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DETECTION OF TOXIGENIC FUSARIUM FROM FOODS BY PCR

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Two PCR methods have been evaluated for the successful identification of Fusarium species. The PCR was performed using a primer specifically targeted to Tri5 gene. The sensitivity of the latter method was determined using the reference strain of Fusarium graminearum CCM F-683 and the selectivity was examined using reference cultures of different genera of fungi naturally occurring in food and feedstuff. Fifty food and feedstuff samples were examined for the presence of trichothecenes-producing Fusarium. In total, seven Fusarium species were isolated, of which five reacted positively with the primer, resulting in the expected size amplicon of 260 bp. Macroscopic and microscopic features were observed on the pure culture of Fusarium and on fungi isolated in relation to their growth in different laboratory media. Morphological features are important for species classification where the PCR can distinguish between toxigenic and non-toxigenic fungi of Fusarium. Thus the PCR technique has proved itself a reliable and rapid method for the detection of the toxigenic species Fusarium.

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Introduction

The genus *Fusarium* is one of most economically important fungi due to its plant pathogenic capabilities and production of potent mycotoxins which are known to affect animals and humans. In addition, some species can also infect humans, especially the immunosuppressed.

Fusarium species have been frequently isolated from whole cereals and cereal-based foods and feedstuffs, particularly from corn and wheat. The ability to produce trichothecenes and other toxic compounds which are dangerous to human and animal health is the main reason for the close attention paid to *Fusarium* species occurrence in foods and feedstuffs. All *Fusarium* species capable of producing trichothecenes carry the *Tri5* or *Tox5* gene [1,2]. The genus of *Fusarium* includes the species of *F. graminearum* and *F. culmorum* as the major trichothecenes producers.

Traditional diagnostic methods for detection and identification of *Fusarium* species in pure culture or in infected food and grain are based on micro- and macro-morphological features developing on a non-selective agar medium. This process is time consuming and requires extensive training. Therefore, the modern and rapid PCR method was developed as highly sensitive and specific to detect fungi. In principle, the PCR is based on the amplification of the target nucleic acid fragments in vitro encoding mycotoxin production [3].

The objective of the present study was to optimize the PCR method using the primer sequence for the trichothecene regulatory gene (*Tri5*) to confirm the specificity and selectivity of the PCR method as a feasible tool for a reliable and rapid detection of the trichothecene-forming *Fusarium* species isolated from foods and feedstuffs [4,5].

Materials and Methods

Cultivation Media

The cultivation media used were MALT agar (Himedia, Bombay, India), Czapek–Dox agar (Imuna, Šarišské Michaľany, Slovakia), GKCH agar (Imuna, Šarišské Michaľany, Slovakia), Y.G.C. agar (Bio Pro, Prague, the Czech Republic), potato dextrose broth and agar (Himedia, Bombay, India).

Buffers and Reagents

All the chemicals were purchased from Sigma unless otherwise stated. The 70 % ethanol (molecular biology grade) was obtained from Top–Bio (Prague, the Czech

Republic).
Buffer I – The TE buffer (pH 8) consists of an aqueous solution of Tris–HCl (10 mmol l^{-1}) and EDTA (1 mmol l^{-1}).
Buffer II – The extraction buffer (pH 8.5) contains Tris–HCl (200 mmol l^{-1}), NaCl (250 mmol l^{-1}), ethylenediamine tetraacetate (EDTA, 25 mmol l^{-1}) and sodium dodecylsulfonate (SDS, 0.5 %).
The PCR buffer (pH 8.3) was an aqueous solution of Tris-HCl (10 mmol l^{-1}) and KCl (50 mmol l^{-1}).
The TBE buffer consists of Tris–HCl (0.089 M), boric acid (0.089 M) and EDTA (0.002 M). The sodium acetate solution (3M) was adjusted to pH 5.3 using acetic acid.
The dNTP mixture contained dATP, dTTP, dCTP and dGTP (2.5 mmol l^{-1} of each). The mixture and the polymerase (Taq DNA polymerase) were purchased from Takara (Shiga, Japan).
A REDExtract–N–AmpTH Plant PCR Kit (Sigma, St. Louis, USA) was used for the DNA isolation. Primers HA Tri/R and HA Tri/F according to Edwards (2001) and DNA marker 67–501 (Top Bio, Prague, the Czech Republic) and agarose for electrophoresis (Serva, Heidelberg, Germany) were used.

