Fungal Contamination and the Levels of Mycotoxins (DON and OTA) in Cereal Samples from Poland and East Slovakia

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Abstract

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The cereal samples were taken immediately after harvest from the selected localities of Poland (45 samples) and East Slovakia (60 samples). Fungal contamination of these samples was investigated and subsequently the presence of two important mycotoxins, deoxynivalenol (DON) and ochratoxin A (OTA), was quantitatively examined. Concerning mould contamination, no difference was observed between the samples from Poland and East Slovakia. The highest incidence was observed of *Fusarium, Aspergillus,* and *Penicillium* genera. However, most of the investigated samples of wheat, rye, and barley contained less than 10^4 cfu/g. The limit 750 ppb for DON in cereals and their products, recommended by the European Mycotoxin Awareness Network (EMAN), was exceeded only by one wheat sample (4.5%) from Poland, but by seven wheat samples (14.6%) from Slovakia. None cereal sample investigated for OTA exceeded the allowed limit – 5 μg/kg.

Keywords: wheat; rye; barley; microscopic fungi; ochratoxin A (OTA); deoxynivalenol (DON)

Microscopic fungi and their metabolites, mycotoxins, are often found as contaminants in agricultural products before or after harvest as well as during transportation or storage. Fungal growth and mycotoxin production in cereals is influenced by various factors. Climatic conditions, especially temperature and humidity, play a very important role in this process. Poland and Slovak Republic are situated in a temperate climatic region where hot sunny weather often changes into colder thundery weather also during the harvest (July–August) period. These climatic variances support the growth of several kinds of microscopic fungi. Fungi growing in cereals could be divided in general into two groups, field and storage fungi. Genera *Aspergillus* and *Penicillium* are typical storage fungi, however, their occurrence in cereals during harvest is also possible. *Fusarium* species are representatives of field fungi and their higher amounts in cereals are connected with a higher humidity and colder weather (Šimerda 1996). There are more than 100 000 species of mould producing more than 300 metabolites which have a toxic potential for humans and animals (Hintz 1990). It is known

that fungi of the genera *Aspergillus* and *Penicillium* produce carcinogenic mycotoxins, aflatoxins and ochratoxins, while *Fusarium* species produce the oestrogenic toxin zearalenone and the structurally related group of trichothecenes. Therefore, they can significantly decrease the quality of cereals. The effects of moulds and their metabolites are investigated from the point of the relationship to human and animal health. Many mycotoxins in food and feed are nephrotoxic, hepatotoxic, immunosuppressive, or carcinogenic (Ostrý 1998). That is why many countries accept the recommended limits for fungal contamination and mycotoxin levels in food and feed.

Fungal contamination of cereals after harvest in 2001 in selected localities in Poland and Slovakia directed our attention also to the investigation of two important mycotoxins, deoxynivalenol (DON) and ochratoxin A (OTA).

MATERIALS AND METHODS

Fungal investigation. Cereal samples were taken after harvest from lorries during haulage of cereals into buyer companies before their storage in silos. Forty five of Polish samples originated from four regions of Poland (Mazowieckieho – 21, Lubelskieho – 12, Kujawsko-pomorskieho – 10 and Lodzskieho – 2). Sixty Slovak samples exhibited the situation in East Slovakia, in the farms of Košice region.

Quantitative evaluation of the fungal contamination was performed in the samples according to ISO-norms STN ISO 7954 (1997) – Microbiology – General guidance for enumeration of yeasts and mould – Colony count technique at 25°C, and STN ISO 6887 (1997) – Microbiology – General guidance for the preparation of dilutions for microbiological examination, which are based on international norms.

