Mycotoxin-Producing Ability and Chemotype Diversity of *Aspergillus* Section *Flavi* from Soils of Peanut-Growing Regions in Iran

S. Amani · M. Shams-Ghahfarokhi ·
M. Banasaz · M. Razzaghi-Abyaneh

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Abstract Invasion of crops with *Aspergillus flavus* may result in contamination of food and feed with carcinogenic mycotoxins such as aflatoxins (AF) and cyclopiazonic acid (CPA). In the present study, distribution and toxigenicity of *Aspergillus flavus* and *A. parasiticus* in soils of five peanut fields located in Guilan province, Northern Iran was investigated. From a total of 30 soil samples, 53 strains were isolated which all of them were finally identified as *A. flavus* by a combination of colony morphology, microscopic criteria and mycotoxin profiles. Chromatographic analysis of fungal cultures on yeast extract sucrose broth by tip culture method showed that 45 of the 53 *A. flavus* isolates (84.9 %) were able to produce either CPA or AFB₁, while eight of the isolates (15.1 %) were non-toxicogenic. The amounts of CPA and AFB₁ produced by the isolates were reported in the range of 18.2–403.8 µg/g and 53.3–7446.3 µg/g fungal dry weights, respectively. Chemotype classification of *A. flavus* isolates based on the ability for producing mycotoxins and sclerotia showed that 43.4 % were producers of CPA, AFB₁ and sclerotia (group I), 13.2 % of CPA and AFB₁ (group II), 9.4 % of AFB₁ and sclerotia (group III), 13.2 % of AFB₁ (group IV), 5.7 % of CPA and sclerotia (group V) and 15.1 % were non-toxicogenic with no sclerotia (group VI). No strain was found as producer of only CPA or sclerotia. These results indicate different populations of mycotoxigenic *A. flavus* strains enable to produce hazardous amounts of AFB₁ and CPA are present in peanuts field soils which

can be quite important regard to their potential to contaminate peanuts as a main crop consumed in human and animal nutrition.

Keywords *Aspergillus flavus* · Cyclopiazonic acid · Aflatoxin · Chemotype diversity · Soil · Peanuts

Introduction

Aflatoxins (AF) are fungal secondary metabolites which, if ingested, can evoke a wide range of toxic responses and disease conditions in higher vertebrates [1]. Members of the genus *Aspergillus* as the most important AF producers can infect commodities such as corn, peanuts, cotton, tree nuts, sorghum, and other oil seeds under suitable conditions of temperature and relative humidity [2]. *Aspergillus* section *Flavi* members such as *A. flavus*, *A. parasiticus* and *A. nomius* can produce AF, but only *A. flavus* is able to produce cyclopiazonic acid (CPA). Besides *A. flavus* as the main producer, CPA is also produced by many other *Aspergillus* and *Penicillium* species [3]. Recently, Chang et al. showed that CPA is produced by *A. flavus* in a close relationship with AF and the gene cluster of CPA is in the adjacent of AF gene cluster.

CPA is an indole tetramic acid that is toxic to animals and humans [4–6]. From several evidences, it has been shown that CPA can also be considered as a natural contaminant of food, feed and agricultural crops [7–10]. AF and CPA contamination can occur together in the same food and feed due to the presence of producing fungi from *A. flavus* populations [4, 7, 11–13]. In general, the ability of *A. flavus* isolates to produce CPA varies greatly depending on geographic conditions and strains isolated. The incidence of toxigenic *A. flavus* strains in agricultural soil as

S. Amani · M. Shams-Ghahfarokhi (✉)
Department of Mycology, Faculty of Medical Sciences,
Tarbiat Modares University, 14115-331 Tehran, Iran
e-mail: shamsm@modares.ac.ir; mshamsgh@yahoo.com

M. Banasaz · M. Razzaghi-Abyaneh
Department of Mycology, Pasteur Institute of Iran,
13164 Tehran, Iran

the main reservoir of the fungus is important not only from the view point of mycological research, but also for their potential to contaminate susceptible crops and agricultural commodities.

