IDENTIFICATION AND PREVALENCE OF *ASPERGILLUS* SPECIES IN SOILS OF FARS AND KERMAN PROVINCES OF IRAN AND EVALUATION OF THEIR AFLATOXIN PRODUCTION

A.H. MOHAMMADI, Z. BANIHASHEMI* and M. HAGHDEL

Department of Plant Protection, College of Agriculture, Shiraz University and Iranian Pistachio Research Institute

Received: 16.09.2008 Accepted: 27.05.2009

Abstract

Aspergillus species are major causes of pre- and post-harvest degradation of agricultural products. During 2004-07, the presence and population of *Aspergillus* species were studied in various fields and orchards of Fars and Kerman Provinces. Soil samples were collected from 0-40 cm depth. Isolates were recovered from soil using modified Czapek, AFPA and SPDA media. Based on macroscopic and microscopic criteria, five sections (*Flavi*, *Nigri*, *Circumdati*, *Fumigati*, *Terrei*) and 13 following species were identified: *A. alliaceus*, *A. auricomus*, *A. carbonarius**, *A. flavus*, *A. fumigatus*, *A. japonicus* var. *japonicus*, *A. niger* var. *niger*, *A. ochraceus*, *A. oryzae**, *A. parasiticus*, *A. sclerotiorum*, *A. sojae* and *A. terreus*. The species with asterisk are new to Iran. SPDA and AFPA were more efficient for isolation and enumeration of *Aspergillus* colonies especially aflatoxigenic

* Corresponding author (E-mail: ziabani@shirazu.ac.ir)

aspergilli. In Fars Province: *A. flavus*, *A. parasiticus*, *A. niger* var. *A. niger*, and in Kerman Province: *A. flavus*, *A. parasiticus* and *A. niger* var. *A. niger* were predominant, respectively. In Fars, pistachio orchard and uncultivated soils showed the highest and lowest *Aspergillus* population, respectively. In Kerman Province, soils collected from Rafsanjan and Zarand had the highest and lowest *Aspergillus* population, respectively. Out of 20 and 52 isolates of *A. flavus* obtained from soils in Fars and Kerman Provinces, 14 and 33 isolates produced aflatoxin, respectively.

Key words: Population density, Pistachio, Mycotoxin

Introduction

Aspergillus is a hyphomycetous genus with approximately 250 recognized species (GEISER *et al*. 2007). Members of the genus inhabit various niches, but mainly occur as saprophytes in soils, stored food and feed (DOMSCH *et al.* 1980, KLICH & PITT 1988). The genus was primarily described by MICHELI in 1729. GAMS & SAMSON (1985) have considered LINK (1809) as the validating author (KLICH 2002). After 1965, teleomorph names for sexual states of *Aspergillus* have become firmly established (MALLOCH & CAIN 1972, WILEY & SIMMONS 1973). At present, the genus has been divided into seven subgenera, each containing one or more sections to fit the Botanical Code (KLICH & PITT 1988a, KLICH 2002). These sections are equal to groups established by RAPER & FENNELL (1965). KLICH (2002) has questioned the validity of some groups and the species involved.

Although the majority of aspergilli are saprotrophs, some are parasitic on insects, plants and animals. Aspergilli are rarely directly pathogenic to plants in fields. *Aspergillus flavus* Link and *A. niger* Van Tieghem have been reported as seedling pathogen and crown rot of peanut, respectively (KLICH 2002). *Aspergillus* species are one of the major causes of pre- and post- harvest degradation of agricultural products. The greatest positive economic impact of aspergilli has been in the exploitation of the enzymes and acids produced by a number of species. For example, amylase and citric acid are two of the most important products produced

by various *Aspergillus* species (KLICH 2002). The negative economic impact of aspergilli involves the production of harmful mycotoxins by some species. Mycotoxins are products of secondary metabolism which are harmful to humans and/or animals. Aflatoxin is the most economically important mycotoxin throughout the world that is produced by *A. flavus*, *A. parasiticus* Speare and *A. nomius* Kurtzman, B.W. Horn & Hesseltine. The most toxic form of aflatoxin is aflatoxin B1 (DIENER *et al.* 1987). Other species of *Aspergillus* produce various mycotoxins (KLICH 2002).

Thirteen species of *Aspergillus* have been reported from Iran by MOJTAHEDI *et al.* (1979). They reported *A. niger* as the predominant species on pistachio kernels. HEIDARIAN *et al*. (2005) also isolated *A. phoenicis* (Corda) Thom & Currie and *A. puniceus* Known-Chung & Fennel from pistachio kernels in Iran. Eleven species of *Aspergillus* were isolated by RAHIMI *et al.* (2007) from pistachio nuts and litter.

Materials and Methods

During 2004-06, the frequency of occurrence of *Aspergillus* species was investigated in various fields and orchards in Fars and Kerman Provinces. In each orchard/field (= plot), four soil samples (500 g) taken from 0-40 cm depth and were pooled as one samples per orchard/field after passing through 2 mm, 40 and 60 mesh sieves in Fars Province. Likewise, four soil samples in each orchard were collected from eight sampling locations (four orchards per each) in Rafsanjan, Sirjan and Zarand of Kerman Province. In each plot, four samples were mixed together and finally, four subsamples (10 g) were suspended in 90 ml of 0.1% water-agar containing 100 ppm NPX (STEINER & WATSON 1965) and shaken for 30 min. Four replicates (0.5 ml) of diluted suspension (10^{-2}, 10^{-3}) in each subsample were flooded on the plates containing Czapek (RAPER & FENNEL 1965) with 250 ppm ampicillin. Plates were incubated at 25-27º C for 3-5 days and recovered colonies were purified using single-spore method. In this study, two other media including AFPA (*Aspergillus flavus-parasiticus* agar) (GOURAMA & BULERMAN 1995), and SPDA (sucrose-pepton-dichloran agar) (DHINGRA & SINCLAIR 1985) were also used for isolation and enumeration of toxigenic *Aspergillus* species from soil.

