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IDENTIFICATION OF *ASPERGILLUS* SPECIES IN CENTRAL EUROPE ABLE TO PRODUCE G-TYPE AFLATOXINS

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The occurrence of potential aflatoxin producing fungi was examined in various agricultural products and indoor air in Central European countries including Hungary, Serbia and Croatia. For species identification, both morphological and sequence based methods were applied. *Aspergillus flavus* was detected in several samples including maize, cheese, nuts, spices and indoor air, and several isolates were able to produce aflatoxins. Besides, three other species of *Aspergillus* section *Flavi*, *A. nomius, A. pseudonomius* and *A. parasiticus* were also isolated from cheese, maize and indoor air, respectively. This is the first report on the occurrence of *A. nomius* and *A. pseudonomius* in Central Europe. All *A. nomius, A. pseudonomius* and *A. parasiticus* isolates were able to produce aflatoxins B_1, B_2, G_1 and G_2. The *A. nomius* isolate came from cheese produced very high amounts of aflatoxins (above 1 mg ml^{-1}). All *A. nomius*, *A. pseudonomius* and *A. parasiticus* isolates produced much higher amounts of aflatoxin G_1 then aflatoxin B_1. Further studies are in progress to examine the occurrence of producers of these highly carcinogenic mycotoxins in agricultural products and indoor air in Central Europe.

Keywords: Aflatoxins – *Aspergillus* – cheese – indoor air – sequence-based identification

INTRODUCTION

Aflatoxins are decaketide-derived secondary metabolites which are produced through a complex biosynthetic pathway. These compounds exhibit hepatocarcinogenic and hepatotoxic properties, and referred to as the most harmful naturally occurring carcinogens [3, 40]. From this reason, aflatoxins are among the economically most important mycotoxins produced by various *Aspergillus* species mainly belonging to *Aspergillus* section *Flavi* [40]. The most important producers of aflatoxins are *A. flavus, A. parasiticus* and *A. nomius* [40]. While *A. flavus* produces B-type aflatoxins, *A. nomius* and *A. parasiticus* are able to produce also G-type aflatoxins [40, 41]. *Aspergillus flavus* and *A. parasiticus* are identified worldwide from various substrates [40]. *Aspergillus nomius* is an aflatoxin producing filamentous fungus which was described only in 1987 [21]. Isolates of this species were originally isolated from

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insects and wheat in the USA, and *Cycas* sp. from Polinesia [21]. Later it was also identified in various agricultural soils in the USA, and the presence of the sexual cycle was also confirmed in this species [16]. Recently, a species closely related to *A. nomius* named *A. pseudonomius* has also been described [41], which was found to be an important source of aflatoxin contamination on Brazil nuts [23]. However, to our knowledge, these species have never been reported from Central Europe.

In this study, we examined the occurrence of aflatoxin-producing species in various food products and indoor air in Central Europe, including Hungary, Serbia and Croatia, using sequence-based and analytical methods.

MATERIALS AND METHODS

Chemicals

Methanol, acetonitrile, dichloromethan, ethyl-acetate and n-hexane for sample preparation and eluents were purchased from VWR (Hungary). The formic acid, trifluoroacetic acid and aflatoxins B_1, B_2, G_1, G_2 standards were obtained from Sigma-Aldrich (Budapest, Hungary). Deionized water both for sample preparation and high-performance liquid chromatography (HPLC) runs was produced by Millipore Milli-Q Gradient A10 water purification equipment (Millipore, Hungary).

Sample collection

The cheese samples (n = 15), surface sterilized maize samples (n = 80), onions (n = 16), nuts (n = 7) and spice samples (n = 16) were plated onto dichloran rose bengal chlortetracycline (DRBC) media [19]. Indoor air in Croatian apartments (n = 50) was sampled using a Mas-100 Eco microbiological air sampler (Merck, Darmstadt, Germany) with 400 holes (hole to agar impactor) and dichloran 18% glycerol agar (DG-18) plates [27]. In Hungary (n = 26), the plate sedimentation method was used [34]. Plates were incubated at 25 °C in darkness and monitored periodically for characteristic mycelium growing from the kernels. Outgrowing mycelia were purified and transferred to malt extract agar (MEA). The isolates were subcultured on malt extract agar (MEA) and Czapek yeast autolysate (CYA) media for morphological identification [34]. Assignment to *Aspergillus* section *Flavi* was confirmed after subculturing on CYA, MEA and Potato Dextrose agar (PDA), according to standard procedures [9, 28, 31].