Peanut (*Arachis hypogaea*) is a very susceptible crop to contamination with AF and closely related mycotoxins. It is a main legume cultivated in different parts of Iran as a nutrient consumed in human and animal nutrition. It is grown mainly in Northern parts of Iran specially Guilan province (around 90 % of peanut cultivation). The main objective of this study was to examine distribution and toxigenicity of *A. flavus* strains in peanut field soils of Astaneh Ashrafieh, Guilan province as a main center of peanut production in Iran with special attention to their chemotype classification based on the types and amounts of mycotoxins produced.

Materials and Methods

Sampling, Isolation and Identification of Fungi

Thirty soil samples were collected from the 5 cm top of the surface soil at random locations within five peanut fields (A–E, each six soil samples) of Astaneh Ashrafieh, the main site of peanut cultivation in Iran (Guilan province, Northern Iran) during June 2009. Each soil comprised from ten subsamples each of approximately 100 cm³ which were obtained using a sterile trowel at 10 m intervals from the 5 cm top of the surface soil and then mixed thoroughly in a Nylon bag. For isolation of *A. flavus* and *A. parasiticus*, the dried soil samples were passed through testing sieves (1 mm mesh) [14]. 2 g of each sieved soil was diluted with 6 ml distilled water in a test tube, mixed vigorously for 20 min., and serially diluted to 10^{−5} in sterile distilled water. One tenths ml of each dilution was spread onto 9.0 cm petri-dishes containing isolation culture media i.e., Dichloran rosebengal chloramphenicol agar (DRBC) and *Aspergillus flavus/parasiticus* agar (AFPA) [15, 16]. The cultures were incubated at 28 °C for DRBC and 30 °C for AFPA and checked periodically for colonies with brilliant orange-yellow reverse coloration on AFPA (during 48–72 h) and yellowish-green colonies on DRBC. The putative *Aspergillus* colonies were purified after sub-culturing on potato dextrose agar plates. *Aspergillus* species were identified based on microscopic morphology and macroscopic criteria on czapek dox agar (CZ) and czapek yeast extract agar (CYA) plates for 7 days at 25 °C [2, 17].

Detection of CPA and AFB₁ Production by TLC

For assessment of CPA and AFB₁, all the isolates were cultured in triplicate on 1 ml tips containing 250 µl yeast

extract 2 %, sucrose 20 % (YES) broth at 28 °C for 4 days by the tip culture method [18]. After separating the culture medium from the tips using centrifugation, the mycelia weight was calculated. CPA was extracted from the culture broth and assessed by TLC using toluene–ethyl acetate–formic acid (5:4:1, vol/vol/vol) as mobile phase [16, 19]. For detecting AFB₁, spotted TLC silica gel 60 F₂₅₄ plates were developed by chloroform–ethyl acetate–formic acid 90 % (6:3:1, vol/vol/vol) as mobile phase. AFB₁ were visualized under UV light (365 nm) and photographed with a TLC scanner CAMAG Reprostar 3 (CAMAG, Switzerland).

Quantitation of CPA and AFB₁ by HPLC

For quantitation of AFB₁ and CPA, chloroformic extract of the culture broth was injected into a HPLC (KNAUER D-14163 UV–VIS system, Germany) according to Razzaghi-Abyaneh et al. [20]. 50 µl of each chloroformic extract was injected into the HPLC column (TSKgel ODS-80TS; 4.6 mm ID × 15.0 cm, TOSOH BIOSCIENCE, Japan) and eluted at a flow rate of 1 ml/min by methanol/water (70:30) contained 300 mg ZnSO₄·7H₂O for CPA and by water/acetonitrile/methanol (60:25:15, v/v/v) for AFB₁. The amounts of CPA and AFB₁ were measured at wavelengths of 284 and 365 nm, respectively. The elution time of the samples was compared with pure AFB₁ and CPA standards and quantified on the basis of the ratio of the peak area of samples to those of the standards.