For identification of *Aspergillus* species, four media and two temperatures were applied: CYA (PITT 1973) at 25 and 37º C, CYA20S (CYA+20% sucrose) and MEA (KLICH & PITT 1988a) at 25º C. Inoculated plates were incubated in the dark for seven day. Isolates were examined both macroscopically and microscopically. For macroscopic examination, colony diameter, exudates and soluble pigment, surface and reverse colors and texture were considered. Diameter, shape, color and surface texture of conidia, phialides, metulae, vesicle, stipes, sclerotia and seriation (uniseriate or biseriate) were examined using microscope. All isolates were identified at species level (KLICH & PITT 1988a, KLICH 2002). In Fars Province, 20 isolates of *A. flavus* were randomly selected and examined for production of aflatoxin B1 and B2. In Kerman Province, 20, 16 and 16 isolates (2 isolates from orchards) of *A. flavus* from Rafsanjan, Sirjan and Zarand were also evaluated for their ability to produce aflatoxins, respectively, using WEI & JONG (1986) method. Ten ml of sterile distilled water was added to Petri dishes containing seven-day-old colonies of *A. flavus* and spore suspension was added to 500 ml flask containing 50 g of sterilized rice flour at 25º C. The flasks were incubated at 25° C in darkness for two weeks. To extract aflatoxin, four g of NaCl, 250 ml methanol 55 % and 100 ml n-hexan were added to the flasks. After 45 min shaking, the mixture was passed through the Whatman paper (No. 4). Fifty ml of the suspension was added to the same amount of chloroform and mixed thoroughly. The chloroform phase was transferred into new flasks and evaporated in rotary (40° C) with vacuum condition. Samples were dissolved in 200 µl of banzen-acetonitril (98:2) and 5 µl were spotted on silicagel TLC plate (60F254 Merck). Standard aflatoxin samples were spotted on the edges of the plates. Plates were then placed in developing tank containing developing solvent (chloroform-aceton, 9:1 v/v) for 15-20 min. Plates were air-dried in dark cabinet. Samples were quantified by comparing to standard aflatoxin spots using CAMAG TLC Scanner 3.

Results

All species of *Aspergillus* and other soil fungi such as *Penicillum, Rhizopus, Fusarium* and *Rhizoctonia* were easily isolated on Czapek medium; nevertheless discrimination of *Aspergillus* species was difficult on the medium. Growth and

development of soil fungi except *Aspergillus* species was very low on SPDA and AFPA. *A. flavus* and *A. parasiticus* were easily identified on Czapek by the production of yellow to olive green spores three days after inoculation on AFPA and SPDA media. *A. niger* colonies developed dark brown to black conidia after three days and the reverse of colonies became yellow, but not orange. *A. ochraceus* grew slowly at 30° C and yellow colors appeared after three days. Applying these media shortened the time required for isolation and identification of potentially aflatoxigenic *Aspergillus* species. SPDA and AFPA displayed lower population of other fungi than Czapek medium. SPDA also showed lower population of soil bacteria compared with AFPA and Czapek.

In the present study, 340 isolates of *Aspergillus* were recovered from soils in Fars and Kerman Provinces from which 280 isolates were selected and identified to species. Based on macroscopic and microscopic examinations, 13 different species were identified in *Aspergillus* subgenera *Circumdati*, *Fumigati* and *Nidulantes* (Table 1).

Table 1. Identification of the recovered isolates in *Aspergillus* subgenera *Circumdati*, *Fumigati* and *Nidulantes* in Fars and Kerman Provinces

Subgenus	Section	Soil	Species	Soil
Circumdati	*Circumdati*	88.23, 100 [a]	*A. alliaceus*	41.18, 55.26 [b]
			A. auricomus	29.41, 44.74
			A. ochraceus	64.71, 60.53
			A. sclerotiorum	29.41, 44.74
	Flavi	100, 100	*A. flavus*	100, 92.11
			A. oryzae	52.94, 63.16
			A. parasiticus	94.12, 86.84
			A. sojae	41.18, 50
	Nigri	100, 100	*A. carbonarius*	58.82, 81.58
			A. japonicus var. *japonicus*	41.18, 44.74
			A. niger var. *niger*	100, 94.74
Fumigati	*Fumigati*	76.47, 65.79	*A. fumigatus*	76.47, 65.79
Nidulantes	*Terrei*	23.53, 36.84	*A. terreus*	23.53, 36.84

[a,b] Percentage of soils in which sections and species of *Aspergillus* were isolated in Fars and Kerman Provinces, respectively.

Description of selected *Aspergillus* species

***Aspergillus carbonarius* (Bain.) Thom, J. Agric. Res. 7: 12** (Fig. 1)

Thirty-one isolates of the species were used for identification.

Macroscopic characteristics**:** Colony diameter on CYA, 78 mm; conidia black and mycelium white; reverse pale yellow; sclerotia were not observed. Colony diameter on CYA20S, 85 mm, other characters similar to those on CYA except the stipes that was longer than those on CYA with usually yellow reverse. Colony diameter on MEA 55 mm, conidia black and uncrowded; mycelium white; reverse uncolored, Sclerotia were not observed. Colony diameter 45 mm at 37º C.

Microscopic characteristics: Conidial heads radiate; stipes thick-walled, finely roughened and colorless; vesicles globose 72-87 µm; biseriate; phialides 10-16 × 5-7 µm; metulae over the entire vesicle surface 23-45 × 6-14 µm; conidia very large 6-10 µm in diameter, walls extremely rough with tubercles and globose.

***Aspergillus oryzae* (Ahlburg) Cohn, Jahresbericht Schles. Ges. Vaterl. Kult. 61: 226** (Fig. 2)

Twenty isolates of the species were used for identification

Macroscopic characteristics: Colony diameter on CYA 63 mm; conidia olive to brown and mycelium white; reverse uncolored; dark sclerotia occasionally formed; colony texture generally floccose. Colony diameter on CYA20S 67 mm; conidia olive brown to olive yellow, in some isolates conidia were abundant; mycelium white, dense; reverse uncolored and colonies were more floccose compared to other media. Colony diameter on MEA 58 mm; conidia olive or occasionally green; mycelium white, not as dense as on CYA; reverse colorless; colony floccose. Colony diameter 70 mm at 37º C.

