Characterization of *Aspergillus* section *Flavi* from pistachio soils in Iran

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Abstract: During 2012, soil samples from commercial pistachio orchards in three major production regions include Rafsanjan (Kerman Province, center of Iran), Damghan (Semnan Province, north-central Iran) and Feyz-Abad (Khorasan-e Razavi Province, north-eastern Iran), were assayed on Dichloran Rose-Bengal Chloramphenicol agar (DRBC) and *Aspergillus flavus-parasiticus* agar media to quantify populations of *Aspergillus* species from the section *Flavi*. The mean propagule density of *Aspergillus* species from the *Flavi* section [$\log_{10}$ (CFU/g soil)] was higher in Feyz-Abad (3.06, 2.88–3.24), compared to Damghan (2.55, 2.44–2.65) and Rafsanjan (2.40, 2.26–2.54). *A. flavus* (69.7, 65.3 and 57.9%), *A. parasiticus* (19.6, 25.4, and 29.3%), and *A. nomius* (10.7, 9.3, and 12.8%) were the predominant species in the regions of Rafsanjan, Damghan, and Feyz-Abad, respectively. There were significant differences among sclerotia producing isolates of *A. flavus* in the sampling regions ($p < 0.05$). The percentage of sclerotium-producing isolates of *A. flavus* from Rafsanjan (14.5%) was much lower than Damghan (39.5%) and Feyz-Abad (41.4%). The *A. flavus* isolates from Damghan, Rafsanjan, and Feyz-Abad were toxigenic at 53.7%, 61.6%, and 60.4%, respectively. In Rafsanjan, aflatoxin B_1 (AFB1), and AFB1 + AFB2 (aflatoxin B_2) ranged from 274 to 553 ppb (393±17.11) and 394 to 3745 ppb, respectively, while AFB1, and AFB1 + AFB2 ranged from 257 to 392 ppb (285±13.18) and 415 to 1658 ppb, respectively, in Damghan. We found 16 and 20 vegetative compatibility groups (VCGs) for 41 and 37 *nit* mutant producing isolates of *A. flavus* from Rafsanjan and Damghan, respectively. From Damghan the VCG diversity for *A. flavus* isolates was greater (54%) than from Rafsanjan (39%). Because there were a few number of sclerotium-producing isolates of *A. flavus*, we did not determine the relationships between sclerotium production with VCGs and/or geographical distribution in the three pistachio production regions. This study was the first to determine the strain and VCG diversity of *A. flavus* soil isolates from Iranian pistachio orchards.

Key words: aflatoxin, *Aspergillus flavus*, pistachio, sclerotia production, vegetative compatibility

Introduction

Pistachio (*Pistacia vera* L.) is one of the most important horticultural products of Iran with special economic importance. The main pistachio producing countries are Iran and the United States (US), which together contribute to nearly 75% of the total global pistachio market (Bui-Klimke *et al.* 2014). In Iran, pistachio is cultivated in 380,000 ha. with 70% bearing and 30% non-bearing orchards and production of about 300,000 t/year (Sedaghat 2011). Pistachio is cultivated in the dry regions of Iran which have a low rainfall (Kerman, Semnan and Khorasan-e Razavi provinces). Fungi in the *Aspergillus* section *Flavi*, especially *A. flavus* Link, *A. parasiticus* Speare, and *A. nomius* Kurtzman, Horn and Hesseltine are responsible for producing aflatoxins, potent carcinogenic mycotoxins (IARC 1993; Manonmanti *et al*. 2005; Oktay *et al*. 2011). *A. flavus* is the most commonly isolated causal agent of aflatoxin contamination (Bayman and Cotty 1993; Moradi *et al.* 2010) of many crops, including maize, cotton, peanut, and tree nuts (Robens and Brown 2004).

The main combinations of aflatoxins are that of B_1, B_2, G_1, and G_2. The most toxic form of aflatoxin is aflatoxin B_1 (AFB1) (Diener *et al*. 1987).