Statistical Analysis

The results were analyzed by SPSS version 16 programme for Windows (SPSS Inc., Chicago, IL). Analyses of variance (ANOVA) and Tukey's multiple comparison tests at 5 % significance level were used to compare the means of fungal incidence and toxin production data. *P* < 0.05 was considered significant.

Results

Isolation of *A. flavus* and *A. parasiticus*

All the 53 *Aspergillus* isolates produced yellow-green colonies on CZ, and CYA and showed yellow-orange reverse coloration on AFPA. Microscopically, uni- and biserial conidial heads, long conidiophores and globose conidia with smooth to finely echinulated walls in variable sizes were observed indicating that all strains were *A. flavus*. No isolate of *A. parasiticus* were found in analyzed samples. Table 1 shows detailed information about 53 *A. flavus* strains isolated. A total of 9, 5, 20, 16 and 3 *A. flavus* strains were isolated from peanut fields A, B, C, D and E,

Table 1 Distribution and toxigenicity of *A. flavus* strains in peanuts field soils

Peanut field	<i>A. flavus</i> strain	Fungal dry weight (μg)	CPA (μg/g DW)	AFB ₁ (μg/g DW)
A	PICC-AF59	41.0 ± 4.2	203.9 ± 33.2	808.9 ± 83.1
	PICC-AF60	43.8 ± 8.0	71.6 ± 10.2	905.8 ± 106.6
	PICC-AF61	34.5 ± 3.7	34.6 ± 6.0	1478.3 ± 173.2
	PICC-AF62	39.1 ± 3.5	39.5 ± 3.2	558.1 ± 62.2
	PICC-AF63	35.6 ± 7.2	ND	90.4 ± 13.5
	PICC-AF64	53.3 ± 9.9	ND	ND
	PICC-AF65	33.4 ± 5.5	ND	ND
	PICC-AF66	52.0 ± 7.6	58.5 ± 8.3	943.2 ± 99.1
B	PICC-AF67	35.1 ± 6.3	ND	615.4 ± 47.3
	PICC-AF68	30.6 ± 4.4	149.0 ± 17.7	ND
	PICC-AF69	46.9 ± 4.8	403.8 ± 55.3	ND
	PICC-AF70	46.3 ± 6.5	ND	444.3 ± 60.0
	PICC-AF71	52.8 ± 8.1	18.2 ± 3.0	291.1 ± 30.2
C	PICC-AF72	42.4 ± 7.1	255.9 ± 29.2	1900.1 ± 131.7
	PICC-AF73	44.8 ± 9.7	339.4 ± 41.1	7446.3 ± 869.5