Microscopic characteristics: Conidial heads radiate; stipes colorless, walls finely rough; vesicles subglobose 15-62 µm; aspergilla in some isolates predominantly biseriate, other predominantly uniseriate; phialides 7-13 × 2.5-4.5 µm; metulae cover more surface of the vesicle surface measuring 6-15 × 3.5-5 µm; conidia globose, 3.5-5.5 µm in diameter, with smooth to finely roughened walls.

***Aspergillus sclerotiorum* Huber, Phytopathology 23: 306** (Fig. 3)

Twenty four isolates of the species were used for identification

Macroscopic characteristics: Colony diameter on CYA 55 mm, conidia light yellow, mycelium white, reverse brownish yellow. Colony diameter on CYA20S 70 mm, characters similar to those on CYA except reverse that was lake red. Colony diameter on MEA 55 mm, conidia light yellow, mycelium white, not dense, reverse light yellow. Colony diameter 26 mm at 37º C.

Microscopic characteristics: Conidial heads radiate; stipes rough-walled, yellow; vesicles spherical 25.5-46 µm; biseriate; phialides 5-7.5 × 2.5-4 µm; metulae usually cover the entire vesicle surface, 11-14 × 3-4 µm; conidia spherical 2-4 µm in diameter, with smooth to finely roughened walls.

Flavi and *Nigri* sections were found in 100% whereas *Terrei* in 23.53% of collected soils in Fars Province (Table 1). In Kerman Province, *Circumdati*, *Flavi* and *Nigri* sections were recovered from 100% of soils and *Terrei* in 36.84% of the soils (Table 1). Among the *Aspergillus* species, *A. flavus* and *A. niger* var. *niger* were found in all collected soils of Fars Province whereas in Kerman Province, *A. niger* var. *niger* and *A. flavus* were recovered from 94.74 and 92.11% of the soils. *A. terreus* had the lowest frequency in soil samples of both provinces (Table 1).

Frequency of occurrence (%) of the *Aspergillus* sections are shown in Table 2. For each case, *Flavi* and *Terrei* sections had the highest and lowest frequency, respectively (Table 2). *A. flavus* (21.35%) and *A. parasiticus* (19.45%) in *Flavi* section and *A. niger* var. *niger* (18.97%) in *Nigri* section were the most prevalent species in Fars Province. Other species including *A. oryzae* (7.72%), *A. carbonarius* (6.86%), *A. fumigatus* Fres. (5.65%), *A. sojae* (4.76%), *A. ochraceus* Wilhelm (4.63%), *A. sclerotiorum* (3.30%), *A. japonicus* Saito var. *japonicus* (2.26%), *A. auricomus* (2.02%), *A. alliaceus* (1.75%) and *A. terreus* Thom (1.29%) showed low frequency in various fields and orchards. *Aspergillus* population was variable in various soils of Fars Province. Pistachio orchard soils with 5280 and uncultivated soils with less than 1000 propagules/g of soil, showed the highest and lowest *Aspergillus* population, respectively.

Table 2. Colony counts and frequency of occurrence (%) of *Aspergillus* sections in field and orchard soils of Fars Province

Substrate (Location)	Sections of *Aspergillus*				
	Flavi	***Nigri***	***Circumdati***	***Fumigati***	***Terrei***
Pistachio (Neiriz)	2550[a] (5.8)[b]	1910 (4.4)	570 (1.3)	250 (0.6)	0 (0.0)
Cotton (Kheer-Darab)	2185 (5)	900 (2.1)	215 (0.5)	200 (0.5)	0 (0.0)
Sugarbeet (Rooniz)	1800 (4.1)	800 (1.8)	330 (0.8)	180 (0.4)	360 (0.8)
Corn (Saadatshar-Bajgah-Abadeh)	1563 (3.6)	1023 (2.3)	437 (1.0)	160 (0.4)	33 (0.4)
Grapevine (Kavar)	1470 (3.4)	400 (0.9)	850 (1.9)	160 (0.4)	0 (0.0)
Alfalfa (Bajgah-Abadeh)	1355 (3.1)	465 (1.1)	400 (0.9)	165 (0.4)	70 (0.2)
Citrus (Darab)	1370 (3.1)	850 (1.9)	150 (0.3)	250 (0.6)	0 (0.0)
Sesame (Kheer)	1520 (3.5)	600 (1.4)	350 (0.8)	0 (0.0)	100 (0.2)
Tomato (Neiriz)	1250 (2.9)	690 (1.6)	500 (1.1)	100 (0.2)	0 (0.0)
Fig (Estahban)	1140 (2.6)	760 (1.7)	280 (0.6)	260 (0.6)	0 (0.0)
Melon (Maharloo)	1590 (3.6)	400 (0.9)	250 (0.6)	200 (0.5)	0 (0.0)
Almond (Maharloo)	1330 (3.1)	560 (1.3)	250 (0.6)	250 (0.6)	0 (0.0)

Substrate (Location)	Sections of *Aspergillus*				
	Flavi	***Nigri***	***Circumdati***	***Fumigati***	***Terrei***
Potato (Safashahr)	950 (2.2)	740 (1.7)	250 (0.6)	200 (0.5)	0 (0.0)
Wheat (Safashahr-Sepidian)	1050 (2.4)	475 (1.1)	175 (0.4)	100 (0.2)	0 (0.0)
Ornamental (Shiraz-Bajgah)	760 (1.7)	610 (1.4)	110 (0.3)	0 (0.0)	0 (0.0)
Apple (Bajgah)	900 (2.1)	750 (1.7)	0 (0.0)	0 (0.0)	0 (0.0)
Uncultivated soil (Kavar-Abadeh-Safashahr-Sepidan)	574 (1.3)	364 (0.8)	0 (0.0)	0 (0.0)	0 (0.0)

[a] Data are the means of soil samples of four plots.
[b] Frequency of occurrence (%) of the *Aspergillus* sections are shown in parentheses. The data are based on the proportion of the total isolates of recovered *Aspergillus* (175166).

