Survey on *Aspergillus* Section *Flavi* from Peanut Cropped-Soils in Iran

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Abstract: During post-sowing (PS) and pre-harvesting (PH) periods, 248 and 232 *Aspergillus* strains were isolated from a total of 120 soil samples from two major peanut production regions included Astaneh-e Ashrafieh (AA) and Minoodasht (MD), respectively, which 160 strains (PS, N=64 and PH, N=96) and 144 (PS, N=55 and PH, N=89) were identified as *Aspergillus parasiticus* as the most abundant species of *Aspergillus* section *Flavi*, respectively. In total, a great proportion of *A. flavus* strains (68.8% and 58.4%) from AA and MD were able to produce sclerotia at 30°C, respectively. Approximately 66.4% and 61.1% of the *A. flavus* strains from AA and MD were capable to produce aflatoxins, respectively. The amounts of *A. flavus* toxin (AFB1) produced by the *A. flavus* strains from AA and MD were in the range of 312-1247 ppb and 164-1147 ppb, respectively. The *A. flavus* populations, either toxigenic or non-toxigenic, should be considered as potential threats for Iranian peanut cultivation and public health. Vegetative compatibilities were determined through complementation assays between nitrate-nonutilizing (nit) mutants. Twenty-eight VCGs included six multi-member VCGs and 22 single-member VCGs were identified from 80 *A. flavus* isolates. The *A. flavus* isolates from Astaneh-e Ashrafieh or Minoodasht could be grouped into one of Minoodasht or Astaneh-e Ashrafieh VCGs, respectively (except for VCG1, VCG3 and VCG5). The diversity index of VCGs was 57.5% for *A. flavus* VCGs in peanut regions of Iran. It is concluded that, populations of *A. flavus* from peanut fields of Iran showed a random distribution of soil isolates based on their toxigenicity, sclerotial production and VCGs.

Key words: Peanut · *Aspergillus* section *Flavi* · Aflatoxins · *Nit* mutants · Sclerotia production · Vegetative compatibility · Iran

INTRODUCTION

Peanut or groundnut (*Arachis hypogaea* L.) is one of the most important food and oil seed crops cultivated and utilized in most parts of the world. In Iran, peanut growing area is more than 5000 hectares. Guilan (Astaneh-e Ashrafieh) and Golestan provinces (north and north-east of Iran) with 16800 and 3500 tons productions are the major peanut producers in Iran.

The section *Flavi* fungi are ubiquitous in soil [1-6]. Peanuts produce seed-bearing pods below the soil surface, so their pods are in direct contact with soil populations of *Aspergillus* species and the developing pods and seeds can often be invaded by these species in the field before harvest [5, 7, 8, 9, 10, 11]. The major mycotoxins found in Iranian peanuts are aflatoxins [12, 13, 14, 15]. Aflatoxins are carcinogenic fungal secondary metabolites produced by *A. flavus* and other closely related species [16 17, 18,19]. The *A. flavus* isolates were identified as members of either the L strain which produces sclerotia that are >400 μm in diameter, or the S strain, which produces numerous small sclerotia that are <400 μm in diameter.

Populations of both L and S strains comprise numerous subpopulations called Vegetative Compatibility Groups (VCGs). *A. flavus,* populations are diverse genetically and comprise large numbers of VCGs, even within a restricted geographic area [20, 21, 4, 22,]. VCG analysis based on complementation between nitrate-nonutilizing (nit) mutants was used to study A. flavus populations in Georgia peanut fields [4, 23]. VCG 1 of *A. parasiticus* appears to be widely distributed in peanut-growing regions throughout the United States [5].

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In Iran, peanut is cultivated across northern and north-eastern agroecological zones (Guilan and Golestan provinces). There are no information about the prevalence, structure and VCGs of *A. flavus* populations in section *Flavi* from peanut fields of Iran. The present investigation, the first one in Iran, was conducted to isolate a range of *Aspergillus* section *Flavi* strains from peanut soils in two major peanut production regions of Iran and obtain information on the relative role of the key species, ability to produce aflatoxins, sclerotia and characterize the distribution of several *A. flavus* VCGs.