	PICC-AF74	43.5 ± 5.1	103.6 ± 12.4	234.5 ± 26.6
	PICC-AF75	45.6 ± 8.0	21.0 ± 3.6	480.5 ± 35.2
	PICC-AF76	52.0 ± 9.2	ND	244.7 ± 18.1
	PICC-AF77	50.8 ± 10.5	83.8 ± 10.7	2677.2 ± 358.1
	PICC-AF78	46.6 ± 7.5	ND	607.3 ± 50.3
	PICC-AF79	49.0 ± 6.0	ND	53.3 ± 10.1
	PICC-AF80	47.1 ± 8.4	300.1 ± 23.5	1017.2 ± 99.1
	PICC-AF81	34.5 ± 5.5	176.6 ± 19.4	397.8 ± 42.5
	PICC-AF82	54.4 ± 6.8	150.8 ± 14.3	658.3 ± 61.0
	PICC-AF83	44.9 ± 4.0	241.6 ± 30.1	1582.6 ± 190.8
	PICC-AF84	45.9 ± 7.1	ND	185.5 ± 20.2
	PICC-AF85	52.6 ± 5.6	ND	ND
	PICC-AF86	50.4 ± 6.8	173.1 ± 14.2	126.4 ± 18.6
	PICC-AF87	46.0 ± 8.2	99.9 ± 13.4	2047.4 ± 255.2
	PICC-AF88	38.5 ± 2.7	234.1 ± 27.9	2127.0 ± 343.3
	PICC-AF89	41.7 ± 3.3	313.6 ± 40.1	1043.5 ± 102.7
	PICC-AF90	43.5 ± 4.8	88.6 ± 12.0	306.7 ± 35.2
	PICC-AF91	45.0 ± 6.3	60.5 ± 9.1	670.8 ± 59.0
D	PICC-AF92	38.8 ± 7.0	79.3 ± 11.1	143.1 ± 43.2
	PICC-AF93	50.0 ± 8.8	22.2 ± 5.0	2913.3 ± 200.8
	PICC-AF94	34.2 ± 5.5	177.0 ± 15.7	1360.8 ± 155.1
	PICC-AF95	43.5 ± 5.6	231.2 ± 39.5	143.5 ± 43.5
	PICC-AF96	45.8 ± 3.7	311.9 ± 40.8	774.3 ± 50.7
	PICC-AF97	37.6 ± 4.3	55.1 ± 9.8	1183.9 ± 165.4
	PICC-AF98	44.5 ± 6.6	ND	2024.8 ± 179.3
	PICC-AF99	35.0 ± 4.7	ND	930.1 ± 101.8
	PICC-AF100	39.1 ± 2.2	ND	ND
	PICC-AF101	41.1 ± 6.9	ND	ND
	PICC-AF102	36.0 ± 4.4	ND	1606.1 ± 195.2
	PICC-AF103	36.6 ± 3.6	119.4 ± 18.2	455.8 ± 61.5
E	PICC-AF104	45.3 ± 5.2	143.1 ± 23.8	ND
	PICC-AF105	36.5 ± 4.5	ND	ND
	PICC-AF106	42.9 ± 3.9	ND	ND
	PICC-AF107	37.0 ± 5.1	ND	505.8 ± 45.6
	PICC-AF108	51.5 ± 8.0	250.2 ± 20.6	887.3 ± 66.0
	PICC-AF109	54.1 ± 7.5	65.0 ± 10.6	1615.9 ± 150.3
	PICC-AF110	55.0 ± 4.8	ND	ND
	PICC-AF111	41.5 ± 6.2	ND	613.1 ± 15.2
Standard strains	CMI102566	39.9 ± 3.3	377.5 ± 29.7	9450.6 ± 892.4
	CMI93803	35.6 ± 6.0	ND	ND

