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Mycological Analysis of Cereal Samples and Screening of *Fusarium* Strains' Ability to form Deoxynivalenole (DON) and Zearalenone (ZEA) Mycotoxins – a Pilot Study

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A b s t r a c t

Filamentous fungi are cosmopolitan microorganisms found in almost all environments. It should be pointed out that occurance of moulds on food or feed may cause health disorders in humans and animals. Mycoflora appears as a source of toxic methabolites, mycotoxins, which hepatotoxic, genotoxic, nefrotoxic and carcinogenic abilities were already proven in several studies. Hense mycological analysis of cereal grains raises as an important manner in evaluation of food and feed health features. Among the most frequent cereal contaminants *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium* strains are mentioned. Due to their ability to grow on cereals during both its field growth and storage, *Fusarium* moulds occure to be an important contamination factors in food and feed industry. In this study *Fusarium* strains isolates from wheat and maize were examined in order to recognize their abilities to produce two toxins: zearalenon (ZEA) and deoxynivalenole (DON). Mycological analysis shown differentiation within fungal microflora occuring in samples of different storage conditions, where *Fusarium* strains represented aproximately 20–70% of all mould species present. In purpose of *Fusarium* strains species evaluation, isolates were mycologically analysed. In the second step of the project, toxicological screening of isolates was performed using Thin Liquid Chromatography (TLC) evaluating toxigenic potential of single strains' production of ZEA and DON. This data gives the possibility of pointing the most toxigenic strains and also shows differentiations in their occurance in cereals. This paper presents introductory research data, which can be useful in recognition of cereal contamination with moulds and their toxic methabolites.

K e y w o r d s: mycoflora of cereal grains, *Fusarium* sp., toxins, ZEA, DON

Introduction

Filamentous fungi genus *Fusarium*, first described by Link in 1809, consists of various groups of species, with ascomycete teleomorphs known for several of them. Broad range of hosts, ecological flexibility and wide geographical distribution are characteristic for this genus. Fusaria are common soil microflora, frequently pathogenic to plants. Their occurrence in plants is associated with toxin formation, especially trichothecenes, *e.g.* deoxynivalenol (DON) and it's derivatives, nivalenol (NIV), T-2 toxin and other, like fumonisins and zearalenone (ZEA) (EU Commission Regulation No 856/2005, Kwasna *et al.*, 1991). Deoxynivalenol is wide spread mycotoxin implicated in human and animal toxicity. DON may cause liver, kidney and alimentary canal disorders as well, it's nick name – 'vomitoxin' describes one of the consequences of DON exposure in humans and animals. Zearalenone is fungal hormone responsible for perythecium formation. It's estrogen-like chemical structure gives an opportunity to deceive estrogen receptors, causing 'estrogenic syndrome' affecting urogenital system (Marin *et al.*, 2004). European Union directive regarding levels of mycotoxin concentrations in foods was actualized on 6 of June 2005 in Brussels (EU Commission Regulation No 856/2005).

Compounds in question are secondary metabolites of high toxicity, widely distributed in food chain. Main sources of *Fusarium* mycotoxins are cereals and cereal products made from wheat and corn (Leblanc *et al.*, 2002). *Fusarium* contamination of cultivated plants rises to be a topic of great importance in terms of food/feed quality and safety.

There were a few aims of the work. Wheat samples were analyzed in order to state fungal contamination levels and percentage of *Fusarium* strains amongst the whole range of filamentous fungi present. Occurrence frequency of Fusaria was described in chosen samples. Secondly, toxin formation ability screening was performed and percentage of DON- , ZEA- , DON & ZEA-forming isolates was settled.

Experimental

Materials and Methods

Nine wheat samples from European Union countries were obtained from Abteilung Analytikzentrum IFA-Tulln, Austria. Samples were collected in 2002 in Germany (3 samples: 7607, 7610, 7613), France (2 samples: 7630, 7635) and Great Britain (4 samples: 7645, 7646, 7649, 7651). Levels of DON and ZEA were known thanks to previous HPLC analysis performed in IFA-Tulln.

