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A critical evaluation of cultural methods for the identification of atoxigenic *Aspergillus flavus* isolates for aflatoxin mitigation in pistachio orchards of Iran

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Abstract Aflatoxin contamination of tree nuts is a growing concern for pistachio producing countries. Development of competitive exclusion strategies through application of atoxigenic *Aspergillus flavus* isolates is a highly effective route of natural aflatoxin mitigation. Aflatoxin assays conducted on a high number of native *A. flavus* isolates are a first step to identify potential biological control isolates. Many cultural methods for the rapid and visual identification of atoxigenic *A. flavus* isolates have been described. The current study identified atoxigenic *A. flavus* isolates from Iranian pistachio orchards using and contrasting cultural, analytical and molecular methods. Ammonium vapour (AV) and fluorescence detection (FD), two rapid aflatoxin assays, were directly compared using various media preparations to screen 524 *A. flavus* isolates obtained from Iranian pistachio orchards. Percentages of false negatives were high using FD assays for all media preparations ranging from 13 to 15 %. This in contrast to AV assays. Here incidences of false negatives ranged from 0 % (using coconut agar medium) to 7.2 % (using potato dextrose agar). Aflatoxin-producing ability of all isolates was further confirmed using thin layer- and high-performance liquid chromatography. Sixty three atoxigenic *A. flavus* isolates were identified as atoxigenic in all assays. For these isolates, five loci across the aflatoxin biosynthesis cluster pathway were compared to identify genetic defects explaining atoxigenicity. Genetic deletions in at least one of five loci in the aflatoxin biosynthesis pathway were found for 97 % of isolates. Frequencies of atoxigenic strains ranged from 7.1 to 37.5 % with the lowest incidence detected in the Kerman province. Proper identification of atoxigenic isolates is considered a first step in the development of biological control strategies. Ability of identified isolates to competitively exclude aflatoxin-producing fungi has to be further investigated.

Keywords Aflatoxin · Atoxigenic · *Aspergillus flavus* · Pistachios · Fluorescence detection · Ammonium vapor

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Introduction

Members of the fungal genus *Aspergillus* are notorious contaminants of agricultural commodities. As a result, crops frequently become infected with aflatoxins, highly carcinogenic secondary fungal metabolites (Amaike and Keller 2011). These infections diminish crop value and export chances and, moreover, impose health risks for both humans and domesticated animals (Williams et al. 2004). Iran harbours some of the oldest pistachio orchards in the world. In recent years, export of pistachios (*Pistacia vera* L.), a tree nut frequently contaminated with aflatoxins, from Iran to the European Union has been limited due to stricter aflatoxin regulations (Dini et al. 2012; Zheng et al. 2012). Aflatoxin mitigation strategies are urgently needed to ensure that Iranian pistachio growers can maintain food quality and safety for both domestic consumption and international trade.

In general, aflatoxin-producing potential among isolates of *Aspergillus flavus* is highly variable ranging from potent aflatoxin producers to completely atoxigenic isolates. Consequently, the severity of aflatoxin contamination found on crops is dictated by frequencies of toxigenic to atoxigenic isolates (Moradi and Javanshah 2006; Cheraghali et al. 2007; Cotty et al. 2008; Dini et al. 2012; Probst and Cotty 2012).

