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INCIDENCE OF *Aspergillus* SPECIES AND MYCOTOXINS IN DRIED FIGS IN SOUTHERN IRAN*

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Abstract

During 2005-2007, soil, leaf and fig fruit samples from fig plantation in Estahban in Fars Province and several samples of dried figs from markets in Shiraz city were collected. Ninety four isolates of *Aspergillus* species were recovered from soil, leaf and fig fruit samples. Based on cultural characteristic, temperature and morphological criteria the following *Aspergillus* species with their percentage frequencies were identified: *A. alliaceus* (3.13), *A. carbonarius* (7.44) *A. flavus* (35.1), *A. fumigatus* (6.38), *A. japonicus* (4.25), *A. niger* var *niger* (39.6), *A. ochraceus* (2.13), and *A. terreus* (2.13). *A. japonicus* and *A. ochraceus* are as a new report for Iran and Fars province mycoflora respectively. The genetic diversity of a population of *A. flavus* from various substrates using *nit* mutants and their aflatoxin production was determined. Seven vegetative compatibility groups (VCGs) were identified among the *nit* mutants. The *in vitro* ability of *A. flavus* isolates in aflatoxin production was assessed using yeast extract sucrose agar medium supplemented with 0.2% methylated -β-cyclodextrin. There was a relationship between a VCG and the amount of mycotoxin produced by the isolate.

Keywords: *Ficus carica*, Fars, Estahban, Aflatoxin, Genetic diversity.

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Introduction

The fig (*Ficus carica*) is a native of south western Asia, and probably was first cultivated in Saudi Arabia and neighboring countries (Ferguson *et al.* 1990, Ogawa and English 1991). The approximately over 20,000 hectares of rain-fed commercial dried fig orchards in Estahban in Fars province, accounted for most of the dried fig production in Iran (Faghih and Sabet-Sarvestani 2001). Iran after Turkey is the second in world production (14%) of dried fig (Isin, *et al*. 2003, www.irasdriedfruit.com/dried-fig phd). The fig fruit with unusual type of multiple fruit known as a "Syconium" has ostiole that allows access to the tiny flowers on the inside. The main fig cultivar in Estahban is smyrna type that requires the fig wasp (*Blastophaga psenes* L.) to pass through the ostiole for pollination. Fungi and insects can also enter through the ostiole and results in contamination by various molds including many species of *Aspergillus*, which grow in a wide range of agricultural crops in Iran including pistachio (Rahimi *et al.* 2008), sesame (Habibi and Banihashemi, 2008), pomegranates (Ogawa and English, 1991) and other agricultural commodities in the world (Perrone *et al*. 2007).

Many *Aspergillus* spp. produce mycotoxins harmfeul to humans and animals (Doster *et al.* 1996). Aflatoxins are produced by four species of *Aspergillus*, *A. flavus, A. parasiticus, A. nomies* and *A. tamarii* (Payne and Brown 1998). *Aspergillus flavus* and *A. parasiticus* are agronomically important. *Aspergillus flavus* is the predominant species on the major commodities contaminated with aflatoxin. *Aspergillus parasiticus* may also be prevalent on some crops but it is rarely the dominant species (Payne and Brown 1998). Research on aflatoxin in figs(Buchanam *et al.* 1975, Karaka and Nas 2008) has shown that aflatoxin is produced in figs inoculated with *A. flavus* and was detected in naturally infected figs from Turkey (Doster *et al*. 1996).

In spite of large acreage of rain-fed fig plantations in Iran, no comprehensive studies have been done on identification of *Asperigillus* species in dried fig and their mycotoxin production. The objective of this research were to determine the incidence of *Aspergillus* species in soil, leaf and fig fruits in commercial Estahban rain-fed fig orchards and also fig fruits in different fig markets in various cities in Fars Province. The genetic diversity of *A. flavus* and aflatoxin production by different isolates from various substrates were also studied. The preliminary results of this work have been presented earlier (Farjood and Banihashemi 2008, 2010a, 2010b).

Materials and methods

Sample collection

Dried figs and caprifig fruits (used as pollinators) were collected from fig orchards in Estahban and different markets in Shiraz and other cities in Fars Province. Leaves of fig tree in Estahban fig plantation were collected from various parts of the tree. Soil samples (5-10 cm deep) under each fig tree were also collected from different parts of the orchard. All samples were brought to the laboratory for further studies.

