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FUNGI ON WHEAT BRAN AND THEIR TOXINOGENITY

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Abstract: To survey the overall fungal contamination and toxicological potential of isolated fungi, a mycological investigation of 56 samples of wheat bran was carried out during March–June 2004 in Slovakia. Despite of low values of water activity (a_w) found in the samples reaching an average of 0.54, the individual samples were contaminated with fungi in a range from 1.82×10^1–3.42×10^4 colonies forming units per gram of sample (CFU/g). A total number of 65 fungal species pertaining to 23 genera were recovered. The most dominant and diverse genus was *Penicillium* (20 spp.) followed by *Aspergillus* (10 spp.) and *Cladosporium* (3 spp.) with 100%, 89% and 72% frequency, respectively. Isolates of potentially toxinogenic species recovered from the samples were found to produce various mycotoxins, namely citrinin (23 isolates), cyclopiazonic acid (43 isolates), griseofulvin (23 isolates), ochratoxin A (14 isolates), patulin (30 isolates), penitrem A (18 isolates) and sterigmatocystin (7 isolates). Furthermore, fumonisin B_1 as well as moniliformin producing *Fusarium* strains, were recovered. It is evident that this type of cereal commodity is a significant source of toxicologically relevant species of which majority are true toxinogenic.

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Key words: agar plug method, fungal contamination, *Aspergillus*, *Fusarium*, *Penicillium*, mycotoxin screening, wheat bran.

INTRODUCTION

Wheat bran, being rich in fibre and mineral content, has attracted attention as a promising component of various cereal products intended for human consumption. However, as wheat grain layers are separated during the milling process, surface-adhering contaminants, including fungi, are concentrated in the end product bran, wheat germ and pollard, which comprise the outer layers of the grain [5]. Moreover, it is also well known that small-grain cereals, including wheat, may constitute a toxicological risk due to the occurrence of various types of mycotoxins [9, 18, 21, 22, 34, 38] following fungal contamination either in the field or during storage under inappropriate conditions. In milling or other separation procedures, for example, ochratoxin A will be concentrated or reduced in the resulting components. By-products such as "cleanings" or bran may contain high concentrations [38, 39]. Unfortunately, there is a scarcity of information available dealing with the mycological assessment of quality of the cereal bran proper. The main goal of this pilot study, therefore, was to investigate some randomly selected samples of wheat bran collected and processed in the Slovak Republic in order to determine the overall fungal contamination with an emphasis on toxicologically relevant fungi.

MATERIAL AND METHODS

The samples. A total of 56 samples of wheat bran were collected during a period from March–June 2004 from the Ivanka pri Nitre mill (Slovakia). The mill applies technology by Bűchler (Germany), i.e. dry repeated grinding. The wheat originated from various regions of south-west Slovakia. The samples were collected immediately during the

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milling process after wheat conditioning, and were stored in paper bags at 4–5°C prior to analysis.

Moisture content of wheat bran samples. Samples (2 g) were oven dried with forced air circulation for 16 hours at 80°C. The samples were then re-weighed and the initial water content determined.

Water activity of wheat bran samples. The water activity of all samples was measured using a Novasina Humidat-RC analyzer (Novasina, Zurich, Switzerland).

Isolation of the fungi. The dilution plate technique as described by Samson *et al.* [36] was used for isolation of fungi from the samples. 20 g of sample was mixed with 180 ml of saline solution (0.85% sodium chloride) with 0.05% Tween 80 on a horizontal shaker for ca 30 min. One ml of the diluents (made up to 10^{-5}) was then inoculated on Dichloran Chloramphenicol Peptone agar (DCPA, [4]) in triplicate for *Fusarium* species, and *Aspergillus flavus*/*A. parasiticus* medium (AFPA, [35]) for potentially aflatoxinogenic species. Dichloran Rose Bengal Chloramphenicol agar (DRBC) was used for isolation of the other fungi and overall quantitative enumeration of fungal propagules referring to CFU (colony forming units) per gram of sample. After 5–7 days of incubation at 25°C in the dark, resulting colonies were counted and transferred to appropriate identification media. All formulae used here are those given in Samson *et al.* [35].

Identification of *Aspergillus* and *Eurotium* species. Conidial suspensions were inoculated at 3 equidistant points both on Czapek-yeast extract agar (CYA), Czapek-yeast with 20% Sucrose (CY20S) and malt extract agar (MEA), and incubated in the dark at 25°C, for 7–14 days. Species identification was carried out according to Klich and Pitt [20], Klich [19], Pitt and Hocking [32], and Samson *et al.* [35].

