Aspergillus parasiticus from Wheat Grain of Slovak Origin and its Toxigenic Potency

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Abstract

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During the mycological investigation of the wheat grain originating in Poltár (Central Slovakia), an endogenous aspergillus producing aflatoxins was encountered. Morphology, physiology and extrolites indicated the species *Aspergillus parasiticus* Speare. The amounts of aflatoxins detected by Liquid Chromatography/Tandem Mass Spectrometry on a synthetic medium were: B_1 15.7, G_1 23.4, B_2 0.52, G_2 0.68, and M_1 0.18 mg/l. Compared to other screened strains, the amount of B_1 produced was 5.6 mg/l lower than in *A. parvisclerotigenus* NRRL 3251 and 0.5 and 3.15 mg/l higher than in *A. nomius* I and *A. nomius* II, respectively. The production of G_1 was 22.25 and 18.65 mg/l lower than in *A. nomius* I and II, respectively. The yields of other aflatoxins were lower and the yield of kojic acid, 227.0 mg/l, was higher. It is the first finding of both an aflatoxin producer and of *A. parasiticus* on a food commodity of Slovak origin within the last 20 years. The yields produced indicate rather a high toxigenic potency.

Keywords: *Aspergillus* section *Flavi*; toxigenicity; mycotoxins; food safety; wheat grain

During the mycological investigation of the wheat grain originating in Poltár (Central Slovakia), an endogenous isolate was encountered producing aflatoxins B_1 and G_1, and not producing cyclopiazonic acid. From the research carried out in Slovakia in the last 20 years, only two published papers have appeared regarding the isolation of species known to be potential producers of both types of aflatoxins from commodities or substrates of Slovak origin (Lizoň & Bacigálová 1998; Franková & Šimonovičová 1999; Šimonovičová 2001, 2008; Čerňanský *et al*. 2006). No data regarding the toxigenic potency of these isolates is available. Out of the other so far known potential producers of aflatoxins, the only species isolated, regularly from commodities and substrates of Slovak origin, is *A. flavus*. Frisvad *et al*. (2006) summarise that only about 40% of the known isolates of the species are able to produce aflatoxin. The isolates of Slovak origin that were tested in the studies by Piecková

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and Jesenská (2001), Labuda and Tančinová (2006), Tančinová and Labuda (2009) did not produce aflatoxin.

The production of aflatoxins B_1 and G_1, and no production of cyclopiazonic acid has so far been found in mitotic species *Aspergillus arachidicola*, *A. bombycis*, *A. nomius*, *A. parasiticus* (Pildain *et al.* 2008), *A. parvisclerotigenus*, *A. toxicarius* (Frisvad *et al.* 2005) as well as in meiotic species *Petromyces nomius* (Horn *et al.* 2011), and *P. parasiticus* (Horn *et al.* 2009). It is difficult to distinguish between particular species; misidentification can easily occur (Frisvad *et al.* 2006).

Precise species identification is crucial for further studies and for the use of a specimen (Fassatiová 1979). Strains producing aflatoxins of more types could be potentially used in the production of reference substances either for further research or in the control of food quality and safety, as described by Labuda *et al.* (2009). In the case of a microorganism isolated from a food commodity, the data about its anthropocentrically malicious potency can contribute to an accurate estimation of the hazards in terms of food safety and human health, as aflatoxins are carcinogenic mycotoxins (JECFA 1987, 2001).

This study summarises the physiological, morphological, and chemotaxonomical characteristics of an isolated aspergillus, which led to its species identification. The toxigenic potency as assessed on a minimal liquid medium, and compared with three other strains which produce aflatoxins in high amounts, is included.

MATERIAL AND METHODS

Strains. *A. parasiticus* strain is preserved in the Fungal Collection at the Department of Microbiology, Slovak University of Agriculture in Nitra, Slovakia, assigned as MD 502. *A. parvisclerotigenus* NRRL 3251, *A. nomius* I and II are the property of Romer Labs Division Holding GmbH (Tulln, Austria). The strains were selected for comparison with *A. parasiticus* MD 502 because of their production of high amounts of aflatoxins.

