kernel, 2007	–
F. aywerte	CBS 395.96	Australia, Northern Territory, Little Palm Creek, soil under *Plectrachne* sp., 1992	–
F. cerealis	CBS 195.80	Colombia, burnt páramo soil	–
	CBS 623.85	Netherlands, *Solanum tuberosum*, tuber, buried in soil	–
F. chlamydosporum var. chlamydosporum	CBS 220.61	South Africa, soil	–
	CBS 445.67	Australia, *Triticum aestivum*	–
	CBS 511.75	Peru, seedling of *Gossypium* sp.	–
F. culmorum	CBS 256.51	Netherlands, soil	–
	CBS 129.73	Portugal, *Populus nigra*	–
F. equiseti	DDPP 0603	Poland, winter wheat kernel, 2006	–
	DDPP 05282	Poland, winter wheat kernel, 2005	–
	CBS 406.86	Germany, soil	–
F. graminearum	CBS 110263	Iran, corn	–
	CBS 110247	New Caledonia, orange twig	–
	DDPP 07406	Northern Poland, winter wheat kernel, 2007	–
	DDPP 07504	Northern Poland, winter wheat kernel, 2007	–
F. heterosporum	CBS 737.79	Ethiopia, *Cynodon dactylon* (Gramineae), ear	–
	CBS 119845	Australia, *Claviceps paspali* sclerotia on *Paspalum* sp., 1983	–
F. langsethiae	DDPP 061301	Southern Poland, winter wheat kernel, 2006	–
F. nurragi	CBS 393.96	Australia, Victoria, Wilson's Promontory National Park, soil in heathland, 1991	+
F. oxysporum	CBS 620.87	Denmark, kernel of *Hordeum vulgare*	–
F. poae	CBS 317.73	Australia, bud of *Dianthus* sp.	–
	CBS 186.96	Poland, wheat	–
	CBS 623.87	Denmark, straw of *Hordeum vulgare*	–
	DDPP 07410	Northern Poland, winter wheat kernel, 2007	–
	DDPP 07407	Northern Poland, winter wheat kernel, 2007	–
F. proliferatum	DDPP 05512	Poland, winter wheat kernel, 2005	–
F. pseudograminearum	CBS 109953	Australia, uncultured pasture soil	–

Table 1 cont.

1	2	3	4
F. sambucinum var. sambucinum	CBS 161.57	Canada, Manitoba, *Solanum tuberosum*	–
	CBS 135.73	Egypt, *Lycopersicon esculentum*	–
	CBS 665.86	Iraq, *Glycine soja*	–
F. sporotrichioides var. sporotrichioides	CBS 178.64	*Nicotiana tabacum*, seedling root	–
	CBS 447.67	Germany, Niedersachsen, *Pinus nigra* seed, 1965	–
	CBS 534.96	Unknown, 1965	–
	CBS 412.86	Denmark, Řřrvig, Skansehage, Juncus (Juncaceae), stipe, 1986	–
	CBS 413.86	Denmark, *Hordeum vulgare*, ear, 1986	–
	DDPP 061605	Poland, winter wheat kernel, 2006	–
	DDPP 07403	Northern Poland, winter wheat kernel, 2007	–
	DDPP 07408	Northern Poland, winter wheat kernel, 2007	–
	DDPP 07414	Northern Poland, winter wheat kernel, 2007	–
F. sporotrichioides var. minus	CBS 249.61	USSR (country unknown)	–
F. venenatum	CBS 148.95	Sweden, *Spinacia oleracea*	–
	CBS 485.93	Austria, *Orchis santa*	–
F. verticillioides	CBS 734.97	Germany, *Zea mays*	–

+ presence of the expected PCR product; - absence of the expected PCR product

cycler Mastercycler gradient (Eppendorf, Hamburg, Germany) with an initial denaturation step (5 min at 94°C), followed by 35 cycles of denaturation (10 s at 92°C), annealing (30 s at 65°C), extension (55 s at 72°C) and the final extension step (3 min at 72°C). Aliquots (8 µL) of each PCR product were analyzed by electrophoresis in a TBE buffer in 1.5% agarose gels.

Table 2. PCR results and morphological analysis of wheat seed samples contaminated with MON and ANT Y from different parts of Northern Poland