Reference Strains and Food or Feed Samples

Culture strains (Tables I and III) were inoculated onto malt agar slants and after 4-7 days of cultivation at 25 °C were kept in a refrigerator. The collection of samples consists of whole cereal grains, cereal flours and cereal-based products partially obtained from local markets and from a local baking company. Samples of feedstuff were obtained from The Central Institute for Supervising and Testing in Agriculture (Brno, the Czech Republic). All the samples were stored in a dark and dry place until use.

The Extraction of DNA

The DNA Extraction according to Cenis [6]

Reference strains and strains of *Fusarium* isolated from foods and feedstuffs were inoculated into 500 µl potato dextrose broth and incubated at 25 °C for 4-7 days. After the incubation, the broth was centrifuged (19 460 g, 5 min), 500 µl of the buffer I was added and the centrifugation of the final mixture was repeated. The supernatant was removed and 300 µl of the buffer II was added and then ultrasonicated for 30 min. After adding 150 µl of the sodium acetate solution, the test tube was kept at –20 °C for 10 min. DNA was collected by centrifugation and

the pellet was washed with 300 µl ethanol, then dried and dissolved in 50 µl buffer I.

Table I Selectivity testing of PCR with *Tri5* specific primer

Species	Presence of amplion size 260 bp
Acremonium ochraceum CCM F –365	-
Acremonium strictum CCM F – 761	-
Botrytis cinerea CCM F – 16	-
Cylindrocarpon destructans CCM F – 50	-
Fusarium avenaceum CCM 8015	-
Fusarium culmurum CCM F – 163	+
Fusarium graminearum CCM – 683	+
Fusarium incarnatum CCM F – 794	-
Fusarium oxysporum CCM F – 45	-
Fusarium poae CCM F – 169	+
Fusarium poae CCM F – 584	+
Fusarium poae CCM F – 658	-
Fusarium solani CCM 8014	-
Fusarium sporotrichioides CCM F – 164	-
Fusarium sporotrichioides CCM F – 352	+
Fusarium sporotrichioides CCM F – 554	-
Fusarium tricinctum CCM 8072	-
Gliocladium roseum CCM 8070	-
Paecilomyces variotii CCM F – 566	-
Trichothecium roseum CCM F – 372	-
Verticillium fusisporum CCM 8021	-
Verticillium psalliotae CCM F – 235	-

Table II Samples of food and feed

No.	Sample	No.	Sample
1	groundnut	26	Mixture for buns and rolls with vegetable
2	flaxseed	27	wheat flour for bread
3	sunflower shelled	28	Mixture for buns and rolls
4	gingili shelled	29	Mixture for fried pastry
5	poppy	30	corn 1
6	mixture	31	barley 1
7	caraway	32	barley 2
8	Feed for broiler with salinomycin	33	maize
9	Feed for chicken	34	corn 2
10	Permix – feed for broiler	35	birdfood
11	Permix – feed for calf	36	Muesli bar
12	corn MAVE – Jičín	37	cornbread
13	barley MAVE – Jičín	38	pinole
14	air 1	39	pinole
15	air 2	40	wholemeal roll 1
16	air 3	41	wholemeal roll 2
17	air 4	42	Corn MINI
18	air outside 1	43	Corn roll
19	air 5	44	Bread – Moskva
20	air outside 2	45	walnuts
21	air 6	46	pollard
22	wheat flour	47	cornflakes
23	Mixture for pastry	48	bread
24	bake mixture for wheaten dough	49	roll
25	rye flour for bread	50	spice

Table III Detection o total mycoflora and *Fusarium* species in food and feedstuff samples

Sample No.	Genus	Viable count of total fungi cfu g^{-1}		Viable count of *Fusarium* fungi cfu g^{-1}		Presence of amplicon size 260 bp
		MALT	Y.G.C	MALT	Y.G.C.	
12	*Fusarium poae*	1×10^2	1×10^3	1×10^2	1×10^2	+
15	*Fusarium graminearum*	1×10^2	0	1×10^2	0	+
16	*Fusarium graminearum*	1×10^2	0	1×10^2	0	+
21	*Fusarium chlamydosporum*	1×10^2	0	1×10^2	0	+
38	*Fusarium sacchari* var. *subglutinans*	0	1×10^2	0	1×10^2	-
39	*Fusarium sacchari* var. *subglutinans*	1×10^2	1×10^2	0	2×10^2	-
50	*Fusarium semitectum*	1×10^2	0	1×10^2	0	+