CPA cyclopiazonic acid, AFB₁ aflatoxin B₁, DW fungal dry weight, ND not detected

respectively. All the soils in fields A, C and D, 5/6 soils of field B and 2/6 soils of field E were contaminated with *A. flavus* strains (Table 1).

Sclerotia Production

The presence of sclerotia was observed macroscopically as an identifiable criterion for *A. flavus* isolates. Sclerotia size was evaluated by a measuring reticule with a Nikon Microscope at 40× magnification. Based on the results obtained, 30 of 53 *A. flavus* strains (56.6 %) were capable to produce sclerotia all from L-type on CZA medium at 30 °C (Table 2). They were classified in chemotypes I, III and V.

CPA-Producing Ability

As shown in Fig. 1, 33 of 53 *A. flavus* strains (62.3 %) were capable to produce CPA. The amounts of CPA produced by the strains were reported in the range of 18.2–403.8 µg/g fungal dry weight. The strains were classified in five classes including class 1 (non-CPA producers; 20 strains), 2 (<100 µg/g; 14 strains), 3 (100–200 µg/g; 8 strains), 4 (200–300 µg/g; 6 strains) and 5 (>300 µg/g; 5 strains).

Table 2 Chemotype patterns of *A. flavus* strains based on sclerotia, aflatoxins, and CPA production

Chemotype	No. of strains	CPA	AFB1	AFB2	Sclerotia
I	23	+	+	–	+
II	7	+	+	–	–
III	5	–	+	–	+
IV	7	–	+	–	–
V	3	+	–	–	+
VI	8	–	–	–	–

Fig. 1 Classification of *A. flavus* strains from peanut field soils in relation to CPA production on YES broth

AF-Producing Ability

As indicated in Fig. 2, a total of 42 of 53 *A. flavus* strains (79.2 %) were capable to produce AFB₁. None of the strains produced AFB₂ or AFG series. The amounts of AFB₁ produced by the strains were reported in the range of 53.3–7446.3 µg/g fungal dry weight. In relation to the amounts of AFB₁ produced, the strains were classified in five classes including class 1 (non-producers; 11 strains), 2 (<100 µg/g; 2 strains), 3 (100–1,000 µg/g; 25 strains), 4 (1,000–5,000 µg/g; 14 strains) and 5 (>5,000 µg/g; 1 strain).

Chemotype Classification of *A. flavus* Strains

As shown in Table 2, *A. flavus* strains were classified into six chemotypes based on the ability for producing mycotoxins and sclerotia. The strains capable to produce CPA, AFB₁ and sclerotia (23/53) were classified as chemotype I. They comprised the most prevalent chemotype with an incidence of 43.4 %. Other 30 strains were distributed in chemotypes II–VI including CPA and AFB₁ producers (chemotype II; 13.2 %), AFB₁ and sclerotia producers (chemotype III; 9.4 %), AFB₁ producers (chemotype IV; 13.2 %), CPA and sclerotia producers (chemotype V; 5.7 %) and 15.1 % non-producers of mycotoxins with no sclerotia (chemotype VI).

Discussion

In the present study, different communities of toxigenic and non-toxicogenic *A. flavus* strains isolated from peanuts field soils were identified that were capable of producing various amounts of CPA and/or AFB₁. Co-contamination of agricultural commodities with AF and CPA has been reported by different researchers [7, 9, 12, 21, 22]. Although CPA mycotoxicoses has not been reported in the field, its association with “kodo poisoning” after

Fig. 2 Classification of *A. flavus* strains from peanut field soils in relation to AFB₁ production on YES broth

