

ROLE OF *Aspergilli* AND *Penicillia* IN MYCOTOXIN CONTAMINATION OF MAIZE IN HUNGARY

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Aspergillus and *Penicillium* species and their mycotoxins, including aflatoxins, ochratoxins, fumonisins and patulin, are frequently encountered on cereal products. The occurrence of these species and their mycotoxins on maize was investigated in Hungary after harvest in two consecutive years. Surface-sterilized cereal seeds were placed on selective media, and the isolated fungal strains were identified using morphological methods. In 2010 and 2011, 81.94% and 14.33%, respectively, of the samples were found to be contaminated with potentially toxigenic isolates. The species identification of selected isolates was carried out using sequence-based methods. Several *Aspergillus flavus* isolates were identified, which are potential aflatoxin producers. Other mycotoxin-producing species were also isolated, including black *Aspergilli*, which potentially produce ochratoxins and fumonisins, and *A. clavatus*, which produces patulin. In 2010 a large number of *Penicillium* species occurred in the samples, producing a wide range of mycotoxins. The mycotoxin content of the samples was analysed using the ELISA and HPLC techniques. Aflatoxins were not detected in any of the samples, while ochratoxins and fumonisins were successfully identified in some of the maize seeds.

Key words: maize, *Aspergillus flavus*, aflatoxins, fumonisins, ochratoxins, *Penicillium*, sequence-based identification, climate change

Introduction

Aspergillus and *Penicillium* are among the economically most important fungal genera. *Aspergillus* isolates are used for the production of soy sauce, several organic acids and enzymes, and biologically active metabolites such as lovastatin, while *Penicillia* are used in the pharmaceutical industry to produce penicillin, and in the food industry for the production of various cheeses and salami. Although not considered to be major causes of plant disease, both *Aspergillus* and *Penicillium* species may be responsible for several disorders in various plants (Varga et al., 2004). The most notorious plant pathogens are

Aspergillus niger, *A. flavus* and *Penicillium expansum*, which may cause various plant diseases. In contrast with specialized plant pathogens such as powdery mildews, rusts or most *Fusarium* species, these species are opportunistic pathogens without host specialization, as proved in *A. flavus* (Varga et al., 2004).

The most important aspect of food spoilage caused by these organisms is the formation of mycotoxins, which may have harmful effects on human and animal health. Several mycotoxins have been identified that may contaminate foods, including various agricultural products such as cereals, fruit and vegetables. Economically the most important of these are aflatoxins, ochratoxins and fumonisins. EU limits exist for all of these *Aspergillus*/*Penicillium* mycotoxins in cereals. In the case of aflatoxins, 4 µg/kg total aflatoxin content is allowed in all cereal products. For ochratoxins, 5 µg/kg is the limit for unprocessed cereals, and 3 µg/kg for cereal products intended for direct human consumption. In the case of fumonisins, 4000 µg/kg is the limit for unprocessed maize, and 200 µg/kg for foods intended for infants and children.

Although *Fusarium* toxins, including trichothecenes, zearalenone and fumonisins, are considered to be the most important in cereals in regions with a temperate climate, mycotoxins produced by *Aspergilli* and *Penicillia* are also frequently identified in cereal products. In Croatia aflatoxin-producing isolates were identified in maize and flour (Halt, 1994; Halt et al., 2004). Serious *A. flavus* field infection and aflatoxin contamination were detected in northern Italy in 2003 (Giorni et al., 2007). In Romania, although aflatoxins were not detected in cereal samples in 1998 (Curtui et al., 1998), about 30% of the cereal samples examined between 2002 and 2004 were found to be contaminated (Tabuc et al., 2009). In 20% of these samples, the aflatoxin levels exceeded the EU limits. More recently, Borbély et al. (2010) examined the mycotoxin levels in cereal samples and mixed feed samples collected in eastern Hungary, and detected AFB1 levels above the EU limit in some of the samples. In addition, Dobolyi et al. (2011) recently identified aflatoxin-producing *A. flavus* isolates from maize kernels collected in various parts of Hungary.

