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Vegetative compatibility groups among isolates of *Aspergillus flavus* from sesame seed in Iran and mycotoxin production

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Summary. The genetic diversity of a population of *Aspergillus flavus* isolated from sesame seeds collected in 2004 and 2005 from various parts of Iran was studied through vegetative compatibility, and their mycotoxin production was determined. Sixteen vegetative compatibility groups (VCGs) were identified among the *nit* mutants. VCGs were not evenly distributed through Iran. With few exceptions, there was a relationship between a VCG and the amount of mycotoxins produced by its isolates .

Key words: aflatoxin, genetic diversity, *Sesamum orientalis*, toxin.

Introduction

Sesame (*Sesamum orientalis* L.) grows in tropical and semitropical areas of the world and is one of the oldest plants grown in Iran, where its cultivation started around 2000 BC. Currently this plant is used as food and for medical purposes (Hue, 1996). Sesame seeds can be infected or contaminated with various pathogenic and saprophytic fungi. Recent studies on fungi infecting sesame seeds from different parts of Iran found that *Aspergillus flavus* was the dominant fungus (Basirnia and Banihashemi, 2004). *Aspergillus* species are common saprophytes in soil, in stored food and feed products, and in the vegetation of tropical and subtropical regions (Diener, 1960). *A. flavus* has no sexual stage, but its genetic diversity can be examined through vegetative compatibility groups (VCGs). VCGs have been associated with many morphological and physiological features (Bayman and Cotty, 1991). Although there are several reports on the VCGs of *A. flavus* in a single field (Bayman and Cotty, 1991) and on the occurrence of *A. flavus* VCGs in many crops such as corn, cotton seeds, almond, pistachio nuts and peanut (Klich and Pitt, 1988; Horn *et al*., 1996, Barros *et al*., 2000), very little is known about *A. flavus* in sesame seeds.

Many isolates of *A. flavus* produce aflatoxins, which are potent carcinogenics toxic to animals and humans (Pildain *et al*., 2004). Several studies have reported that aflatoxin B_1, which is produced by *A. flavus,* occurs in sesame seeds and tahini, which is the main constituent of halva (Jonsyn, 1988; Bahkali and Moslem, 1996; Nilufer and Boyacioglu, 2002; Var *et al.,* 2007). Although sesame seeds are widely used in Iran for its oil, halva, ardeh and for traditional breads, little is known about toxicogenous aspergilli contaminating these seeds. An earlier summary of this work was published in Habibi and Banihashemi (2006a, b).

Materials and methods

Isolation and identification

In 2004 and 2005, sixty samples of sesame seeds were collected from various regions of Iran: Kerman,

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Jiroft, Shiraz, Sari, Mashhad and Ahvaz. *Aspergillus* species were isolated from sesame seeds on rose-bengal streptomycin agar containing 10 mg ml^{-1} Botran 75% WP (dichloran: 2, 6-dichloro-4-nitroanilin) (King *et al*., 1979) and incubated at 25°C and 28°C in the dark (Bell and Crawford, 1967; Bothast and Fennel, 1974). Single-spore isolates were grown on Czapek yeast extract agar (CYA), malt extract agar, CYA+20% sucrose, and G25N medium. Isolates on CYA were incubated at 37°C and the other isolates at 25°C, and all isolates were identified to species level based on their morphological characteristics and colony color (Klich and Pitt, 1988). In addition *A. flavus* and *A. parasiticus* agar (AFPA) was used and incubated at 30°C to determine specific color formation by *A. flavus* (Bell and Crawford, 1967).

Vegetative compatibility groups

Nit mutants were obtained using the method of Bayman and Cotty (1991a). Mycelial disks of each isolate cultured on PDA were transferred to three media containing 30 g l^{-1} potassium chlorate (PDC, CZC and MMC). Each Petri dish was inoculated with a conidial disk of *A. flavus* at three or four points and was incubated at 30°C. The edges of the colonies were examined daily for fast growing sectors. Hyphal tips of sectors from different colonies were transferred to minimal medium Petri dishes. Sectors with sparse mycelium unable to utilize nitrate were taken to be *nit* mutants and were stored.

The strains were grown on media with different sources of nitrogen including $NaNO_2$ (nitrite medium), ammonium titrate (ammonium medium) and hypoxanthine (hypoxanthine medium) according to Papa (1986). Three classes of mutants were recovered: *nia*D (nitrate nonutilizing), *nir*A (nitrate and nitrite nonutilizing) and *cnx* (nitrate and hypoxanthine nonutilizing).

Pairs of complementary mutants were inoculated 15 mm apart in the center of MM dishes and incubated for 7–14 days at 30°C. Compatibility was indicated by a strip of wild-type growth occurring between the two mutants. Compatibility pairings of *nia*D, *nir*A and *cnx* mutants within each isolate were also made in order to determine self-incompatibility.

