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AFLATOXIGENIC *ASPERGILLUS FLAVUS* AND *ASPERGILLUS PARASITICUS* STRAINS IN HUNGARIAN MAIZE FIELDS

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Due to the climate change, aflatoxigenic *Aspergillus* species and strains have appeared in several European countries, contaminating different agricultural commodities with aflatoxin. Our aim was to screen the presence of aflatoxigenic fungi in maize fields throughout the seven geographic regions of Hungary. Fungi belonging to *Aspergillus* section *Flavi* were isolated in the ratio of 26.9% and 42.3% from soil and maize samples in 2013, and these ratios decreased to 16.1% and 34.7% in 2014. Based on morphological characteristics and the sequence analysis of the partial calmodulin gene, all isolates proved to be *Aspergillus flavus*, except four strains, which were identified as *Aspergillus parasiticus*. About half of the *A. flavus* strains and all the *A. parasiticus* strains were able to synthesize aflatoxins. Aflatoxigenic *Aspergillus* strains were isolated from all the seven regions of Hungary. *A. parasiticus* strains were found in the soil of the regions Southern Great Plain and Southern Transdanubia and in a maize sample of the region Western Transdanubia. In spite of the fact that aflatoxins have rarely been detected in feeds and foods in Hungary, aflatoxigenic *A. flavus* and *A. parasiticus* strains are present in the maize culture throughout Hungary posing a potential threat to food safety.

Keywords: *Aspergillus flavus*, *Aspergillus parasiticus*, SOS chromotest, aflatoxin biosynthesis genes

Introduction

The genus *Aspergillus* is a quite diverse group including 339 species belonging to 19 sections based on partial calmodulin gene sequences [1]. *Aspergillus* section *Flavi* contains species (e.g., *Aspergillus oryzae*, *Aspergillus*

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sojae, and *Aspergillus tamarii*) used in the food industry as well as mycotoxin-producing species (*Aspergillus flavus* and *Aspergillus parasiticus*) [2–5]. Mycotoxins are secondary metabolites produced by filamentous fungi having acute or chronic toxic effect on humans and animals. One of the most important groups of mycotoxins are aflatoxins, which were discovered in the United Kingdom in the 1960s when they were identified as the causative agent of "turkey X" disease. Investigation cleared that the feed consumed by turkeys contained *A. flavus* contaminated peanut with a South American origin [6]. The toxin was named after its producer (***A**spergillus **fla**vus* **toxin**), and based on the color of the intense fluorescence in ultraviolet light aflatoxin B (AFB) (**B**lue) and aflatoxin G (AFG) (**G**reenish yellow) can be distinguished [7]. AFB_1 and AFB_2 are mainly produced by *A. flavus* and *A. parasiticus*, but the latter produces AFG_1 and AFG_2 as well [8]. Among these toxins, AFB_1 is the most toxic, causing carcinogenic, teratogenic, and immunosuppressive effects, and it was classified in Group 1 by the International Agency for Research on Cancer [9].

Generally, *A. flavus* and *A. parasiticus* are unspecialized saprophytes; these fungi can degrade biopolymers as cellulose, pectin, lignin, and lipids in soil with their catabolic enzymes [10–13]. They have been isolated from the soil of different biomes (forest, grassland, wetland, and desert) [14]; however, it was found that cultivated soils contain higher proportion of *A. flavus* than natural habitats [15]. Previously, it was thought that only tropical and subtropical regions are favorable for the aflatoxin-producing fungi [5], but due to the climate change these fungi found their niche also in Europe. Since the first Italian aflatoxin outbreak (2003), aflatoxin contamination has not confined only to imported commodities and aflatoxin and/or the producing fungi have been detected in the agricultural commodities of several European countries [16–21].

In Hungary, Richard et al. examined first the mycotoxin-producing ability of the *A. flavus* isolates obtained from various sources (e.g., corn, soya, and wheat) [22]. They found that none of the 32 isolates was able to produce AFB_1. Borbély et al. examined 83 cereal samples from 2005 to 2008 for aflatoxin content and three samples proved to be over the allowable level [23]. Dobolyi et al. collected 104 maize samples from different maize growing regions in Hungary between 2009 and 2010. *A. flavus* was isolated from 63.5% of the samples and 18.8% of these isolates were found to be able to produce aflatoxins above 5 μg/kg on maize kernels. It should be noticed that 43 of the tested 69 strains also produced AFB_1 under the level of 5 μg/kg official EU limit [24]. Tóth et al. investigated the occurrence of *Aspergillus* species and their mycotoxins on maize in Hungary between 2010 and 2011. Several potentially aflatoxigenic

A. flavus strains were isolated but none of the maize samples contained aflatoxins [25].

Our aim was to study the occurrence of aflatoxigenic *Aspergillus* section *Flavi* in maize fields throughout Hungary (a) with the screening for the presence of the fungi in soil, as a reservoir for the infection of crops, and maize kernels and (b) with the analysis of the aflatoxin-producing ability of the isolated strains.