Aflatoxin mitigation strategies, including physical (Doster and Michailides 1994), chemical (Park 2002), cultural (Jaime-Garcia and Cotty 2006), and biological control (Mehl et al. 2012) have been developed. Applicability of methods depends on in-country legislation, agricultural commodity, financial and practical capabilities (Dorner 2004; Moradi and Hokmabadi 2011). Much focus has been laid on biological control agents such as bacteria, yeasts, and atoxigenic strains of *A. flavus* for their abilities to modify natural *A. flavus* populations, to interfere with aflatoxin biosynthesis, or to competitively exclude aflatoxin-producers from crops (Dorner 2004; Cotty and Mellon 2006). Among all biological control agents, application of native atoxigenic isolates of *A. flavus* is the most promising strategy for aflatoxin mitigation through competitive exclusions (Cotty 1990; Michailides et al. 2007; Dorner 2008). To achieve sustainable biological control, it is necessary to characterize native fungal community structures. Subsequently aflatoxin-producing abilities of identified species and strains must be assessed to find atoxigenic isolates that could be suitable for biological control. This step is labour and time intensive but crucial for any following investigations. High-number aflatoxin screens are conducted using cultural and/or analytical methods (Abbas et al. 2004a). In particular, analytical methods are proven accurate and reliable, but require expensive laboratory equipment and supplies (Nasir and Jolley 2002). Availability of these methods may be limited especially in developing countries. Here, qualitative, cultural methods provide a cheaper alternative to screen large number of fungal isolates for aflatoxin production. Among the many cultural methods, visual indictors are thought to be highly predictive for aflatoxin development (Hara et al. 1974; Davis et al. 1987). Media enhancements with natural (e.g. peanut-, or coconut extract, corn steep liquor) and/or chemical substances (e.g. methyl β-cyclodextrin) further trigger the natural ability of fungal isolates to produce aflatoxins (Hara et al. 1974; Davis et al. 1987; Fente et al. 2001). These cultural methods have been developed many decades ago and their precision and reliability are widely criticized. For example, coconut agar medium is very popular in developing countries due to its cheap and ready available ingredients. However, its reliability is considered controversial (Ritter et al. 2009; Yazdani et al. 2010). On the other hand, aflatoxin assays using coconut agar medium can be conducted in several ways which have been described or evaluated independently previously but have not been contrasted in the same study using a large number of *A. flavus* isolates.

The aims of this study were: i) to screen a high number of *A. flavus* isolates obtained from pistachio orchards in Iran for their aflatoxin producing abilities by employing cultural and analytical methods; ii) to identify potential biological control agents and assess the origin of atoxigenicity in these isolates with molecular methods; and iii) to assess precision and reliability of ammonia vapour and fluorescence aflatoxin detection assays using different standard media preparations in detecting atoxigenics by direct comparison with two analytical methods (TLC and HPLC).

The results of this study provide guidance for researchers depending on readily available cultural methods to assess the role of members of the fungal community structures in contamination processes and for their use as potential biological control agents.

Fig. 1 Sites in seven Provinces of Iran from which pistachio nut and soil samples were collected and isolates of *Aspergillus flavus* were obtained

Material and methods

Fungal isolates and identification

A total of 205 pistachio nut and 20 soil samples were collected from commercial pistachio orchards in Kerman, Esfahan, Semnan, Khorasan Razavi, Qom, Markazi and Yazd Provinces, Iran, during 2010–2012 (Fig. 1). Sampling followed a diagonal pattern in each orchard. Each soil sample was composed of 15 sub-samples taken from the top 5 cm of the soil surface. Samples were kept in paper bags, were thoroughly homogenized and sieved to obtain a 100 g sample. To obtain *Aspergillus* section *Flavi* isolates, ten grams of each soil sample was blended with 0.1 % sterile peptone water (90 ml). For pistachio nuts, 500 nuts were randomly collected from 10 pistachio trees in each orchard (50 nuts per tree). Samples were cooled and transported in paper bags to the laboratory for further analysis. One hundred pistachio nuts were submerged in 0.1 % peptone water (500 ml) and shaken vigorously. The resulting suspension was immediately used for fungal isolations. For each sample (soil and nuts), a serial dilution

of 10^{-1} to 10^{-3} in 0.1 ml aliquots were spread on Aspergillus Flavus and Parasiticus Agar (AFPA) medium (Gourama and Bullerman 1995). The plates were incubated at 30 °C for 2 to 3 days. Appearing colonies displaying an orange color on the reverse side of the growing colony were subsequently cultured on Potato Dextrose Agar (PDA). 524 *A. flavus* isolates were identified based on macroscopic and microscopic characteristics (Raper and Fennell 1965; Klich 2002) and stored as single spore isolates on slant agar at 4 °C until further analysis. Both soil and nut isolations were repeated three times per sample.