AFB1 is the most important and toxic to human beings from the public health point of view (Stark and Demain 1980). It is the most toxic and potent carcinogen, teratogen, and mutagen to humans and animals (Sweeney and Dobson 1998; Shahidi 2004), causing damage such as toxic hepatitis, hemorrhage, edema, immunosuppression, and hepatic carcinoma (Speijers and Speijers 2004; Woo *et al.* 2011).

In Iran, pistachio nut contamination to *Aspergillus* species and their toxins are the most serious problems in pistachio production, consumption, and export process-

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ing (Mojthahedi *et al.* 1978; Moradi and Javanshah 2005). Individual isolates of *A. flavus* have varying characteristics, including aflatoxin-producing ability, sclerotia production, morphology of sclerotia, and sporulation (Geiser *et al*. 2000; Horn 2003; Giorni *et al*. 2007; Atehnkeng *et al*. 2008). Sclerotia are considered small if the diameter is less than 400 μm and large if greater the 400 μm (Horn 2003; Pildain *et al*. 2004). There is no known sexual stage of *A. flavus*; consequently, most studies on its genetic variability have been evaluated mainly by characterizing isolates based on vegetative compatibility (VC) a process that vegetatively compatible hyphae fuse to form heterokaryons. Phenotypic characteristics (i.e. size of sclerotia and aflatoxin-producing ability) are usually conserved within vegetative compatibility groups (VCGs) (Bayman and Cotty 1993; Horn *et al*. 1996; Novas and Cabral 2002). It was assumed that VC was controlled by multiple gene loci, is a useful marker for identifying the genetic diversity of populations (Leslie 1996). Sequence data confirm that isolates within a VCG are closely related and distinct from other VCGs (Ehrlich *et al.* 2007; Grubisha and Cotty 2009). A better understanding of population structure and genetic diversity of *A. flavus* from pistachio orchards will faciliate the development of effective biocontrol strategies. This study was conducted to determine, for the first time, the population density of *Aspergillus* section *Flavi*, aflatoxin production, sclerotium size, and VCG diversity of *A. flavus* isolates collected from naturally infected soils of pistachio orchards in the three major pistachio production regions included the Kerman (Rafsanjan), Semnan (Damghan), and Khorasan-e Razavi (Feyz-Abad) provinces of Iran.

Material and Methods

Collection and preparation of soil samples

In June 2012, soil samples were collected randomly from 75 commercial pistachio orchards (1–2 ha) in main pistachio production regions included Rafsanjan (Kerman province, southeastern Iran), Damghan (Semnan province, central-northern Iran) and Feyz-Abad (Khorasan-e Razavi province, northeastern Iran). Pistachio orchards were selected from the upper, middle, and lower areas of the regions in order to reflect the region's variable geography and soil type. Twenty soil cores were taken randomly from each of orchard as seen in figure 1. Cores from each orchard were bulked and mixed as a single sample (approximately 2 kg) immediately after collection. Samples then were sieved through a 2-mm mesh screen., and composed samples 200 g collected on a surface-disinfested plastic container, dried (48°C for 48 h) and stored at 4°C. The dominant soil series at the sampled regions were sandy-loam, loam-clay-silt, and/or loam-clay (data not shown).

Determination of section *Flavi* populations in soil samples

Dilution plating was used as the enumeration technique (Pitt and Hocking 1997). Briefly, 10 g of each combined soil sample was placed in a 250-ml Erlenmeyer flask containing 90 ml of autoclaved 0.1% water agar and the suspension was stirred for 5 min on an electric stir plate. The mixture was diluted 10^2, 10^3, and 10^4 folds. Aliquots consisting of 0.1 ml of each dilution were spread (in triplicate) onto individual 9-cm Petri dishes containing Dichloran Rose-Bengal Chloramphenicol agar medium (DRBC; King *et al*. 1979). Colonies exhibiting *Aspergillus* morphology characteristics were identified and counted following incubation for 5 days at 30°C in dark. One of the three sets of dilutions averaging between 10 and 60 colonies per Petri dish was selected for enumeration. The average number of colonies per plate was used to calculate an estimate of the population density of the *Aspergillus* species of the *Flavi* section in the soil collected from each sampled orchard. The propagule densities were reported as a $\log_{10}$ (CFU/g soil, CFU/g).