MATERIALS AND METHODS

Collection and Preparation of Soil Samples: During 2012, one-hundred twenty soil samples (0-5 cm depth) were collected randomly throughout the peanut fields (0.2-0.3 ha) from Astaneh-e Asharfieh (AA, Guilan province, northern Iran, 3 main districts Kyashahr, Kisoom-e Chahardeh and Central) and Minoodasht (MD, Golestan province, north-eastern Iran, two main districts Galikesh and Central) at post-planting (PP; 2 weeks after sowing) and pre-harvesting (PH; 2 weeks before harvesting) periods (Fig. 1). The soil samples from each peanut field were mixed thoroughly into a single composite sample (500 g) for section *Flavi* species isolations.

Determination of Populations of Section *Flavi* in Soil Samples: Soil sub-samples 1g each were decimal diluted in water agar 0.2% (10^{-1} to 10^{-3}) and spread onto Dichloran Rose Bengal Chloramphenicol (DRBC) agar plates (Merk, Germany) prior to incubation at 25ºC for 7 days in the dark and then examined daily for fungi growth [5, 24]. Colonies of section *Flavi* fungi presumptively identified based on morphological characteristics [25]. Soil densities of *Aspergillus* species from section *Flavi* were evaluated based on colony-forming units per g soil (CFU/g soil). For this, the numbers of CFUs were calculated from the number of colonies obtained on DRBC plates chosen at dilution levels.

Fungal Identification: Taxonomic identification of fungal colonies was carried out by morphological, macro and microscopic characteristic, according to standard methods [24, 26, 27]. When necessary, culturing was repeated using APFA Agar (Oxoid- CM0731, UK) for exact identification.

Aflatoxin Detection: A preliminary screening for aflatoxin production by the strains was performed by growing them on conducive Yeast Extract Sucrose (YES) medium (20 g/l Yeast extract, 150 g/l Sucrose, 15 g/l Agar) based on fluorescence under long-wave UV light (365 nm). All strains were incubated at 25°C for 2 to 3 days in the dark condition. Three plugs (5 mm diameter) were cut out across the diameter of the colony and placed in 2 ml Eppendorf tubes and weighed. Aflatoxins were extracted by adding 1 ml of chloroform and shaking for 1 hour. The biomass was discarded after centrifugation and chloroform was evaporated to dryness. The residue was derivatized using TFA (Triflouroacetic acid) as described in the AOAC (2000). Chromatography was performed on thin layer chromatography (TLC) silica gel 60F254 (20×10 cm), aluminum sheets by Camag Linomat-5 applicator, with mobile phase condition acetone: chloroform (1: 9) [28]. Finally the plates were scanned in CAMAG HPTLC scanner-3 under 366 nm wavelength to determine the concentrations of aflatoxin B1 (AFB1) and B2 (AFB2) in the samples. The Limit of detection by HPTLC was 0.5 ppb.

Sclerotia production; Sclerotium production was recorded for single spore cultures (after 21 d dark incubation at 30°C) on Czapek agar (CZ) containing 3% $NaNO_3$ (three replicate for each isolate). Sclerotia larger than 400 µm were classified as large and sclerotia smaller than 400 µm were classified as small [29-34]. Some isolates did not produce sclerotia was also noted. All isolates belong to the L-strain morphotype of *A. flavus*, which produce sclerotia that are >400 ìm in diameter [35].

Vegetative Compatibility Analysis of *A. flavus* Isolates: Nitrate-nonutilizing mutants (*nit* mutants) of each wild type isolate were generated on potassium chlorate (30 gL^{-1}) supplemented CZ. Four plates of each chlorate medium (sole nitrogene source = NO_3) were inoculated with mycelial plugs of each isolate and incubated at 30°C in dark for at up to two weeks and monitored for the growth of fast-growing chlorate-resistant sectors [4, 36]. The colonies with fine and expansive growth with little or no sporulation and aerial mycelium were considered *nit* mutants [22]. These mutants were identified as *nia*D (nitrate non-utilizing, nitrate reductase mutant), *nir*A (nitrate and nitrite non-utilizing, nitrate reductase mutant) and *cnx* (hypoxanthine and nitrate non-utilizing permease mutant) based on their growth on a hypoxanthine (0.2 gL^{-1}), ammonium tartrate (1 gL^{-1}) or sodium nitrite (0.5 gL^{-1}) medium [22, 37]. Spores of *nit* mutants were inoculated on PDA and incubated at 28°C for 2 days. An agar plug of each wild-type isolate growing on CZ was also included as a wild-type control for each experiment.