Media composition

Laboratory grade chemicals and ultra-pure water were used for media preparation.

Cultural media were: Coconut agar medium (CAM; per l: 200 g desiccated or shredded coconut homogenized for 5 min and filter through four layers of cheese cloth, 20 g agar, pH 6.5) (Davis et al. 1987); Yeast extract sucrose medium (YES; per l: 20 g yeast extract, 200 g sucrose, 20 g agar, pH 6.5) (Davis et al. 1966); YES amended with 0.3 % methyl β-cyclodextrin (YES-MβC) (Fente et al. 2001); and Potato dextrose agar (PDA, per l: 39 g potato dextrose agar, pH 6.5).

Assessment of aflatoxin production with cultural methods

Fluorescence detection (FD) assays The basic principle for FD is the diffusion of the toxins into the media (Fente et al. 2001).

For FD analyses, CAM and YES-MβC were used. *A. flavus* isolates (n=524) were cultured on the respective medium and incubated at 30 °C for 3–5 days in the dark. Presence (=aflatoxin were produced) or absence (=no aflatoxins were produced) of a characteristic blue fluorescence in the agar surrounding the colonies was visualized under UV light (365 nm) using a transilluminator (UVP, Upland, CA 91786, USA). The results were expressed as positive or negative for aflatoxin production and isolates were grouped as either toxigenic or atoxigenic. Each isolate was tested three times and experiments were replicated twice.

Ammonia vapour (AV) assays The basic principle of visual colour changes is the production of yellow anthraquinone pigments during aflatoxin biosynthesis by aflatoxigenic *A. flavus* isolates (Wiseman et al. 1967). The yellow pigments turn to plum-red in the presence of ammonia hydroxide (Saito and Machida 1999; Abbas et al. 2004b; Bhatnagar et al. 2003; Shier et al. 2005; Yazdani et al. 2010).

For Ammonium vapour analyses, PDA, YES and CAM were used. *A. flavus* isolates (n=524) were cultured on the respective medium and incubated at 30 °C for 3 days in the dark. After incubation, media dishes were placed upside down and a drop (0.2 ml) of 25 % ammonia solution (Merck, Germany) was placed into the lid of each culture dish to release ammonium vapour (Saito and Machida 1999). Colour development (pink pigmentation) and colour intensity upon contact with ammonium vapour were indicative for aflatoxin synthesis. Consequently, absence of colour development is indicative for absence of aflatoxin development. The plates were scored as positive or negative and isolates were grouped as either toxigenic or atoxigenic. Each isolate was tested three times and experiments were replicated twice. Toxigenicity was classified visually based on the intensity of pink colour from strongest to no colour changes. Isolates were assigned one out of five groups based on colour changes (Fig. 2).

Assessment of aflatoxin production with two analytical methods

TLC assay Ability of isolates to produce aflatoxins was determined on autoclaved rice (*Oryza sativa* L.) powder (10 g/ 250 ml flask) (Wei and Jong 1986). Briefly,