Genotypic studies

Isolation of genomic DNA from mycelia grown in liquid YPD medium (1% Bacto yeast extract, 1% Bacto peptone, 1% D-glucose) for five days were performed by the

Masterpure™ yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. A fragment of the calmodulin gene was amplified with primers cmd5 and cmd6 as described by Hong et al. [15]. DNA sequences were determined at Agowa GmbH (Berlin, Germany). Sequence analysis was performed by nucleotide-nucleotide BLAST similarity search at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) [1], and sequences were also compared with our own sequence database. Species identification was determined from the lowest expect value of the BLAST output. Partial calmodulin sequences were deposited in the GenBank sequence database under accession numbers KP310011-KP310014.

Examination of aflatoxin producing abilities

During the examination of aflatoxin production the *A. parasiticus* isolates were cultivated on Czapek yeast autolysate (CYA) agar in darkness at 25 °C for 15 days. Aflatoxin extraction was performed from 4 agar plugs (in diameter of 6 mm) with 1 ml of mixture of methanol/dichloromethane/ethyl-acetate (1/2/3, v/v/v) supplemented with 1% of formic acid [37]. The extracts were centrifuged at 10,000 *g* for 10 min and the organic phases were removed and evaporated to dryness under slight stream of nitrogen. For the measurement of aflatoxins, the *A. nomius* and *A. pseudonomius* isolates were cultivated in darkness for 7 days on 2 ml of yeast extract – sucrose (YES) media at 25 °C [34, 38]. Aflatoxin extraction was performed with 2 ml of dichloromethane, the extracts were centrifuged at 10,000 *g* for 10 min and the organic phases were dried.

After the evaporation the samples were derivatized with trifluoroacetic acid as described previously [12]. For the analysis the modular HPLC system (Shimadzu, Japan) equipped with a DGU-14A vacuum degasser, two LC-20AD pumps, SIL-20A autosampler, CBM-20A column thermostat RF-20A fluorescence detector and CBM-20A system controller, which was controlled by ClassVP 6.2 software. Separations were achieved on a Lichocart 250 × 4 mm, 5 μm (Merck, Hungary) column with coupled with a Lichospher 100 RP-18 (Merck, Hungary) guard column and the injection volume was 5 μl. The composition of the isocratic mobile phase were water/methanol/acetonitrile 65/17.5/17.5 (V/V/V), and the flow rate was maintained at 1 ml/min, while column temperature was 40 °C. The fluorescence detector was set to an excitation wavelength of 365 nm and an emission wavelength of 430 nm. For the quantification of aflatoxins, linear calibration curves were used in the concentration range of 50–1000 ng/ml in the case of aflatoxins B_1 and G_1 and 14–722 ng/ml for aflatoxins B_2 and G_2, and the equations of resulted calibration curves were $y = 0.0049x + 14.8$, $y = 0.0012x + 15.6$, $y = 0.0034x + 4.2$ and $y = 0.0008x + 4.5$ for aflatoxins G_1, B_1, G_2 and B_2, respectively.