Mycological analysis. 2 g of each sample were added to 18 mL of saline with 0.01% Tween 80 and shaken for 15 min on a magnetic shaker (BMM21 DHNI PAN-WABEL) at 1000 rpm. 0.1 mL of the suspension was applied on an agar plate filled with chloramphenicol agar. Dilutions 10^{-1} to 10^{-5} were examined in 3 replications. Plates were incubated in 23–25°C in darkness for 5–7 days. Filamentous fungi and exclusively Fusaria were counted and expressed as colony forming units per 1 gram of wheat sample. Occurrence frequency and relative species proportions were defined for *Fusarium* isolates. This procedure was replied three times for each sample (Labuda *et al.*, 2003).

Taxonomic identification was performed on PDA agar during 14–28 days incubation in daylight in 18–23°C. For conidia observations and evaluations light microscopes were used (Domsch *et al.*, 1993; Fassatiova, 1983; Hoog and Guarro, 1995; Kwasna *et al.*, 1991; Samson *et al.*, 1996).

Toxin formation ability analysis. Isolates from *Fusarium* colonies on chloramphenicol agar were inoculated on malt extract agar slants with sterilized wheat grains for 7 days in 23–25°C in darkness and stored in 4°C afterwards. Wheat grain played important role in preventing Fusaria from changing metabolic abilities, their morphology and becoming sterile. *Fusarium* cells covering wheat grains were used for toxicological analysis procedures. One wheat grain covered with mould was added to 50 mL of saline with 0.01% Tween 80 and shaken for 15 min on magnetic shaker. Conidia concentration was checked using Thoma counting chamber, suspension was diluted to the level of 10^6 cfu/mL if needed.

In order to assure natural conditions, medium for *Fusarium* cultivation was wheat of low mycological contamination (about 10^{-2} cfu/g) and level of *Fusarium* toxins beneath 1 ppb. Cultivations dishes were 250 mL flasks covered with antiseptic gauze and foil. 50 g of dry wheat and water (to set grain humidity level at 35–40% to enable effective sterilization) (Jelen, 2001) were added to each flask. Grain was sterilized for 15 min in 121°C. 5 mL suspension of 10^6 cfu/mL conidia concentration was added to each flask and shaken in order to achieve equal cultivation conditions in the flask. After 14 days incubation in daylight in 18–23°C cultures were neutralized by 45 min pasteurization and dried to the stable weight in 50°C afterwards. Dry wheat cultures were grinded for 15 s in a Bosch mill (MKM6003, type KM13). Toxins were extracted when 5 g of grinded wheat culture was added to 20 mL 80% methanol and shaken for 90 min on shaker (Premed Universal Shaker type 327, amplitude 4, frequency 2). This procedure was performed 4 times per isolate strain. 5 μL of toxin dilution were applied on TLC plate (silica gel 60 without fluorescent marker; A07084 by Merck), put in TEF solvent (toluene/ethyl acetate/90% formic acid 5:4:1), visualized with 20% $AlCl_3$ in 60% ethanol and heated for 5 min at 130°C. DON and ZEA presence was stated according to the toxin standard observed in UV light (Samson *et al.*, 1996; Mubatanhema *et al.*, 1999; Leblanc *et al.*, 2002).

Results and Discussion

The reference for this pilot study were the results obtained in IFA-Tulln, stating levels of *Fusarium* toxins in analyzed samples (Fig. 1). ZEA concentrations ranged from 6 to 244 mg/kg of wheat sample and did not exceed acceptable levels for food components (EU Commission Regulation No 856/2005). DON concentrations appeared much higher, ranging from 366 to 9915 mg/kg. Levels of DON exceeded acceptable rate for foodstuffs in two of nine wheat samples and for cereal components of food without preparation in four of nine samples. Fungal contamination reached from 420 cfu/g to 12 000 cfu/g in analyzed samples

Table I
Summary of results obtained during mycological analysis of wheat samples from IFA-Tulln, Austria

Sample number	Toxin concentration [μg/kg]		Level of fungal contamination [cfu/g]	Percentage of *Fusarium* strains occurrence [%]
	DON	ZEA		
7607	513	14	2.3×10^3	89.13
7610	395	8	3.7×10^3	63.43
7613	390	9	3.2×10^3	71.31
7630	8249	244	1.2×10^4	44.21
7635	9915	17	1.1×10^4	77.88
7645	268	206	4.2×10^2	24.40
7646	721	16	5.0×10^2	13.24
7649	366	n.n.	9.8×10^2	34.94
	460	6	1.0×10^3	43.75