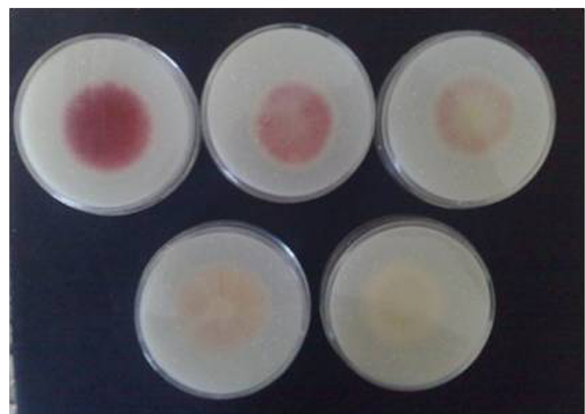

Fig. 2 Intensity of color development (presence of aflatoxin development) on coconut agar medium upon contact with ammonium vapor in *Aspergillus flavus* isolates. Top left to right: *Dark red*, *Red* and *Pink*; Bottom left to right: *Pale pink* and *No color* (atoxigenic isolate)

fungal isolates were cultured on PDA and incubated for 10 days at 25 °C in the dark. Spores were washed with 10 ml of sterile distilled water, collected in a sterile container and adjusted to 2×10^5 spores/ml. Spore suspension (4 ml) was added aseptically to each flask containing sterile rice powder. Moisture of the rice powder was equal to 32 % by inoculations. Flasks were incubated for 10 days at 28 °C in the dark. Thin-layer chromatography (TLC) was used to confirm presence or absence of aflatoxin production by *A. flavus* isolates; To extract aflatoxins, 125 ml methanol: water (55:45, v/v) and 3 g sodium chloride were added into each flask, incubated on a rotary shaker for 30 min and filtered through Whatman No. 4 filter paper. Filtrates (50 ml) were poured in a separator funnel, an equal amount of chloroform was added and the mixture was rotated for 1 min. The extraction was repeated with 50 ml portions of chloroform. Chloroform extracts were combined and evaporated in a rotary evaporator to a final volume of 2 ml. The extracts were transferred to screw capped borosilicate vial and evaporated to dryness under a gentle stream of nitrogen and dissolved in 200 μl benzene: acetonitrile (98:2, v/v), spotted on TLC plate adjacent to known aflatoxin standards (Sigma-Aldrich, Milan, Italy). The plates were developed in chloroform: acetone (9:1, v/v) and visualized under 365 nm UV light. Among the four aflatoxins (B1, B2, G1 and G2), only aflatoxin B_1 (the predominant aflatoxin detected in all isolates) was quantified directly on TLC plates with a scanning densitometer (TLC Scanner 3; Camag Scientific Inc., Wilmington, NC). The limit of detection was 0.5 ng/g.

HPLC assay HPLC assays were conducted to confirm atoxigenicity in isolates grouped as atoxigenic by cultural and TLC assays. Standard solutions of aflatoxins B1, B2, G1 and G2 were prepared using commercially available solid toxin preparations (Sigma-Aldrich, Milan, Italy). These were dissolved in toluene: acetonitrile (9:1, v/v) to a final concentration of 10 mg/ml. The exact concentration of each standard aflatoxin solution was determined according to AOAC official method 971.22 (AOAC Official methods of analysis 2000). Stock solutions containing 1 mg/ml of aflatoxin B1 or G1 and 0.25 mg/ml of aflatoxin B2 or G2 were prepared accordingly. Aliquots of the stock solution were transferred to 4 ml amber glass vials and evaporated to dryness under a stream of nitrogen at 50 °C. The residue was dissolved with water: methanol (40:60, v/v) to obtain a desired final concentration of 0.4, 1.2, 2.0, 3.6, 5.0, 6.2 and 7 ng/ml of aflatoxin B_1 and G_1, or 0.08, 0.24, 0.40, 0.72, 1.0, 1.24, and 1.4 ng/ml of aflatoxin B_2 and G_2. Standardized solutions were stored at −20 °C and warmed to room temperature before use.

Average recoveries of AFB_1 and AFB_2 from agar media spiked at levels of 5.0, 10.0 and 100 μg/kg and ranged from 80 to 91 % for PDA medium, with relative standard deviations (RSDs) of less than 20 % (triplicate experiments). PDA medium with no inoculation was used as a control.