Morphological characterization of the isolates

The colonies of *Aspergillus* species from the *Flavi* section were selected from DRBC plates and sub-cultured on 9 cm

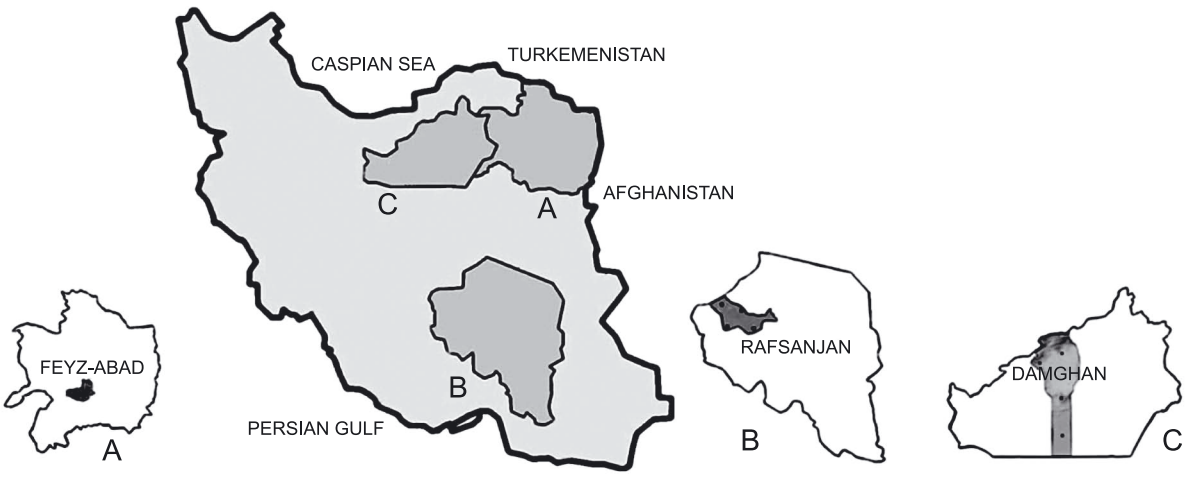

Fig. 1. Map of the Islamic Republic of Iran showing the Kerman, Semnan and Khorasan-e Razavi pistachio producing provinces from which *Aspergillus* section *Flavi* soil isolates were obtained: A – Feyz-Abad, Khorasan-e Razavi province; B – Rafsanjan, Kerman province; C – Damghan, Semnan province

diameter Petri dishes containing Malt-Extract agar (MEA) and Czapek-Dox agar (CZ). Cultures were incubated at 25°C for 7 days in the dark, and then analyzed for colony color, presence, and size of sclerotia, head seriation, and conidial morphology. For micromorphological observations, the isolates were examined under the microscope (10×, 40× and 1000× magnifications). The identification of *Aspergillus* species included *A. flavus*, *A. parasiticus*, and *A. nomius* and was based on the morphological criteria of Pitt and Hocking (1997) and Klich (2002). All colonies were re-cultured on *Aspergillus flavus-parasiticus* agar (AFPA) at 30°C for 3–5 days to verify group identification by colony reverse color (Gourama and Bulerman 1995).

Aflatoxigenic ability of isolates

Detection by fluorescence on YES agar medium

Five-day-old Potato Dextrose Agar (PDA) cultures of *A. flavus* isolates were plated on Yeast Extract Sucrose agar (YES) containing 0.3% methyl *β*-cyclodextrin and were incubated at 28°C for 3 days in the dark (Cepeda *et al.* 1996; Fente *et al.* 2001). Then, the cultures were examined under UV light (365 nm) for detection of the florescence signal. A fluorescent area around each colony of the aflatoxinogenic isolates was observed.