Identification of *Penicillium* species. The penicillia belonging to *Aspergilloides*, *Furcatum* and *Biverticillium* subgenera were inoculated at 3 equidistant points on Czapek-yeast extract agar (CYA) and on malt extract agar (MEA), and incubated in the dark at 25°C. Sub-cultivation on CYA at 37°C was also used. In addition, Creatine Sucrose agar (CREA) was used for isolates belonging to *P. glabrum*, *P. spinulosum* and *P. purpurascens*. The penicillia representing subgenus *Penicillium* were inoculated on CYA, MEA, CREA and Yeast Sucrose agar (YES), and incubated at 25°C in the dark. Identification to species was made after 7 days by Pitt [30, 31], Pitt and Hocking [32], Samson *et al.* [35], Frisvad and Samson [16].

Identification of *Fusarium* species. Potato Dextrose agar (PDA) was used for observation of colony characteristics. Synthetic agar (Synthetischer nährstoffarmer agar, SNA) was used for micro-morphological features. Cultures were incubated at 25°C in the dark (PDA) and UV-light 365 nm (SNA). Identification to species was made after 7 days by the criteria of Burgess *et al.* [9], Nelson *et al.* [28], Pitt and Hocking [33] and Samson *et al.* [35].

Identification of other fungi. CYA and MEA were used for identification to species of all other fungi. An additional medium, PDA was used for *Epicoccum nigrum* identification and Potato-carrot agar (PCA) for *Alternaria* species. Cultures were incubated at 25°C for 7 days. Identification to species was made by the criteria of Domsch *et al.* [12], Pitt and Hocking [33], Samson *et al.* [35] and Simmons [40, 41].

Mycotoxins screening by a modified agar plug method. Only isolates of potentially toxinogenic species were screened. The *Aspergillus* and *Penicillium* mycotoxins, i.e. aflatoxin B_1 and G_1 (AB_1, AG_1), citrinin (C), cyclopiazonic acid (CA), griseofulvin (G), ochratoxin A (OA), patulin (P), penitrem A (PA) and sterigmatocystin (S) were screened by the method adapted from Samson *et al.* [33]. For AB_1, AG_1, C, G, OA and P screening, 3 small pieces (each ca 3 × 3 mm) were cut from the colony growing on YES at 7 and/or 14 days and placed into small 4 ml screw cap vials. For CA, PA and S screening, the plugs were taken from colonies growing on CYA or CY20S. Then 0.5 ml of extraction solvent (chloroform:methanol 2:1 v/v) was added to the vial containing the agar plugs and shaken on a vortex at least for 1 min. Twenty microliter aliquots, along with the standards, were applied afterwards as spots to the TLC plate (Silicagel 60, Merck, Germany) 1 cm apart. Consequently the spots were dried, and the plates developed in a solvent system toluene:ethylacetate:formic acid (5:4:1 v/v/v) that gave an average R_f value of 0.3 for AB_1, 0.2 (AG_1), 0–0.68 (C), 0.53 (G), 0.45 (OA), 0.58-0.90 (CA), 0.48 (P), 0.7 (PA) and 0.6 (S). The *Fusarium* mycotoxins, i.e. fumonisin B_1 (FB_1) and moniliformin (M) were screened according to Mubatanhema *et al.* [27]. The procedure was essentially the same as above, except the YES plugs were extracted with 1 ml of acetonitrile:water (1:1 v/v) and gave an average R_f value of 0.64 for FB_1 and 0.68 for M using butanol:acetic acid:water (20:10:7 v/v/v) as the solvent system.

Mycotoxin visualisation. Under UV- light (365 nm) some of the mycotoxins were directly detectable as coloured spots, namely AB_1 (blue), AG_1 (green), C (yellow-green tailed), G (blue), OA (bluish-green) and S (reddish). CA was visualized by spraying with Ehrlich reagent [23], and after drying detected as a violet tailing-spot in daylight. P was visualized by spraying with 0.5% methylbenzothiazolone hydrochloride (MBTH, Merck, Germany) in methanol, heated at 130°C for 8 min, and then detectable as a yellow-orange spot. PA was visualized by spraying with 20% $AlCl_3$ in 60% ethanol, heated at 130°C for 8 min, and then viewed under daylight as a dark-green to black spot.

Table 1. Total fungal counts on DRBC, dry matter content and water activity values in Slovakian wheat bran samples investigated during March–June 2004.

