ECOLOGICAL CHEMISTRY AND ENGINEERING A

Vol. 17, No. 1 2010

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ENDOGENOUS CONTAMINATION OF WHEAT BY SPECIES OF GENERA *Aspergillus* AND *Penicillium*

ENDOGENNE ZANIECZYSZCZENIA PSZENICY PRZEZ GATUNKI RODZAJU *Aspergillus* I *Penicillium*

Abstract: The aim of this study was to analyse the endogenous mycobiota of superficially sterilised wheat grains with the focus on *Aspergillus* (including two teleomorphs) and *Penicillium* genera. The Slovak wheat samples (*Triticum aestivum* L.) were harvested in the season 2006. The total of 6 wheat samples grown under conditions of the conventional and 12 of the ecological farming system were investigated for the presence of microscopical fungi. A total of 17 genera were recovered as members of the endogenous mycobiota on Dichloran Rose Bengal Chloramphenicol agar (DRBC) and Dichloran Yeast Extract 18 % Glycerol agar (DYSG). On DRBC were detected *Aspergillus* and *Penicillium* species only from the ecological agriculture, namely *A. candidus*, *A. flavus, A. niger*, *Emericella nidulans*, *Eurotium amstelodami*, *E. chevalieri*, *Eurotium* sp., *Penicillium aurantiogriseum*, *P. chrysogenum*, *P. corylophilum, P. crustosum, P. griseofulvum, P. viridicatum* and *Penicillium* sp. On DYSG were detected *Eurotium* species (*E. amstelodami*, *E. chevalieri*, *E. repens*, *E. rubrum*) and *Penicillium* species (*P. griseofulvum*, *P. hordei*) both from ecological and conventional agriculture. From the ecological wheat was isolated a wider spectrum of fungi on DYSG in comparison with the conventional agriculture, namely *A. flavus*, *A. ochraceus*, *A. sydowii*, *Emericella nidulans, E. amstelodami, E. chevalieri, E. repens, E. rubrum, Eurotium* sp. *P. aurantiogriseum*, *P. crustosum*, *P. solitum* and *Penicillium* sp. The isolates of potentially toxigenic species of *Aspergillus*, *Emericella* and *Penicillium* were tested for their ability to produce particular toxic metabolites, ie mycotoxins *in vitro* by means of a thin layer chromatography (TLC). All the tested isolates were obtained from the samples of ecological agriculture. Out of 18 screened isolates 11 produced at least one mycotoxin and a production was vague in 2 isolates. One isolate (out of one) produced sterigmatocystin, 6 (out of 11) cyclopiazonic acid (production was vague in 2 isolates), and patulin 3 (out of 3). Conversely, none of potentially aflatoxinogenic isolates (*Aspergillus flavus*) tested in this study produced aflatoxins. Two isolates were tested for the production of ochratoxin A, *Aspergillus niger* did not produce ochratoxin A and in *A. ochraceus* production was unclear.

Keywords: fungi, *Aspergillus*, *Penicillium*, *Triticum aestivum* L., wheat

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Mould growth in grain normally occurs both in the field and in the storage. Mould growth can spoil the nutritional aspects of the grain and can also result in the production of secondary metabolites that are highly toxic to animals and humans. *Aspergillus* and *Penicillium* species belong to the group of the storage fungi [1]. The members of the genus *Aspergillus* are common contaminants of diverse substrates [2]. The most important possible consequence of their presence in foods and feeds is mycotoxin contamination [3]. Several potentially toxigenic species have been reported to be dominant on cereal grains at low water activities: *A. candidus*, *A. flavus*, *A. niger*, *A. versicolor*, *A. penicillioides* and *Eurotium* spp. (Lacey et al. 1991; Sauer et al. 1992, [4]). The most important mycotoxins, potentially produced by food-borne aspergilli, are aflatoxins, ochratoxin A, sterigmatocystin, cyclopiazonic acid and patulin [5, 6]. Out of genus *Penicillium*, dominant fungi in wheat kernels are: *P. aurantiogriseum*, *P. hordei*, *P. verrucosum*, *P. cyclopium*, *P. polonicum* [7]. According to Pitt and Leistner (1991, [1]) *Penicillium* species can produce 27 different mycotoxins, with three being the most important: ochratoxin, patulin, and citrinin. Ochratoxin A is a potent nephrotoxin, teratogen, and carcinogen. The main, if not the only producer of OTA in European cereals is *P. verrucosum* [8]. Patulin produces adverse neurological and gastrointestinal effects and is produced eg by *P. expansum*, *P. griseofulvum* (Damoglou, Campbell, 1986, [1]). Finaly, citrinin is a nephrotoxin and is produced mainly by *P. citrinum*, *P. expansum*, and *P. verrucosum* [9].