Fig. 1: Maps of Golestan (a) and Guilan (b) provinces (north and north-east of Iran, respectively) with sampling locations Minoodasht (M) and Astaneh-e Ashrafieh (A) where soil samples were collected

Compatibility based on complementation of *nit* mutants was conducted by cutting a mycelial plugs (5 mm in diameter) containing mycelia from the edge of *cnx* and *nir*A mutants (if not *nia*D and *nir*A mutants or *nia*D and *cnx*) of different isolates in petri dishes containing CZ (with nitrate as the nitrogen source). The one pair (sometimes two pairs) of complementary and compatible mutants that was the most efficient in stable heterokaryon formation at the contact zone between the colonies was chosen as representative of that isolate. Also complementation tests between the same isolate (self-fusion), which were included for each test run on the same plate as a negative control representing no complementation of mutation. Three mutants were placed about 2 cm apart on each dish and incubated at 28°C for 7-14 days [38]. Then, one *cnx* and one *nir*A mutant were selected from each group and paired with the remaining *nia*D mutants. Additionally all *cnx* and *nir*A mutants that did not fall into a VCG were also paired with the remaining *nia*D mutants and grown for three weeks. Diversity of VCGs of *A. flavus* isolates was calculated as the number of groups divided by the total number of isolates.

Statistical Analysis: For statistical analysis SPSS version 11.5 (SPSS Institute Inc. Chicago, Illinois) was used. Data on soil population of *Aspergillus* species belong to section *Flavi* and sclerotia production (%) were often not normally distributed and therefore, were log transformed prior to analysis. To evaluate the differences in the proportions of VCGs frequencies and aflatoxigenic strains during the sampling period, a t-test was used [39].

RESULTS

DISCUSSION

Isolation of *Aspergillus* Section *Flavi* from Soil Samples:
The two studied regions and sampling periods evaluated showed variations in the soil density of *Aspergillus* species from section *Flavi* (Table 1). During periods PS and PH, 248 and 232 *Aspergillus* strains were isolated from a total of 120 soil samples from AA and MD peanut fields, respectively, which 160 (PS, n=64 and PH, n=96) and 144 (PS, n=55 and PH, n=89) strains were identified as *Aspergillus parasiticus* as the most abundant species of *Aspergillus* section *Flavi*, respectively. The *A. flavus* strains produced sclerotia varied in size and number (Table 2).

Production of Aflatoxins and Sclerotia by *A. flavus* Isolates: A total of 174 strains of *A. flavus* (Astaneh-e Ashrafieh No=84, Minoodasht No=88) isolated from Iranian peanut soils were grown on YES medium supplemented with 0.3% ß-cyclodextrin and fluorescence was used to assess aflatoxin. IT was found that ca. 33.7% and 38.6% strains *A. flavus* from AA and MD were not able to produce aflatoxins, respectively. Twenty-five toxigenic strains of *A. flavus* were examined for production of aflatoxin B. The AFB1 and AFB1 + AFB2 were in the range 312-1247 and 578-2392 ppb for strains of *A. flavus* from AA (38 and 62%, respectively). In MD, five *A. flavus* strains (20%) produced 164-1147 ppb AFB1 and twenty strains produced AFB1 + AFB2 varying from 252-1632.5 ppb.