Mycotoxin analysis

Aspergillus flavus isolates were screened to determine whether they produced toxins *in vitro*. The isolates were grown on Czapek agar in the dark for seven days and mycelial disks were transferred to each 6 cm glass (not plastic) Petri dishes (one disk per dish) containing yeast extract sucrose agar 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 320 nm generated from Gel document apparatus. A white halo around a colony indicated aflatoxin production (Fente *et al*. 2001; Ordaz *et al*., 2003; Rojas *et al*., 2004). To validate the accuracy of the method, an aflatoxin (B1) producer and an aflatoxin non-producer of *A. flavus* were included in all assays as a positive and negative control respectively. The isolates were supplied by the Iranian Pistachio Institute and had been screened for aflatoxin production using HPLC by a general procedure. The intensity of the halo around each colony indicated the amount of aflatoxin produced. No attempts were made to characterize the type of aflatoxin formed by each isolate.

Results

***Nit* mutant production**

Since *nit* mutant production on Czapek-dox, MM and PDA was 81, 65.1 and 55.4% respectively, the CZC medium was used in all further trials.

A. flavus colonies started sectoring after 7 days and continued sectoring until the 14th day.

Three isolates never produced *nit* mutants and were discarded. From all the *A. flavus* isolates, 643 sectors were obtained, of which 570 were *nit* mutants

Of the *nit* mutants generated, 72.2% (412) were *nia*D, 20% (114) *nir*A, and 7.7% (44) *cnx* (Fig.1).

Vegetative compatibility groups

Four isolates were self-incompatible and were excluded from further analysis. Based on the complementation tests, 16 VCGs were obtained from all the isolates evaluated (Table 1).

In some cases pairings initially defined as positive later turned out to be negative. This coincided with a phenotype change in one of the testers, expressed as an increase in sporulation and aerial mycelium.

Complementation tests revealed 16 VCGs among the 230 *A. flavus* isolates recovered from sesame seeds. These VCGs were designated A to P. The largest VCG (K) comprised five isolates, while two VCGs (I and N) had one isolate each (Table 1).

The VCG diversity of *A. flavus* was expressed as

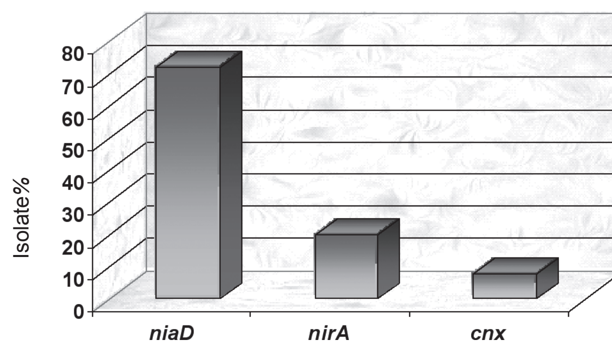

Fig. 1. *Aspergillus flavus nit* mutant phenotypes.

Table 1. Vegetative compatibility groups of *Aspergillus flavus* isolated from sesame seeds collected from various regions of Iran.

Isolate	Geographical region	Toxicogenicity[a]	VCG
sar1	Sari	+	A
sar7	Sari	+	
sar2	Sari	+/-	B
sar4	Sari	+/-	
sar6	Sari	-	
sar3	Sari	++	C
sar5	Sari	++	
k1	Kerman	+	D
k16	Kerman	+/-	
sh3	Shiraz	+	
ma4	Mashhad	+	
k3	Kerman	+/-	E
sh1	Shiraz	+/-	
jir3	Jiroft	+/-	
jir4	Jiroft	-	F
jir6	Jiroft	-	
jir7	Jiroft	-	
k4	Kerman	++	G
ma3	Mashhad	++	
tox	Rafsanjan	++	
k13	Kerman	+	
k5	Kerman	+	H
k9	Kerman	+	
k6	Kerman	+	I
k7	Kerman	+	J
k21	Kerman	+	
sh4	Shiraz	+	

(continued on the next page)

(Table 1 continued)

k2	Kerman	-	K
k8	Kerman	-	
ma1	Mashhad	-	
k19	Kerman	+	
k17	Kerman	+/-	
jir1	Jiroft	-	L
Ahv	Ahvaz	-	
k10	Kerman	+	M
k15	Kerman	+	
k18	Kerman	+	
k20	Kerman	+	
k11	Kerman	+	N
k12	Kerman	-	O
jir2	Jiroft	-	
jir5	Jiroft	+/-	
K14	Kerman	+	P
sh2	Shiraz	+	
ma5	Mashhad		
ma6	Mashhad		-
ma2	Mashhad		Self-incompatible
esf	Kerman		Self-incompatible
Semn	Semnan		Self-incompatible
sh01	Shiraz		-
sh0	Shiraz		-

[a] + +, strong halo; +, normal halo; +/-, faint halo; -, no halo.

the number of VCGs divided by the total number of the isolates, giving an index of 0.36.

With few exceptions, all the isolates grouped in the same VCG produced the same combination of mycotoxins, but individual VCGs differed in the toxins they produced.