The aim of this study was to investigate endogenous microscopic filamentous fungal contamination of wheat grains grown under conditions of the so-called conventional and ecological agriculture in Slovakia in year 2006 with the focus on genera *Aspergillus* and *Penicillium*. The ability of isolates of potentially toxigenic species to produce the most important mycotoxins was determined by the means of thin layer chromatography.

Materials and methods

The total of 18 samples of wheat grains (*Triticum aestivum* L.) grown under conditions of the so-called conventional (6 samples) and the so-called ecological (12 samples) agriculture and harvested in year 2006 in Slovakia was mycologicaly investigated for the endogenous presence of *Aspergillus* and *Penicillium* species. For this purpose, direct plating method was used. Out of each sample, the amount of 200 morphologically indefectible grains was superficially sterilised and plated on DRBC (*Dichloran Rose Bengal Chloramphenicol* agar; Merck, Germany; 100 grains) and DYSG (*Dichloran Yeast Extract 18 % Glycerol* agar [8]; 100 grains). The superficial sterilisation was achieved by poring grains into 0.4 % solution of chloramine for 2 minutes; grains were consequently 3 times rinsed in sterile distilled water and dried on sterile filter paper. The members of genera *Aspergillus* and *Penicillium* including their perfects were consequently isolated on diagnostic media of CYA (*Czapek Yeast Extract* agar [10]), MEA (*Malt Extract* agar [10]), CY20S (*Czapek Yeast Extract* agar with 20 % Sucrose [10]) and CYA, MEA, CREA (*Creatine-Sucrose* agar [11]), YES (*Yeast Extract* agar [2]; 1000 cm^3 of distilled water), respectively. In all cases, cultivation proceeded for 5–7 days in the dark at 25 ± 1 ^oC. To determine particular species,

diagnostic literature was used as follows: Pitt [12], Klich [10], Samson et al [2] and Kubatova [13] for aspergilli and Ramirez [14], Pitt et Hocking [9], Samson et al [2], Samson et Frisvad [15] for penicillia. The ability of selected isolates of potentially toxigenic species to produce relevant mycotoxins *in vitro* conditions was screened by the means of thin layer chromatography (TLC) according to Samson et al [16] modified by Labuda et Tancinova [17]. The cultivation for screening of extracellular metabolites (griseofulvin, patulin, aflatoxin B_1, ochratoxin A) was carried out on YES and for intracellular (sterigmatocystin, cyclopiazonic acid, penitrem A, roquefortin C) on CYA; the conditions of cultivation as described above. In each tested isolate, 3 pieces of mycelium together with the cultivation medium of area of approximately 5 × 5 mm were cut from colonies and extracted in 1000 cm^3 of chloroform-methanol (2:1, v/v) on vortex for 2 minutes. 20 mm^3 of liquid phase of extracts along with standards (Sigma, Germany) were applied on TLC plate (Marchey-Nagel, Germany) and consequently developed in solvent system toluene:ethylacetate:formic acid (5:4:1, v/v/v). The visualisation of extrolites was carried out as follows: cyclopiazonic acid directly in daylight after spraying with the Ehrlich reagent (violet-tailed spot); patulin by spraying with 0.5 % methylbenzothiazolone hydrochloride (MBTH, Merck, Germany) in methanol, heated at 130 ^oC for 8 min and then detectable as a yellow-orange spot; penitrem A after spraying with 20 % $AlCl_3$ in 60 % ethanol, heated at 130 ^oC for 8 min and then detectable as a dark green to black spot on daylight; roquefortin C after spraying with $Ce(SO_4)_2 \cdot 4\ H_2O$ visible as orange spot. Directly under UV light (λ = 365 nm) were visualised following mycotoxins: sterigmatocystin (reddish spot), ochratoxin A (bluish-green), griseofulvin (blue).