DNA Extraction using REDExtract-N-AMPTH Plant PCR Kit

Reference strains and strains of *Fusarium* isolated from food and feedstuff were inoculated into 500 µl potato dextrose broth and incubated at 25 °C for 4-7 days. After the removal of the broth supernatant (19 460 g, 5 min), the mycelium mass was transferred into a sterile test tube to which 100 µl extraction solution was added and incubated at 95 °C for 10 min. DNA was dissolved in 100 µl a diluent solution.

PCR Optimization

PCR Procedure [1]

The reaction mixture (25 µl) contained 2 µl DNA solution (see The DNA Extraction by Cenis), 4 µl dNTPmix, 2.5 µl PCR buffer (10× diluted), 2 µl $MgCl_2$ (2 mmol l^{-1}), Taq DNA polymerase 0.1 µl and 0.25 µl of each primer. The reaction mixture in the micro tube was overlaid with 10 µl mineral oil (*MJ Research*, San Francisco CA, USA). Temperatures of annealing from 52 to 66 °C were tested in a gradient thermocycler (Robocycler 2000 Gradient, Stratagene, La Jolla, CA, USA). The final optimized procedure consisted of initial denaturation for 3 min 15s at 94 °C and 30 cycles of denaturation (1 minute at 94 °C), followed by annealing (1min at 61 °C) and extension (1min 45 s at 72°C). In the last cycle,

extension of 4 min 15 s at 72 °C was used [1].

The PCR product was analysed by electrophoresis in agarose (1%, Serva, Heidelberg, Germany) in the TBE buffer. Before solidification, ethidium bromide (0.015 %) was added to the gel. Electrophoresis was run at 100 V for 30 min and the results were observed on Bio Print (*Vilber Lourmat*, Marne-La-Vallee Cedex 1, France).

PCR Procedure using REDExtract-N-AMPTH Plant PCR Kit

Plant PCR Kit was used according to the manufacturer's instructions, the annealing temperature was optimized in the laboratory.

PCR Selectivity Testing

The fungal strains (Table I) were used to test the selectivity of the primer. Fungal DNA was isolated using REDExtract-N-AMPTH Plant PCR Kit and the modified PCR process according to Edwards (2001) was applied. A volume of 20 µl of the reaction mixture for the PCR contained 1.5 µl cell lysate, 10 µl PCR reaction mixture and 0.25 µl of each primer. The reaction mixture in the micro tube was overlaid with 10 µl mineral oil (*MJ Research*, San Francisco CA, USA). Different temperatures of annealing (from 47 to 67 °C) were tested for the optimization of the PCR method using a gradient thermocycler (Robocycler 2000 Gradient, Stratagene, La Jolla, CA, USA). The amplification was performed in the thermal cycler for an initial denaturation for 3 min 15 s at 94 °C and 30 cycles of denaturation (1 minute at 94 °C), annealing (1min at 56 °C), and amplification (1 min 45s at 72 °C). The last cycle was followed by a final extension at 72 °C for 4 min.

The PCR product was analysed by electrophoresis in agarose (1 %, Serva, Heidelberg, Germany) in the TBE buffer. Before solidification, ethidium bromide (0.015 %) was added to the gel. The electrophoresis was run at 100V for 30 min. Visualization was carried out on Bio Print (*Vilber Lourmat*, Marne-La-Vallee Cedex 1, France).

Isolation of Fungi from Real Sample

To determine the presence of fungi, 10 g of food or feedstuff was transferred into a sterile plastic bag and homogenized with 90 ml saline and peptone mixture. The homogenate was incubated at 25 °C for 5-7 days, then suspicious fungal colonies were isolated and inoculated onto a potato dextrose agar plate at 25 °C for 5d.

After the incubation period, PDA plates were used to isolate fungal DNA as mentioned above.

PCR Sensitivity Testing

The reference strain of *Fusarium graminearum* (CCM F-683) was used to test the sensitivity of the primer. To determine the correlation between the isolated DNA concentration and the mycelial mass, the extraction of fungal DNA by the Plant PCR Kit and the subsequent PCR process were applied in one-day intervals (up to 7 days) using the PCR reaction mixture and the temperature profile as described above (PCR Procedure using REDExtract-N-AMPTH Plant PCR Kit). Eleven test tubes with the stated amount of the PCR reaction mixture and the extracted DNA were prepared for each of these reactions.

