Journal of Nuts 5(1):15-22, 2014
ISSN: 2008-9937

Analysis of Soil Populations of *Aspergillus flavus* Link. from Pistachio Orchards in Iran for Vegetative Compatibility

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Received: 22 September 2013 Accepted: 18 November 2013

Abstract

One hundred-thirty soil isolates of *A. flavus* were collected from commercial pistachio orchards in two main production regions including Rafsanjan (Kerman Province, south-eastern of Iran) and Damghan (Semnan Province, central north of Iran) and assayed for vegetative compatibility groups (VCGs). The sixteen and twenty VCGs were identified for 41 and 37 nit-mutant producing isolates of *A. flavus*. The variability in morphology was found among *A. flavus* isolates from different VCGs, but no variability among isolates from the same VCG. VCG diversity for *A. flavus* isolates from Damghan and Rafsanjan was 54 and 39%, respectively. The high VCG members in small size-isolates of *A. flavus* from Damghan indicated high genetic variability in fungal population. Because of the small number of sclerotium-producing isolates of *A. flavus*, we did not determine the relationships between sclerotium production with VCG in two pistachio production regions. This study was the first to determine the strain and VCG diversity of soil isolates of *A. flavus* from pistachio orchards in Iran.

Keywords: *Aspergillus flavus*, Damghan, Pistachio, Rafsanjan, Vegetative compatibility.

Introduction

Pistachio (*Pistacia vera* L.) is one of the most important horticultural products of Iran and has special economic importance. Iran is the largest producer of pistachio in the world, accounting for about two-thirds of global planted area and slightly more than one-half of world production in recent years. In Iran, pistachio is cultivated on 380000 has with 70% bearing and 30% non-bearing orchards and production of about 300000 tonnes/year (Sedaghat, 2011). Historically, pistachio has been grown in monoculture in the two major pistachio production regions including Kerman and Semnan provinces. Rafsanjan is the largest pistachio-producing area in Kerman province, with about 51 per cent of pistachio farmers producing 44 percent of the pistachio output (Ministry of Jihad-e-Agriculture, 2004). Fungi in *Aspergillus* section Flavi, especially *A. flavus* Link, *A. parasiticus* Speare and *A. nomius* (Kurtzman), Horn and Hesseltine are responsible for producing aflatoxins, which are potent carcinogenic mycotoxins. *A. flavus* is the most commonly isolated causal agent of aflatoxin contamination of many crops, including maize, cotton, peanut, and tree nuts (Schroeder and Buller, 1973; Bayman and Cotty, 1993; Diener *et al*., 1987; Robens and Brown, 2004). The most toxic form of aflatoxin is aflatoxin B_1 (Diener *et al*., 1987). In Iran, contamination due to *Aspergillus* species and their toxins is the most serious problem in pistachio production, consumption and export processing (Emami *et al*., 1977; Mojthahedi *et al*., 1978; Thomson and Mehdy, 1978; Moradi and Javanshah, 2005). Individual *A. flavus* isolates vary in many characteristics, including aflatoxin-producing ability, production and morphology of sclerotia, and sporulation (Schroeder and Buller, 1973; Cotty, 1989; Orum *et al*., 1997; Geiser *et al*., 2000; Horn, 2003; Abbas *et al*., 2005; Giorni *et al*., 2007; Rodrigues *et al*., 2007; Atehnkeng *et al*., 2008). *A. flavus* Link has no known sexual stage; consequently, most studies on its genetic variability have been evaluated mainly by characterizing isolates based on vegetative compatibility (VC) a process in which vegetatively compatible hyphae fuse to form heterokaryons. Complementary nitrate-nonutilizing (nit) mutants are commonly used to identify compatible isolates (Bayman and Cotty, 1991*a*, *b*). It was assumed that the VC was controlled by multiple gene loci, making it a useful marker to identify the genetic diversity of populations (Leslie 1993, 1996). Therefore, in *A. flavus*, the VC of two isolates of different origins indicates a degree of genetic relatedness (Leslie, 1993 and 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). The present study was conducted to determine for the first time the VCG diversity of *A. flavus* soil isolates collected from naturally infected soils of pistachio orchards in the Kerman and Semnan provinces of Iran.