The sample suspension was prepared by the weight of the investigated material in peptone water in ratio 1:10 and it was subsequently shaken for 2 hours. One ml of the suspension was diluted with physiological solution to provide a dilution series between 10^{-2} and 10^{-4}. From each dilution, one ml was inoculated on Petri dishes and overlayered with 15 ml of the nutrient medium (GKCH – Chloramphenicol Yeast Glucose Agar). Medium sterility was confirmed by a control dish. The inoculated plates were incubated aerobically at the temperature of 25°C for 5 days. The number of moulds in 1 g of the analysed sample was calculated on the basis of the colony numbers grown on the plates from two subsequent (following) dilutions. It was calculated according to formula:

$$\Sigma c/(n_1 + 0.1n_2) \times d$$

where:

Σc – sum of colonies on all plates used for the calculation

n_1 – number of plates from the first dilution used for the calculation

n_2 – number of plates from the second dilution used for the calculation

d – the first dilution used for the calculation

The samples were evaluated also qualitatively on the fifth day of cultivation. The individual genera of moulds were determined on the basis of their macroscopic and microscopic morphology.

DON analysis. The methodology for the DON determination was described by Czerwiecki and Wilczyńska (2003). DON was extracted with methanol followed with water from the cereal samples (50 g) ground in the laboratory mill. The extracts were purified on immunoaffinity columns. The mycotoxin was determined by high-performance liquid chromatography on C_{18} column with UV-detection at 218 nm. The mean recovery of DON was about 68%. The limits of detection (LOD) and quantification (LOQ) were 10 and 50 μg/kg, respectively. To improve the identification, co-chromatography was performed.

OTA analysis. For ochratoxin A, another method was used (Czerwiecki *et al.* 2004). Ochratoxin A was extracted using methanol and water from the ground cereal grain samples (50 g), the extracts were purified on immunoaffinity colums. The ochratoxin A content was determined by high-performance liquid chromatography on C_{18} column using fluorometric detection with excitation at 330 nm and emission at 460 nm. The mean recovery of ochratoxin A was 69–78%. The limits of detection (LOD) and quantification (LOQ) were 0.015 and 0.025–0.03 μg/kg, respectively. The positive results were confirmed by the reaction with BF_3 complex in CH_3OH.

Statistical analysis. The results obtained were analysed by ANOVA test (InStat). The comparison of the individual groups and the significance were evaluated by Tukey-Kramer Multiple Comparison Test.