No. of sample	CFU · g^{-1}	Dry matter %	a_w	No. of sample	CFU · g^{-1}	Dry matter %	a_w
1	2.3×10^1	88.47	0.60	31	6.6×10^1	87.92	0.55
2	2.9×10^2	86.92	0.59	32	1.2×10^2	87.39	0.56
3	3.4×10^4	88.71	0.58	33	4.2×10^1	87.61	0.57
4	2.4×10^1	88.33	0.61	34	7.4×10^1	86.93	0.56
5	4.7×10^1	88.63	0.46	35	4.8×10^2	86.74	0.54
6	2.7×10^1	87.39	0.47	36	6.6×10^1	86.24	0.56
7	1.6×10^2	86.65	0.47	37	1.1×10^3	88.16	0.56
8	2.9×10^2	86.76	0.46	38	1.8×10^3	88.50	0.57
9	3.3×10^2	86.61	0.45	39	3.0×10^2	86.47	0.54
10	7.5×10^2	84.40	0.47	40	9.1×10^1	87.79	0.56
11	2.1×10^2	89.14	0.47	41	4.8×10^1	86.43	0.59
12	3.8×10^2	89.14	0.47	42	2.5×10^2	87.54	0.56
13	3.8×10^1	86.69	0.47	43	7.8×10^1	88.25	0.55
14	1.5×10^2	88.16	0.50	44	8.7×10^3	88.47	0.54
15	2.5×10^3	88.05	0.51	45	2.3×10^2	86.03	0.57
16	1.4×10^2	87.85	0.49	46	1.3×10^1	86.73	0.56
17	1.1×10^2	88.07	0.50	47	5.8×10^2	87.42	0.59
18	1.4×10^2	86.58	0.45	48	1.3×10^2	86.87	0.59
19	5.2×10^1	87.87	0.45	49	1.1×10^2	86.39	0.59
20	1.8×10^1	88.30	0.51	50	3.2×10^2	86.40	0.57
21	2.0×10^1	89.06	0.52	51	3.4×10^2	86.35	0.59
22	5.0×10^1	89.56	0.51	52	4.4×10^2	88.28	0.59
23	2.2×10^2	88.51	0.51	53	5.4×10^2	87.75	0.57
24	1.8×10^1	87.96	0.51	54	5.4×10^2	87.75	0.56
25	5.7×10^1	88.42	0.49	55	8.6×10^2	86.98	0.59
26	5.5×10^2	87.92	0.56	56	1.2×10^3	86.19	0.59
27	5.2×10^2	87.16	0.63				
28	9.7×10^1	88.17	0.55	x*	1.08×10^3	87.5	0.54
29	1.3×10^2	86.51	0.59	Min.	1.82×10^1	84.4	0.45
30	1.2×10^2	86.64	0.64	Max.	3.42×10^4	89.6	0.64

* mean

FB_1 was visualized by spraying with 0.5% p-anisaldehyde in methanol:acetic acid:conc. sulfuric acid (17:2:1 v/v/v), heated at 120°C for 2–3 min., and giving a reddish-purple spot under daylight. M was visualized by spraying with the MBTH, heated at 120°C for 2–3 min., and appeared as very faint yellowish-orange spot under daylight and showing orange fluorescence under UV. In addition to the screening technique, isolates of *Aspergillus flavus* group were concurrently tested for their ability to produce aflatoxins using the method by Abarca *et al.* [1]. After 14 days incubation of *A. flavus* group strains in a liquid medium consisting of 20% sucrose and 2% yeast extract at 28°C in the dark, 20 µl aliquots of the broth were applied directly onto the TLC plate and developed as described above. Identities of the mycotoxins detected on TLC matching with appropriate standards used (i.e. Rf value and colour/fluorescence under daylight and/or UV light) were further no confirmed by other chromatographic methods.

Mycotoxin standards. Mycotoxin standards, with the exception of patulin (Calbiochem, USA), were obtained from Sigma (Germany).