Experimental Analysis

Sampling soils and maize kernels

Both soil and ear samples were collected from 196 maize fields of Hungary in the period of July 2013 to October 2014. Number of the samples per region is presented in Table I. Ten ears were collected from each maize field and approximately 100 g soil samples (consisted of five subsamples) were taken from the upper 1 cm layer of the soil surface with a sterile trowel into clean zip-lock bags. Samples were stored at 4 °C until the analysis.

Fungal isolates identification

Ears were shredded and the surface of 20 kernels per sample was disinfected, then aseptically cut into halves and placed on culture media with its cut surface (five pieces per Petri dish). Soil subsamples were mixed in the bag, and then 10 g of each soil sample were suspended in 90 ml of sterile distilled water. Soil suspensions were bath sonicated at 37 kHz for 5 min to disperse soil aggregates and vortexed for 5 s before aliquots of 100 µl was spread (in triplicate) on Petri dishes with pentachloronitrobenzene-rose bengal-glycerol agar [26]. This media was originally introduced for culturing *Fusaria* from environment; however, it was also found to be suitable for obtaining isolates of *Aspergillus* section *Flavi* [24]. The inoculated plates were incubated at 37 °C for 5 days. Typical green colonies were transferred to malt extract agar and incubated at 26 °C for further morphological characterization [1]. Beside the classical identification, the partial calmodulin gene sequence was analyzed as follows. DNA was extracted using the Masterpure™ yeast DNA purification kit (Epicentre Biotechnologies, USA) according to the instructions of the

manufacturer. Partial calmodulin gene was amplified using primers cmd5 and cmd6 [27]. The amplified DNA fragments were purified by NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany). The purified polymerase chain reaction (PCR) products were used in sequencing reactions using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing was performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA). Sequences were compared to the NCBI GenBank Sequence Database (http://www.ncbi.nlm.nih.gov). The representative strains of *Aspergillus* section *Flavi* were maintained in 20% (w/w) glycerol solution at −80 °C in our culture collection for further examinations.

Detection of aflatoxin-producing ability of isolated strains

AFB_1-producing ability of the strains was studied with the presence of two structure genes (*nor-1* and *omt-1*) and a regulator gene (*aflR*) involved in the aflatoxin biosynthesis, and with the measurement of the genotoxic effect of AFB_1 produced on maize kernels by the strains. The *nor-1*, *omt-1*, and *aflR* genes were amplified according to Erami et al. [28]. PCR products were visualized on ethidium bromide-stained 1% agarose gels.

Twenty-five grams of dry forage maize were soaked in water for 1 h, then poured off the water, and autoclaved at 121 °C for 15 min two consecutive days. The sterilized maize kernels were inoculated with conidium suspensions (1 ml, 10^6 CFU/ml) of each strain previously grown on potato dextrose agar medium for 7 days. The inoculated maize was incubated for 21 days at 26 °C. The moldy kernel samples were autoclaved at 100 °C for 30 min after incubation and grounded using mortar and pestle. Aliquot of 1 g of the grounded kernels was placed in a test tube and 4 ml of 70% methanol was added. The content of the test tube was mixed for 2 h on a reciprocating shaker (180 rpm) and then centrifuged at 14,000 rpm for 10 min. The genotoxic effect of aflatoxin was detected from the supernatants with SOS chromotest™ kit (Environmental Bio-detection Products Inc., Ontario, Canada) according to the instructions of the manufacturer. Induction factor (IF) was calculated according to the report of Krifaton et al. [29]. Samples with 1.5 or higher IF were considered genotoxic.

The concentration of aflatoxins (B_1, B_2, G_1, and G_2) was also measured in the case of seven *A. flavus* strains having different IF values (1.51–4.49) and all the *A. parasiticus* strains with high-performance liquid chromatography (HPLC) (AflaTest WB, VICAM, USA) by an accredited laboratory.