Detection by TLC

The *A. flavus* isolates were examined for the production of AFB1 and AFB2 based on the thin-layer chromatography (TLC) method (Chang *et al*. 2004). For this, four cores (16-mm in diameter) corresponding to 2 g of fungal biomass, were collected from each replicate of YES cultures and placed in a 2-ml microtubes. From these samples, aflatoxins (AFs) were extracted by adding 0.5 µl of chloroform per 100 mg mycelia. Extracts were allowed to dry and then re-suspended in 30–50 µl of chloroform. A volume of 15 µl of each extract was applied on a silica gel G_{60} plate (20 × × 20 cm, 0.25 mm thick, Merck 5721, Germany), along with the standard AFB mixture (containing AFB1 at 0.5 µg/ml and AFB2 at 0.15 µg/ml). The plates were developed in a acetone: chloroform (10 : 90, v/v) solvent system. After development, the plates were dried and observed under short (254 nm) and long wavelengths (365 nm). The detection limit was 0.05 µg/g for all the AFBs. The AFBs were detected as an intense blue fluorescence spot with the relative fraction values (Rf) 0.8 and 0.6 for AFB1 and AFB2, respectively. Aflatoxins were quantified directly on TLC plates with a scanning densitometer (TLC Scanner 3; Camag Scientific Inc, Wilmington, NC, USA) (Pons 1966). Each isolate was subjected to three replications. In all the experiments, two toxigenic and atoxigenic *A. flavus* strains including K49 (NRRL 30797) and F3W4 (NRRL 30796) were used as positive and negative controls, respectively.

Sclerotia production

Sclerotium production was recorded for single spored cultures after 21 days in dark incubation at 30°C on CZ containing 3% $NaNO_3$ (three replicates for each isolate). For each isolate the diameters of at least ten sclerotia were measured. The sclerotial isolates were classified according to the sclerotial size; L strain isolates produced very few sclerotia with a diameter greater than 400 µm, and S strain isolates produced numerous sclerotia with a diameter under 400 µm as previously reported (Cotty 1988; Geiser *et al*. 2000; Horne 2003; Abbas *et al*. 2005; Giorni *et al*. 2007; Atehnkeng *et al*. 2008).

Vegetative compatibility analysis of *A. flavus* isolates

Generation and characterization of nit mutants

Nitrate nonutilizing mutants (*nit* mutants) of each wild type isolate were generated on potassium chlorate (30 g/l) 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 the dark for up to two weeks, and monitored for the growth of fast-growing chlorate-resistant sectors (Bayman and Cotty 1993; Cotty 1994; Horn and Greene 1995).

Chlorate-resistant mutants were putative *nit* mutants and were purified by transferring the growing tip of mycelia onto fresh chlorate-CZ plates for single colony isolation. The colonies with fine and expansive growth with little or no sporulation and aerial mycelium were considered *nit* mutants (Papa 1986). These mutants were identified as *niaD* (nitrate non-utilizing, nitrate reductase mutant), *nirA* (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 g/l), ammonium tartrate (1 g/l) or sodium nitrite (0.5 g/l) medium (Cove 1976; Papa 1986). 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.

Complementation tests

Compatibility based on complementation of *nit* mutants was conducted by cutting mycelial plugs (5 mm in diameter) containing mycelia from the edge of *cnx* and *nirA* mutants (if not *niaD* and *nirA* mutants or *niaD* and *cnx*) of different isolates. 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 a 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. Complementation between *nit* mutants was tested in 9-cm diameter Petri dishes containing CZ (with nitrate as the nitrogen source). Four mutants were placed, about 2 cm apart on each dish, and incubated at 28°C for 7–14 days. A complementary reaction was determined by evidence of developing dense aerial mycelial growth and sporulation at the zone of hyphal contact. If one or more mutants from a given isolate formed heterokaryons with one or more mutants from another isolate, the isolates were assigned to the same VCG

(Cotty 1994). Then, one *cnx* and one *nirA* mutant were selected from each group and paired with the remaining *niaD* mutants. Additionally all *cnx* and *nirA* mutants that did not fall into a VCG were also paired with the remaining *niaD* mutants and grown for three weeks. The diversity of VCGs of *A. flavus* isolates was calculated as the number of groups divided by the total number of isolates.

Statistical analysis

The quantities of the population density of *Aspergillus* species from section *Flavi* were subjected to analysis of variance (SAS 1999). The means of data for the provinces were separated at the 5% level of significance using Fisher's protected Least Significant Difference (LSD) test. Data from *A. flavus* strains (%) were arcsine transformed prior to analysis.