Flavi and *Terrei* sections showed the highest (44.97%) and lowest (2.69%) frequency of occurrence in Kerman Province, respectively (Table 3). *A. niger* var. *niger* (22.43%) and *A. carbonarius* (10.38%) in *Nigri* and *A. flavus* (18.42%) and *A. parasiticus* (18.18%) in *Flavi* sections dominated in various soils. *A. oryzae* (4.37%), *A. fumigatus* (4.05%), *A. sclerotiorum* (3.78%), *A. ochraceus* (3.76%), *A. sojae* (3.61%), *A. alliaceus* (3.10%), *A. auricomus* (2.73%), *A. terreus* (2.69%) and *A. japonicus* var. *japonicus* (2.11%) showed low population. Results also showed that soils collected from Rafsanjan and Zarand had the highest and lowest *Aspergillus* population, respectively. *Aspergillus* population in soils with routine cultural practices collected from Rafsanjan, Sirjan and Zarand was 4860, 4332 and 3777 propagules/g soil, respectively (Table 3). Pistachio soils with low manure application in Rafsanjan, Sirjan and Zarand had lower *Aspergillus* population compared to other pistachio soils (1784, 1326 and 1269 propagules/g soil). In these soils, no manure or low amounts of manure had been used by the growers.

In Fars Province, 14 (70%) isolates out of 20 *A. flavus* isolates produced aflatoxin, 13 isolates producing AFB1+AFB2 and one isolate producing AFB1 (Table 4). *A. flavus* isolates of corn (Bajgah), alfalfa (Abadeh), wheat (Saadatshahr), uncultivated soil (Sepidan) and citrus (Darab) produced no aflatoxin. Isolates capability in aflatoxins production was inconsistent and varied from 4.13 to 625.32 ppb for AFB1 and 1.54 to 17.89 ppb for AFB2.

In Kerman Province, 17 and 16 isolates out of 52 isolates of *A. flavus* produced AFB1 + AFB2 and AFB1, respectively (Table 3). Fifteen (75%) isolates out of 20 *A. flavus* from Rafsanjan, produced aflatoxins. Among these, seven isolates (35%) produced 353-226 ppb AFB1 and eight (40%) AFB1 + AFB2 ranging from 382 to 4536 and 15.6 to 225 ppb.

From 16 isolates of *A. flavus* from Sirjan, four isolates (25%) produced 256-1254 ppb AFB1 and five isolates (31.25%) AFB1 + AFB2 varying from 514 to 2346 and 49 to 563.2 ppb (Table 4).

Table 3. Colony counts and frequency of occurrence (%) of *Aspergillus* sections in pistachio orchard soils with routine and low manure application in Kerman Province

Location	Sections of *Aspergillus*				
	Flavi	***Nigri***	***Circumdati***	***Fumigati***	***Terrei***
Orchard soils with routine manure application					
Rafsanjan	2128[a] (13.6)[b]	1508 (9.7)	701 (4.5)	283 (1.8)	240 (1.5)
Sirjan	1940 (12.4)	1443 (9.3)	651 (4.2)	159 (1)	139 (0. 9)
Zarand	1784 (11.4)	1394 (8.9)	475 (3)	95 (0.6)	29 (0.2)
Orchard soils with low or irregular manure application					
Rafsanjan	803 [c] (3.9)	708 (3.4)	178 (0.9)	78 (0.4)	17 (0.1)
Sirjan	588 (1.9)	600 (1.9)	88 (0.3)	50 (0.2)	0 (0.0)
Zarand	538 (1.7)	543 (1.7)	163 (0.5)	25 (0.1)	0 (0.0)
Average	1297 (45)	1033 (35)	376 (13.4)	115 (4.1)	71 (2.7)

[a] Data are the means of four plots per location. Twenty to 40 tones of manure are used every other year.
[b] Frequency of occurrence (%) of the *Aspergillus* sections are shown in parentheses. The data are based on the proportion of the total isolates of recovered *Aspergillus* (124815).
[c] In soils with low manure application, data are the means of six, four and four locations in Rafsanjan, Sirjan and Zarand, respectively. (in each location, four plots were considered as replicates).

In Zarand, five isolates (56.25%) produced only 989 to 775 ppb AFB1 and four (25%) produced AFB1 +AFB2 ranging from 1942 to 3654 and 87 to 652ppb (Table 4). In Kerman Province, 19 isolates of *A. flavus* (5, 7 and 7 isolates from Rafsanjan, Sirjan and Zarand, respectively) were unable to produce aflatoxin.

Table 4. Aflatoxin production by *Aspergillus flavus* isolates from various soils in Fars and Kerman Provinces

Isolate No.	Host Location	Aflatoxin Concentration (ppb)	
		B1	B2
1	Corn-Saadatshahr	389.95 [a]	4.32
2	Corn-Bajgah	ND	ND
3	Corn-Abadeh	415.3	9.45
4	Alfalfa-Abadeh	215.67	6.8
5	Alfalfa-Abadeh	ND	ND
6	Wheat-Safashahr	ND	ND
7	Wheat-Sepidan	447.35	12.31
8	Uncultivated soil-Sepidan	ND	ND
9	Cotton-Darab	4.13	ND
10	Cotton-Darab	240.15	5.32
11	Citrus-Darab	202.47	9.3
12	Citrus-Darab	ND	ND
13	Grapevine-Kavar	219.2	8.79
14	Grapevine-Kavar	489.65	12.356
15	Fig-Estahban	165.89	5.78
16	Fig-Estahban	510.32	10.68
17	Sugarbeet-Rooniz	105.96	1.54
18	Pistachio-Neiriz	79.7	1.73
19	Pistachio-Neiriz	625.32	17.89
20	Pistachio-Neiriz	ND	ND
21	Pistachio-Rafsanjan	353	ND
22	Pistachio-Rafsanjan	ND	ND
23	Pistachio-Rafsanjan	986	ND
24	Pistachio-Rafsanjan	382	15.6
25	Pistachio-Rafsanjan	ND	ND
26	Pistachio-Rafsanjan	1784	ND
27	Pistachio-Rafsanjan	2236	ND
28	Pistachio-Rafsanjan	1887	203.4
29	Pistachio-Rafsanjan	ND	ND
30	Pistachio-Rafsanjan	578.3	ND