Results and discussion

Table 1 shows the results from investigation of the endogenous contamination of wheat grains. From 6 samples of wheat grains grown in the so-called conventional agriculture, no member of genus *Aspergillus* and its perfects was isolated on DRBC and on DYSG were recovered 23 isolates of 4 species of genus *Eurotium*, what represented 3.83 % infestation from the total of 600 investigated kernels. *E. amstelodami* with total number of 17 isolates appeared to be the most encountered species. However, all the isolates were recovered from a single sample of wheat. From 12 samples obtained from the so-called ecological farming system were isolated 19 isolates of 8 species of 3 genera *Aspergillus*, *Eurotium* and *Emericella* on DRBC. That represented 0.42 %, 0.08 % and 1.1 % infested kernels, respectively out of 1185 investigated. On DYSG were detected 46 isolates of 9 species of the same three genera, what represented 0.58 %, 0.17 % and 3.08 % infested grains, respectively out of 1200 tested. In the case of samples from ecological agriculture, the most encountered species on DRBC was *Eurotium chevalieri* and *E. amstelodami* on DYSG. These species are xerophilic and they can be considered as members of typical mycobiota of wheat grains [2, 5, 7]. Genera *Eurotium* and *Emericella* are perfect microscopic filamentous fungi, which have their imperfects in the genus *Aspergillus* [2]. From the list of isolated, all the species except one (*A. sydowii*) are known to produce at least one toxic product in their

Table 1

The endogenous contamination of wheat grains by species of genera *Aspergillus* (and relevant teleomorphs) and *Penicillium* on DRBC and DYSG

Species and genera	DRBC[1]		DYSG[2]	
	Conventional farming system	Ecological farming system	Conventional farming system	Ecological farming system
	Number of isolates	Number of isolates	Number of isolates	Number of isolates
Aspergillus candidus		1		
Aspergillus flavus		2		5
Aspergillus niger		2		
Aspergillus ochraceus				**1**
Aspergilus sydowii				**1**
Aspergillus	**0**	**5**	**0**	**7**
Emericella nidulans		1		2
Emericella	**0**	**1**	0	**2**
Eurotium amstelodami		5	17[3]	20
Eurotium chevalieri		6	1	8
Eurotium repens		1	1	3
Eurotium rubrum			4	2
Eurotium sp.		1		4
Eurotium	**0**	**13**	**23**	**37**
Penicillium aurantiogriseum		12		11
Penicillium chrysogenum		2		
Penicillium corylophilum		2		
Penicillium crustosum		1		3
Penicillium griseofulvum		3	1	
Penicillium hordei			2	
Penicillium viridicatum		4		
Penicillium solitum				1
Penicillium sp.		5		5
Penicillium	**0**	**29**	**3**	**19**
Number of tested wheat grains	**600**	**1185**	**600**	**1200**

DRBC[1] – Dichloran Rose Bengal Chloramphenicol agar; DYSG[2] – Dichloran Yeast Extract 18 % Glycerol agar; [3] only from one sample of wheat.

secondary metabolism. All of the identified species except one (*Emericella nidulands*) are common on grains of cereals including wheat [2, 18]. According to several studies the species of the genus *Penicillium* belong to the dominant cereals microbiota [19–21]. *Penicillium* spp. isolates were not detected in any of the 6 wheat samples from

conventional agriculture on DRBC and on DYSG were found only two species: *P. griseofulvum* and *P. hordei*, what presented 0.5 % ratio from 600 kernels. They are terverticillate species from subgenus *Penicillium* and their primary occurrence is in cereals. According to Pitt et Hocking [9] many species in Section *Penicillium* appear to have their primary natural habit on cereal grains. Taxa in *Penicillium* subg. *Penicillium* are very important in foods and feedstuffs, too because of their widespread occurrence and their ability to produce several potent mycotoxins (Frisvad, 1986, [22]; Mantle, 1987, [22]). From 12 ecological wheat samples 7 *Penicillium* species, namely *Penicillium aurantiogriseum*, *P. chrysogenum*, *P. corylophilum*, *P. crustosum*, *P. griseofulvum*, *P. viridicatum* and *Penicillium* sp. were isolated on DRBC agar. The highest number of these isolates was diagnosed to the species *P. aurantiogriseum* (12), likewise on DYSG (11), where were recovered 4 *Penicillium* species: *P. aurantiogriseum, P. crustosum, P. solitum* and *Penicillium* sp. Out of 1185 tested wheat kernels *Penicillium* spp. isolates presented 2.4 % infestation found on DRBC and 1.6 % on DYSG agar.