Wheat seed sample	Origin	PCR results *F. tricinctum*	MON	ANT Y	Number of *Fusarium* spp. identified by morphological analysis
1	Chrząstowo	–	n.d.	n.d.	*F. equiseti* (1), *F. poae* (1)
2		+	< 300	n.d.	*F. poae* (18)
3		+	< 300	n.d.	*F. poae* (8)
4		–	n.d.	n.d.	*F. poae* (3)
5	Radostowo	+	< 300	< 120	*F. avenaceum* (1), *F. poae* (4)
6		+	< 300	< 120	*F. poae* (2), *F. culmorum* (2)
7		+	n.d.	< 120	*F. avenaceum* (3), *F. poae* (9), *F. sporotrichioides* (1), *F. tricinctum* (1)
8		+	< 300	< 120	*F. poae* (7), *F. sporotrichioides* (1), *F. tricinctum* (1)
9	Białogard	+	n.d.	n.d.	F. spp. (1)
10		–	n.d.	n.d.	*F. poae* (1)
11		–	n.d.	n.d.	*F. poae* (1)
12		–	< 300	n.d.	*F. poae* (1)
13	Karżniczka	–	n.d.	n.d.	*F*. spp.(1)
14		–	n.d.	n.d.	*F*. spp.(1)
15		–	n.d.	n.d.	*F. avenaceum* (1), *F. poae* (4)
16		–	n.d.	n.d.	*F. poae* (3)
17	Ruska Wieś	+	< 300	n.d.	*F. poae* (3)
18		–	n.d.	n.d.	*F. poae* (2)
19		–	n.d.	n.d.	*F. poae* (7)
20		+	n.d.	n.d.	*F. poae* (3), *F. tricinctum* (1)
21	Wrocikowo	+	< 300	< 120	*F. culmorum* (1), *F. poae* (3)
22		+	< 300	< 120	*F. culmorum* (1), *F. poae* (9)
23		+	n.d.	< 120	*F. graminearum* (4), *F. poae* (6), *F. sporotrichioides* (2)
24		+	< 300	< 120	*F. avenaceum* (2), *F. culmorum* (1), *F. graminearum* (1), *F. poae* (9)

MON (LOD = 150 µg kg^{-1}, LOQ = 300 µg kg^{-1}); ANT Y (LOD = 60 µg kg^{-1}, LOQ = 120 µg kg^{-1})

Results

Sequence analysis of partial IGS rDNA and the design of primers

The alignment of the IGS rDNA sequence data obtained in this study revealed nucleotide polymorphism on which the *tri1/tri2* primers were designed. A single nucleotide polymorphism differentiated all isolates of *F. tricinctum* from *F. heterosporum* (CBS 737.79), while three nucleotide polymorphisms differentiated *F. tricinctum* from *F. acuminatum* (CBS 334.75) and *F. avenaceum* (CBS 115957, 408.86, 119834, 241.94).

PCR assay

The specificity of the *tri1/tri2* primers was evaluated on genomic DNA extracted from 68 isolates representing 21 *Fusarium* species (Table 1). A control PCR assay employing the P58SL/P28SL primers (Hue et al. 1999) was used to confirm that a lack of amplification with the primers tested was a result of primer specificity rather than failure of the PCR reaction (data not shown). Positive PCR results (215 nt) were obtained from all 12 *F. tricinctum* isolates tested in this study. With two exceptions, no cross-reactivity was found with any other *Fusarium* species tested. Isolates CBS 618.87 (*F. acuminatum*) and CBS 393.96 (*F. nurragi*) also gave unexpected positive results.

Detection of target species from wheat seed samples

A total of 13 wheat seed samples tested gave positive results using the PCR assay developed in this study (Figure 1), of which 11 were contaminated with moniliformin and/or antibiotic Y. *Fusarium tricinctum* cultures were only identified in three samples analyzed morphologically (Table 2).

Discussion

Fusarium tricinctum is one of the causal agents of FHB of cereals in Europe. Although considered a weak parasite, several studies demonstrated that this species is a major member of the causal agents of FHB of cereals under certain environmental conditions (Bottalico and Perrone 2002). On the basis of morphological characteristics, this species may be confused with other members of the section *Sporotrichiella*, such as *F. poae*, *F. sporotrichioides* and *F. chlamydosporum* (Leslie and Summerell 2006). In contrast to morphology, the metabolite profile of *F. tricinctum* is similar to *F. avenaceum* classified in the section *Roseum*. Both species have been reported as producers of chlamydosporol, enniatins and moniliformin (Desjardins 2006). Phylogenetic analysis has shown a surprisingly close genetic relationship between these two species (Tan and Niessen 2003) and difficulties have been encountered in generating specific sets of primers for *F. avenaceum*. For example, the first diagnostic primers designed on the basis of the ITS2 rDNA region for the specific detection of *F. avenaceum* (Schilling et al. 1996) were found in later studies to cross-react with *F. tricinctum* (Turner et al. 1998). Among the two other sets of primers derived on the basis of RAPD analysis for the specific detection of *F. avenaceum*, the first Faf/r (Doohan et al. 1999) was found in later studies to cross-react with *F. acuminatum* (Demeke et al. 2005) and *F. tricinctum* (unpublished results from our laboratory), while another one JIAf/r developed by Turner et al. (1998) was shown to give negative results with target strains of *F. avenaceum* (Yli-Mattila et al. 2004).

In this study, primers enabling the detection of *F. tricinctum* were designed on the basis of polymorphism between isolates of *F. tricinctum* and *F. avenaceum*, *F. acuminatum* and *F. heterosporum*.