Isolate No.	Host Location	Aflatoxin Concentration (ppb)	
		B1	B2
31	Pistachio-Rafsanjan	ND	ND
32	Pistachio-Rafsanjan	1369	87.9
33	Pistachio-Rafsanjan	421.5	ND
34	Pistachio-Rafsanjan	1056	ND
35	Pistachio-Rafsanjan	1425.6	92
36	Pistachio-Rafsanjan	986.8	87.9
37	Pistachio-Rafsanjan	4536	225
38	Pistachio-Rafsanjan	1026	103
39	Pistachio-Rafsanjan	ND	ND
40	Pistachio-Rafsanjan	985.6	87.9
41	Pistachio-Sirjan	514	85.3
42	Pistachio-Sirjan	1483	563.2
43	Pistachio-Sirjan	587.3	49
44	Pistachio-Sirjan	ND	ND
45	Pistachio-Sirjan	ND	ND
46	Pistachio-Sirjan	ND	ND
47	Pistachio-Sirjan	1542	215.6
48	Pistachio-Sirjan	256.4	ND
49	Pistachio-Sirjan	ND	ND
50	Pistachio-Sirjan	2346	207.8
51	Pistachio-Sirjan	ND	ND
52	Pistachio-Sirjan	1225	ND
53	Pistachio-Sirjan	ND	ND
54	Pistachio-Sirjan	475	ND
55	Pistachio-Sirjan	ND	ND
56	Pistachio-Sirjan	1254	ND
57	Pistachio-Zarand	756	ND
58	Pistachio-Zarand	ND	ND
59	Pistachio-Zarand	ND	ND

Isolate No.	Host Location	Aflatoxin Concentration (ppb)	
		B1	B2
60	Pistachio-Zarand	1942	87
61	Pistachio-Zarand	98	ND
62	Pistachio-Zarand	546	ND
63	Pistachio-Zarand	ND	ND
64	Pistachio-Zarand	3098	201
65	Pistachio-Zarand	ND	ND
66	Pistachio-Zarand	2457	146
67	Pistachio-Zarand	ND	ND
68	Pistachio-Zarand	ND	ND
69	Pistachio-Zarand	775	ND
70	Pistachio-Zarand	3654	652
71	Pistachio-Zarand	598.6	ND
72	Pistachio-Zarand	ND	ND

ND: Not Detected

[a] Isolates were grown on 50 g rice flour and the presence of aflatoxin was determined by TLC.

Discussion

It seems that variation among the species and population of *Aspergillus* spp. in soil samples is due to variation of plants and cultural practices such as applying different manures and soil tillage. *A. flavus*, *A. parasiticus* and *A. niger* var. *niger* were ranked as first, second and third highest frequent species in Fars Province. These species were also found in the majority of soils (Table 1). Whereas, the order of ranks changed to *A. niger* var. *niger* > *A. flavus* > *A. parasiticus* in Kerman Province. These species were isolated from all pistachio orchard soils with routine manure application. In some soils with low manure application, a number of *Aspergillus* species were absent. These pistachio soils had lower *Aspergillus* population compared with soils with routine manure application. *Aspergillus alliaceus* produced no sclerotium on MEA as reported by KLICH & PITT (1988) and KLICH (2002). Isolates of *A. auricomus* produced many orange sclerotia in small clusters on MEA. The average size of vesicle (14.5 µm) was in agreement with KLICH & PITT (1988a) and KLICH (2002). Isolates of *A. carbonarius* obtained from Fars and Kerman Provinces had long stipes (up to several

millimeters) and large and tuberose conidia as reported by KLICH & PITT (1988a) and KLICH (2002). Most of the isolates of *A. flavus* were distinguished from *A. oryzae* by less floccose texture and greener color of colonies. Smaller and smoother conidia were the most important criteria to discriminate *A. flavus* from *A. parasiticus*. Based on other reports (KLICH and PITT 1988a,b, GOURAMA & BULLERMAN 1995, KLICH 2002), conidial ornamentation is the most diagnostic criterion to differentiate these fungi. Isolates of *A. fumigatus* had uniseriate conidial heads with curving phialides that were parallel to each other and stipe axis. The color of the colonies in this species quickly turned into turquoise to dark green (KLICH & PITT 1988a, KLICH 2002). Uniseriate conidial heads with black spinose conidia were a good criteria to identify *A. japonicas* var. *japonicus* that was in agreement with KLICH & PITT (1988a) and KLICH (2002). Based on KLICH & PITT (1988a), *A. japonicas* var. *japonicus* is distinguished from *A. japonicus* var. *aculeatus* primarily by the globose conidia and smaller vesicle in the former. Nevertheless, both varieties have been elevated to one species by KLICH (2002). Isolates of *A. niger* var. *niger* had roughened conidia and dark brown to black colonies. Based on KLICH (2002) two former described varieties (KLICH & PITT 1998a), *A. niger* var. *awamori* and *A. niger* var. *niger*, have been elevated to two species. The average colony diameter at 37° C (zero to 30 mm) and yellow buff colonies of *A. ochraceus* was in agreement with the KLICH & PITT (1988a) and KLICH (2002) and made a distinction between this species and *A. alliaceus*. Colonies of *A. oryzae* were usually floccose with olive colors. These characters in addition of producing large conidia distinguished this species from *A. flavus*. Isolates of *A. parasiticus* had predominantly uniseriate conidial heads, more olive colonies and rough-walled conidia. These criteria differentiated this species from *A. flavus*. Isolates of *A. sclerotiorum* produced no sclerotia on CYA at 25° C as reported by KLICH & PITT (1988a) and KLICH (2002). Isolates of *A. sclerotiorum* had also yellow stipes with pale yellow and smooth-walled, small conidia (2.5 µm). Isolates of *A. sojae* had also predominantly uniseriate conidial heads with rough-walled conidia. *A. sojae* is differentiated from *A. parasiticus* by its larger conidia (7.5 µm compared to 4.2 µm) and more floccose colonies. Isolates of *A. terreus* had compact columnar conidial heads and very small (2.5 µm) and smooth-walled

conidia. This species also possessed hyaline aleurioconidia and lateral cells on hyphae. These characters were in accordance with KLICH & PITT (1988a) and KLICH (2002).