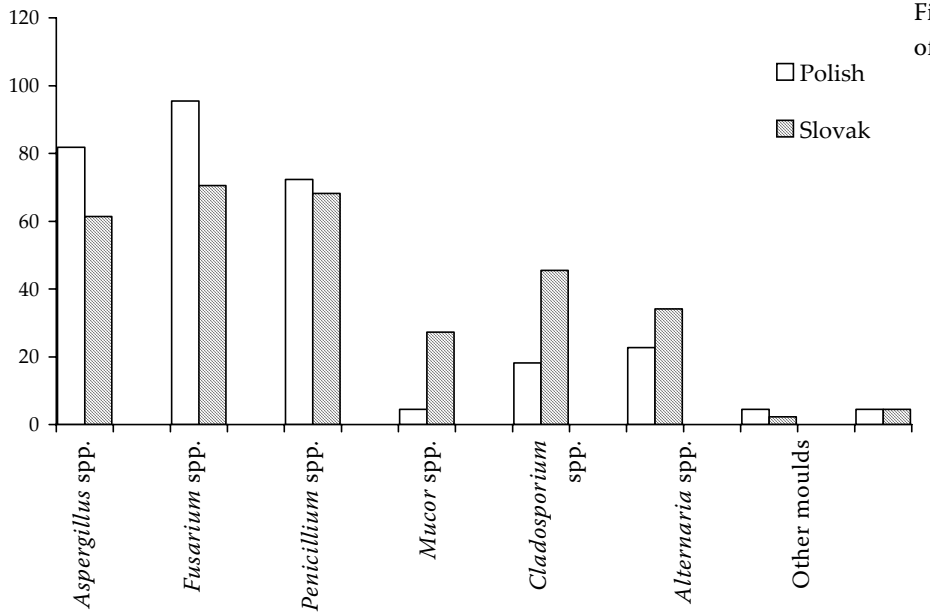

Figure 1. Fungal contamination of wheat

RESULTS

Fungal contamination of the investigated wheat samples from Poland and Slovakia is shown in Figure 1. The wheat samples from both countries were strongly contaminated by *Fusarium* spp. (95.5% and 70.5% of samples, respectively). As concerns other fungal contaminants, a high percentage of the Polish samples contained also *Aspergillus* spp. (81.8%) and *Penicilium* spp. (72.3%). Except *Fusarium* spp., also *Penicillium* spp. (from 68.2% of samples), *Aspergillus* spp. (61.4%), and *Cladosporium* spp. (45.5%) were isolated from the Slovak wheat. In comparison with the Polish samples, a higher contamination by *Alternaria* spp. (34.1% compared to 22.7%) and *Mucor* spp. (27.3% compared to 4.5%) was detected in the Slovak samples.

Fusarium spp. dominated also in the rye samples from both countries (Figure 2) with 86.9% of the

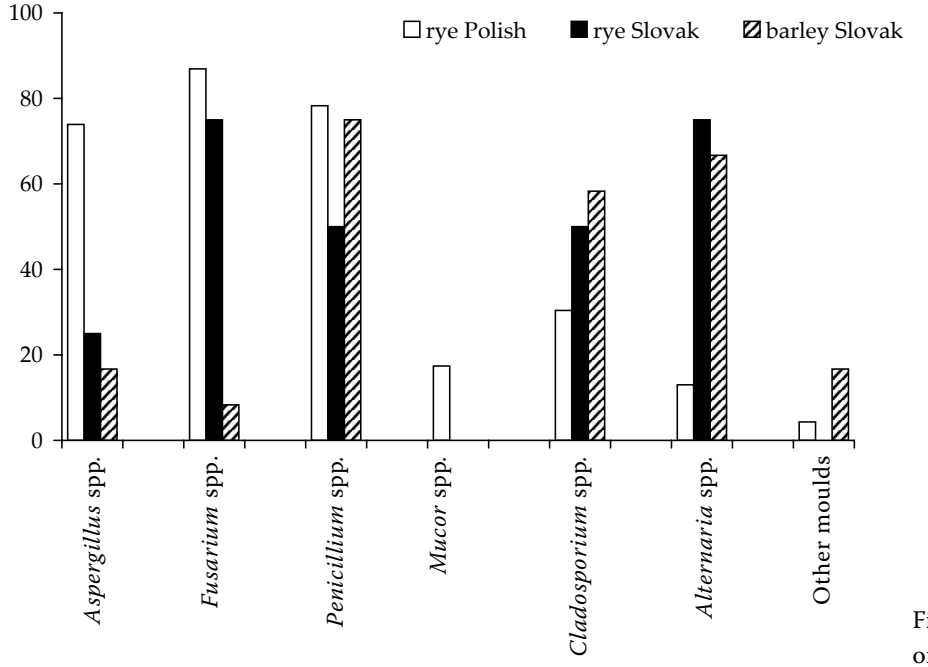

Figure 2. Fungal contamination of rye and barley

Table 1. Fungal contamination of Polish (P) and Slovak (S) cereal samples

Cereal sample kind		Total amount of investigated samples	$< 10^3$ cfu/g		$< 10^4$ cfu/g		$< 10^5$ cfu/g		$< 10^6$ cfu/g		Contamination log cfu/g			Median	Signification
			n	(%)	*n*	(%)	*n*	%	*n*	(%)	min.–max.	$\bar{x}$	SD		
Wheat	P	22	8	36.4	9	40.9	4	18.2	1	4.5	2.3–5.04	3.39	0.70	3.10	
	S	48	1	2.1	47	97. 9	–	–	–	–	2.8–3.80	3.27	0.72	3.43	ns; $P > 0.05$
Rye	P	23	9	39.1	12	52.2	2	8.7	–	–	2.5–4.60	3.09	0.87	3.11	
	S	4	–	–	4	100.0	–	–	–	–	3.3–3.73	3.50	0.19	3.49	ns; $P > 0.05$
Barley	S	8	–	–			–	–	–	–	3.11–3.69	3.41	0.20	3.43	