Isolation

Fruits

Each fruit was cut into halves and surface sterilized for two minutes in 0.5% sodium hypochlorite and rainsed in sterilized distilled water (SDW), cut into 6-8 pieces depending on fruit size, and plated on acidified PDA or Rose Bengal streptomycin agar containing Dichloran (1ppm) (King *et al.* 1979). From each sample, 50-80 pieces were used.

Leaves

Five to seven leaves from each sample were cut into small pieces and placed in five 250 ml flasks containing fifty ml SDW and shaken for 30-60 min on a reciprocal shaker. One ml of the washing liquid was drawn from each flask and flooded on the above media. Five ml were used from each flask.

Soil

Twenty grams of each soil sample was used and diluted with SDW at different proportions. From each dilution series, one ml was flooded on the surface of each Petri plates containing above media and incubated at 25°C and 28°C (Bell and Crawford 1967).

Purification

Growing colonies identified as *Aspergillus* were purified by single spore method on water agar

containing 1% dextrose, and sub cultured further for identification to the species level.

Identification

Purified *Aspergillus* isolates were transferred to Czapek Yeast Extract Agar (CYA), Malt Extract Agar (MEA), G25N and CY20s (CYA + 20g sucrose).

Isolates on CYA were incubated at 37°C and the other media at 25°C for seven days. All isolates were identified to the species level based on growth rate at different temperature, colony appearance and color, morphological criteria like dimension of sporangiospores, metula, phialides, vesicle and vesicle pedicel and sclerotial formation.

Generation and characterization of *nit* mutants

Nit mutants of isolates of *A. flavus* were generated following the method of Bayman and Cotty (1991). Mycelial discs of each isolate grown for 7days on PDA, were transferred to PDA and CYA containing $40gl^{-1}$ potassium chlorate and incubated at 30°C for 30days. Each colony examined daily for the appearance of fast growing sectors. Hyphal tips of the sectors from different colonies were transferred to Petri dishes containing minimal medium (MM) (Bayman and Cotty 1991). Sectors with sparse mycelium unable to use nitrate were taken to be *nit* mutants and were stored at 4°C. These mutants were further identified as *nit*1, *nit*3 and *Nit* M based on their growth on a nitrate, nitrite, ammonium tartarate and hypoxanthine media according to Correll *et al.* (1987).

Complementation tests

The self compatibility of each isolate was determined by pairing different complementary *nit* mutants (*nit* 1with *nit* 3, *nit* 3 with *Nit* M and *nit* 1with *Nit* M) on MM. Isolates that did not form *Nit* M, the complementation was done with *nit* 1 and *nit* 3. Small block of each isolate was paired 15mm apart in the center of MM Petri dishes and incubated for 7-14 days at 30°C. Complementation was evident if there was formation of a dense, aerial growth of mycelia from the two mutants which had met and formed a prototrophic heterakaryon (Papa 1986).

Mycotoxin analysis

Selected isolates of *A. flavus* were screened to determine whether they produce toxin *in vitro*. The isolates were grown on PDA in the dark for 4-5 days and each mycelial disc was transferred to each 6cm glass (not plastic) Petri dishes containing yeast extract sucrose agar (CY20s) supplemented with 0.2% methylated β-cyclodextrin (Sigma, St. Louis, Mo, USA) and incubated for two days in the dark at 28°C. Aflatoxin production was monitored by exposing the colonies to UV at 320nm generated from Gel document apparatus. A white ring (halo) around each colony indicated aflatoxin production (Fente et al. 2001, Ordaz et al. 2003, Rajas et al. 2004, Habibi and Banihashemi 2008). To retard the colony diameter of the fungus surfactant NPX (nonyl phenyl polyethylene glycol ether containing 10.5 moles of ethylene oxide) at $100\mu g\ ml^{-1}$ (Steiner and Watson 1965) was used. To validate the accuracy of the method, an aflatoxin producer and non-producer were included as reported earlier (Habibi and Banihashemi 2008). No attempts were made to characterize the type of aflatoxins formed by each isolate. The intensity of halo formation around each colony (using -, +/-, +, + +, + ++) indicated the amount of aflatoxin produced.