Morphological Features of *Fusarium* Species

All tested reference strains of *Fusarium* species were grown on four different agar plates at 25 °C (CZ-DOX, MALT, BA and Y.G.C.). Distinctive macroscopic and microscopic features were observed in one day intervals; i.e. the growth rate, the mycelia appearance, the pigment formation and particularly the macroconidial form and the presence or absence of microconidia.

Results and Discussion

PCR Optimization

Modification of PCR Process according to Cenis (1992) and according to Edwards et al. (2001)

F. graminearum CCM F-368 reacted positively with the *Tri5* primer resulting in an amplicon size of 260 bp. The volume of the primer had to be increased from 0.025 µl to 0.25 µl and the concentration of Mg^{2+} ions was optimized (2 mM) in order to observe a clear indicator band in this region. If the concentration of Mg^{2+} in the reaction mixture decreased, the illumination of the indicator band decreased or did not give any amplicon. Two amplicons (260 and 180 bp) were observed using the PCR reaction mixture with the Mg^{2+} concentration higher than 2 mM, in which the latter indicator band resulted in a non-specific reaction. The temperature of the primer annealing was evaluated using gradient thermocycler with the temperature ranging from 52 to 63 °C and from 56 to 67 °C (Fig. 1.). The temperature of 61 °C was found to be suitable for the primer hybridization, giving

one amplicon the size of 260 bp. The PCR method itself, consisting of the DNA extraction, the reaction mixture preparation, the amplification and the electrophoresis of PCR product, takes approximately ten hours. To speed up the identification of *Fusarium* species, the PCR process with the time profile was performed according to Edwards *et al.* (2001). However, the primer and the Mg^{2+} concentration, as well as the annealing temperature had to be adapted, as mentioned above. It was proved that the PCR method used in this study gives inconsistent results, which is probably due to a rigid wall of *Fusarium* cells. It can be stated that the modified method by Cenis (1992) does not provide a proper procedure to isolate DNA from tested fungus. Hence, it was necessary to find another, suitable extraction technique to isolate fungal DNA.

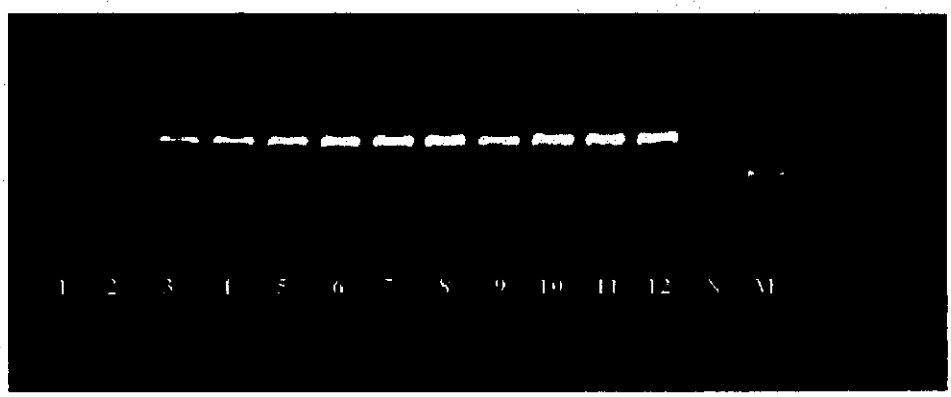

Fig. 1 Annealing temperature optimization to obtain maximum specificity and selectivity using *F. graminearum* CCM F – 683. Annealing temperature: 1 – 67 °C; 2 – 66 °C; 3 – 65 °C; 4 – 64 °C; 5 – 63 °C; 6 – 62 °C; 7 – 61 °C; 8 – 60 °C; 9 – 59 °C; 10 – 58 °C; 11 – 57 °C; 12 – 56 °C; N – negative control (53 °C); M – DNA marker

Extraction of DNA using REDExtract-N-AMPTH Plant PCR Kit and Modified PCR Fungal Process according to Edwards et al. (2001)

The commercial PCR kit, primarily designed for the isolation and amplification of plant DNA, was used to detect *Fusarium* species based on the assumption that fungi cell wall has a composition similar to that of plant leaves. After DNA of *F. graminearum* was extracted using the original reagents available in the Plant PCR Kit, the PCR process according to Edwards *et al.* (2001) was applied. To obtain clear indicator bands after electrophoresis, annealing temperature had to be optimized to 56 °C. If annealing temperature fluctuated, non-specific reaction occurred, resulting in the formation of another indicator band.