consumption of CPA-contaminated kodo millet has been proposed in India [23, 24]. It has been shown that the ability of *A. flavus* isolates to produce CPA and AF vary greatly which affects by several parameters such as genetics of isolates, the source of isolation and geographic conditions [3, 10]. So, different results have been reported for the isolates from various regions of the world [9, 13, 16, 21, 25]. Although toxigenic *Aspergillus* strains have been reported from various crops and agricultural commodities, agricultural soil serves as the main reservoir of these fungi all over the world [26–28]. In the present study, *A. flavus* was isolated as sole species of *Aspergillus* section *Flavi* from peanut field soils. In total, 45 of the 53 peanuts field soil isolates of *A. flavus* (84.9 %) were able to produce either CPA or AFB₁. In a study on the occurrence of *Aspergillus* section *Flavi* in 300 soil samples from three peanut-growing regions of Argentina, both *A. flavus* and *A. parasiticus* were isolated [29]. In a similar study by these authors on soil samples of peanut-growing region in Argentina during 1998–2001, it was shown that 80 % of 369 *A. flavus* isolates produced both AF and CPA [30]. Examination of peanut seeds in five regions of Egypt in the year 2010 showed that among a total of 88 *Aspergillus* section *Flavi* isolates, 95 % were *A. flavus* as the only AF-producing species [31]. 28/32 *A. flavus* (87 %) isolated in the year 2008 and 51/56 (91 %) isolated in the year 2007 produced AFB₁ in the range of 12.8–75,843 and 10.9–248,460 ng/g respectively. All aflatoxigenic isolates produced AFB₁ and AFB₂, but not AFG series. Analysis of soil samples from four peanuts production regions in the State of São Paulo, Brazil in the year 2012 showed that *A. flavus* was the most frequent species of the genus *Aspergillus* (13.4 %) [32]. Nesci et al. (2011) showed that among the *Aspergillus* populations recovered from peanut seeds in Argentina, *A. flavus* was the most frequently isolated (79 %), and *A. parasiticus* (5.9 %) was the third prevalent species [33]. Totally, 79 *A. flavus* and 2 *A. parasiticus* were isolated. AFB₁ produced by 52 strains out of 79 of *A. flavus* ranging from <0.002 to 225 µg/ml and 2 strains of *A. parasiticus* ranging from <0.002 to 25 µg/ml. From a total number of 25 peanut samples and 11 farm soils tested in Vietnam, *A. flavus* was found in 9 (36.0 %) and 3 (27.3 %) samples, respectively. Totally, 36 *A. flavus* were isolated from contaminated soils (7 isolates) and peanuts (29 isolates) of which 2 (28.6 %) and 11 (37.9 %) produced AF [21]. From the study of Razzaghi-Abyaneh et al. [16] on corn field soils, it was shown that 25 of 58 *A. flavus* isolates (43.1 %) produced CPA; while 16 isolates (27.6 %) were aflatoxigenic and only 4 isolates (6.9 %) produced sclerotia. The authors classified the isolates in 5 chemotypes based on their ability to produce CPA, AF and sclerotia. Rodrigues et al. [34] showed that among 13 strains of *A. flavus* isolated from almonds, 10 (77.0 %) were

atoxigenic and only 2 (15.0%) were capable to produce CPA and AFB₁. Vaamonde et al. [9] reported that *A. flavus* strains from various crops may be different in their aflatoxin producing ability. They showed that 29 % of the total *A. flavus* isolates were able to produce aflatoxin. Horn and Dörner [13] showed that the majority of *A. flavus* isolates (more than 80.0 %) from soils of USA produced both CPA and AF. Different results of aflatoxigenicity among *Aspergillus* section *Flavi* population may be attributed to differences in prevailing climatic conditions in the regions analyzed, the cultivars grown, local agricultural practices, the incidence of insect damage and seasonal variations in affecting factors.

Sclerotia are compacted bodies of aggregated hyphae, which resist unfavorable environmental conditions due to their resistance to chemical and biological degradation [35]. Production of sclerotia by *A. flavus* isolates in relation to mycotoxin production has been a matter of controversy for many years. According to the description of Horn et al., sclerotia size is a phenotypic character within *A. flavus* strains that can be used to create two different groups: the large strains (L) having sclerotia >400 µm in diameter and the small strains (S) with sclerotia <400 µm. In the present study, all 30 sclerotia positive *A. flavus* strains produced CPA (3 strains), AFB₁ (5 strains) or both (23 strains) [36]. This is in accordance to the data from other researchers implying a positive correlation between CPA, AF and sclerotia production [9, 13, 37]. All sclerotia producing strains examined in our study were classified as L strains because of producing sclerotia >400 µm in diameter. We classified *A. flavus* isolates into six chemotypes (I–VI) based on CPA, AF and sclerotia production patterns. The group I (CPA and AFB₁ positive) was the most prominent chemotype which comprised nearly 40 % of *A. flavus* strains. In our previous study on *A. flavus* strains isolated from corn field soils, the group of non-producers of both AF and CPA was the most abundant chemotype reported [16].

Overall, the data from present study clearly show that diverse populations of CPA- and AF-producing *A. flavus* strains are present in peanut field soils as potential threats for agriculture and public health. These fungi have considerable variation in the types and amounts of mycotoxins produced. So, studying of their genetic diversity and community structures can help us to make a better understanding about population dynamics of the fungus in relation to prevent mycotoxin contamination of crops and agricultural commodities in practice.

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