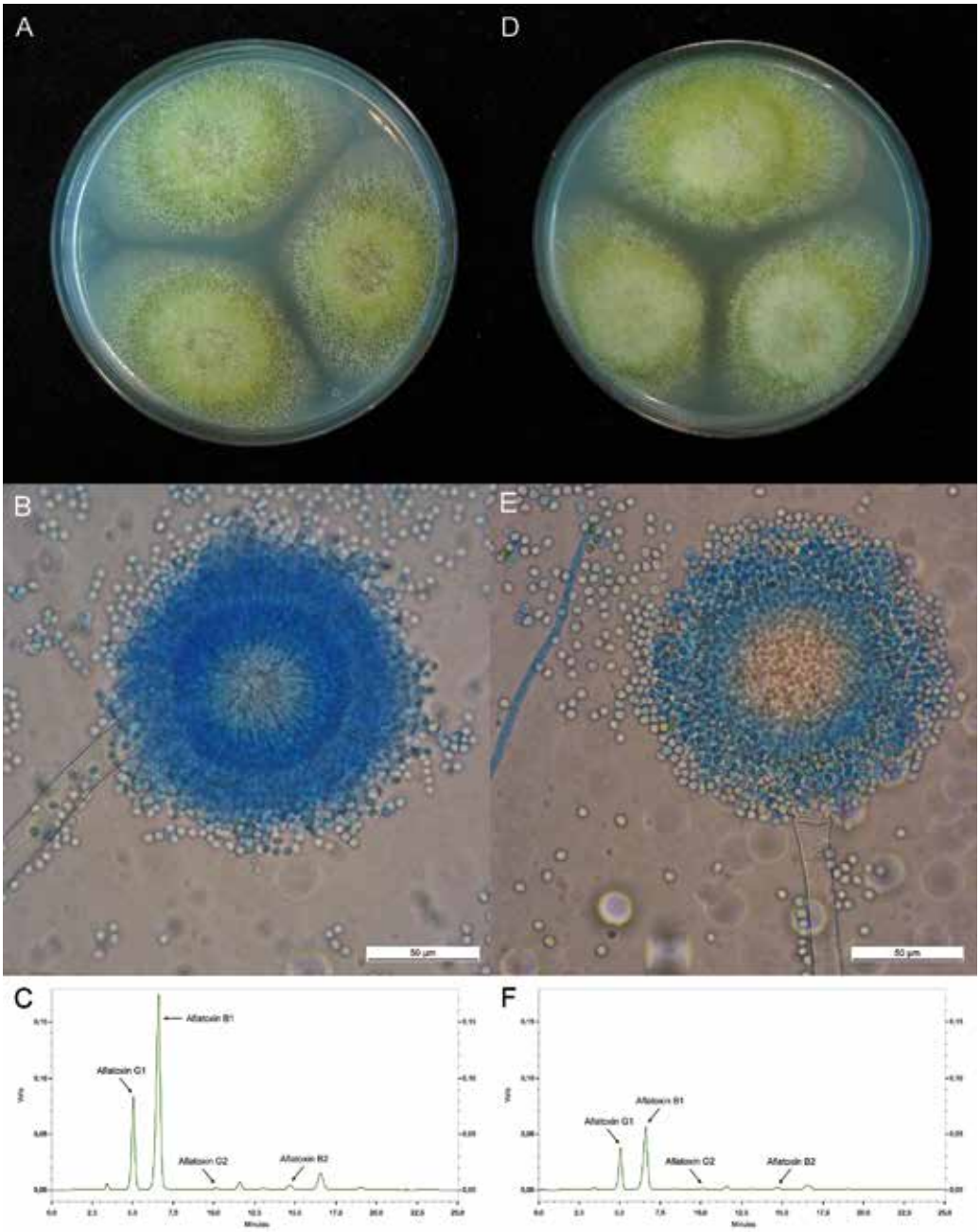

Fig. 1. Colony morphologies on PDA plates (A, D), light-microscopic pictures (B, E), and HPLC chromatograms of aflatoxins (C, F) of *A. nomius* SZMC 22631 and *A. pseudonomius* SZMC 22273, respectively

RESULTS AND DISCUSSION

Large numbers of samples were analyzed from maize, dried fruits, cheese, nuts, wheat and spices (Hungary), from maize (Serbia), and from indoor air (Croatia, Hungary) [42]. Species identifications were carried out using sequence analysis of part of the calmodulin gene. This gene is suitable for sequence-based identification of isolates belonging to *Aspergillus* section *Flavi* [41]. Potential aflatoxin producing species were not identified on dried fruits including raisins, figs and dates (data not shown). However, aflatoxigenic species have been found in the remaining samples (Table 1). *Aspergillus flavus* was identified in many of the samples (Table 1), while *A. nomius* was detected in only one sample of cheese from Hungary (Fig. 1). Both the morphological features and calmodulin sequence data indicated that this isolate belongs to *A. nomius* (Fig. 1). To our knowledge, this is the first report on the occurrence of *A. nomius* in Central Europe. This species was originally isolated from mouldy wheat in the USA, and later from various substrates in India, Japan and Thailand. Recently, Olsen et al. [26] and several other authors have observed that *A. nomius* is an important producer of aflatoxins in Brazil nuts [2, 5, 14, 33], on cocoa [7], almond [17], Indonesian nutmegs [25], maize, rice, black beans and cassava from Thailand [29, 30], and from various soil samples in Thailand, Iran and Japan [11, 20, 32]. It was also identified in house dust samples from Mexico [43]. This species has also been found to be able to cause human infections including onychomycosis in Italy [44], keratitis in India [22], invasive aspergillosis, cavitary and fibrosing pulmonary and pleural aspergillosis in Hong Kong [39] and breakthrough pneumonia in Italy [4]. Besides, it was also identified as the causative agent of stonebrood disease in honeybees in Great Britain [13].