Results

Aspergillus section Flavi in maize fields of Hungary

Propagula of the fungi belonging to *Aspergillus* section *Flavi* were found in 21 (26.9%) soil samples and 33 (42.3%) maize samples from the total of 78 maize fields throughout Hungary in 2013. Both sample types (soil and maize) of 11 sites proved to be positive for the presence of the fungi. Soil samples of further 10 and maize samples of further 22 sites contained these molds. In the case of 35 maize fields, no potentially aflatoxigenic fungi could be detected. The proportion of contamination differs significantly in different regions (Table I). Soil samples from Central Transdanubia contained the lowest proportion (12.5%) of contaminated soil samples and the highest proportion (52.9%) was found in samples from Southern Great Plain. None of the maize samples of Northern Hungary was infected with *A. flavus*. The highest proportion (62.6%) of contaminated maize samples was experienced in the region Central Transdanubia. However, the infection rate of 50.0% was reached or exceeded by the maize samples from the regions Central Hungary, Southern Transdanubia, Western Transdanubia, and Southern Great Plain.

In 2014, 118 soil and the same number of maize samples were analyzed for the presence of the fungi. Member(s) of *Aspergillus* section *Flavi* could be isolated from 19 (16.1%) soil samples and 41 (34.7%) maize samples. The fungi could be isolated from both soil and maize samples of six maize fields, further 13 soil and 35 maize samples contained the propagula of the fungi. Soil and maize samples of 64 sites were found to be free of the propagula of the fungi. The lowest level (soil: 10.0%, maize: 10.0%) of the contaminated samples was experienced in the region

Table I. Number (ratio) of soil and maize samples contaminated with *Aspergillus* strains of section *Flavi* in the regions of Hungary in 2013 and 2014

Region	2013			2014		
	Soil and maize samples	Positive sample		Soil and maize samples	Positive sample	
		Soil	Maize		Soil	Maize
Central Hungary	6	1 (16.7%)	3 (50.0%)	9	3 (33.3%)	6 (66.7%)
Central Transdanubia	8	1 (12.5%)	5 (62.5%)	11	2 (18.2%)	2 (18.2%)
Northern Great Plain	13	2 (15.4%)	3 (23.1%)	26	3 (11.5%)	11 (42.3%)
Northern Hungary	11	2 (18.2%)	0 (0.0%)	10	1 (10.0%)	1 (10.0%)
Southern Great Plain	17	9 (52.9%)	10 (58.8%)	28	5 (17.9%)	10 (35.7%)
Southern Transdanubia	10	4 (40.0%)	5 (50.0%)	19	3 (15.8%)	3 (15.8%)
Western Transdanubia	13	2 (15.4%)	7 (53.8%)	15	2 (13.3%)	8 (53.3%)
Total	78	21 (26.9%)	33 (42.3%)	118	19 (16.1%)	41 (34.7%)

Northern Hungary. The fungi occurred in the highest rate (soil: 33.3%, maize: 66.7%) in the region Central Hungary.

Strain determination

Colonies of the 114 strains were yellow-green after a 7-day incubation at 26 °C, but the color of the colonies of four strains was found to be greyish green. Several isolates produced dark sclerotia. After the microscopic observation, it could be concluded that the conidiophores of the strains were hyaline and roughened, and the globose to subglobose vesicles were often biseriate, but heads with only phialides were also common. Conidia were globose to subglobose and finely rough-walled.

According to the sequence analysis of their partial calmodulin gene, all isolates proved to belong to the species *A. flavus* (99%–100% sequence similarities to strain CBS 100927^T, accession number: KJ175534), except the four ones of darker colony which were identified as *A. parasiticus* (99%–100% sequence similarities to strain CBS 100926^T, accession number: KJ175553).

Aflatoxin-producing ability of strains

The ranges of induction factor of aflatoxigenic *A. flavus* strains are shown in Table II. The SOS chromotest indicated genotoxic response (IF > 1.5) in case of 50 (45.5%) of the tested 110 *A. flavus* strains. Strains isolated in 2013 from soil and maize samples contained aflatoxigen strains in the ratio of 21.1% and 15.6%, respectively. These ratios significantly increased (to 50.0% and 78.0%) in the case of the strains isolated in 2014.