Table 2. Ochratoxin A (OTA) in Polish (P) and Slovak (S) cereal samples

Cereal sample kind		Total amount of investigated samples	Negative samples		Positive samples		Min.–max. (µg/kg)	Contamination		Median	Signification
			n	(%)	*n*	(%)		$\bar{x}$	SD		
Wheat	P	22	21	95.5	1	4.5	0–0.015	0.0007	0.003	0	
	S	48	42	87.5	6	12.5	0–2.940	0.0063	0.420	0	ns; $P > 0.05$
Rye	P	23	19	82.6	4	17.4	0–0.038	0.0055	0.012	0	
	S	4	4	100.0	–	–	–	–	–	–	ns; $P > 0.05$
Barley	S	8	8	100.0	–	–	–	–	–	–	

Table 3. Deoxynivalenol (DON) in Polish (P) and Slovak (S) cereal samples

Cereal sample kind		Total amount of investigated samples	Negative samples		DON < 100 µg/kg		DON ≥ 100 µg/kg		Min.–max. (µg/kg)	Contamination		Median	Signification
			n	(%)	*n*	(%)	*n*	(%)		$\bar{x}$	SD		
Wheat	P	22	15	68.2	1	4.5	6	27.3	0–970	195.9	336.4	0	
	S	48	35	72.9		–	13	27.1	0–2770	274.4	592.9	0	ns; $P > 0.05$
Rye	P	23	19	82.6	–	4.3	3	13.0	0–240	23.2	58.3	0	
	S	4	4		1	–	–	–	–	–	–	–	ns; $P > 0.05$
Barley	S	8	3	37.3	–	–	5	62.5	0–530	187.5	194.0	180.0	

Polish and 75% of the Slovak samples being contaminated. As to other moulds, *Penicillium* spp. (in 78.3% samples), *Aspergillus* spp. (in 73.9%), *Cladosporium* spp. (in 30.4%), *Mucor* spp. (in 17.4%), and *Alternaria* spp. (in 13%), were found in the Polish rye. The Slovak rye samples were contaminated with *Alternaria* spp. (75% samples), *Penicillium* spp. (50% samples), *Cladosporium* spp. (50% samples) and *Aspergillus* spp. (25% samples).

Barley samples were taken only in East Slovakia. The examination of their fungal contamination confirmed (Figure 2) the occurrence of *Penicillium* spp. (75% samples), *Alternaria* spp. (66.7% samples) and *Cladosporium* spp. (58.3% samples). Moreover, the occurrence of yeasts was also ascertained (in 25% samples).

The abundance of fungi in the cereal samples investigated is shown in Table 1. The occurrence of moulds in the Polish wheat was between 1.8×10^2 and 2.7×10^4 cfu/g and in the Polish rye between 2.7×10^3 and 1.1×10^5 cfu/g. In the wheat from Slovakia, the occurrence of moulds was in the range between 6×10^2 and 5.3×10^3 cfu/g. The Slovak rye contained 1.8×10^3–3.5×10^3 cfu/g and barley 1.3×10^3–4.5×10^3 cfu/g of moulds. As concerns the comparison of the total mould contamination, no significant difference between the Polish and the Slovak samples was detected.

The ochratoxin A (OTA) levels in the Polish and the Slovak cereal samples are shown in Table 2. Most of the cereal samples were OTA-negative. OTA was detected only in one sample (4.5%) of the Polish wheat with average levels of 0.0007 µg/kg (median 0), in 6 samples (12.5%) of the Slovak wheat with a mean of 0.063 µg/kg (median 0), and in 4 samples (17.4%) of the Polish rye with average levels of 0.0055 µg/kg (median 0). No sample of the Slovak rye or barley was OTA-positive. As shown in Table 2, OTA levels in all investigated samples were under the limit of 5 µg/kg. No difference between the Polish and the Slovak samples was ascertained.