MOHAMMADI & BANIHASHEMI (2006) previously isolated *A. alliaceus*, *A. auricomus*, *A. carbonarius*, *A. flavus*, *A. niger* var. *niger*, *A. oryzae*, *A. parasiticus*, *A. sclerotiorum*, *A. sojae* and *A. terreus* from soil in Fars Province. Therefore, *A. carbonarius* and *A. oryzae* are new species to the mycoflora of Iran. *A. japonicus* var. *japonicus*, *A. carbonarius*, *A. oryzae*, *A. sclerotiorum* and *A. sojae* are new for pistachio orchard mycoflora in Kerman Province. MOJTAHEDI *et al.* (1979) isolated *A. niger*, *A. flavus*, *A. fischeri* Wehmer, *A. tamari* Kita, *A. terreus*, *A. nidulans* (Eidam) G. Winter, *A. umbrosus* Bainier & Sartory, *A. vesicolor* (Vuill.) Tiraboschi, *A. sydowii* (Bain. & Sart.) Thom & Church, *A. ochraceus*, *A. petrakii* Voros-Felkai, *A. fumigatus* and *A. parasiticus* from pistachio kernels. They reported that among the isolated species, *A. niger* had the highest population (MOJTAHEDT *et al.* 1979). HEIDARIAN *et al.* (2005) reported seven *Aspergillus* species from dry pistachios which *A. phoenicis* and *A. punvieus* were new to pistachio mycoflora of Iran.

RAHIMI *et al.* (2007) isolated 11 *Aspergillus* species from pistachio nuts in Kerman, Rafsanjan and Isfahan. They reported that *A. alliaceus*, *A. unguis* and *A. wentii* are new for mycoflora of Iran. *A. alliaceus*, *A. candidus*, *A. niveus*, *A. unguis* and *A. wentii* were also reported as new species for pistachio mycoflora (RAHIMI *et al.* 2007).

It seems that, SPDA and AFPA media are more efficient for isolation and enumeration of *Aspergillus* species especially *A. flavus* and *A. parasiticus*. In addition to quick identification and enumeration of these fungi, population of other soil mycoflora was low due to dichloran. Applying antibiotics, Rosebengal and streptomycine, in SPDA resulted in lower population of soil bacteria compared to AFPA and Czapek media.

Aspergillus population was totally variable in soil samples from Fars Province. Among the field and orchard soils, pistachio, cotton, sugar beet, corn and wheat, ornamental plants, apple, rose had the highest (3180 to 5280) and lowest (1080 to 1950) *Aspergillus* population/g soil, respectively. The lowest population of

Aspergillus occurred in uncultivated soils with a mean less than 1000 propagules/g soil. *Aspergillus* species were diverse in soils of Fars Province. Species related to sections *Flavi* and *Nigri* were found in all collected soils whereas section *Circumdati* and *Fumigati* were not isolated from ornamental, apple and uncultivated soils. *Terrei* section was only isolated from sugarbeet, corn, alfalfa and sesame soils and had the lowest frequency in Fars Province. *A. flavus*, *A. parasiticus* (*Flavi* section) and *A. niger* var. *niger* (*Nigri* section) were three species with highest frequency in Fars Province and showed variation in the population in various soils.

Other studies showed variation in populations of *A. flavus*/*A. parasiticus* in soils. GRIFFIN & CARREN (1974) found that *A. flavus* population in Virginia field soils was around 0.5-57.3 propagules/g soil. BELL &CRAWFORD (1967) reported significantly greater amount of propagules in naturally infested soils in Georgia (1.5×10^4 propagules/g soil). MARTINUK & WAGNER (1978) and SHEARER *et al.* (1992) reported 2.8×10^3 and zero to 256 propagules of *A. flavus*/ *A. parasiticus* from soils that were fertilized and cropped to continuous corn and from cultivated fields on three years rotation of wheat, red clover and corn in Missouri, respectively.

Aspergillus species obtained from Kerman Province were placed in three groups based on their population pattern in soils. First group included *A. niger* var. *niger* (*Nigri* section), *A. flavus* and *A. parasiticus* (*Flavi* section) representing more than 590 propagules/g soil (736, 616 and 597 propagules/g of soil, respectively). Second group included *A. carbonarius* (342 propagules/g soil) and third group included other *Aspergillus* species (86 to 141 propagules/g soil). Among the third group, *A. terreus* (*Terrei* section) and *A. oryzae* (*Flavi* section) had the lowest and highest population, respectively. Species belonging to the first group are important because of colonization of pistachio nuts, production of aflatoxins and decreasing pistachio quality. Among members of this group, *A. niger* var. *niger* may be potentially important due to its role in biological control of aflatoxigenic species. DOSTER & MICHAILIDES (1994) reported that *A. niger* var. *niger*, *A. japonicus*, *A. flavus*, *A. parasiticus*, *A. tamari*, *A. melleus*, *A. ochraceus* and *A. wentii* can colonize pistachio litter in pistachio growing areas in California. Among identified species, members of *Nigri* section (*A. niger* and *A. japonicus*)

were more common in pistachio litter and soil (DOSTER & MICHAILIDES 1994) but in Kerman Province, *Flavi* and *Terrei* sections showed the highest (1297) and lowest (71) *Aspergillus* population, respectively. Although *A. niger* var. *niger* had a higher population than *A. flavus* or *A. parasiticus*, but *Nigri* section had a lower population compared with *Flavi* section.