RESULTS AND DISCUSSION

Water activity (a_w) in the bran samples analyzed was found to range from 0.45–0.64 a_w with an average of 0.54 a_w. According to Magan and Lacey [25], a content of free water less or equal to 0.70 a_w in a substrate causes inhibition of fungal growth. Since the a_w values in the samples were far below this limit, the wheat bran analyzed here may, logically, be considered as being a low risk substrate for fungal growth when further appropriately stored. However, each of the samples investigated showed fungal contamination ranging from 1.82×10^1–3.42×10^4 CFU/g of sample. In addition to this, a comparatively large number of isolated fungi of toxicologically relevant *Aspergillus*,

Penicillium or *Fusarium* species were encountered here. The maximal acceptable amount of fungal counts for wheat bran destined for food processing has been estimated by Berghofer *et al.* [5] as 1×10^4 CFU/sample. Our results demonstrate that only in a single case (sample No. 3) fungal counts exceeded this value by 3.42×10^4 CFU/sample. Total fungal counts, water activity and dry matter values are given in Table 1. Altogether, 6,562 isolates were recovered and assigned to 65 fungal species within 23 genera. In addition, 6 species were determined to genus level only. The incidence and a total number of fungal species isolated from the wheat bran samples are given in Figure 1 and Table 2. The most frequently occurring and diverse genus was *Penicillium*, found in all 56 samples (100%) and represented by 20 species. *Aspergillus* was the second most frequent genus found in 50 samples (89%) and represented by 10 species. The third most common fungi were members of the genus *Cladosporium* isolated from 72% of samples investigated. Within the genus, and even among all the fungi recorded, *C. cladosporioides* appeared to be the most prevalent species, being present in 84% of samples. In contrast, *Aureobasidium*, *Beauveria*, *Botrytis* and *Eupenicillium* were found in only 2% of samples of each being represented by a single species. In their study dealing with wheat bran, Berghofer *et al.* [5] reported the highest occurrence frequency of *Aspergillus* and *Penicillium* species, albeit in less percentage of positive samples then in the samples studied here. In general, these genera are usually regarded as storage fungi invading cereals stored under unsuitable conditions such as high humidity and temperature [14]. From a toxicological point of view, attention should be focused on the nephrotoxinogenic *Penicillium* species, such as *P. verrucosum*, *P. citrinum*, *P. aurantiogriseum* and *P. viridicatum* which are known as being very common on stored cereals in temperate regions of the world [14, 24, 26]. All of the above mentioned species were recovered in relatively high frequency. As for *P. verrucosum*, 7 out of 8 isolates screened here produced citrinin and ochratoxin A, simultaneously. A summary of mycotoxin producing fungi recovered from wheat bran samples during this study is given in Table 3. The ability to produce ochratoxin A was found in 5 *Aspergillus ochraceus* isolates and 2 *A. alliaceus* isolates tested. According to Lund and Frisvad [24], the presence of *Penicillium verrucosum* in more then 7% of cereal grains may indicate contamination of the samples by ochratoxin A. Hence, the findings of the ochratoxin A producers on wheat bran samples in the course of this study are notable. The same authors consider this species as the main producer of ochratoxin A in cereals of European origin. Within the cereal grains, the highest amounts of ochratoxin A were found in bran [38].

What should be considered worthy of attention is the comparatively high incidence of *P. crustosum* isolates. This species is a potential producer of the powerful neurotoxin penitrem A [15, 16, 35]. According to Pitt and Hocking [32], the presence of *P. crustosum* in foods or feeds should

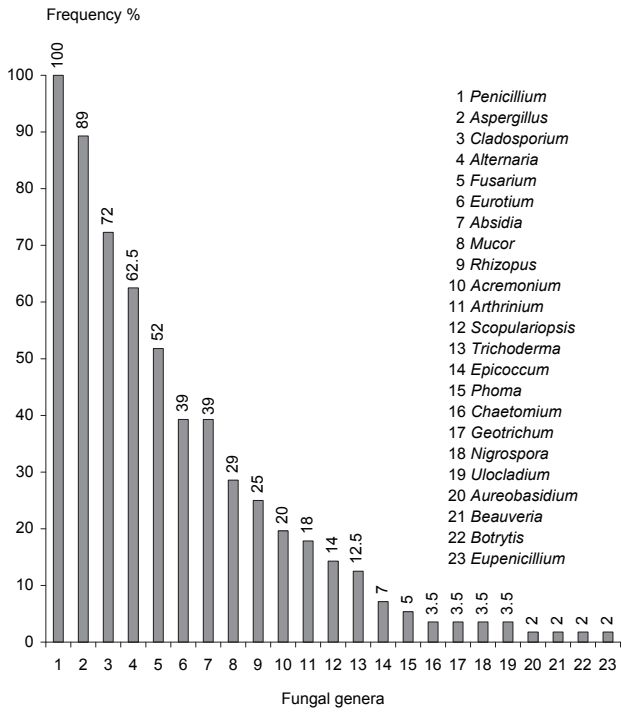

Figure 1. Occurrence frequency of fungal genera recovered from Slovakian wheat bran samples investigated during March–June 2004.

be regarded as a warning signal, since nearly all isolates of this species produce penitrem A at high levels. In fact, all of the *P. crustosum* isolates tested here produced penitrem A.