Ochratoxin A has also been frequently identified in cereal samples in several European countries, including Hungary, Croatia, Spain and Portugal. According to a recent survey of mycotoxins worldwide, aflatoxins were detected in 6% of cereal samples collected in Central Europe, while fumonisins and ochratoxins were found in 29% and 41% of the samples, respectively (<http://www.biomin.net/mycoreport/mtsurvey.html>). Ochratoxins are produced by *P. verrucosum* on cereals under colder climatic conditions (e.g. in northern parts of Europe; Lund and Frisvad, 2003), while *Aspergilli* dominate in the tropical and subtropical regions (Magnoli et al., 2007).

Maize products are frequently contaminated by fumonisins, which are principally produced by *Fusarium* species, although black *Aspergilli* also occur frequently on maize kernels. Black *Aspergilli* are suspected to be responsible for

the fumonisin contamination of several agricultural products, including grapes, onions and figs (Varga et al., 2010; 2012).

These observations led us to examine the occurrence of mycotoxin-producing *Aspergillus* and *Penicillium* species in Hungarian maize. It was hoped to discover: 1. the frequency with which aflatoxigenic *Aspergilli* occur in Hungarian maize; 2. the species responsible for the OTA contamination of maize in Hungary; and 3. whether black *Aspergilli* contribute to the overall fumonisin contamination of maize.

Materials and methods

Sample collection

Samples were collected in 9 and 10 maize growing regions in 2010 and 2011, respectively. The samples were surface sterilized using ethanol, and plated onto dichloran rose bengal (DRBC) medium (King et al., 1979). The plates were incubated at 25°C in darkness and monitored periodically for the growth of characteristic mycelia on the kernels. The outgrowing mycelia were purified and transferred to malt extract agar (MEA) medium. Isolates were subcultured as single conidia on MEA, Czapek-yeast extract agar (CYA) and potato dextrose agar (PDA) plates (Samson et al., 2004).

Identification of fungal isolates

The morphological identification of *Aspergilli*/*Penicillia* isolated from maize grains was done according to standard textbooks and monographs (Raper and Fennell, 1965; Samson et al., 2004; 2010). For sequence-based identification, the cultures used for the molecular studies were grown on malt peptone (MP) broth for 2 days, and DNA was extracted from the mycelia using the Masterpure™ yeast DNA purification kit (Epicentre Biotechnol.) according to the instructions of the manufacturer. Part of the calmodulin gene was amplified and sequenced as described previously (Pildain et al., 2008). The calmodulin sequences were compared using nucleotide-nucleotide BLAST (blastn) with default settings (<http://blast.ncbi.nlm.nih.gov>; Altschul et al., 1990) to the Genbank database, and to our own sequence database. Species identification was determined from the lowest expected value of the BLAST output.

Mycotoxin content analysis

The aflatoxin and fumonisin contents of the maize samples were detected with the HPLC-MS technique. The toxins were extracted with a mixture of methanol:distilled water (70:30) in a vertical shaker for 2.5 h. The membrane-filtered extracts (1 µl) were directly examined using high-performance liquid chromatography (HP 1090 HPLC) coupled with electrospray ionisation tandem mass spectrometry (Varian 500-MS) operating in positive ion mode. The components were separated with the use of a gradient of formic acid-methanol and distilled water on a reversed phase column (Polaris C18-A, 150 × 3 mm × 3 µm). The aflatoxins were differentiated with the use of characteristic fragment ions (AFB1: m/z 313.0>285.1, aflatoxin B2: m/z 315.0>287.1) produced by cleavage of the protonated molecule ion in MS/MS mode. In order to determine the quantity of toxins the external standard method was applied. The ochratoxin contents of selected samples from 2010 were determined with ELISA tests according to the instructions of the manufacturer (RIDASCREEN®, R-Biopharm AG, Darmstadt, Germany).