Another species identified in the samples was *A. pseudonomius,* which was described recently in insects and soil from the USA [41], and was found to be partly responsible for aflatoxin contamination of Brazil nuts in Brazil [23]. Recently this species was also identified from house dust samples in Thailand and Micronesia [43].

Table 1
Occurrence of *Aspergillus* isolates from various substrates

Source	Number of *Aspergillus* isolates	Isolates of *Aspergillus* section *Flavi*	*A. flavus*	*A. nomius*	*A. pseudonomius*	*A. parasiticus*
Maize	258	111	110	0	1	0
Cheese	5	1	0	1	0	0
Nuts	18	12	12	0	0	0
Onion	105	0	0	0	0	0
Spices	28	5	5	0	0	0
Indoor air	171 (Croatia), 42 (Hungary)	67 (Croatia), 6 (Hungary)	65 (Croatia), 6 (Hungary)	0	0	2 (Croatia), 0 (Hungary)

Table 2
Aflatoxin producing abilities of the identified *A. parasiticus, A. nomius* and *A. pseudonomius* isolates

	Aflatoxin B_1 (μg ml^{-1})	Aflatoxin B_2 (μg ml^{-1})	Aflatoxin G_1 (μg ml^{-1})	Aflatoxin G_2 (μg ml^{-1})	Total aflatoxins (μg ml^{-1})
A. parasiticus MFBF10887 (SZMC 22727)[a]	18.16	0.28	194.61	3.71	216.76
A. parasiticus MFBF 11062B (SZMC 22728)	9.95	0.17	129.42	2.01	141.54
A. nomius III/4/2 (SZMC 22631)	370.88	9.89	814.44	19.63	1214.84
A. pseudonomius NS 640 B/22 (SZMC 22273)	283.95	5.89	446.93	7.76	744.53

[a]MFBF: Microbial Collection, Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia; SZMC: Szeged Microbiological Collection, Szeged, Hungary

In this study, *A. pseudonomius* was found in maize samples collected in Serbia. This is the first report on the occurrence of this species in Europe.

Besides, another species able to produce both B and G-type aflatoxins, *A. parasiticus* was also detected in indoor samples from Croatia, which species was also detected previously in similar habitats [8, 27] (Table 1). However, the isolates were previously identified using only morphological methods. More recently, *A. parasiticus* was also detected in wheat in Slovakia [10], and in maize in Hungary [36].

Regarding the mycotoxin producing abilities of the isolates, the *A. nomius, A. pseudonomius* and *A. parasiticus* isolates were able to produce aflatoxins B_1, B_2, G_1 and G_2 in different amounts (Table 1, Fig. 1). The amounts observed were extremely high in the case of the *A. nomius* isolate came from cheese (Table 1, Fig. 1). Usually aflatoxin B_1 concentrations exceed that of aflatoxin G_1 in these species [18]. However, in our studies, all the *A. parasiticus, A. nomius* and *A. pseudonomius* isolates produced higher amounts of aflatoxin G_1 than aflatoxin B_1 at 25 °C (Table 2). These data are in agreement with some previous studies [6, 24, 35]. These studies indicated that the ratio of aflatoxins B and G is greatly influenced by temperature. Further studies are needed to clarify the significance of these findings.

CONCLUSIONS

Large numbers of agricultural products and indoor air samples were analyzed in this study to clarify the species distribution of potential aflatoxin producing fungi in our region. Besides *A. flavus*, *A. parasiticus* was also identified in indoor samples from

Croatia, *A. nomius* from cheese from Hungary, and *A. pseudonomius* from maize from Serbia. All four isolates were able to produce aflatoxins B_1, B_2, G_1 and G_2. *Aspergillus pseudonomius, A. nomius* and *A. parasiticus* isolates produced aflatoxin G_1 in larger quantities than aflatoxin B_1 under the conditions used, while the *A. nomius* isolate produced these mycotoxins in very high amounts. Further studies are in progress to examine the occurrence of producers of these highly carcinogenic mycotoxins in agricultural products and indoor air in Central Europe.

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