The plant PCR Kit was proved by this experiment to be a suitable tool for the detection of *Fusarium graminearum*. The PCR procedure takes approximately

four hours (including the DNA exctraction, the reagent mixture preparation and amplification). The separation process in particular was significantly simplified, thanks to the direct application of the PCR product onto the electophoresis gel as opposed to other experiments using the contrast buffer solution prior to sampling, in which case the entire PCR procedure takes five hours. Thus, more samples could be analyzed, and a higher concentration of DNA was obtained when using the Plant PCR Kit than when using the PCR process published by Cenis (1992). A comparison of the above mentioned methods is presented in Fig. 2.

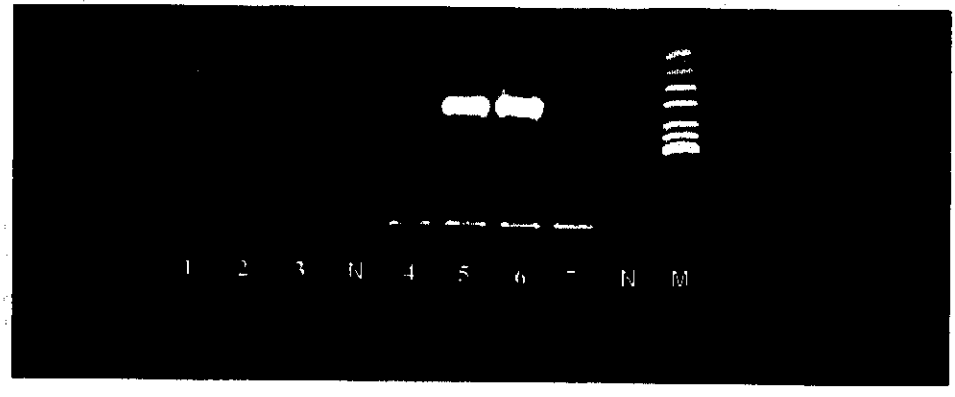

Fig. 2 Comparison of different DNA extraction techniques (annealing temperature 61 °C). DNA isolation using modified procedure by Cenis (1992): 1, 2 - *F. graminearum* CCM F – 683; 3 – *F. culmorum* CCM F – 163; N – negative control. DNA isolation using REDExtract-N-AMPTH Plant PCR Kit: 4, 7 – *F. solani* CCM 8014 (non-trichothecenes-forming strain); 5 – *F. culmorum* CCM F –163; 6 - *F. graminearum* CCM F – 683; M – DNA marker

An amplicon size of 170 bp (see sample no. 5 and 6, Fig. 2) is the result of a non-specific reaction, probably due to the relatively high annealing temperature (61 °C) used. The amplification of extracted DNA, using both the Plant PCR Kit and the process described by Cenis (1992), depends on the annealing temperature; 61 °C was found to be suitable for multiplying a DNA molecule obtained by the procedure previously described by Cenis (1992). Using the PCR Plant Kit, the annealing process requires a broad range of temperatures (47-67 °C) to provide desired PCR products. Bright indicator bands above sample no. 4-7 (Fig. 2) resulted from the excess of extracted DNA molecules.

PCR Method Sensitivity Testing

A minimal and optimal extracted DNA volume added to the PCR reaction mixture was determined. There was a correlation between the extracted fungus DNA amounts and mycelial mass. Thus, a relation between the template DNA amounts

obtained in different growth phases of *F. graminearum* CCM F-368 and the illumination level of indicator band was determined. Our results show that the visible amplicon size of 260 bp was obtained using the lowest DNA volume of 0.2 μl, so that results could be falsely evaluated by analysts. The optimal fungus DNA volume of 1.5 μl giving the expected size amplicon was defined. Moreover, a sufficient DNA amount of *F. graminearum* CCM F-368 was isolated after 24 h of appropriate incubation using the optimal template DNA volume in the PCR reaction mixture. A minimal extracted DNA volume of 0.2 μl pipeted onto the PCR reaction mixture gave a faint indicator band. Our results suggested that trichothecene-forming *Fusarium* strains should be successfully detected using the Plant PCR Kit after 48 h incubation. The longer the incubation period, the greater the amount of template DNA obtained, which resulted in an increase in indicator band illumination.