Toxin-producing micromycetes are widespread in nature, and when occurring in grains they often reduce both the yield and the quality of grains [23]. Table 2 shows results from screening of selected isolates for *in vitro* production of mycotoxins by means of TLC. The most important mycotoxins in general are aflatoxins [5]. The main causal agent of their presence in cereals is *Aspergillus flavus* (Cotty et al 1994, Cotty 1997, [3]). Beside B aflatoxins, *A. flavus* is also a potential producer of another mycotoxin,

Table 2

In vitro production of mycotoxins by endogenous aspergilli and penicillia isolated from wheat grains tested by means of thin layer chromatography

Species	Number of tested isolates	Detected toxin	Evaluation		
			+	±	–
Aspergillus flavus	7	Af B_1			7
	7	CPA	2	2	3
Aspergillus niger	2	OA			2
Aspergillus ochraceus	1	OA		1	
Emericella nidulans	1	SC	1		
Penicillium crustosum	4	PA	4		
	4	ROC	4		
Penicillium griseofulvum	3	CPA	3		
	3	GRI	3		
	3	P	3		
	3	ROC	3		

Af B_1 – aflatoxin B_1; CPA – cyclopiazonic acid; GRI – griseofulvin; OA – ochratoxin A; P – patulin; PA – penitrem A; ROC – roquefortin C; SC – sterigamtocystin; + production of mycotoxin confirmed; ± production of mycotoxin is not clear; – no production of mycotoxin.

cyclopiazonic acid [2, 18, etc.]. In this study, all of the 7 isolates of this species were tested for the production of both of these mycotoxins by means of TLC. None of isolates showed potential to produce aflatoxin B_1. That is in an agreement with previous results of authors, who were dealing with commodities of Slovak origin, where none of the screened isolates of *A. flavus* was a producer of aflatoxins [21, 24–26]. All these results are in understanding with the authoritative publication of Frisvad et al [18], according to which aflatoxin producing isolates of *A. flavus* are typical for tropics and subtropics. The production of cyclopiazonic acid was found in 2 isolates, 3 isolates did not show the production of CPA and the production by 2 isolates was unclear. Production of another mycotoxin, which is important in cereals, nephrotoxic and carcinogenic ochratoxin A (JECFA, 2001, [18]), was tested in 2 isolates of *A. niger* with negative results and in single isolate of *A. ochraceus* with vague result. Single isolate of *Emericella nidulans* showed itself to be a producer of sterigmatocystin (Table 2). Terverticillate penicillia are very efficient mycotoxin producers [22], what was confirmed also with our results. Four isolates of *P. crustosum* produced penitrem A and neurotoxin roquefotrin C and 3 tested isolates of *P. griseofulvum* produced cyclopiazonic acid, griseofulvin, patulin and roquefortin C.

Conclusion

During the investigation of endogenous presence of members of genera *Aspergillus* and *Penicillium* in wheat grains of Slovak origin grown in the so-called conventional and ecological farming system in year 2006 in general higher infestation was found in samples from ecological agriculture. In samples from conventional agriculture following species were detected: *Eurotium amstelodami*, *E. chevalieri*, *E. repens*, *E. rubrum*, *Penicillium griseofulvum*, *P. hordei*. Higher diversity of both *Aspergillus* and *Penicillium* species was observed in ecological samples: *Aspergillus candidus*, *A. flavus*, *A. niger*, *A. ochraceus*, *A. sydowii*, *Emericella nidulans*, *Eurotium amstelodami*, *E. chevalieri*, *E. repens*, *E. rubrum*, *Eurotium* sp., *Penicillium aurantiogriseum*, *P. chrysogenum*, *P. corylophilum*, *P. crustosum*, *P. griseofulvum*, *P. viridicatum*, *P. solitum* and *Penicillium* sp. Out of 18 isolates screened for *in vitro* production of mycotoxins 11 produced at least one mycotoxin and production was vague in 2 isolates. One isolate (out of one) produced sterigmatocystin, 6 (out of 11) cyclopiazonic acid (production was vague in 2 isolates), and 3 patulin (out of 3). Conversely, none of potentially aflatoxinogenic isolates (*Aspergillus flavus*) tested in this study produced aflatoxins. Two isolates were tested for production of ochratoxin A, *Aspergillus niger* did not produce ochratoxin A and in *A. ochraceus* was production unclear.