Table 3 summarises the DON levels in the Polish and the Slovak cereal samples. Contamination ≥ 100 µg/kg was observed in 6 samples (27.3%) of the Polish wheat and in 13 samples (27.1%) of the Slovak wheat. Similarly, high levels of DON (≥ 100 µg/kg) were determined in 3 samples (13%) of the Polish rye and in 5 samples (62.5%) of the Slovak barley. The average mean of DON concentration in the Polish wheat was 195.9 µg/kg (median 0), in the Slovak wheat 274.4 µg/kg (median 0), in the Polish rye 23.2 µg/kg (median 0), and in the Slovak barley 187.5 µg/kg (median 180.0). However, no significant difference between the Polish and the Slovak samples was ascertained in this case either.

DISCUSSION

Different countries have different grain quality evaluation criteria. These can be related to the amount of spores or mycotoxins contamination. The mycological infestation of wheat can be considered as acceptable when fungi cfu is within the range 10^3–10^5 per gram (Schnürer & Jonnson 1992).

There are only few sources describing fungal and mycotoxin contamination of cereals in Slovakia. Šrobárová *et al.* (2002) reported the occurrence of *Fusarium* species and related mycotoxins in maize ear rot samples collected in the most important areas of maize growing in Slovakia. Maize ears were contaminated by fumonisins, beauvericin and fusaproliferin. The natural occurrence of fungi in feeding wheat after harvest and during storage in 7 agricultural farms, located in the South-west and middle Slovakia, was investigated by Tančinová *et al.* (2001). These authors detected the highest fungal population densities immediately after harvest (10^4–10^5 cfu/g wheat grain). For comparison, the occurrence of moulds in our cereal samples after harvest was lower (between 1.8×10^2 and 1.1×10^5 cfu/g in the Polish samples; between 6×10^2 and 5.3×10^3 cfu/g in the samples from East Slovakia). However, we did not examine the possible changes in the fungal populations during the cereals storage period.

Similar situation exists also in Poland; however, some reports describing fungi and their toxins were published recently (Logrieco *et al.* 1998; Krysinska-Traczyk *et al.* 2001; Perkowski *et al.* 2003). Significant correlations between the concentrations of the individual toxins (trichothecens and zearalenone) and the dominant *Fusarium* species were found.

Mycotoxins, which are given most attention in the food safety issues, all belong to the metabolites of the genera *Aspergillus, Penicillium* and *Fusarium* (Olsen 2001). The most important mycotoxins are aflatoxin B_1, ochratoxin A, fumonisin B_1, zearalenone, deoxynivalenol, and T2 toxin (Galvano *et al.* 2001). Especially

ochratoxin A, vomitoxin (deoxynivalenol) and zearalenone dominate in West and East Europe (Devegowda *et al*. 1998).

The objective of our study was to evaluate the fungal contamination of the selected cereal samples and to determine subsequently the possible contamination of these samples by mycotoxins.

The substrate contamination with microscopic filamentous fungi need not result in the presence of mycotoxins. The production of mycotoxins depends upon numerous external factors (relative humidity, temperature), as well as upon the properties of the substrate (composition, a_w, degree of contamination). Cereals represent an ideal medium for the growth and multiplication of moulds and also for the production of the secondary toxic metabolites (Laue *et al*. 1988).