PCR Method Specificity Testing

To test the specificity of *Tri5* primer, related and naturally occurring fungi, which may contaminate food and feedstuff together with the *Fusarium* species, were examined. The extraction of DNA from reference strains of *Fusarium* species was performed using the Plant PCR Kit. The above-mentioned PCR procedure is suitable to discern between trichothecene and non-trichothecene producing *Fusarium* species. Five of thirteen reference strains gave an indicator band in the desired region (Table I), indicating that under such environmental circumstances they are able to produce trichothecene.

Morphological Features of the *Fusarium* Group of Fungi

No selective medium exists for the identification of toxigenic *Fusarium* species, for example those based on colony pigmentation, so typical macroscopic and microscopic morphological features of *Fusarium* species have to be determined in order to distinguish them from other of fungi.

An increase in colony diameter was measured after incubation at 25 °C in one-day intervals using all the tested agar plates (BA, CZ-DOX, MALT, Y.G.C.). The growth of fungi depended both on the nutrient agar and on the fungi genus used in this study. Moreover, despite the same incubation conditions, differences were also observed between several species of fungus genera.

Poor growth in the *Fusarium* group of fungi was detected on Czapek-Dox agar plates, probably due to its high salt content. Uniform growth rate was found in the rest of the laboratory media tested in this study. Each species was clearly distinguished by pigment formation on the reverse side of the colony; however,

several passages of fungus inoculum are needed to fully develop their distinctive features when freeze-dried or on gelatine discs. So incorporated strains were used. Nevertheless, macroscopic features variability was observed between different strains belonging to one species.

Macroscopic variability of tested fungi growing on a variety of nutrient agar plates was observed, thus the use of PCR method for detection of *Fusarium* species is advisable. It should be noted that HA Tri/F and HA Tri/R primers applied in polymerase chain reactions amplified a specific fragment of *Tri5* gene encoding the trichothecene formation, hence only the DNA extracted from toxigenic *Fusarium* species reacted positively. As far as the detection of the *Fusarium* group of fungi, including non-trichothecene forming species is concerned, application of the PCR method alone as described in this paper seems to be insufficient. For example, a *Fusarium* species was isolated from corn flour (sample no. 38, Table II) with its identification based on characteristic growth on MALT, Y.G.C., CZ-DOX and BA agar plates after the cultivation under appropriate conditions. However, the DNA of this fungus did not react with the selected primers used in the current study, indicating the absence of Tri5 gene.

Real Sample Analysis

A total of 50 samples of food and feedstuff were examined in order to isolate trichothecene-producing fungi. Of the seven samples contaminated with *Fusarium* fungi, only five possessed *Tri5* gene. Presumably, when appropriate conditions for growth are provided, including water activity, temperature, etc., the production of mycotoxins may occure (Table II).

Two different *Fusarium* species were isolated from corn flour (sample no. 39), but the amplicon size of 260 bp was not found after the PCR procedure. Thus, three corn-based food samples (no. 37, 47 and 47) were investigated to determine the presence of *Fusarium* species, and the samples tested were found negative. However, due to a limited number of corn-based samples, no conclusion could be made concerning the *Fusarium* incidence in this commodity.

Trichothecene-producing *Fusarium* was isolated from spice and wheat samples, as well as from air deposition. In such matrices, a frequent occurrence of *Fusarium* species is generally known. Macroscopic features of fungi isolated from samples no. 13, 14, 17, 18, 19, 20 and 25 on MALT and Y.G.C. agar plates were after incubation similar to those of *Fusarium* species, hence the PCR method was applied showing that isolated fungi are not able to produce trichothecene. In mount of tested fungi, sterile mycelium and the absence of typical macroconidium even after long incubation period was observed.

Fusarium species were not isolated from the rest of grain samples, foods and feedstuffs. The sampling was performed during winter, which was the possible

cause of the low frequency of *Fusarium* isolation in the current study. Seasonal incidence of fungi is generally known. Spring and autumn months are considered to be most common for fungi occurrence (Table III).

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