It is also interesting to note the findings of toxinogenic *P. griseofulvum* (all isolates tested produced griseofulvin, patulin and cyclopiazonic acid) and *P. expansum* isolates (patulin and citrinin producing strains). Andersen *et al.* [3] reported 98% patulin and 85% citrinin toxinogenity for *P. expansum* isolates, which is in agreement with the results obtained in this study. Concerning *Aspergillus* species in relation to patulin production, the capability to produce the toxin was observed in all of the *Aspergillus clavatus* isolates tested. Potential aflatoxin producers, i.e. *Aspergillus flavus* isolates, were recovered with a relatively high occurrence frequency of 62.5%, but none of the 108 *A. flavus* isolates screened appeared to produce either aflatoxin B_1 or G_1. Similarly, Piecková and Jesenská [29] tested 136 *A. flavus* isolates recovered from various maize products, including maize grains of Slovakian origin, for their ability to produce aflatoxin B_1, but all with negative results. However, each of the 18 *A. flavus* isolates screened showed a consistent ability to produce cyclopiazonic acid. In addition, the evidence of cyclopiazonic acid production may serve as a consistent tool in *A. flavus* species identification, e.g. from a morphologically close related *A. nomius* [18, 32]. Vaamonde *et al.* [46] recently reported 13% aflatoxinogenic *A. flavus* isolates recovered from wheat samples, with 93% of them producing cyclopiazonic acid. Along with the other cyclopiazonic acid producers, including *P. griseofulvum* (20 isolates) and/or *P. palitans* [5], *A. flavus*

Table 2. Occurrence and number of isolates of individual fungal species recovered from Slovakian wheat bran samples investigated during March–June 2004.

Fungi	Samples	Frequency (%)	Isolates *
Absidia corymbifera	16	29	41
Absidia glauca	1	2	1
Absidia sp.	6	11	124
Acremonium strictum	3	5	3
Acremonium sp.	8	14	14
Alternaria alternata group	29	52	68
Alternaria tenuissima group	2	3.5	2
Arthrinium phaeospermum	10	18	14
Aspergillus alliaceus	2	3.5	2
Aspergillus candidus	42	75	861
Aspergillus clavatus	4	7	8
Aspergillus flavus	35	62.5	108
Aspergillus fumigatus	9	16	241
Aspergillus niger	1	2	1
Aspergillus ochraceus	7	12.5	12
Aspergillus sydowii	1	8	5
Aspergillus terreus	3	5	4
Aspergillus versicolor	5	9	8
Aureobasidium pullulans	1	2	3
Beauveria bassiana	1	2	1
Botrytis cynerea	1	2	1
Chaetomium sp.	2	3.5	3
Cladosporium cladosporioides	47	84	789
Cladosporium herbarum	2	3.5	2
Cladosporium macrocarpum	1	2	2
Epicoccum nigrum	4	7	4
Eupenicillium cinnamopurpureum	1	2	1
Eurotium amstelodami	10	18	61
Eurotium chevalieri	2	3.5	3
Eurotium repens	10	18	41
Fusarium proliferatum	18	32	787
Fusarium subglutinans	14	25	299
Geotrichum candidum	2	3.5	2
Mucor hiemalis	5	9	9
Mucor plumbeus	1	2	1
Mucor racemosus	10	18	15
Mucor sp.	1	2	2
Nigrospora oryzae	2	3.5	2
Penicillium aurantiogriseum	43	77	2 057
Penicillium brevicompactum	3	5	5
Penicillium capsulatum	1	2	1
Penicillium chrysogenum	28	32	229
Penicillium citrinum	24	43	46
Penicillium crustosum	20	36	145
Penicillium expansum	13	23	63
Penicillium fellutanum	1	2	1
Penicillium griseofulvum	27	48	121
Penicillium janczewskii	1	2	1
Penicillium hordei	2	3.5	2
Penicillium janthinellum	2	3.5	2
Penicillium palitans	5	9	16
Penicillium pulvillorum	1	2	1
Penicillium raistrickii	10	18	34
Penicillium simplicisimum	1	2	1
Penicillium spinulosum	1	2	1
Penicillium steckii	1	2	1
Penicillium verrucosum	8	14	34
Penicillium viridicatum	15	27	290
Phoma herbarum	3	5	5
Rhizopus stolonifer	14	25	22
Scopulariopsis brevicaulis	7	12.5	20
Scopulariopsis candida	5	9	5
Trichoderma cf. *atroviride*	4	7	5
Trichoderma cf. *pseudokoningii*	3	5	7
Ulocladium sp.	2	3.5	2