Briefly, isolates were cultured on PDA for 10 days at 30 °C in the dark. PDA agar (1 g at diagonal sections) was extracted with a solution of methanol: water (80:20, v/v) on a shaker for 60 min, using a volume three times higher than the weight of each sample. Extracts (500 μl) were transfer into 2-ml eppendorf tubes (Eppendorf, Hamburg, Germany), diluted with an equal amount of sterile distilled water and filtered through a 0.45 μm regenerated cellulose (RC) filter (Alltech, Deerfield, IL, USA). One hundred microlitres of the extract was injected into the HPLC apparatus (Agilent 1100 Series, Agilent Technology, Santa Clara, CA, USA) with post-column photochemical derivatization (UVE™, LCTech GmbH, Dorfen, Germany) with a full loop injection system. The analytical column was a Luna PFP (2) (150×4.6 mm, 3 μm) (Phenomenex, Torrance, CA) preceded by a SecurityGuard™ (PFP, 4×3.0 mm, Phenomenex). The column was thermostated at 30 °C. The mobile phase consisted of a mixture of water: acetonitrile (70:30, v/v) eluted at a flow rate of 1.0 ml/min. Aflatoxin production was measured in ng/g of culture medium. The limit of detection was 0.2 ng/g for aflatoxins B_1 and G_1 and 0.04 ng/g for aflatoxins B_2 and G_2 based on a signal to noise ratio of 3:1. The fluorometric detector was set at wavelengths, ex= 365 nm, em=435 nm. Aflatoxins B_1, B_2, G_1 and G_2 were measured by comparing peak areas with a calibration curves obtained with aflatoxin standard solutions (Sigma-Aldrich, Milan, Italy). The linearity of the analytical response was checked by analyzing the calibration standards and using seven concentrations over the range 0.4–7.0 ng/ml aflatoxins B_1 and G_1; 0.08–1.4 ng/ml aflatoxins B_2 and $G_{2.}$

Molecular detection of atoxigenic isolates

Species-specific primers were used to validate species affiliation of selected atoxigenic *A. flavus* isolates

(Sardiñas et al. 2011; a single fragment of about 100 bp). *Nor1* (=*aflF*; 1.15 kb; encoding a putative aryl alcohol dehydrogenase), *aflR* (=*aflR*; 1.33 kb; encoding the aflatoxin pathway transcription factor), *estA* (=*aflJ*; 1.0 kb; encoding an esterase), *avnA* (=*aflG*; 1.61 kb; encoding a cytochrome P450 monooxygenase), and C3 (part of the 5′ flanking region) genes were assayed to confirm presence or absence of deletions and/or premature stop codons in the aflatoxin gene cluster leading to atoxigenicity. All primers used to amplify the genes C3, *afl*F, *afl*R, *afl*J and *afl*G were previously described by Chang et al. (2005).

PCR assays were performed in 25 μl using 2 μl (5–50 ng) genomic DNA (Moradi et al. 2010), 1 μl of each primer (20 μM), 2.5 μl of 10× PCR buffer, 1 μl of MgCl2 (50 mM), 0.2 μl of dNTPs (100 mM), 0.15 μl of Taq DNA polymerase (5 U/μl) and 17.15 μl millipore water supplied by the manufacturer (CinnaGene, Tehran, Iran) with: 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C (denaturalization), 30 s 55 °C (annealing), 1.5 min at 72 °C (extension) and a final 7 min extension step at 72 °C. A 5 μl aliquot of each PCR product was visualized under UV light on 1.0 % agarose gel (Bio Rad, Richmond, California, USA) in Tris–borate–EDTA buffer, stained with ethidium bromide (0.5 mg/ml) for 10 min. In aflatoxin-producing isolates, primers for *aflF, aflR, aflJ, aflG* and C3 will result in single fragment amplifications with a known size of about 150, 608, 529, 540, 622 bp, respectively. On the contrary, atoxigenicity in isolates is caused by deletions or insertions of base pairs (e.g. causing premature stop codons) at unknown locations and size in the aflatoxin biosynthesis pathway. Hence, primers may not be able to amplify specific regions where changes have occurred. Outlining presence and absence of amplifications gives first insight about causes of atoxigenicity in specific isolates.