Results

Recovery of *Aspergillus* section *Flavi* from soil samples

Our results revealed there was a significant variation in populations of *Aspergillus* species from section *Flavi* in the pistachio soils of the studied regions ($F = 40.94$; $df = 2$; $p < 0.05$). The propagule densities of the *Aspergillus* species (section *Flavi*) on the surface of 5-cm soil were found to range from 2.26 to 2.54, 2.44 to 2.65, and 2.88 to 3.24 (based on $\log_{10}$ CFU/g soil) in Rafsanjan, Damghan, and Feyz-Abad, respectively (Table 1). Amongst 832 *Aspergillus* isolates obtained from a total of 225 pistachio soil samples collected from the three studied regions of Rafsanjan, Damghan, and Feyz-Abad, the predominant species were, respectively *A. flavus* (69.7, 65.3, and 57.9%), *A. parasiticus* (19.6, 25.4, and 29.3%) and *A. nomius* (10.7, 9.3, and 12.8%).

Production of sclerotia and aflatoxins by *A. flavus* isolates

The majority of the soil isolates of *A. flavus* produced no sclerotia (Semnan, n = 129, Kerman, n = 156 and Khorasan-e Razavi, n = 93) (Table 1). Based on the fluorescence on YES agar, 61.1%, 53.7%, and 60.4% of *A. flavus* isolates from Rafsanjan, Damghan, and Feyz-Abad were aflatoxigenic. It was revealed through TLC analysis of toxigenic *A. flavus* isolates that 29 (39.7%) and 37 (50.7%) isolates from Rafsanjan produced AFB1, and AFB1 + AFB2 in Rafsanjan while, 19 (27.9%) and 31 (45.6%) of the *A. flavus* isolates from Damghan produced AFB1, and AFB1 + + AFB2, respectively. In Rafsanjan, AFB1, and AFB1 + + AFB2 ranged from 274 to 553 ppb (mean value 393± ±17.11 ppb), and 394 to 3745 ppb, respectively, while AFB1, and AFB1 + AFB2 ranged from 257 to 392 ppb (mean value 285±13.18 ppb), and 415 to 1658 ppb, respectively, in Damghan.

Classification of VCGs and VCG diversity

Out of 130 toxigenic isolates of *A. flavus* (65 from each region), 248 and 225 *nit* mutants were generated from the 41 (63.1%) and 37 (56.9%) isolates from Kerman (Rafsanjan) and Semnan (Damghan) provinces, respectively. From Rafsanjan, 59.4% and 31.9% of *nit* mutants and from Damghan, 61.7% and 30% of *nit* mutants were *niaD* and *nirA* types, respectively, but only 8.7% and 8.3% of the *nit* mutants from Rafsanjan and Damghan were *cnx* type, respectively. Based on complementation between *nit* mutants, 16 and 20 VCGs were identified for isolates of Kerman (RS01 to RS16) and Semnan (DN01 to DN20) provinces, respectively (Table 2).

The non-producing sclerotia isolates of *A. flavus* from Rafsanjan (n = 33) and Damghan (n = 29) in 11 and 15 VCGs, respectively, all produced AFB1 in the AFB1 low quantity category (AFB1 ≤ 25 ppb). Isolates in VCG RS04 (three *A. flavus* S strain isolates) and DN06 (four *A. flavus* S strain isolates) produced AFB1 in the very high quantity category (AFB1 ≥ 2,500 ppb).

Complementation of *nit* mutants showed ca. 39% (16 VCGs) and 54% (20 VCGs) VCG diversity for Rafsanjan and Damghan, respectively, which are expressed as the ratio of VCGs to isolates examined.