* A total of 6,562 isolates were recovered.

is regarded as a serious source of this mycotoxin in cereals from temperate world regions. The mycotoxin has been detected in naturally contaminated feeds, corn, peanuts, and other foods and feeds [33]. Another very toxic and highly carcinogenic mycotoxin, sterigmatocystin [42], was detected in all of the *Aspergillus versicolor* and the 2 *A. sydowii* isolates isolated and screened. The sterigmatocystin production by commonly occurring taxa within the genus *Eurotium* has also been reported by Cole and Cox [11] and/or Smith and Ross [42]. However, no production of sterigmatocystin was confirmed in any of the *Eurotium* isolates tested here.

The occurrence of *Fusarium* species contamination of small-grain cereals, including wheat, resulting in severe *Fusarium* mycotoxin contamination during growth of cereals for Europe and their conditions has been reviewed by Bottalico [7] and Bottalico and Perrone [8]. The most frequently encountered species on wheat with head scab within European cereal-growing areas appears to be *F. graminearum*, *F. avenaceum*, *F. culmorum* and *F. poae*. Hence, deoxynivalenol and its derivatives, along with zearalenone and moniliformin, are among the mycotoxins most frequently encountered in small-grain cereals throughout European countries [6, 8, 45], including Slovakia [43].

Table 3. Mycotoxin production by fungi recovered from Slovakian wheat bran samples investigated during March–June 2004.

Fungi	AB_1	AG_1	C	CA	FB_1	G	M	OA	P	PA	S
A. alliaceus								2/2			
A. flavus	0[2]/108[1]	0/108		18/18							
A. clavatus									5/5		
A. niger								0/1			
A. ochraceus								5/5			
A. sydowii											2/2
A. terreus			0/1						0/1		
A. versicolor											5/5
F. proliferatum					12/12		0/12				
F. subglutinans					0/8		2/8				
P. citrinum			10/11								
P. crustosum										18/18	
P. expansum			6/6						5/6		
P. griseofulvum				20/20		20/20			20/20		
P. palitans				5/5							
P. raistrickii						3/4					
P. verrucosum			7/8					7/8			

A. – *Aspergillus*, F. – *Fusarium*, P – *Penicillium*, AB_1 aflatoxin B_1, AG_1 aflatoxin G_1, C citrinin, CA cyclopiazonic acid, FB_1 fumonisin B_1, G griseofulvin, M moniliformin, OA ochratoxin A, P patulin, PA penitrem A, S sterigmatocystin; [1] number of screened isolates; [2] number of positive isolates.

In the wheat bran samples tested in this study, however, only 2 *Fusarium* populations were encountered, namely, *F. proliferatum* and *F. subglutinans* with nearly equal occurrence frequency. All 12 *F. proliferatum* isolates tested showed the ability to produce fumonisin B_1. According to Frisvad and Thrane [17], *F. proliferatum* is the most important potential source of this dangerous mycotoxin, especially in maize. A 100% toxinogenity for this mycotoxin has been reported, for instance, by Castella *et al.* [10], Sanchis *et al.* [37], Šrobárová *et al.* [44] and Vesonder *et al.* [47]. Moniliformin production was observed in 2 out of 8 *F. subglutinans* isolates recovered here, which constitutes a lesser toxinogenity then that reported by Farber *et al.* [13] and Mubatanhema *et al.* [27]. No fumonisin production was detected in the *F. subglutinans* isolates recovered here, which is in accordance with the findings of Mubatanhema *et al.* [27] and Šrobárová *et al.* [44]. Without doubt, as claimed by e.g. Filtenborg *et al.* [14], the presence of potential toxinogenic species on food products does not always mean that these products contain mycotoxins. Concerning stored cereals, temperature, water activity and atmospheric composition, all play an essential role in fungal growth and mycotoxin production [48]. Yet, the outcomes of this study clearly confirm that wheat bran is a significant source of important toxinogenic fungi, and that a potential for mycotoxin production exists here. Hence, more detailed mycotoxin surveys dealing with the detection of the appropriate mycotoxins in wheat bran proper are needed to ensure the overall safety of this cereal waste commodity prior to its eventual exploitation in the human diet.

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