The results of the SOS chromotest and that of HPLC showed high correlation ($R^2 = 0.89$). The amounts of AFB_1 produced by the seven strains (having IF value between 1.51 and 4.49) ranged from 1.00 to 56.0 mg/kg. (All of the tested

Table II. Number of aflatoxigenic *Aspergillus flavus* strains isolated from the soil and maize samples collected in 2013 and 2014, based on the genotoxic results of SOS chromotest

Strain isolated		Range of induction factor (IF)	Number of aflatoxigenic *A. flavus* strains
in	from		
2013	Soil	1.55–2.74	4
	Maize	1.51–2.96	5
2014	Soil	1.54–4.49	9
	Maize	1.80–5.04	32

Table III. Aflatoxin production (mg/kg) of the *Aspergillus parasiticus* strains

Strain	Origin	ΣAF	AFB_1	AFB_2	AFG_1	AFG_2
Pf054	Soil, 2013	15.8	2.35	0.19	12.4	0.84
Pf079	Soil, 2013	62.7	41.6	2.34	17.8	0.99
Pf148	Maize, 2013	14.0	2.36	0.32	10.2	1.16
Pf425	Soil, 2014	20.4	12.9	1.08	6.04	0.36

strains produced at least tenfold more AFB_1 than AFB_2 and none of them produced AFG_1 or AFB_2.) Thus genotoxic response indicated by the SOS chromotest was indeed caused by AFB_1.

Aflatoxin-producing abilities of the four *A. parasiticus* strains were also measured. All the *A. parasiticus* strains produced not only aflatoxin B_1 and B_2, but also aflatoxin G_1 and G_2 detected with HPLC (Table III).

The presence of three *aflatoxin biosynthesis genes*, the norsolorinic acid reductase encoding gene *nor-1*, the sterigmatocystin O-methyltransferase encoding gene *omt-1*, and the regulatory gene *aflR* was detected by PCR. All the three genes could be amplified in the case of 74 (64.9%) strains. The *nor-1* gene was the most, while *aflR* the less representative between the three tested genes.

After comparing results on aflatoxin biosynthesis genes and SOS chromotest, it was found that the strains whose genome lacks at least one of the three analyzed aflatoxin biosynthesis genes did not manifest genotoxicity, so presumably did not produce aflatoxin, and most strains which contained the regulatory and the two structural genes of aflatoxin production were able to manifest the genotoxicity of aflatoxin. Interestingly, in spite of the presence of the three genes, some strains were found to be non-aflatoxin producer according to the SOS chromotest.

Aflatoxigenic strains in maize fields in Hungary

The presence of aflatoxin-producing *Aspergillus* strains in 2013–2014 in Hungary is shown in Figure 1. Aflatoxigenic *Aspergillus* strains were isolated from all the seven regions of Hungary. By 2014, the aflatoxigenic *A. flavus* had appeared in the region Northern Hungary. The highest rate (20.0%) of aflatoxigenic *Aspergillus* strains in the soil samples was found in the Central Hungarian region. Concerning maize samples, region Western Transdanubia contained the propagula of aflatoxin-producing *Aspergillus* strains in the highest rate, but similarly high rates (20%–23%) were experienced in the maize samples of the regions Central Hungary, Southern Great Plain, and Northern Great Plain. *A. parasiticus* strains were found in the soil of the regions Southern Great Plain and Southern Transdanubia and in a maize sample of the region Western Transdanubia.

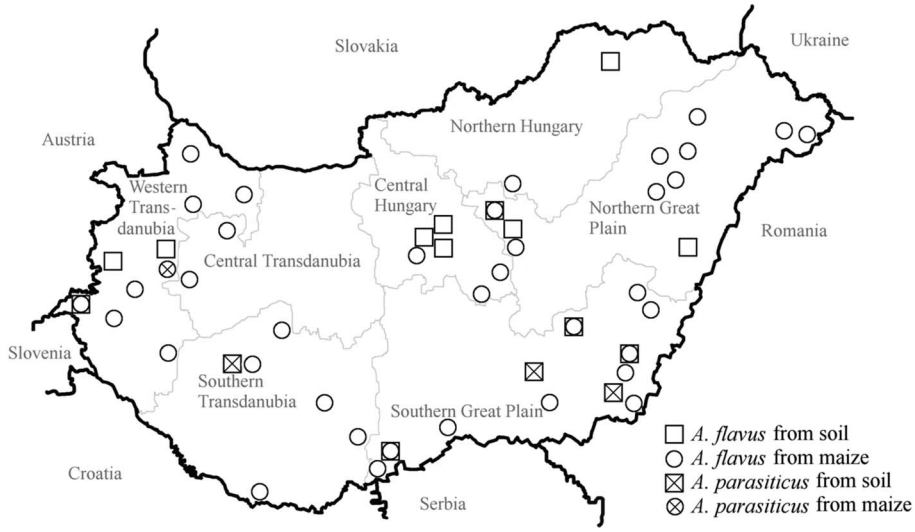

Figure 1. Distribution of aflatoxin-producing *Aspergillus* strains in Hungary, 2013–2014. (Only those sites are indicated where aflatoxigenic strains were found.) Soil and maize samples that contained aflatoxigenic *Aspergillus* strain are indicated by squares and circles, respectively