Table 1. Characterization of *Aspergillus* species belonging to section *Flavi* from pistachio soil samples collected in 2012

Sampling location	Range, mean of propagule density [$\log_{10}$ CFU/g soil][a]	*A. flavus* frequency [%]	Sclerotia production [%]	*A. flavus* strains [%]	
				L strain	S strain
Rafsanjan	2.26–2.54 2.40 b	69.7 a	14.5 b	62.9 a	37.1 a
Damghan	2.44–2.65 2.55 ab	65.3 ab	39.5 a	79.7 b	20.3 a
Feyz-Abad	2.88–3.24 3.06 a	57.9 b	41.4 a	57.6 a	42.4 b

[a] $\log_{10}$ colony forming units *Aspergillus* species of section *Flavi* per g soil. Mean of three replicates determined by serial dilution and plating on Dichloran Rose-Bengal Chloramphenicol (DRBC) agar colonies were counted after a 5-day incubation

Values followed by the same letter in a given column do not differ significantly at the 95% confidence level using Fisher's Least Significant Difference (LSD) test

Table 2. Production of sclerotia and Vegetative Compatibility Groups (VCGs) of *A. flavus* isolates from pistachio orchards in the Kerman (Ker) and Semnan (Sem) provinces[e], Iran

Isolate	VCG	Sclerotial type	Isolate	VCG[d]	Sclerotial type
Ker102	RS03	none[a]	Sem101	DN06	none
Ker103	RS01	none	Sem102	DN03	S
Ker105	RS05	none	Sem103	DN07	none
Ker106	RS01	none	Sem105	DN05	none
Ker107	RS05	none	Sem107	DN03	S
Ker1016	RS03	none	Sem1010	DN08	S
Ker1012	RS01	none	Sem1011	DN02	none
Ker1013	RS06	L[b]	Sem1013	DN04	L
Ker1015	RS02	none	Sem1014	DN09	none
Ker1010	RS07	none	Sem1018	DN02	none
Ker1017	RS05	none	Sem1020	DN05	none
Ker1019	RS08	none	Sem1023	DN03	S
Ker1021	RS04	S[c]	Sem1024	DN10	none
Ker1022	RS02	none	Sem1025	DN05	none
Ker1024	RS09	none	Sem1028	DN11	S
Ker1025	RS05	none	Sem1033	DN06	none
Ker1026	RS10	L	Sem1029	DN12	none
Ker1029	RS03	none	Sem1031	DN02	none
Ker1030	RS05	none	Sem1035	DN13	S
Ker1031	RS03	none	Sem1040	DN14	none
Ker1032	RS06	L	Sem1030	DN04	L
Ker1034	RS01	none	Sem1047	DN02	none
Ker1036	RS03	none	Sem1041	DN15	L
Ker1037	RS11	none	Sem1044	DN06	none
Ker1038	RS04	S	Sem1053	DN16	S
Ker1039	RS02	none	Sem1055	DN17	none
Ker1040	RS12	L	Sem1039	DN03	S
Ker1041	RS02	none	Sem1051	DN06	none
Ker1042	RS13	none	Sem1054	DN02	none
Ker1043	RS05	none	Sem1048	DN01	S
Ker1045	RS02	none	Sem1057	DN18	S
Ker1046	RS14	none	Sem1042	DN03	S
Ker1049	RS01	none	Sem1059	DN19	none
Ker1050	RS05	none	Sem1060	DN02	none
Ker1051	RS04	S	Sem1064	DN20	L
Ker1052	RS02	none	Sem1062	DN01	S
Ker1058	RS15	none	Sem1061	DN02	none
Ker1059	RS01	none			
Ker1061	RS05	none			
Ker1063	RS16	L			
Ker1064	RS01	none			

[a] sclerotia were not produced under our cultural conditions; [b] L strain produce sclerotia > 400 μm in diameter; [c] S strain produce sclerotia < 400 μm in diameter (Cotty 1989); [d] VCG designation correlative with Novas and Cabral (2002); [e] location was the province where the isolate were collected

Discussion

The structure of *Aspergillus* section *Flavi* residing in pistachio soils was determined by analyzing 225 soil samples from 75 orchards located in three main pistachio production regions of Iran.