Preparation of spore suspension. The strains were grown for 5 days on Potato Dextrose agar used was prepared from chemical compounds according to Samson *et al.* (2002a) at 25°C in the dark. The cultures were then overlaid with the sterile distilled water and the surface gently rubbed with a sterile loop. The whole amount was transferred to a centrifuge tube. The spore concentration was calculated using a Neubauer counting chamber. The stock solution was diluted using 0.1% aqueous peptone to prepare a conidial suspension of 2×10^5 conidia/ml concentration.

Isolation of Aspergillus parasiticus MD 502. The strain was isolated from a sample of wheat (*Triticum aestivum* L.) harvested in Poltár (Central Slovakia) and obtained from the bulk store situated in Trnava (Western Slovakia) in 2008. A single isolate of *A. parasiticus* was encountered after direct plating of superficially sterilised grains (0.4% chlorine solution, 2 min) on Dichloran Rose Bengal Chloramphenicol Agar (Merck, Darmstadt, Germany) as described by Hocking *et al.* (2006).

Thin Layer Chromatography. TLC was performed according to Samson *et al.* (2002b) and as modified and described by Labuda and Tančinová (2006).

Species determination. For the species determination, the isolate was grown for 7 days on Czapek Yeast Extract agar (CYA) at 25, 37, 42°C, on CYA with 20% sucrose (CY20S), Malt Extract agar (MEA; Klich 2002), Yeast Extract agar (YES), Creatine Sucrose agar (CREA), *Aspergillus flavus*/*A. parasiticus* selective medium (AFPA) at 25°C and for 30 days on Czapek slant (Samson *et al.* 2002a) with 0.05% *p*-anisaldehyde (CZpa) at 30°C in the dark. Microscopic slides of 7 days old cultures on MEA were prepared in lactophenol (Števlíková *et al.* 2001). The measurements were taken using an Olympus BX51TF equipped with Micro Image software (Olympus C&S, Prague, Czech Republic). The pictures were taken using an Olympus BX51 with QuickPhoto Camera 2.3 software and an Olympus SP-500 UZ camera (Olympus SK, Bratislava, Slovak Republic). The data produced was compared to that given by Murakami (1971), Kurtzman *et al.* (1987), Singh *et al.* (1991), Peterson *et al.* (2001), Klich (2002), Samson *et al.* (2002a), Pildain *et al.* (2008), and Horn *et al.* (2009).

Liquid Chromatography/Tandem Mass Spectrometry. LC-MS/MS was carried out according to Sulyok *et al.* (2007). The jars containing 50 ml of a synthetic minimal liquid medium, Adye et Mateles (A&M; Johnson *et al.* 2008) modified by KCl, were inoculated with 100 µl of the spore suspension and cultivated still at 25°C in dark for 7 days. The whole volume was extracted with an equal volume of ethyl acetate on a horizontal shaker (approx. 0.0112 *g*,

1 h). An amount of 100 µl of filtered (syringe with cotton stopper) ethyl acetate phase was transferred into an HPLC vial, allowed to evaporate, redissolved in 1000 µl of acetonitrile/water (1:1, v/v) and filtered again (Acrodisc® CR 4 mm Syringe Filters; Pall Life Sciences, Farlington, UK) prior to analysis.

Statistics. The average and SD figures were derived from single replication using Microsoft Office Excel.

RESULTS AND DISCUSSION

TLC of the fresh isolate described here revealed the production of aflatoxins B_1 and G_1, whereas cyclopiazonic acid was not produced. The isolate grew well on all cultivation media at all temperatures. The morphological and physiological characteristics observed are shown in Table 1. Considering the good growth at 42°C, the presence of pinkish conidia on CZpa, a positive reaction on AFPA, the fact that the colony colour did not turn brown with age, and the lack of any evidence of sclerotial formation helped to exclude the diagnoses of *A. bombycis* (Peterson *et al.* 2001), *A. toxicarius* (Murakami 1971), *A. arachidicola* (Pildain *et al.* 2008), *A. nomius* (Kurtzman *et al.* 1987), and *Petromyces* species (Horn *et al.* 2009, 2011), respectively. The data collected, including the habitat, are consistent with the features of *Aspergillus parasiticus* Speare recorded or described by Murakami (1971), Singh *et al.* (1991), Klich (2002), Samson *et al.* (2002a), Frisvad *et al.* (2006), Pildain *et al.* (2008), and Horn *et al.* (2009).