Most isolates placed in the same VCG came from widely separated regions of Iran. Isolates from the Sari region formed their own VCGs, whereas the other VCGs mostly contained isolates from more than one region.

More than 50% of *A. flavus* isolates from sesame seeds produced toxin. The intensity of the halo was correlated with the amount of toxin produced. Toxicogenic and nontoxicogenic isolates of *A. flavus* checked by HPLC were used as positive and negative controls respectively.

The medium used in the study to identify aflatoxigenic strains of *A. flavus* was fast, reliable and low-cost as compared with other media used to screen aflatoxigenic strains (Hara *et al*., 1974; Cotty, 1988; Abbas *et al*., 2004; Atanda *et al*., 2005).

Discussion

In this study *A. flavus* was the dominant *Aspergillus* species isolated from sesame seed coming from different parts of Iran.

The proportions of *nit* mutants generated were approximately 72.2% *nia*D, 20% *nir*A and 7.7% *cnx*. These proportions were similar to those reported for other isolates of *A. flavus* (Horn and Greene, 1995; Novas and Cabral, 2002). The frequency of the *nia*D type mutants was higher than that of the *cnx* and *nir*A mutants as reported by other authors (Horn and Green, 1995; Novas and Cabral, 2002).

Cnx mutants usually reacted most strongly when paired with compatible *nia*D or *nir*A mutants (Correll *et al*., 1987). These mutants were therefore preferred as testers to identify the VCGs.

Most mutants of the same isolate did not complement with each other; some pairs of isolates also produced a positive or a negative reaction with different combinations of *nit* mutants. These results were similar to those of Brooker *et al*. (1991) who found that each isolate also sectored mutants that were unable to complement with any of the other mutants derived from the same isolate. In addition, some mutants were able to complement with all the other compatible isolates. It has been suggested that the use of mutants might give a negative pairing reaction as a result of other noncomplementary mutations (Novas and Cabral, 2002). We therefore paired the isolates in all possible combinations with each other so as to avoid getting a false negative.

Some isolates were self-incompatible and therefore, could not be assigned to any VCG. Heterokaryon self-incompatible isolates have already been reported in *A. flavus* (Papa, 1986; Horn and Green, 1995), as well as in other species such as *Fusarium oxysporum* (Correll *et al*., 1987), *Gibberella fujikuroi* (Correll *et al*., 1989), *Colletotrichum* spp. (Brooker *et al*., 1991) and *Verticillum albo-atrum* (Korolev and Katan, 1997). The explanation of this phenomenon is still unclear (Papa, 1986).

The high diversity of *A. flavus* obtained was also found in other studies (Bayman and Cotty, 1991b; Horn and Green, 1995). Horn *et al.* (1996) suggested that the high VCG diversity of *A. flavus* in corn, cottonseed and peanut was similar to that of the nonpathogenic soil isolates of *F. oxysporum*. In contrast, many populations of *F. oxysporum* pathogenic to plants have a low VCG diversity due to the selective advantage of some VCGs in infecting a specific host (Larkin *et al*., 1990). Therefore these authors suggested that none of the *A. flavus* VCGs from agricultural produce was selectively adapted to invade crops under field conditions.

The high genetic diversity in the population under study could be explained as due to the widespread aerial dispersal of this fungus as suggested by Horn and Green (1995). Bayman and Cotty (1991b) found significant differences in the soil VCG distribution from year to year in an Arizona cotton field. The authors postulated that the influx of wind-born conidia could account for these shifts in the distribution of the VCGs.

The genetic diversity of a fungal population is an important factor when developing a biocontrol strategy against that fungus (Dorner *et al*., 1992; Dorner and Cole, 2002). Tran-dinh *et al*. (1999) suggested that if genetic exchange occurs, using a more aggressive nonpathogenic isolate as a biocontrol agent could result in the formation of a recombinant that is more aggressive and toxigenic. In the present study, aflatoxigenic and nonaflatoxigenic isolates were grouped in different VCGs. Heterokaryons can only form between vegetatively compatible isolates; therefore, the results of the study indicate that nonaflatoxigenic isolates belonging to a VCG different from aflatoxigenic isolates can be used in the biological control of the aflatoxigenic isolates.

The results demonstrated that VCGs were effective multilocus markers that characterized the genetic structure of *A. flavus* populations in Iran, where problems of cost and a lack of laboratory facilities may limit the number of isolates that can be analyzed by molecular methods. A better understanding of the genetic variability and the population structure within and between field populations may reveal how this important pathogen can be controlled most effectively.

In populations of *Aspergillus* spp. each VCG may be a single clone; therefore, VCGs would be series of clones derived from a common ancestor (Parmer *et al*., 1963; Varga *et al*., 2003). The idea of clonality has been supported by a comparison of the VCGs, by restriction fragment length polymorphism of the mitochondrial genome, and by analysis of random amplified polymorphic DNA (Bayman and Cotty, 1993). Information about the relationship between isolates belonging to the same VCG is continuously increasing. In the present study, each VCG corresponded clearly to the mycotoxin combination production by the isolates of that VCG.

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