Classification of VCGs and VCG Diversity: Eighty aflatoxigenic isolates of *A. flavus* from Astaneh-e Ashrafieh and Minoodasht which have been previously described were tested for vegetative compatibility. *Nit* mutants were isolated on CZ amended with KClO₃ for 47 isolates (ca. 58.8%, from two regions). Out of 69 *A. flavus nit* mutants, 47 (68.1%) were *nia*D, 18 (26.1%) *nir*A and 4 (5.8%) *cnx*. In addition, 47 isolates (29 and 18 from Astaneh-e Ashrafieh and Minoodasht, respectively) were assigned to 6 multi-member VCGs based on the complementation tests. In total, the diversity index of *A. flavus* VCGs was 57.4% in peanut fields of Iran. The genetic diversity of *A. flavus* isolates based on vegetative compatibility from Minoodasht was 72.2% and for Astaneh-e Ashrafieh was 62.1%. There were significant differences among VCG$_s$ in aflatoxin production (p<0.05).

The cultivation of peanut in the AA and MD is as an intensive agricultural system and has a long history, however the soil densities of *Aspergillus* section *Flavi* were relatively low in both major peanut growing regions of Iran. During sampling periods, the means of population densities of section *Flavi* fungi were mostly averaged from 180 to 300 and 210-310 CFU/g soil in AA and MD, respectively. So, we were unable to detect the population density of *Aspergillus* species in section *Flavi* on soil dilution plates at greater decimal dilutions (<10⁻²). The agricultural soil serves as the main reservoir of these fungi all over the world [40, 41, 42, 17]. It was found that [43] *A. flavus* population in soils of Virginia peanut fields was low (ca. 0.5-57.3 propagules/g soil). Bell and Crawford [44] reported significantly greater amount of propagules in naturally infested soils in Georgia (1.5×10⁴ propagules/g soil). Based on field observation, Griffin and Garren [43] suggested that *A. flavus* is capable of colonizing peanut fruits at very low soil densities. Specifically, It could not explain the cause (s) of the low population density of section *Flavi* species within peanut fields of Iran. It may be the section *Flavi* fungi are stimulated less than other microbial groups (fungi and bacteria) in the peanut fields. In addition, possibly, low soil temperature in two peanut production regions, may be is responsible for the relatively low to average rates of *Aspergillus* populations. The *A. parasiticus* has been found to be the most common species isolated from peanut soils in Iran. This was not in according to some reports [45]. The population analysis of *Aspergillus* (section *Flavi*) showed that *A. flavus* and *A. parasiticus* formed 73% and 27% of section *Flavi* species from peanut fields in Argentina [46, 47]. Although *A. parasiticus* appears to be the dominant species in peanut soils, *A. flavus* is the dominant aflatoxigenic species in peanuts in Iran. Also it was found that *A. parasiticus* is more prevalent in peanuts than in other crops [3]. These may be due to the fact that intensive peanut cultivation selects for aflatoxin producing strains [9]. The previous studies have shown that toxigenic isolates of *A. parasiticus* are extremely abundant [48, 23, 49]. Moreover, Barros *et al.* [50, 51] reported that only 94% of *A. parasiticus* strains from peanut were aflatoxigenic. Results revealed that 58 and 68.6% of the *A. falvus* isolates from Minoodasht and Astaneh-e Ashrafieh peanut soils produced sclerotia, respectively. The S strain isolates *A. flavus* were abundant

Table 1: Means of populations *Aspergillus* section *Flavi* (CFU per g soil) from peanut fields of Astaneh-e Ashrafieh (Guilan and province, Iran) and Minoodasht (Golestan province, Iran) at post-sowing and pre-harvesting periods

Sampling Regions	Periods*	CFU/ g soil**	Districts		
			Kyashahr	Kisoom-e Chahardeh	Central
Astaneh-e	Post-sowing		6.2×10^2	5.84×10^2	5.22×10^2
Ashrafieh	Pre-harvesting		4.76×10^2	4.14×10^2	3.92×10^2
			Galikesh		Central
Minoodasht	Post-sowing		7.55×10^2		6.43×10^2
	Pre-harvesting		4.61×10^2		5.37×10^2

* post-sowing: PS pre-havesting: PH

**CFU/g soil of *Aspergillus* species from section *Flavi*. Mean of three replicates determined by serial dilution and plating on dichloronitroaniline rose bengal (DRBC) agar; colonies were counted after a 5-day incubation.