Acknowledgement

This study was financially supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and the Slovak Academy of Sciences (VEGA no. 1/3456/06) and KEGA no. 3/2080/07.

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ENDOGENNE ZANIECZYSZCZENIA PSZENICY PRZEZ GATUNKI RODZAJU *Aspergillus* I *Penicillium*

Abstrakt: Celem badań było rozpoznanie endogennych grzybów ze szczególnym uwzględnieniem rodzajów *Aspergillus* i *Penicillium* na ziarnach pszenicy poddanych powierzchownej sterylizacji. Próbki pszenicy (*Triticum aestivum* L.) pochodziły ze zbiorów z 2006 r. ze Słowacji. Sześć próbek pszenicy pochodziło z upraw konwencjonalnej, a dwanaście próbek z upraw ekologicznych. Rozpoznano 17 rodzajów endogennych

grzybów wyhodowanych na DRBC (*Dichloran Rose Bengal Chloramphenicol* agar) i DYSG (*Dichloran Yeast Extract 18 % Glycerol* agar). Gatunki z *Aspergillus* i *Penicillium*, tj.: *A. candidus*, *A. flavus*, *A. niger*, *Emericella nidulans*, *Eurotium amstelodami*, *E. chevalieri*, *Eurotium* sp., *Penicillium aurantiogriseum*, *P. chrysogenum*, *P. corylophilum*, *P. crustosum*, *P. griseofulvum*, *P. viridicatum* i *Penicillium* sp. wykryte na DRBC pochodziły wyłącznie z upraw ekologicznych. Na DYSG stwierdzono gatunki z rodzaju *Eurotium* (*E. amstelodami*, *E. chevalieri*, *E. repens*, *E. rubrum*) oraz z rodzaju *Penicillium* (*P. griseofulvum*, *P. hordei*). Znajdowały się one na ziarnach z obu typów badanych upraw. Więcej gatunków grzybów wyizolowano DYSG z ziarna pochodzącego z upraw ekologicznych niż z ziarna pochodzącego z upraw konwencjonalnych. Były to: *A. flavus*, *A. ochraceus*, *A. sydowii*, *Emericella nidulans*, *E. amstelodami*, *E. chevalieri*, *E. repens*, *E. rubrum*, *Eurotium* sp. *P. aurantiogriseum*, *P. crustosum*, *P. solitum* i *Penicillium* sp. Wyizolowane gatunki z rodzajów *Aspergillus*, *Emericella* i *Penicillium* zbadano *in vitro* pod kątem produkcji toksycznych metabolitów, tj. mykotoksyn metodą chromatografii cienkowarstwowej (TLC). Wszystkie badane próbki pochodziły z upraw ekologicznych. Spośród 18 próbek wyizolowanych grzybów w 11 stwierdzono obecność przynajmniej jednej mykotoksyny. W 2 próbkach wyizolowanych grzybów obecność mykotoksyny była wątpliwa, w jednej stwierdzono obecność sterigmatocystyny, w 6 (spośród 11) odnotowano obecność kwasu cyklopiazonowego (obecność w dwóch próbkach była wątpliwa), a w 3 próbkach obecna była patulina. W żadnej z wyizolowanych próbek grzybów nie stwierdzono obecności aflatoksyn. Dwie próbki wyizolowanych grzybów zostały poddane testom na obecność ochratoksyny A. *Aspergillus niger* nie produkował ochratoksyny A, natomiast w próbkach *A. ochraceus* obecność ochratoksyny A była wątpliwa.

Słowa kluczowe: grzyby, *Aspergillus, Penicillium*, *Triticum aestivum* L., pszenica