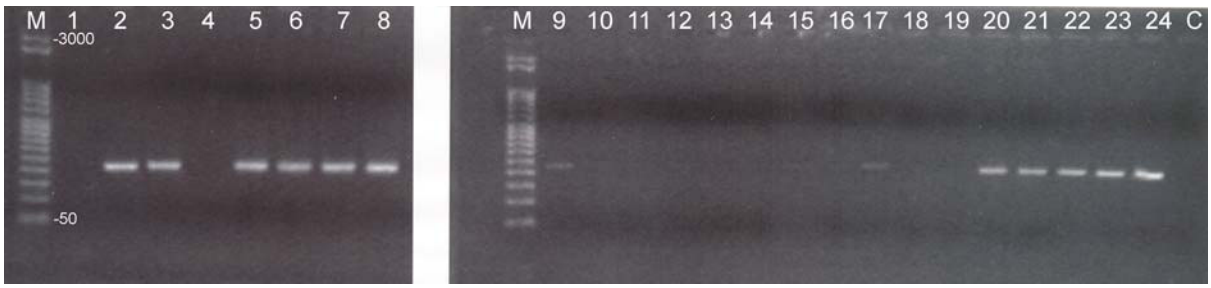

Figure 1. Detection of *F. tricinctum* from wheat seed samples. Lanes: M molecular weight standard Step Ladder, 50 bp (Sigma-Aldrich) ; 1–24 wheat seed samples (numbers represent places of origin described in Table 2); C negative control (no DNA)

Positive PCR results were obtained from all tested isolates of *F. tricinctum*; however, unexpected amplicons were amplified from the templates of *F. acuminatum* (CBS 618.87, originating from Denmark) and *F. nurragi* (CBS 393.96). Additional sequencing and data alignment of the IGS rDNA fragments showed 100% identity of isolate CBS 618.87 (*F. acuminatum*) to CBS 119842 (*F. tricinctum*), while *F. nurragi* (CBS 393.96) showed 99% of identity to CBS 261.51 (*F. tricinctum*).

According to Dr. Ulf Thrane (personal communication), isolates described as *F. acuminatum* from Denmark are similar morphologically and toxicologically (the production of antibiotic Y, aurofusarin, chlamydosporol, enniatins and moniliformin) to *F. tricinctum*. However, several studies have shown that isolates of *F. acuminatum* can produce trichothecenes (Desjardins 2006). The potential for trichothecene synthesis might be revealed by the identification of the tri5 gene, which is present in all fungi capable of producing this group of mycotoxins. A negative result of the PCR assay with primers Tox5-1/Tox5-2 (Niessen and Vogel 1998) targeting conserved regions of tri5 confirmed that the used isolate lacked tri5 and thus was not a trichothecene producer (data not shown). The toxigenic potential of *F. nurragi* has not yet been described; however, its close genetic relationship to *F. avenaceum* and *F. tricinctum* might indicate that it possesses a toxigenic potential similar to these species.

Current problems with the taxonomy of isolates described as *F. acuminatum* are discussed in the recent "The *Fusarium* Laboratory Manual" (Leslie and Summerell 2006). In this study sequencing and data alignment of the partial ef-1α gene of CBS 618.87 in the NCBI database revealed the closest (97%) identity of the isolate analyzed to *Fusarium reticulatum var. negundinis* (DQ295142, DQ295141, DQ295140), *F. tricinctum* (AJ543626, AJ543625, AJ543624, AJ543623, AJ543622, AJ543621, AJ543620, AJ543619, AJ543618, AJ543629, AJ543627, AJ543628) and *F. acuminatum* (EF531698, DQ855948). The above results showed that the taxonomic status of isolate CBS 618.87 could not be resolved on the basis of ef-1α gene data.

The current study demonstrated that 13 of the 24 wheat samples originating from different sites of Northern Poland were positive with the PCR assay developed. Of these 13 positive samples, 11 were contaminated with MON and/or ANT Y. A discrepancy between the two methods was observed only in samples 9, 12 and 20.

A high discrepancy was found between the morphological method and the PCR assay. This could result from different sampling errors, since the morphological analyses were based on plating 100 seeds from each sample, while DNA was extracted from 20 g of seeds (500 kernels). Moreover, different parts of the same sample were analyzed, in which inoculum dispersal could be different. Additionally, *F. tricinctum* growth on PDA may have been inhibited by fast growing fungi such as *F. poae*. Moreover, difficulties associated with the morphological identification of *F. tricinctum*, especially from mixed fungal cultures, may have affected the results. The PCR method is considered as the most sensitive diagnostic technique and extremely low amounts of inoculum of the target pathogen in the analyzed sample result in a positive signal.

The use of the PCR assay developed in this study enabled the discrimination of *F. tricinctum* from the other major FHB agents of cereals; however, cross-reactivity of the marker developed with other minor fungal species will restrict the use of this protocol.

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