Table 2: Aflatoxin and sclerotia production by *A. flavus* soil isolates from Minoodasht and Astaneh-e Ashrafieh peanut fields, Iran

Regions	CFU/g soil*	Sclerotia production (%)	Sampling periods**	Strain isolate*** (%)	
				S	L
Minoodasht	5.98×10^2	58.0 (No=51)	P.S P.H	B23.1 a	A 76.9 a
				B 33.3b	A 66.7 b
Astaneh-e Ashrafieh	5.1×10^2	68.6 (No=59)	P.S P.H	B 35.8b	A 64.2 a
				B 40.5a	A 59.5 b

Means within columns and rows followed by different lower and upper letters, respectively, differ significantly at 5% of probability level according to t-test. The values are the means of three replicates.

** post-sowing: PS pre-havesting: PH

*** L strain produce sclerotia >400 μm in diameter, S strain produce sclerotia < 400 μm in diameter [35]

in peanut-cropped soil at pre-harvesting period (33.3 and 40.5%). Also, it was found that 27% of *A. flavus* sclerotial isolates collected from 30 fields of peanut growing area were S strains [47]. The high frequency of *A. flavus* S strain isolates is not promising because in accordance with some data, the S-type isolates usually produce more aflatoxins compared to L-type isolates [52]. Furthermore, it was reported that about 50% of the *A. flavus* isolates from corn, soil and peanut in Mississippi Delta produced large sclerotia [29]. The more prevalence of the S strain isolates of *A. flavus* in the late growing season postulated that temperature variation has a greater impact on *Aspergillus* population composition. Although sclerotia of *A. flavus* are an important source of primary inoculum, but the survival of *Aspergillus* spp. particulary aflatoxigenic species in peanut growing regions of Iran still is not understood. Furthermore, *A. flavus* populations commonly reach the soil surface through spillage at harvest, sclerotia may represent an important source of these fungal inoculum in field soils where peanut is grown. The present results revealed that 66.3 and 61.4% of the *A. flavus* strains from Astaneh-e Ashrafieh and Minoodasht were toxigenic. Of the toxigenic *A. flavus* strains, all produced AFB1 and ca. 62.7% produced AFB2. Amani *et al.,* [45] showed that 45 of the 53 A. flavus

isolates (84.9 %) were able to produce AFB1, while eight of the isolates (15.1 %) were nonaflatoxigenic. The amounts of AFB1 produced by the *A. flavus* isolates were reported in the range of 53.3–7446.3 ìg/g fungal dry weights. We found a good relation between qualitative (fluorescence) and quantitative levels of aflatoxin as measured by HPTLC. So that, the intensity of the halo around each colony indicated the amount of aflatoxin produced. It is suggested that analysis of aflatoxin production by fluorescence developed by colonies grown on YES agar was a reliable indicator of aflatoxin production.

There was a non-random relationship between the sclerotium type and the production of aflatoxin; that is, aflatoxigenic strains of *A. flavus* from Astaneh-e Ashrafieh (33.3%) and Minoodasht (32.5%) that did not produce sclerotia were more than strains that produced large sclerotia (12.1 and 18.9%), respectively. The present study has provided for the first time the relevant information on distribution, morphological and physiological criteria of different *A. flavus* populations from nonafltoxigenic to highly aflatoxigenic enable to produce hazardous amounts of AFB1 in soils of peanut fields. These fungi, either toxigenic or nonaflatoxigenic, should be considered as potential threats for agriculture and public health.