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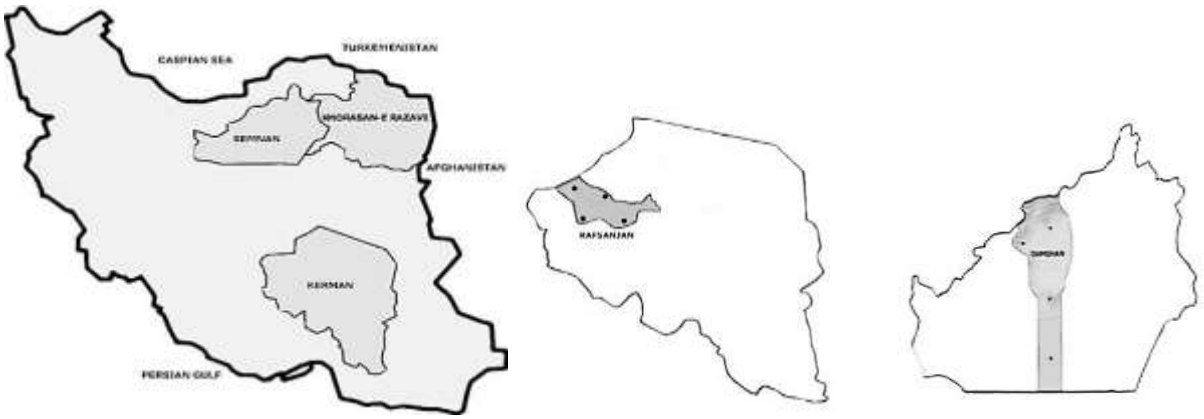
Materials and Methods

Collection and Preparation of Soil Samples

In June 2012, soil samples were collected randomly from 75 commercial pistachio orchards (1-2 ha) in two main pistachio production regions included Rafsanjan (Kerman province, southeastern of Iran) and Damghan (Semnan province, central north of Iran). 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 orchard in three regions as shown in Fig. 1. Cores from each orchard were bulked and mixed as a single sample (approximately 2 kg) immediately after collection. Then, samples were sieved through a 2-mm mesh screen, and specimens of 200 g collected on a surface-disinfested plastic container, air dried and stored at 4°C.

Isolation and Identification of A. flavus Isolates

Ten grams of each combined soil sample was placed in a 250-ml flask containing 90 ml of autoclaved 0.1% agar in water and the suspension was stirred for 5 min. The mixture was diluted and aliquots of 0.1 ml were spread (in triplicate) onto individual 9-cm Petri dishes containing *Aspergillus flavus-parasiticus* agar (AFPA), after which they were kept at 28°C for 3-5 days (Gourama and Bourama, 1995). Identification of *A. flavus* was based on morphological criteria of Pitt and Hocking (1997) and Klich (2002). For micromorphological observations, the isolates were examined under the microscope (10x, 40x and 1000x magnifications).



A **B**

Fig. 1. Map of the Iran Islamic Republic showing Kerman and Semnan pistachio producing provinces from which *Aspergillus* section Flavisoil isolates were obtained. (A) Rafsanjan, Kerman province; (B) Damghan, Semnan province.

Aflatoxin producing Ability of Isolates

Detection by Flourescence on Yeast extracts sucrose Agar (YES) Medium

Five-day-old PDA cultures of *A. flavus* isolates were plated on YES agar 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). Subsequently, plates were examined under UV light (365 nm) for detection of fluorescence. 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 aflatoxin B_1 (AFB1) based on a thin-layer chromatography (TLC) method (Chang, 2004). The AFB1 was detected as an intense blue fluorescence spot with an R_f value of 0.8. The AFB1 was quantified directly on TLC plates with a scanning densitometer (TLC Scanner 3; Camag Scientific Inc, Switzerland) (Pons, 1966). Each isolate was subjected to three replications. Two toxigenic and atoxigenic *A. flavus* strains includedK49 (NRRL 30797) and F3W4 (NRRL 30796) were used as positive and negative controls in all experiments, respectively.

SclerotiumProdudion

Sclerotium production was recorded for single-spore cultures after three to four weeks of dark incubation at 30 °C on CzapeckDox Agar (CDA) 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 sclerotia size; L strain isolates produced very few sclerotia larger than 400 µm in diameter, and S strain isolates produced numerous sclerotia with diameters under 400 µm (Cotty 1989; 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

Nit-mutants of each wild type isolate were generated on CDA supplemented with potassium chlorate (30 g l^{-1}).