Results

In this study, the occurrence of potential mycotoxin producing *Aspergillus* and *Penicillium* was examined in maize in Hungary. Surface-sterilized cereal seeds were placed on selective media, and the isolated fungal strains were identified using morphological and sequence-based methods. The overall fungal contamination rate of the samples was 81.94% in 2010, while only 14.33% of the samples were found to be contaminated in 2011 (Table 1). The lower contamination rate observed in 2011 was possibly due to the weather conditions. While the summer was rainy in 2010, it was dry and hot in 2011. The humid weather conditions may also have been responsible for the fact that *Penicillia* were frequently isolated from the samples collected in 2010, while this genus was virtually absent from the samples collected in 2011. However, *Fusaria* were present in the samples in both years.

The number of primary isolates of each sample was reduced on the grounds of colony and microscopic features and only diverging ones were maintained for further investigations. A total of 340 and 90 isolates were recovered in 2010 and 2011, respectively. Among the samples examined, several isolates were found to be members of the section *Flavi* of the genus *Aspergillus* based on colony morphology and microscopic features (Fig. 1). Although several *Aspergillus* species capable of producing aflatoxins have been identified recently, *A. flavus*, *A. parasiticus* and *A. nomius* are economically the most important species in terms of the aflatoxin contamination of agricultural products (Varga et al., 2009). These species can readily be distinguished using sequence analysis of part of their β -tubulin or calmodulin genes (Varga et al., 2011). The species assignment of the isolates was carried out using partial sequence analysis of their calmodulin genes. All the isolates assigned to section *Flavi* based on morphological features were found to belong to the *A. flavus* species based on sequence data.

Besides *A. flavus*, several other potential mycotoxin producers were identified in the samples. The patulin producer *A. clavatus* and black *Aspergilli* able to produce both ochratoxins and fumonisins were recovered from several samples (Fig. 1). In the case of *Penicillia*, several mycotoxin producers were identified (e.g. *P. crustosum*, *P. brevicompactum*, *P. chrysogenum* and *P. viridicatum*). Other mycotoxin-producing genera were also identified, including *Fusarium* species. Among the *Fusaria*, *F. verticillioides* dominated, although other species (*F. graminearum*, *F. proliferatum*, *F. sporotrichioides*, *F. subglutinans*) could also be identified in some samples. Other genera (*Alternaria*, *Nigrospora*, *Epicoccum*, *Cladosporium*) were found in smaller proportions (data not shown).

Table 1
Occurrence of mycotoxigenic fungi on maize in Hungary in 2010–2011

Year	Origin	Infection of grains, %	% of isolated fungal strains		
			<i>Aspergillus</i> sp.	<i>Penicillium</i> sp.	<i>Fusarium</i> sp.
2010	Szeged	80.83	2.06	26.80	71.13
	Hódmezővásárhely	92.50	2.70	12.60	84.68
	Kétsoprony	68.33	1.22	15.85	82.92
	Karcag	80.00	5.21	10.42	84.37
	Kőszárhegy	75.83	1.09	21.99	75.84
	Pacsá	96.66	0	62.93	37.07
	Iregszemcse	87.50	4.76	1.90	93.33
	Majs	83.33	0	30.00	70.00
	Mosonmagyaróvár	72.50	1.15	65.52	33.33
	Average	81.94	2.02	27.56	70.30
2011	Szeged	15.00	0	0	83.33
	Hódmezővásárhely	1.66	0	0	0
	Jászbódogháza	6.66	0	0	25.00
	Cegléd	10.83	7.69	0	69.23
	Kőszárhegy	6.66	25.00	0	75.00
	Pacsá	10.00	0	0	100
	Iregszemcse	5.00	50.00	0	50.00
	Dalmand	25.83	0	0	29.30
	Kaposvár	25.00	0	0	93.33
	Bruck (Austria)	36.66	0	0	77.27
	Average	14.33	8.27	0.00	60.25