Results

Identification

Ninety four isolates of *Aspergillus* spp. were recovered from different substrates including orchard soil, fig leaves, dried fig fruits and caprifigs. *Aspergillus niger* and *A. flavus* accounted for more than75% of the isolates and the frequencies of the species from various substrates are shown in Table1. All of the species were recovered from soil samples collected under fig trees. *Aspergillus parasiticus* was not recovered from fig fruit and other substrates. *Aspergillus niger*, *A. flavus* and *A. carbonarius* were present in higher density on all substrates. Among *Aspergillus* species recovered, *A. ochraceus* and *A. japonicus* are the first record respectively for Fars Province and Iran mycoflara. Several toxinogenic species of *Aspergillus* including *A. niger* var. *niger*, *A. flavus*, *A. carbonarius*, *A. japonicus* and *A. terreus* were also recovered from fresh fig leaves in Estahban fig orchards (Table 1).

Generation of *nit* mutants

The amount of potassium chlorate was critical for *nit* mutants' formation. No *nit* mutants were generated at $30gl^{-1}$ as reported earlier (Habibi and Banihashemi 2008). Increasing the chlorate

Table 1. Incidence frequency of *Aspergillus* spp. in fig fruit and fig orchards in Estahban.

% *Aspergillus* spp.								
terreus	*orchraceus*	*alliceus*	*japonicus*	*funigatus*	*carbonarius*	*flavus*	*niger var niger*	Source
1.06	10.11	0.00	0.00	3.23	18.22	31.21	36.17	Edible dried fig
0.00	0.00	0.00	0.00	14.29	17.66	28.33	39.72	Caprifig
5.46	0.00	0.00	10.58	0.00	21.88	22.80	39.28	Fig leaves
5.24	6.57	11.14	6.13	10.23	15.96	19.62	25.47	Fig orchard soil
2.13	2.13	3.13	4.25	6.38	7.44	39.6	39.6	Total incidence

concentration to 50gl^{-1} resulted in growth inhibition by many isolates and only 25% of the isolates formed *nit* mutants. Most of the isolates generated *nit* mutant if concentration of chlorate was adjusted to 40gl^{-1} after 10-30 days incubation. Few isolates did not produce *nit* mutants. From thirty three isolates of *A. flavus* used, 446 sectors and 350 *nit* mutants were obtained which consisted of 80.85% *nit*1, 12.12.85% *nit*3 and 6.28% *Nit* M.

Vegetative compatibility group

Based on the complementation tests, seven VCGs were obtained from all of the isolates evaluated (Table 2) and designated as VCGA to VCGG (Table 2). Numbers of VCG identified in each substrate was dependent on the number of isolates used. The numbers of VCG recovered in each substrate were 6, 4, 3 and 3 respectively in dried fig fruit, caprifig, soil and leaf. VCGF was recovered only from soil. Three isolates from leaf, fig fruit and caprifig were self-incompatible. Sclerotial production among VCGS varied, with highest number in VCGE.

Mycotoxin production

The intensity of halo formation varied among isolates and VCGs. Although many isolates in VCGA to VCG F produced toxin, but no toxin was produced in all of the isolates of VCGG. Two isolates designated as VCG A which produced highest amount of toxin, was isolated from dried fig fruit and soil. There was no relationship between sclerotial formation and toxin production. Both of the isolates with maximum mycotoxin production did not produce sclerotium but many sclerotial isolates produced mycotoxin.

Discussion

In this study, ninety four isolates of *Aspergillus* spp. were recovered from dried fig fruits and leaves and soils in fig orchards of which about 37% were *A. flavus* and 42% *A. niger* var. *niger* which were present at high frequency in soil, dried fig fruits and leaves. Other species accounted for less than 7% of the isolates. *Aspergillus japonicus* was not recovered from either dried fig fruits or caprifigs but was isolated from fig leaves and soil. This species is new for Iran mycoflora. *Aspergillus ochraceus* which is new for Fars mycoflora was isolated from soil and dried figs but with lowest incidence.

Among thirty four isolates of *A. flavus* examined, seven VCGs were identified. Habibi and Banihashemi (2008) among 570 *nit* mutants from 230 isolates of *A. flavus* from sesame (*Sesamum orientalis* L.) seeds identified 16 VCGs. Although the sample size might have some effect on the number of VCGs recovered, the sources of sampling are also important. Bayman and Cotty (1990) found higher number of VCG in cotton fields using smaller number of the isolates of *A. flavus* than in cotton field in Yuma Agricultural Center with higher number of isolates. The number of VCGs in a small sample size is the indicative of high genetic diversity among the population. VCGG which recovered with higher frequency among isolates was not isolated from soil. In contrast VCGF was recovered only from soil but not from aerial parts of the plant.