The findings of *A. parasiticus* on commodities or substrates of Slovak origin are scarce. Altogether, two isolations of the species, one from soil (Černňanský *et al.* 2006) and another one from drinking water (Franková & Šimonovičová 1999) are the only records, as listed in papers summarising the biodiversity in Slovakia (Lizoň & Bacigálová 1998; Šimonovičová 2001, 2008).

The species in general produces high amounts of aflatoxins (Frisvad *et al.* 2006). In the study by Parrish *et al.* (1966), the amounts of AFB_1, AFG_1, AFB_2, and AFG_2 varied with the fungal strain. The quantities of extrolites produced by the strains studied here are shown in Table 2. The amount of AFB_1 produced by *A. parasiticus* MD 502 was 5.6 mg/l lower than in *A. parvisclerotigenus* NRRL 3251 and 0.5 and 3.15 mg/l higher than in *A. nomius* I and *A. nomius* II, respectively. The production of AFG_1 was 22.25 and 18.65 mg/l lower than in *A. nomius* I and II, respectively. The yields of other screened types of aflatoxins were lower, and the yield of KA was higher, compared to the other strains. Strain NRRL 3251, formerly recognised as *A. flavus* (Pai *et al.* 1975; Lin *et al.* 1976; Dutton *et al.* 1985, and others) or *A. flavus* var. *parvisclerotigenus*, in a taxonomic study by Frisvad *et al.* (2005), was assigned as one of the representative strains of *A. parvisclerotigenus*. When tested on agar media, the strain produced kojic acid, unlike here or in the study by Lin *et al.* (1976). Aflatoxin M_1 is known mostly to occur in milk and dairy products as a result of the metabolic conversion of AFB_1 ingested by livestock with contaminated feed (JECFA 1987, 2001). Its pres-

Table 1. Recorded characteristics of *Aspergillus parasiticus* MD 502

Colony appearance		Microscopic structures	
Colour	dark green, not turning brown with age	Conidial heads	mostly uniseriate
		Stipes	rough
Reverse	yellow-orange to pinkish on CYA; yellow on CY20S	Vesicles	spherical; width 37.5 µm; length 34.1 µm
Exudate	uncoloured on CYA		fertile on the upper half or on entire surface
Sclerotia	not observed*		
CREA	weak growth; no acid production	Phialides	length 9.5 µm
		Conidia	distinctly rough; visible inner and outer wall
AFPA	positive reaction		
CYA 42°C	21 mm†		5.2·4.9 µm; showing connective tissue
CZpa	pink colouration observed		

*on any agar or liquid medium after 6 weeks of cultivation, †average colony diameter

Table 2. Potency of *Aspergillus parasiticus* MD 502 on seventh day of cultivation on modified A*et*M, in comparison with other strains as assessed by LC-MS/MS

Strain	Production (mg/l)					
	AFB_1	AFG_1	AFB_2	AFG_2	AFM_1	KA†
A. parasiticus MD 502	15.70 ± 3.25	23.40 ± 1.27	0.52 ± 0.07	0.68 ± 0.00	0.18 ± 0.06	227.00 ± 7.07
A. parvisclerotigenus NRRL 3251	20.80 ± 8.06	nd	0.93 ± 0.53	nd	0.12 ± 0.11	nd
A. nomius I	15.20 ± 4.24	45.65 ± 4.60	0.32 ± 0.11	1.15 ± 0.24	0.45 ± 0.16	124.50 ± 84.15
A. nomius II	12.55 ± 0.92	42.05 ± 0.35	0.57 ± 0.04	2.14 ± 0.08	0.33 ± 0.00	152.00 ± 67.88

AF – aflatoxin; KA – kojic acid; average ± SD; nd – not detected

ence in the cultures of aflatoxinogenic strains on aflatoxin production enhancing media are known from the studies by Stubblefield *et al*. (1970), Pai *et al*. (1975), Dutton *et al*. (1985), and others.

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