The high incidence of *Fusarium* in the cereal samples investigated directed our attention to the detection of deoxynivalenol, especially because of the reported widespread occurrence of this mycotoxin as a natural contaminant in grain. High concentrations of deoxynivalenol were also found in North American and European wheats during recent years by Pettersson (2000). Deoxynivalenol (DON), called also vomitoxin, is produced mainly by *Fusarium graminearum, F. culmorum, F. sporotrichioides, F. poe, F. tricinctum* and *F. acuminatum* (Pittet 1998). It belongs to the type B trichothecenes and besides carbonyl function (typical for the type B) has an epoxy moiety, which was shown to play a role in its toxicity (He *et al*. 1992; Binder *et al*. 1997). Its toxic effects are mostly known from a number of serious outbreaks of mycotoxicoses in both humans and animals. Intoxication of animals with vomitoxin is manifested by a decrease in feed intake or its refusal, vomiting and digestive disorders with subsequent reduction of weight gain (Ellend 1998). The ingestion of DON caused outbreaks of acute human mycotoxicoses in India, China and rural Japan with symptoms including nausea, vomiting, abdominal pain, diarrhoea, dizziness and headache. These outbreaks were usually associated with the consumption of infected wheat and corn and the symptoms disappeared after the contaminated food source was removed (Beardall & Miller 1994). DON is not classified as carcinogenic substance for humans. However, the International Agency for the Research on Cancer (IARC) incorporated DON into three category (IARC 1993a). The FAO/WHO Expert Committee established a provisional maximum tolerable daily intake (TDI) of 1 μg/kg of b.w. for DON (WHO 2002). According to EMAN, the acceptable limit for DON is 750 ppb in cereals and their products. Also in many countries of EU, the level of 750 μg/kg has been set and applied as (unofficial) maximum limit for DON in flour used for the human consumption since several years ago (EFSA 2004). This limit was exceeded only by one wheat sample (4.5%) from Poland, but by seven wheat samples (14.6%) from Slovakia.

Since a high occurrence of genera *Aspergillus* and *Penicillium*, described as possible producers of OTA, was detected in cereals, we decided to investigate also the presence of ochratoxin A. It is known that especially *Penicillium verrucosum* participate in OTA production in the colder climatic regions, while in the regions with higher temperatures (in the warmer and tropical parts of the world) OTA is produced mainly by a number of *Aspergillus* species, e.g. by *A. alutaceus* (formerly known as *A. ochraceus*) (Pittet 1998). Recent reports indicate that it is also produced by some isolates of *A. niger, A. carbonarius* and *A. tereus* (CAC 1998). Czerwiecki *et al*. (2002a, b) investigated in 1997 and 1998 the levels of ochratoxin A and fungal flora in cereals from conventional and ecological farms. In 1997, no OTA presence in the wheat samples from conventional farms was detected. However, OTA was detected in the range of 0.2–57 μg/kg in the samples from ecological farms. As regards the mould contamination, *Penicillium cyclopium, P. viridicatum, P. chrysogenum* as well as *Aspergillus alliaceus, A. versicolor, A. glaucus,* and *A. flavus* were dominating. Rye was contaminated only in one case of ecological and in one case of conventional farms. In 1998, it was ascertained that the frequence of contamination of rye and barley in ecological or conventional farms was similar (in the range of 5–12%). The contamination frequence of wheat samples from conventional and ecological farms was 48% and 23%, respectively. *Penicillium cyclopium, P. viridicatum, Aspergillus ochraceus, A. glaucus* and *A. versicolor* were isolated from these samples.

OTA exhibits nephrotoxic (Stoev *et al*. 1998), teratogenic, hepatotoxic (Kuiper-Goodman & Scott 1989), genotoxic (Creppy *et al*. 1985) and immunotoxic (Lea *et al*. 1989) effects which were observed mainly in animals. OTA has been linked also with a human disease – Balkan endemic nephropathy (Castegnaro *et al*. 1987). Some studies suggest the occurrence of OTA in blood serum

also in people outside the Balkan region because of the consumption of food containing OTA (Solti *et al*. 1997; Malíř *et al*. 1998). The International Agency for Research on Cancer (IARC 1993b) classified OTA as a possible human carcinogen (group 2B). It was recommended by the FAO/WHO Expert Committee on Food Additives and Contaminants to establishe a maximum limit for OTA at the level of 5 μg/kg for cereals and cereal products (CAC 2001). All our investigated samples were below this limit.

Despite the fact that the moulds incidence and the levels of both investigated mycotoxins during harvest could be evaluated positively, the investigation of the mycotoxins occurrence is always actual in view of the safety of human and animal health.

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