Populations of *Aspergillus* species belonging to the section *Flavi* differ by region in: population density, species composition as well as sclerotium and aflatoxin producing potentials. The lowest population of *Aspergillus* (section *Flavi*) was recorded in soil samples from Rafsanjan compared to the soil samples from Damghan and Feyz-Abad. Moradi *et al*. (2004) reported that populations of the *A. flavus* group were lower in Rafsanjan (center of Iran) than other parts of Iran. Mohammadi *et al*. (2009) indicated that *Flavi* section fungi had the lowest frequency of the *Aspergillus* population in the pistachio soils of the Kerman province. It seems that variations among the soil propagules of the *Flavi* species section in pistachio soils are due to different cultural practices and climatic conditions. The cold and dry climate and low monthly average temperature of Rafsanjan can be reasons for the low soil population density of the *Flavi* section species.

Five-hundred and eighty isolates (Damghan n = 203, Rafsanjan n = 218 and Feyz-Abad n = 159) were identified as *A. flavus* and as the most abundant species of *Aspergillus* section *Flavi* in pistachio orchards of Iran. Mohammadi *et al.* (2009) reported that *A. flavus*, *A. parasiticus*, and *A. niger* var. were predominant in the soils of Iranian pistachio orchards

All isolates of *A. flavus* were classified as either having none, small (less than 400 μm) or large sclerotia (greater than 400 μm). The percentage of sclerotium-producing isolates of *A. flavus* from Rafsanjan (14.5%) was much lower than from Damghan (39.5%) and Feyz--Abad (41.4%). Mirabolfathy *et al.* (2005) found that 42% of the soil isolates of *A. flavus* recovered from pistachio in Iran (the Kerman and Semnan provinces) could produce sclerotia. Jamali *et al.* (2012) found that 61% of *A. flavus* isolates were able to produce sclerotia. Hua *et al*. (2012) showed that approximately 50% of the *A. flavus* isolates from pistachio produced sclerotia.

The *A. flavus* L strain occurred more often than the strain S, in pistachio orchards of Iran. This is according to previous findings. Horn and Dorner (1999) knew that 70% of sclerotium-producing isolates of *A. flavus* from the soil were L strains. Abbas *et al.* (2005) reported that about 50% of the *A. flavus* isolates from corn, soil, and peanut in the Mississippi Delta produced large sclerotia. Barros *et al.* (2007) found that 73% of *A. flavus* sclerotial isolates collected from 30 fields of the peanut growing area were L strain. In soils of several areas of the southern United States. The L strain incidence average around 70% (Cotty 1992).

Screening of *A. flavus* isolates for aflatoxin producing ability revealed that 53.7–61.1% of *A. flavus* isolates produced aflatoxins. This variability can be used to develop a better understanding of the ecological niches to which strains may be used and to which strains are adapted. Variability in production of aflatoxins among *A. flavus* isolates has often been reported and discussed (Diener and Davis 1966; Clevstrom and Ljunggren 1985). Based on other studies, 41–95% of *A. flavus* could produce aflatoxins (Klich and Pitt 1988). Rahimi *et al.* (2007) found that 63.5% of *A. flavus* isolates recovered from pistachio orchards, produced aflatoxins. They showed that aflatoxin producing *Aspergillus* species from pistachio were more prevalent in the Kerman and Isfahan provinces. Jamali *et al.* (2012) determined the frequency of aflatoxigenic *A. flavus* isolates from soil samples of pistachio orchards, as 59%.

Based on the qualitative assay of AFBs by TLC, the aflatoxin values were categorized into four different classes, low AFB1 greater than zero and less than or equal to 25 ppb AFB1, medium AFB1 greater than 25 ppb and less than or equal to 250 ppb AFB1, high AFB1 greater than 250 ppb and less than 2,500 ppb, and very high AFB1 greater than 2,500 ppb. All non-producing sclerotia isolates were classified in toxin group 1 (AFB1 ≤ 25 ppb). Also, about 40% of *A. flavus* S strain isolates were capable of producing AFB1 greater than 2,500 ppb.