Isolates of *A. flavus* recovered from fig orchard soil, dried fig fruit and fig leaves differed in their ability to produce aflatoxin (Table 2). Both sclerotial and non- sclerotial isolates produced toxin. The size of sclerotia in isolates was above 700µm which are resemble to large type (L) as

Table 2. Characteristic of *Aspergillus flavus* isolates from fig orchards and dried figs and their aflatoxin production.

Isolate code	Source	Location[a]	Sclerotial ormation	VCG	Mycotoxin production[b]
L1	Fig leaf	EO	+	F	+/-
L2	Fig leaf	ER	+	C	+/-
L3	Fig leaf	EO	-	B	-
L4	Fig leaf	EO	+	?	-
S1	Fig Soil orchard	EO	+	F	+/-
S2	Fig Soil orchard	EO	-	A	+/-
S3	Fig Soil orchard	EO	+	F	+/-
S4	Fig Soil orchard	EO	+	C	+/-
S5	Fig Soil orchard	ER	+	F	+
GHT	Dried Fig Fruit	ER	+	B	-
GHR	Dried Fig Fruit	ER	+	G	-
ZR	Dried Fig Fruit	ER	+	D	+
100/1	Dried Fig Fruit	ER	+	G	-
GHO	Dried Fig Fruit	EO	-	A	+ +
KH	Dried Fig Fruit	ER	-	E	+/-
A	Dried Fig Fruit	ER	-	B	+/-
AA	Dried Fig Fruit	ER	-	G	-
B	Dried Fig Fruit	ER	+	?	+
KA2	Dried Fig Fruit	K	-	-	+/-
MOS	Dried Fig Fruit	Sh	-	?	-
MAL	Dried Fig Fruit	Sh	-	G	-
MOL	Dried Fig Fruit	Sh	+	D	+
ATL	Dried Fig Fruit	EO	+	C	+/-
GA2	Dried Fig Fruit	Sh	-	G	-
DK2	Dried Fig Fruit	Sh	+	E	+/-
KA	Dried Fig Fruit	K	+	B	+
AKI	Dried Fig Fruit	EC	+	E	+
BF	Dried Fig Fruit	Black Fig	-	?	-
BG1	Caprifig	ER	-	B	-
BG2	Caprifig	EO	-	D	-
BG3	Caprifig	EO	+	E	+
BG4	Caprifig	Ni	-	G	-
BG5	Caprifig	EO	-	?	-
MO	Pestachio	Rafsingon	-	-	+ + +

(a) EO: Estahban orchard, ER : Estahban Fig Research Station, K = Kazeroon market
Sh : Shiraz market, Ni = Niriz market, EC: Fig cooperative Storage
(b) O : no halo, +/- faint halo, + visible halo , + + normal halo, + + + strong halo

reported by others (Garber and Cotty 1997) and produced aflatoxin. Two isolates from fig fruit and soil belonged to VCGA did not produce sclerotia but produced strongest halo under UV. No aflatoxin was detected in VCGG which was detected only in fig fruits.

Previous reports on the incidence of *Aspergillus* species in soil in Fars Province of Iran had shown that *A. flavus*, *A. parasiticus* and *A. niger* var *niger* were ranked as first, second and third highest frequency of the species recovered (Mohammadi et al. 2009).The majority of *A. flavus* isolates (70%) recovered from soil in Fars Province produced aflatoxin using TLC method, among these, two isolates were recovered from fig soil with high quantity of aflatoxin B1 and B2 (Mohammadi et al. 2009). Although high population of *A. parasiticus* was present in soil in fig orchard in California, low infection was detected in fig fruits (Doster et al. 1996) but none had been recovered from dried fig fruits in Iran (Javanmardi 2010).

Aspergillus management in fig fruit under rain-fed irrigation with high temperature and very low humidity during fruit set could be easily managed by proper harvest and precaution during pollination.

Caprifigs left from the previous year were still found to be contaminated by many *Aspergillus* species which spores could be easily carried to ostiole of the fig fruit. Most of fig infection is probably originate from soil during harvest. The present study showed that soil is the main source of *Aspergillus* species in fig orchards.

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