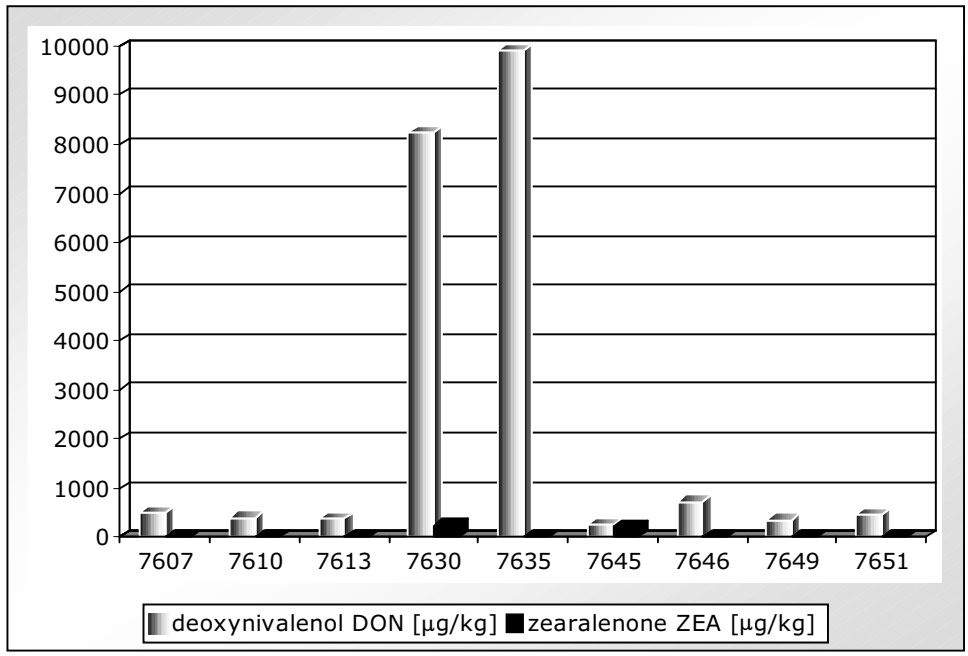

Fig. 1. Deoxynivalenol and zearalenone concentrations in wheat samples analyzed using HPLC method (IFA-Tulln, Austria)