Population of *Aspergillus* species in soil samples of Rafsanjan was globally higher than Sirjan and Zarand (Table 3). These results were not in agreement with MORADI *et al.* (2004) who reported that population density of *A. flavus* and *A. niger* groups were higher in Zarand than Rafsanjan and Sirjan soils. In the present study, we identified five *Aspergillus* sections with 13 species, whereas Moradi *et al.* (*l.c.*), only studied two sections of *Aspergillus*. Moradi *et al.* (*l.c.*) also indicated that sheep and poultry manure had the highest and lowest *A. niger*/*A. flavus* groups population, respectively, and cow manure was intermediate. These manures are mixed with the soils and increased the level of organic substances and subsequently *Aspergillus* population. Cultural practices such as tillage can distribute *Aspergillus* inoculum in manure and soil and increase the number of spores. These spores could be important by reaching to the aerial parts of the pistachio trees through dust (DOSTER & MICHAILIDES 1992). In some orchards of Kerman Province, low amounts of manure had been used or the application of manure was irregular. Very little or no pistachio debris was observed in some of the orchards. These soils supported lower *Aspergillus* population than others. DOSTER & MICHAILIDES (1994) reported that spores of *A. flavus* on male inflorescences are evenly distributed in orchards that can be important in increasing *A. flavus* population in pistachio orchards in California. In pistachio growing areas of Iran, male inflorescences fall from late March to April but male trees are few compared to California pistachio orchards. Therefore, the male inflorescences may not be important in increasing *Aspergillus* population in Iran. Nearly 10% of female inflorescences can be fertilized by pistachio pollen in orchards and the rest are distributed on the ground (CRAINE & EVAKIRI 1981). These female inflorescences can be colonized by *Aspergillus* species and increase fungus population. In pistachio orchards of Iran, pistachio debris is distributed in the vicinity of processing terminals or on orchard ground. Colonization of debris by *Aspergillus* species can build up the fungus population in

the pistachio orchards. Other reports (DOSTER & MICHAILIDES 2004, SHEARER *et al.* 1992) also showed that pistachio litter and cob and stalk pieces of corn may play an important role in infection of pistachio and corn by increasing the amount of *Aspergillus* inocula in soils. Since *Aspergillus* species are able to decompose numerous substrates including plant debris, it is not surprising to find high population of the fungus in soils of various fields and orchards (DOSTER & MICHAILIDES 1994). It seems that type of manures (cow, sheep and poultry), manure application procedure (burying under the soil or spreading on soil surface), presence of plant debris on field and orchard ground and tillage or cultural practices are important factors for increasing population of *Aspergillus* species in Fars and Kerman Provinces.

In corn fields of America, sclerotia of *A. flavus*/*A. parasiticus* are an important source of primary inoculum. These sclerotia can not disperse the fungus at harvest, but remain as a long-term survival structure in soils (WICKLOW 1987, WICKLOW *et al.*1984). The method of survival of *Aspergillus* spp. particulary aflatoxigenic species in Fars and Kerman soils still is not well understood. MIRABOLFATHY *et al.* (2005) reported that 42% of soil isolates, 33% of air isolates and 27% of nut isolates of *A. flavus* recovered from Kerman and Semnan Provinces can produce sclerotia. They also observed a high correlation between production of aflatoxin and sclerotium by *A. flavus* whereas, RAHIMI *et al.* (2007) showed that 10% of pistachio isolates of *A. flavus* produced sclerotia and there is not a direct relationship between sclerotium and aflatoxin production among *A. flavus* isolates.

The majority of *A. flavus* isolates (70%) recovered from soil of Fars and Kerman Provinces produced aflatoxin. It seems that the soil is the major source for contamination of crops to aflatoxins. Based on other studies, 41-95% of *A. flavus*/*A. parasiticus* can produce aflatoxins (DIENER & DAVIS 1966, KLICH & PITT 1988b, TABER & SCHROEDER 1967), but DOSTER & MICHAILIDES (1994) found that most isolates of *A. flavus* from pistachio litter could not produce detectable amounts of aflatoxins. Although many reports revealed that some isolates of *A. flavus* isolates do not produce aflatoxins, the exact percentage of these aflatoxigenic *A. flavus* isolates vary substantially. In the ATCC, only 34-41%

(depending on substrate) of *A. flavus* and 85% of *A. parasiticus* produced aflatoxin (WEI & JONG 1986). RAHIMI *et al.* (2007) reported that 50 and 100% of *A. flavus*/*A. parasiticus* isolates recovered from pistachio orchards produced aflatoxin. Isolates of *A. flavus* recovered in the present study did not produce aflatoxin G. This result was in accordance with DOSTER & MICHAILIDES (1992). COTTY (1989) and TABER & SCHROEDER (1967) who also reported none of the isolates of *A. flavus* produced aflatoxin G but isolates in ATCC (WEI & JONG 1986) and 26% of the *A. flavus* isolates from Africa and Thailand (Cotty 1997), produced the G aflatoxins. In addition to *A. flavus*/*A. parasiticus*, other mycotoxigenic aspergilli were also isolated and identified in various soils of Fars and Kerman Provinces. *A. ochraceus*, *A. auricomus*, *A. sclerotiorum* and *A. alliaceus* can produce an important group of carcinogenic toxins such as ochratoxin and less important mycotoxin penicillic acid (BAYMAN *et al.* 2002). The occurrence of *A. alliaceus* was correlated with the presence of ochratoxin A in figs, against *A. ocharceus* (BAYMAN *et al.* 2004). Although ochratoxin producing species showed low population in Fars and Kerman Provinces, but the presence of these species and the possibility of ochratoxin production must be regarded in field and orchards of Iran.

References

BAYMAN, P., BAKER, J.L., DOSTER, M.A. MICHAILIDES, T.J. and MAHONEY, N.E. 2002. Ochratoxin production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus*. App. Environ. Microb. 68: 2326-2329.

BELL, D.K. and CRAWFORD, J.L. 1967. A Botran-amended medium for isolating *Aspergillus flavus* from peanuts and soil. Phytopathology 57: 939-941.

COTTY, P.J. 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79: 808-814.

COTTY, P.J. 1997. Aflatoxin-producing potential of communities of *Aspergillus* section *Flavi* from cotton producing areas in the United States. Mycol. Res. 101: 698-704.

CRAINE, J.C. and EVAKIRI, B.T. 1981. Morphology and reproduction of pistachio. Hort. Rev. 3: 376-393.

DHINGRA, O.D. and SINCLAIR, J.B. 1985. Basic Plant Pahtology Methods. CRC Press.

DIENER, U.L. and DAVIS, N.D. 1966. Aflatoxin production by isolates of *Aspergillus flavus*. Phytopathology 56: 1390-1393.