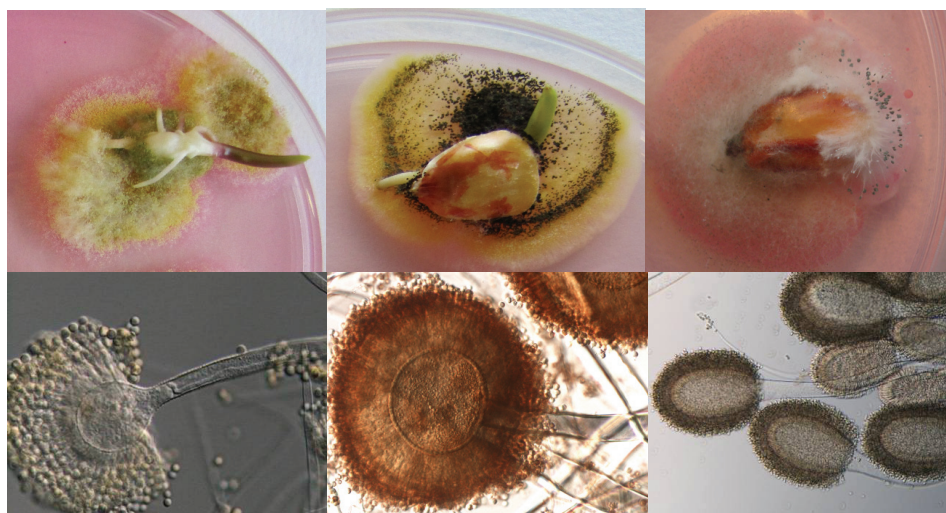


Fig. 1. Occurrence of *Aspergillus flavus*, *A. niger* and *A. clavatus* in maize seeds (top) and microscopic picture of their conidial heads (bottom)

The aflatoxin and fumonisin contents of the samples were analysed using HPLC-MS. None of the samples was found to be contaminated by any of the aflatoxins (B1, B2, G1, G2). Fumonisin (B1 + B2) was observed to be present in quantities exceeding the EU limit in samples collected in Pacsa (4.66 mg/kg), Kőszárhegy (10.15 mg/kg), Majs (5.13 mg/kg) and Szeged (7.55 mg/kg) in 2010. One sample collected in Bruck (Austria) was contaminated by 30.77 mg/kg fumonisins, which is more than seven times higher than the EU limit (4 mg/kg). The ochratoxin content of the samples collected in 2010 was analysed with the ELISA technique. Among the 35 samples analysed, the ochratoxin content of 34 was well above the EU limit (data not shown). The confirmation of these results using HPLC analysis is in progress.

Discussion

During a survey of mycotoxin-producing moulds in Hungarian maize samples in 2010 and 2011, 81.94% and 14.33% of the samples were found to be contaminated with potentially toxigenic isolates. Several *Aspergillus flavus* isolates were identified, which are potential aflatoxin producers. Other mycotoxin-producing species were also identified, including black *Aspergilli* that potentially produce ochratoxins and fumonisins, *Penicillium* species producing a range of mycotoxins, and *Fusarium* species. The data indicated that black *Aspergilli* and *Penicillia* could be responsible for ochratoxin contamination, while black *Aspergilli* had little role in the fumonisin contamination of maize in Hungary. The most contaminated samples were found in 2010, possibly due to the rainy, humid weather conditions. Samples collected in 2011 were found to be less severely infected, though the *Aspergillus* infection was higher than in the previous year. This situation represents a potential risk of the toxin contamination of Hungarian agricultural products, similarly to recent observations in neighbouring countries. The examination of the mycotoxin-producing abilities of the isolates is in progress. A thorough investigation of the mycobiota of other agricultural products would be justified to estimate the potential effects of climate change on the occurrence of mycotoxin-producing species in Hungary.

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