Four plates of each chlorate medium (sole nitrogen source = NO_3) were inoculated with mycelial plugs of each isolate and incubated at 30 °C in the dark for at 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-CDA 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 gl^{-1}), ammonium tartrate (1 gl^{-1}) or sodium nitrite (0.5 gl^{-1}) medium (Cove, 1976; Papa, 1986). Spores of nit-mutants were inoculated on PDA and incubated at 28°C for two days. An agar plug of each wild-type isolate growing on CDA was also included as a wild-type control for each experiment.

Complementation Tests

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 *nirA* mutants (if not *niaD* and *nirA* mutants or *niaD* and *cnx*) of different isolates. 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 nocomplementation of mutation. Complementation between nit-mutants was tested in 9-cm diameter Petri dishes containing CDA 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 vegetative compatibility group (VCG, Cotty 1994). Then, one *cnx* and one *nirA* mutant was 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. Diversity of VCGs of *A. flavus* isolates was calculated as the number of groups divided by the total number of isolates.

Results

Out of one-hundred thirty aflatoxigenic isolates of *A. flavus* (65 from each region), 248 and 225 nit-mutants generated from 41 (63.1%) and 37 (56.9%) soil isolates from Rafsanjan and Damghan, respectively. 59.4% and 31.9% of nit-mutants from Rafsanjan and 61.7% and 30% of nit-mutants from Damghan 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 (Fig.2).

Based on complementation between nit-mutants, 16 and 20 VCGs were identified from soil isolates of Rafsanjan and Damghan regions (Fig. 3). The thirty-one of 41 isolates from Rafsanjan and 23 of 37 isolates from Damghan composed 6 multi-member VCGs (2-8-member), respectively (RS-1 to RS-6, DN-1 to DN-6). The ten and 14 remaining isolates of *A. flavus* from Rafsanjan and Damghan could not be assigned to VCGs and so formed single-member VCGs (Table 1).

Our results revealed that VCGs consisted of isolates with consistent size of sclerotia. VCGs RS-4 and RS-6, RS-10, RS-12, RS-16 from Rafsanjan consisted of three and five isolates which produced small and large sclerotia, respectively. 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 low quantity (AFB1 $\leq$ 25 ppb). Isolates in VCGs RS-4 (three *A. flavus* S strain) and DN-6 (four *A. flavus* S strain) produced AFB1 in very high quantity (AFB1 $\geq$ 2500 ppb).

VCGs diversity for isolates of *A. flavus* from Rafsanjan (16 VCGs) and Damghan (20 VCGs) was about 39% and 54%, respectively, which are expressed as the ratio of VCGs to isolates examined

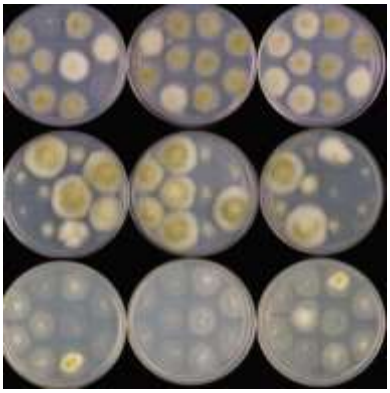


Fig. 2. Growth of nit-mutant phenotypes of *A. flavus* on the CDA containing different nitrogen sources. Rows from top to bottom: Hypoxanthin, nitrite and nitrate, respectively.

Fig. 3. The complementary reaction between A. flavus isolates by developing dense aerial mycelial growth and sporulation at the zone of hyphal contact.

Discussion

Based on the growth pattern on CDA with nitrate, nitrite, ammonium tartrate or hypoxanthine, three types of nit-mutants, *nia D*, *nirA* and *cnx*, were generated according to Cove (1976). The higher frequency of *nia D* and *nir A* mutants than the *cnx* mutants in this experiment had also been reported by several authors (Bayman and Cotty, 1991; Horn and Greene, 1995; Heydarian *et al.*, 2007; Hua *et al.*, 2012).

The VCGs RS-1, RS-2 and RS-5 from Rafsanjan and VCG DN-2 from Damghan were frequently isolated. The single-member VCGs were self-incompatible and did not complement with any other isolate. It is unknown whether these isolates are in single VCG groups or in multiple different VCGs because they were not tested against each other. The number of VCGs varied among fields and crops (Bayman and Cotty, 1991; Cotty, 1997; Horn and Dorner, 1999; Pildain *et al*., 2004; Atehnkeng *et al.*, 2008; Donner *et al.*, 2009). The number of VCGs recovered from a given geographic region was not dependent on its frequency in the *A. flavus* population and rate of reproduction of isolates from that VCG, but on the number and spectrum of the samples examined (Leslie, 1996). Some of studies indicated that some VCGs were frequently isolated, whereas the others were rare (Bayman and Cotty, 1991; Horn and Greene, 1995; Pildain *et al*., 2004). Hua *et al*., (2012) identified 26 VCGs (six multi-memberand twenty single-member groups) among thirty-eight pistachio soil isolates of *A. flavus* (Table 1).