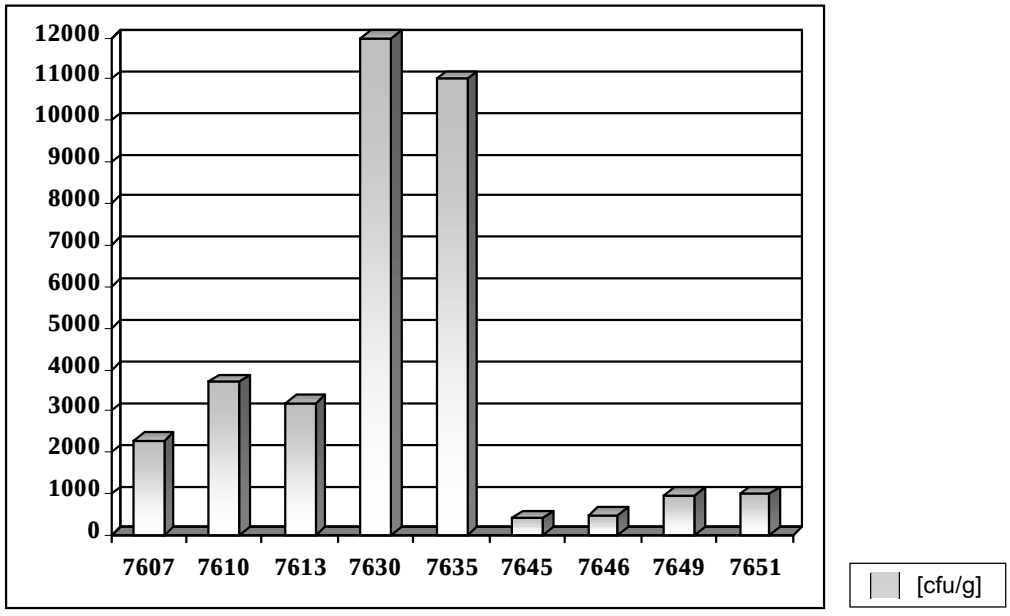

Fig. 2. Level of fungal contamination of wheat samples from IFA-Tulln, Austria

(Fig. 2). The correlation between total level of toxins concentration and fungal contamination was observed, while two samples of highest toxin levels occurred to be fungal contaminated up to the highest extent. No clear correlation between level of contamination and percentage of *Fusarium* strains could be stated (Fig. 3). The results obtained during mycological analysis are shown in Table I. Fusaria occurred at the level from 13 to 89% of total amount of fungi present in samples. There was no correlation between DON and ZEA contamination level and percentage of *Fusarium* as well. For mycotoxin formation ability screening four wheat samples were chosen (7610, 7613, 7630, 7635). Time period of cultivation was chosen as a standard period needed for secondary metabolite formation. The aim of the study was to check whether the fungus forms the toxin or it does not. Proportions between toxigenic and non-toxigenic Fusaria are presented in Table II. Most of isolates formed ZEA (more than 50%), while DON presence in cultures

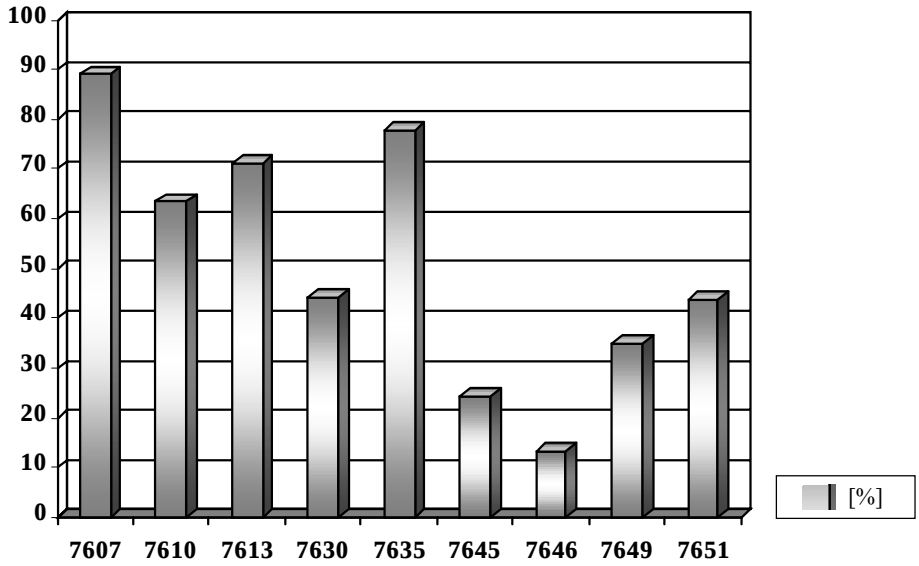

Fig. 3. Percentage of *Fusarium* strains occurrence in wheat samples from IFA-Tulln, Austria

reaches up to 40%. There are about 30% of non-toxigenic strains among all isolates. Mycological analysis shown that there are dominant section representatives in analyzed samples (Table III), but there is no significant correlation between toxin formation and species' composition in wheat. In addition, morphological and metabolic changes of isolates occurred, causing taxonomic identification disturbance. Differences in secondary metabolite profile were observed in isolates, TLC analysis was useful in taxonomic identification of *Fusarium* strains.

This pilot study showed that no significant correlation between level of *Fusarium* contamination and toxin concentration in sample exists. The main role is played by climate conditions while cultivation of cereals and storage conditions as well. Furthermore, special attention shall be paid to those conditions in order to prevent fungal toxin formation.

Table II
Proportions between toxigenic and non-toxigenic *Fusarium* strain isolates from chosen wheat samples

Sample number	Number of strains forming			Number of non-toxigenic strains [%]
	ZEA [%]	DON [%]	ZEA+DON [%]	
7610	20	40	20	60
7613	33	50	25	42
7630	70	50	40	20
7635	68	47	47	32

Table III
Proportions between *Fusarium* section representatives among isolates from chosen wheat samples according to Nelson, Toussoun and Marassas clasiffication (1983) described in Domsch *et al.*, 1993; Kwasna *et al.*, 1991; Samson *et al.*, 1996

Sample number	*Fusarium* Section			
	Arthrosporiella	*Discolor*	*Roseum*	*Sporotrichioides*
7610	0	60	40	0
7613	0	25	33	8
7630	20	40	10	0
7635	0	30	20	40

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