Recovery rates of *nit* mutants, *nia*D, *nir*A and *cnx*, were variable among isolates. All of the *A. flavus* isolates produced *nia*D and/or *nirA* mutants, but only a few isolates produced *cnx* mutants. Complementation occurred between different phenotypes and prototrophic growth of *nit* mutants on CZ often varied among *Aspergillus* isolates and *nit* mutant phenotypes. Within a same VCG, stronger reactions occurred in certain pairings. Isolates *A. flavus* which could not be assigned to any VCG because of complementary pairs of mutants were not obtained or the isolates pairings failed to generate the heterokaryon (vegetative incompatibility). We identified 28 VCGs from 80 *A. flavus* isolates in two studied peanut regions. Twenty two VCGs from *A. flavus* isolates (14 and 8 from Astaneh-e Ashrafieh and Minoodasht, respectively) contained only a single isolate (single-member VCGs). Six VCGs contained three to more *A. flavus* isolates single-member VCGs. Barros *et al.* [50, 51, 53] identified 56 VCG$_s$ from 100 *A. flavus* isolates from peanut-cropped soils in Argentina. They reported Twenty-five VCG$_s$ contained two or more isolates and 31 VCG$_s$ contained only a single isolate. *A. flavus* isolate ast13 from Astaneh-e Ashrafieh could be grouped to Minoodasht VCG6. Also, *A. flavus* strain min36 from Minoodasht could be grouped to Astaneh-e Asharafieh VCG2. One of the VCGs (VCG4) included strains from the two geographical regions. Results showed that there was heterogeneity of *A. flavus* populations in samples of two peanut production regions (Astaneh-e Ashrafieh and Minoodasht). There relatively average to high diversity among the isolates of *A. flavus* placed in VCGs was detected in two regions. The *A. flavus* populations from Minoodasht were more genetically diverse than in Astaneh-e Ashrafieh and included 19 VCGs which were reflected by a diversity value of 70.4%. This result shows that a small area may be inhabited by several too many genetically distinct individuals and it is in agreement with the result shown by Bayman and Cotty [20, 21] Like many filamentous fungi, *A. flavus* populations are highly diverse in numbers of VCGs which vary markedly in relative abundance so that a few VCGs comprise most isolates whereas many VCGs contain very small numbers of isolates [20, 21, 54, 55]. Isolates within each of the VCG are not all sclerotia producers. Furthermore, sclerotia producers within each VCG group are either L or S strain, not both. The isolates belonging to the same VCG are likely to be genetically similar. However, this conclusion must be considered with a caution because the results are based on the small number of isolates. The genetic diversity of *A. flavus* populations is an important factor when developing a biocontrol strategy against that fungus [56, 57]. The isolates within a VCG are similar in their production of aflatoxins, isolates belonging to the same VCG can be detected among isolates that have the same mycotoxin profile. Therefore, there were no significant differences among the VCGs from nine *A. flavus* L strains (VCG1, VCG4, VCG9, VCG15, VCG16, VCG17, VCG20N VCG24, VCG25 and VCG27) or nine *A. flavus* S strains (VCG2,VCG10, VCG11, VCG12, VCG13, VCG18, VCG21, VCG23 and VCG26) in aflatoxin production between two peanut regions. VCG 2 (*A. flavus* S strain) from Astaneh-e Ashrafieh with an average AFB1 production of 196.7 ppb was the highest toxigenic VCGs among other VCGs. In this regard, the S-strain isolates generally produced high levels of AFB1, whereas the L-strain isolates were more variable in aflatoxin production [40].

CONCLUSION

The present study has provided for the first the relevant information on the population density and strain type of *Aspergillus* species (section *Flavi*) from peanut production regions in Iran. The *Aspergillus* section *Flavi* communities resident in different locations of Astaneh-e Ashrafieh and Minoodasht differed in strain composition. Although Astaneh-e Ashrafieh was cultivated in peanut for several long years (continous crop system), the mean populations from *Aspergillus* species in section *Flavi* are low to average rates. The L and none sclerotial strain isolates were more prevalent in the peanut fields of Iran (Guilan and Golestan provincs). Also, we concluded that, VCGs are helpful for characterizing genetic structure in populations of *A. flavus* in two major peanut-growing area of Iran. A better understanding of genetic variability and population structure of *A. flavus* within and among peanut fields may reveal how we can most effectively manage this important pathogen. In fact, knowledge of regional differences in the aflatoxigenicity and genetic variability of *A. flavus* populations may help in understanding the population dynamics and also give important information that could be used in determination of which control measures are most effective in reducing preharvest aflatoxin contamination.

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