Discussion

The occurrence of species belonging to *Aspergillus* section *Flavi* and aflatoxin-producing potential of these fungi was unexplored in Hungarian (and in most European) soil. The important goals of the present work were to investigate the presence of these fungi not only in the kernels but also in the soil of maize fields and to determine their ability to produce aflatoxin by molecular methods and to confirm the aflatoxin production by biological and analytical methods.

Soil and maize samples of all the regions of Hungary (in different proportions) contained propagula of the members of *Aspergillus* section *Flavi*. The occurrence of fungi belonging to *Aspergillus* section *Flavi* in soil had a lower rate than in maize in both years. The presence of fungi, apparently because of their low quantity, could not be proved by culturing from soil in some occasions, but an enrichment of their propagula in maize kernels could be manifested. Though some factors may favorably affect the growth of molds during the transport and storage time, it can be considered that the maize crop for food and feed was infected already in the field.

Ninety-six percent of the strains isolated from Hungarian maize fields belonged to *A. flavus*, and the rest 4% to *A. parasiticus*. These results are in

agreement with a previous work which studied *Aspergillus* section *Flavi* strains isolated in 2003–2004 in northern Italy [17]. The presence of *A. parasiticus* has been reported for decades in several countries, especially in America and Asia [30–33], and recently it was detected in wheat in Slovakia [34], and in indoor air in Croatia [35]. However, regarding the appearance of *A. parasiticus* in maize, it is the first data from Central Europe.

Although the ratio of the presence of *Aspergillus* section *Flavi* was found to be lower in 2014 than in 2013, a greater proportion of the strains isolated in 2014 were able to produce aflatoxin than the isolates of 2013. It can be supposed that aflatoxigenic *Aspergillus* are more competitive than strains not able to produce toxins. Influence of weather conditions on the ratio of AFB_1-producing strains is considered more probable. According to the report of Hungarian Meteorological Service (http://www.met.hu/en/eghajlat/magyarorszag_eghajlata/eghajlati_visszatekinto/elmult_honapok_idojarasa/), the summer of 2013 was warm and dry, which could induce maize stress and promote fungal infection, while that of 2014 broke the series of drought and hot summers. Heat wave and scorching heat were not experienced and more precipitation occurred than in a usual summer. However, such rainy summers used to be cooler and the weather of July 2014 was nearly tropic-like, which favors the aflatoxin biosynthesis.

The aflatoxin-producing ability of isolated strains varied widely which is in agreement with other reports [36–39]. None of the tested strains produced more AFB_2 than AFB_1 which is in accordance with Giorni et al. [17]. However, two of the *A. parasiticus* strains synthesized more AFG_1 than AFB_1.

As the majority (>90%) of *A. parasiticus* strains are known as aflatoxin producers [40, 41] and all the four strains from Hungary were proved to be aflatoxigenic, occurrence of this species brings about the risk of aflatoxin contamination of maize in this country. Due to the ability of producing AFG_1 and AFG_2, beside AFB_1 and AFB_2, this risk can increase.

Conclusions

Contrary to the fact that aflatoxins have rarely been detected in feeds and foods in Hungary, our results demonstrated that aflatoxigenic *A. flavus* strains were presented in maize fields of all the seven regions of Hungary. Moreover, *A. parasiticus* strains have appeared in the soil at the regions of Southern Great Plain and Southern Transdanubia and even in a maize sample of the region Western Transdanubia. Occurrence of *A. parasiticus* among the aflatoxigenic fungi in Hungary is a noticeable result, since its appearance in maize kernels in Central Europe has not been reported. All the *A. parasiticus* isolates were able to

synthesize not only aflatoxin B_1 and B_2, but also aflatoxin G_1 and G_2. Thus, regular examination of feed and food for aflatoxins, mainly after a warm and droughty summer, is essential for food safety.

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Conflict of Interest

The authors declare no conflict of interest.

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