Table 1. Production of sclerotia and vegetative compatibility groups of *A. flavus*isolates from pistachio orchards in Kerman (Ker) and Semnan (Sem) provinces [c], Iran.

Isolate	VCG	Sclerotial type[a,b]	Isolate	VCG	Sclerotial type[a,b]
Ker102	RS03	none	Sem101	DN06	none
Ker103	RS 01	none	Sem102	DN03	S
Ker105	RS 05	none	Sem103	DN07	none
Ker106	RS 01	none	Sem105	DN05	none

Continue of Table1

Ker107	RS 05	none	Sem107	DN03	S
Ker1016	RS 03	none	Sem1010	DN08	S
Ker1012	RS 01	none	Sem1011	DN02	none
Ker1013	RS 06	L	Sem1013	DN04	L
Ker1015	RS 02	none	Sem1014	DN09	none
Ker1010	RS 07	none	Sem1018	DN02	none
Ker1017	RS 05	none	Sem1020	DN05	none
Ker1019	RS 08	none	Sem1023	DN03	S
Ker1021	RS 04	S	Sem1024	DN10	none
Ker1022	RS 02	none	Sem1025	DN05	none
Ker1024	RS 09	none	Sem1028	DN11	S
Ker1025	RS 05	none	Sem1033	DN06	none
Ker1026	RS 10	L	Sem1029	DN12	none
Ker1029	RS 03	none	Sem1031	DN02	none
Ker1030	RS 05	none	Sem1035	DN13	S
Ker1031	RS 03	none	Sem1040	DN14	none
Ker1032	RS 06	L	Sem1030	DN04	L
Ker1034	RS 01	none	Sem1047	DN02	none
Ker1036	RS 03	none	Sem1041	DN15	L
Ker1037	RS 11	none	Sem1044	DN06	none
Ker1038	RS 04	S	Sem1053	DN16	S
Ker1039	RS 02	none	Sem1055	DN17	none
Ker1040	RS 12	L	Sem1039	DN03	S
Ker1041	RS 02	none	Sem1051	DN06	none
Ker1042	RS 13	None	Sem1054	DN02	none
Ker1043	RS 05	none	Sem1048	DN01	S
Ker1045	RS 02	none	Sem1057	DN18	S
Ker1046	RS 14	none	Sem1042	DN03	S
Ker1049	RS 01	none	Sem1059	DN19	none

Continue of Table1

Ker1050	RS 05	none	Sem1060	DN02	none
Ker1051	RS 04	S	Sem1064	DN20	L
Ker1052	RS 02	none	Sem1062	DN01	S
Ker1058	RS 15	none	Sem1061	DN02	none
Ker1059	RS 01	none			
Ker1061	RS 05	none			
Ker1063	RS 16	L			
Ker1064	RS 01	none			

a None = sclerotia were not produced under our cultural conditions.
b L strain produce sclerotia>400 µm in diameter, S strain produce sclerotia< 400 µm in diameter (Cotty, 1989).

The results of these experiments showed that variability in morphology was commonly found among *A. flavus* isolates from different VCGs, but that there was no variability among isolates from the same VCG. This was in accordance to Grubisha and Cotty (2010). Phenotypic characteristics (i.e., size of sclerotia and aflatoxin-producing ability) are usually conserved within VCGs (Bayman and Cotty, 1993; Horn *et al*., 1996; Novas and Cabral, 2002; Pildain *et al.,* 2004).

Research has shown that the high VCG members in small size-isolates of *A. flavus* have high genetic variability in fungal populations (Bayman and Cotty, 1990). According to some reports, *A. flavus* isolates in soils and on crops are composed of many VCGs (Bayman and Cotty 1991; Horn and Greene, 1995; Pildain *et al*., 2004). 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 (Orum *et al.*, 1997; Bock *et al*., 2004; Gaime-Garcia and Cotty, 2006).

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

This is the first investigation on the strain isolate and VCG diversity of *A. flavus* from pistachio soils in Iran.

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