DIENER, U.L., COLE, R.J., SANDERS, T.H., PAYNE, G.A., LEE, L.S. and KLICH, M.A. 1987. Epidemiology of Aflatoxin formation by *Aspergillus flavus*. Ann. Rev. Phytopathol. 25: 249-270.

DOMSCH, K.H., GAMS, W. and ANDERSON, T.H. 1980. Compendium of Soil Fungi. Academic Press, London. 859 pp.

DOSTER, M.A. and MICHAILIDES, T.J. 1994. Development of *Aspergillus* molds in litter from pistachio trees. Plant Dis. 78: 393-397.

GEISER, D.M., KLICH, M.A., FRISVAD, J.C., PETERSON, S.W., VARGA, J. and SAMSON, R.A. 2007. The current status of species recognition and identification in *Aspergillus*. Stud. Mycol. 59: 1-10.

GOURAMA, H. and BULERMAN, L.B. 1995. *Aspergillus flavus* and *Aspergillus parasiticus*: Aflatoxigenic fungi of concern in foods and feeds, a review. J. Food Protect. 58: 1395-1404.

GRIFFIN, G.J. and GARREN, K.H. 1974. Population levels of *Aspergillus flavus* and the *A. niger* group in Virginia peanut field soils. Phytopathology 64: 322-325.

HEIDARIAN, R., JAVAN-NIKKHAH, M., ORMAZ, B. and PEYAMBARI, M. 2005. Study of fungal contamination of pistachio seeds in Kerman Province, Iran and some new fungi for Iranian pistachio mycoflora. IV International Symposium on Pistachio and Almond-ISHS, Tehran, Iran, p. 180.

JONES, R.K. 1979. The epidemiology and management of aflatoxins and other mycotoxins. pp. 381-392. *In*: J.G. Horsfall & E.B. Cowling (eds). Plant Disease. An Advanced Treatise. Vol. 4. How Pathogens Induce Disease?. Academic Press, New York.

KLICH, M.A. 2002. Identification of Common *Aspergillus* Species. CBS, Utrecht, The Netherland.

KLICH, M.A. and PITT, J.I. 1988a. A Laboratory Guide to *Aspergillus* species and their Teleomorphs. CSIRO, Division of Food Processing, North Ryde, NSW, Australia. 116 pp.

KLICH, M.A. and PITT, J.I. 1988b. Differentiation among *Aspergillus flavus* and *Aspergillus parasiticus* and other closely related species. Trans. Br. Mycol. Soc. 91: 99-108.

MALLOCH, D. and CAIN, R.F. 1972. The Trichocomaceae: Ascomycetes with *Aspergillus*, *Paecilomyces* and *Penicillium* imperfect states. Can. J. Bot. 50: 2613-2628.

MARTYNIUK, S. and WAGNER, G.A. 1978. Quantitative and qualitative examination of soil microflora associated with different management systems. Soil Sci. 125: 343-350.

MIRABOLFATHY, M., MORADI GHAHDARIJANI, M. and WALIYAR, F. 2005. Variability in aflatoxicogenic potential and sclerotial production of *Aspergillus flavus* in pistachio of Iran. IV International Symposium on Pistachio and Almond-ISHS. Tehran, Iran. p. 188-189

MOHAMMADI, A.H. and BANIHASHEMI, Z. 2006. Isolation and identification of *Aspergillus* species from soil in Fars Province. 17th Iran. Plant Protect. Cong. Tehran, Iran. p. 452.

MOJTAHEDI, H., RABIE, C.J., LUBEN, A., STEYN, M. and DANESH, D. 1979. Toxic *Aspergillus* from pistachio nuts. Mycopathologia 67: 123-127.

MORADI, M., ERSHAD, D., MIRABOLFATHI, M. and PANAHI, B. 2004. The role of plant debris, soil and manure on population density of *Aspergillus flavus* and *Aspegillus niger* groups in pistachio orchards of Kerman Province (in Persian). Iran. J. Plant Pathol. 40: 221-234

PITT, J.I. 1973. An appraisal to identification methods for *Penicillium* species: novel taxonomic criteria based on temperature and water relations. Mycologia 65: 1135-1157.

RAHIMI, P., SHARIFNABI, B. and BAHAR, M. 2007. *Aspergillus* species isolated from pistachio and determination of their aflatoxin production. Rostaniha 8: 30-42 (In Persian).

RAPER, K.B. and FENNEL, D.I. 1965. The Genus *Aspergillus*. Williams and Wilkins, Baltimore.

SHEARER, J.F., SWEETS, L.E., BAKER, N.K. and TIFFANY, L.H. 1992. A study of *Aspergillus flavus*/*A. parasiticus* in Iowa crop fields: 1988-1990. Plant Dis. 76: 19-22.

STEINER, G.W. and WATSON, R.D. 1965. The effect of surfactants on growth of fungi. Phytopathology 55: 1009-1012.

TABER, R.A. and SCHROEDER, H.W. 1967. Aflatoxin-producing potential of isolates of the *Aspergillus flavus-oryzae* group from peanut (*Arachis hypgaea*). Appl. Microbiol. 15: 140-144.

WEI, D. and JONG, S. 1986. Production of aflatoxins by strains of *Aspergillus flavus* group maintained in ATCC. Mycopathologia 93: 19-24.

WICKLOW, D.T. 1987. Survival of *Aspergillus flavus* sclerotia in soil. Trans. Br. Mycol. Soc. 89: 131-134.

WICKLOW, D.T., HORN, B.W., BURG, W.R. and COLE, R.J. 1984. Sclerotium dispersal of *Aspergillus flavus* and *Eupenicillium ochrosalmoneum* from maize during harvest. Trans. Br. Mycol. Soc. 83: 299-303.

WILEY, B.J. and SIMMONS, E.G. 1973. New species and a new genus of *Plectomycetes* with *Aspergillus* states. Mycologia 65: 934-938.

Addresses of the authors: A.H. MOHAMMADI, Dr. Z. BANIHASHEMI and Department of Plant Protection, College of Agriculture, Shiraz University, Shiraz and M. HAGHDEL, Iranian Pistachio Research Institute, Rafsanjan, Iran.