Our results revealed that there was an irregular relationship between sclerotium size and aflatoxin production in *A. flavus* soil isolates. All the S-type isolates but not all L-strains or non-producing sclerotia of *A. flavus* isolates were toxigenic. Haddadian *et al.* (2004) and Mirabolfathy *et al.* (2005) observed a high correlation between the production of aflatoxin and sclerotium by *A. flavus*, while Rahimi *et al.* (2007) reported that 10% of pistachio isolates of *A. flavus* produced sclerotia and there was not a direct relationship between sclerotium and aflatoxin production among *A. flavus* isolates. On average, L strain isolates produce much lower aflatoxin levels than S strain isolates, and also less sclerotia and more conidia (Saito *et a1.* 1986). A relationship between sclerotia and aflatoxins has been repeatedly suggested (Mehan and Chohan 1973; Sanchis *et al.* 1984; Horn *et al.* 1996).

Based on the growth pattern on CZ with nitrate, nitrite, ammonium tartrate or hypoxanthine, three types of *nit* mutants, *niaD*, *nirA*, and *cnx*, were generated. The higher frequency of *niaD* (59.4 and 61.7%) and *nirA* (31/9 and 30%) mutants than the *cnx* mutants (8.7 and 8.3%) in this experiment had also been reported by several authors (Cove 1976; Horn and Greene 1995; Heydarian *et al.* 2007; Hua *et al.* 2012). Thirty-one of 41 isolates from Kerman (Rafsanjan) and 23 of 37 isolates from Semnan (Damghan) provinces composed six multimember VCGs (2–8-member), respectively (RS01 to RS06, DN01 to DN06). The 10 and 14 remaining isolates of *A. flavus* from Kerman (Rafsanjan) and Semnan (Damghan) provinces could not be assigned to VCGs and so formed single-member VCGs (Table 2) because they were self-incompatible and did not complement with any other isolate. It is unknown whether these isolates are in singleton VCGs or in multiple different VCGs since they were not tested against each other. The VCGs RS01, RS02, and RS05 from Kerman province and VCG DN02 from Semnan province, were frequently isolated. The number of times a VCG is recovered from a given geographic region depends not only on its frequency in the *A. flavus* population and on the rate of reproduction of isolates from that VCG, but also on the number and spectrum of the samples examined (Les-

lie 1996). Some studies indicated that some VCGs were frequently isolated whereas others are rare (Horn and Greene 1995). Hua *et al.* (2012) identified 26 VCGs among 38 pistachio soil isolates of *A. flavus* (six multi-member, two or more, and 20 single-member groups). Some VCGs consisted of isolates with consistent sized sclerotia. From Kerman, RS04 and VCGs RS06, RS10, RS12, and RS16 had three and five isolates, respectively, which produced small and large sclerotia. Physiological and morphological traits are typically much more consistent within a VCG than within the species as a whole (Bayman and Cotty 1993).

Research had shown that high VCG members in small size-isolates of *A. flavus* indicate high genetic variability in fungal population (Bayman and Cotty 1990). According to some reports, *A. flavus* isolates in soils and on crops are composed of many VCGs (Horn and Greene 1995). Hua *et al.* (2012) and Horn and Greene (1995) reported 65% and 56% VCG diversity for the *A. flavus* populations from pistachio and peanuts, respectively. Furthermore, Papa (1986) found 69% VCG diversity for *A. flavus* isolates from corn kernels.

Even though *A. flavus* apparently does not have host specificity (St. Leger *et al*. 2000), distributions of different *A. flavus* lineages suggest that they may be adapted to specialized niches and exhibit competitive advantages in specific soils, hosts, regions, and seasons (Bock *et al*. 2004; Gaime-Garcia and Cotty 2006). The variability in morphology was commonly found among *A. flavus* isolates from different VCGs, but no variability among isolates from the same VCG. This was in agreement with Grubisha and Cotty (2010).

The data in the present study suggest that VCG diversity is not relatively limited, with an even distribution of isolates in the *A. flavus* population from pistachio soils in Iran. This shows that the *A. flavus* population is homogeneous and that isolates are genetically closely related. The *A. flavus* examined strains from two different geographical regions, all belong to the different VCGs, suggesting the presence of a relation between VCG and geographic origin. This indicates that the studied populations of *A. flavus* isolates from two regions were heterogenous and the strains were not genetically related. In this study, since our tester strains were chosen on a local or regional basis, they were effective and VCG analysis was accurate for population diversity of *A. flavus* from pistachio soils.

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