Statistical analysis

Pearson's Chi-squared test with Yates' continuity correction was applied to compare the frequency of strains based on toxigenicity between and within provinces using the statistical package for social sciences (SPSS) and as described by Preacher (2001).

Results

Fungal isolation

Aspergillus section *Flavi* isolates (n=681) were obtained from pistachio nuts (n=571) and orchard soils (n=110) in seven major pistachio growing provinces. Based on cultural and physiological characteristics, 524 isolates (426 and 98 isolates obtained from nuts and soils samples respectively) were identified as *A. flavus*. Isolates of other species were discarded.

Aflatoxin production in *A. flavus* isolates varied significantly among all sampled regions ($X^2_{12,\ 0.05}$= 37.915, p<0.0001). Overall, more toxigenic than atoxigenic isolates were detected ranging from 62.5 to 92.9 % (Table 1). In Kerman, Semnan and Markazi Provinces the prevalence of toxigenic isolates was equal to or greater than 88.9 %. Frequencies of atoxigenic isolates were lowest in Kerman (7.1 %), a province with a high percentage of area under monoculture cropping.

On average, frequencies of atoxigenic isolates found in other provinces were about 2.2 times higher than in Kerman province ($X^2_{6,\ 0.05}$=9.129, p=0.166). Fifty two and 11 atoxigenic strains of *A. flavus* were obtained from nuts and soil samples, respectively, with a proportion of 12.2 (nut) and 11.2 % (soil).

Aflatoxin assays

With fluorescence detection, 144 (27.5 %) and 134 (25.6 %) isolates were initially scored as atoxigenic on YES-MβC and CAM, respectively. In contrast, less isolates were identified as atoxigenic using ammonium vapor assays. Here, only 19.3 % (101 isolates on PDA), 14.7 % (77 isolates on YES), and 12 % (63 isolates on CAM) isolates were initially evaluated as atoxigenic based on the absence of colour changes upon contact with ammonium vapour (Table 2). In general, the accuracy of methods declined in the order YES-AV, PDA-AV, CAM-FD and YES-MβC-FD, respectively. The frequency of false negatives ranged from 2.6 % (AV using YES) to 15 % (FD using YES-MβC) indicating that most methods overestimated the frequency of atoxigenic isolates. Interestingly, CAM was very precise and accurate using ammonium vapour (0 % false negatives) but not with fluorescence detection (13 % false negatives). False positive isolates were never detected.

HPLC and TLC assays found 63 atoxigenic isolates, all of which were correctly identified by Ammonium

Table 1 Frequency of aflatoxin-producing potential of *Aspergillusflavus* communities in different geographical regions

Province	Number of isolates (Nut/soil)				
	Aflatoxin B_1 production (μg/g)[c]			Yates'[*]	
	Highly toxigenic[a]	Low toxigenic[b]	Non-toxigenic	*p*-value	chi-square
Kerman	127/29	43/10	13/3	0.003	11.427
Khorasan Razavi	79/18	40/8	23/2	0.633	0.916
Esfahan	26/6	28/6	12/2	0.008	9.752
Qom	4/1	10/3	2/1	0.007	9.943
Semnan	3/1	5/1	0/1	0.350	2.101
Markazi	5/2	1/0	1/0	0.611	0.984
Yazd	3/1	1/0	1/2	0.248	2.791
Yates' *p*-value	0.036	0.017	0.166		
Yates' chi-square	13.438	15.341	9.129		
Total	247/58	128/28	52/11	$p<0.0001$	37.915

[*] Pearson's Chi-squared test with Yates' continuity correction was applied to compare the frequency of strains

[a] Alatoxin B1 higher than≥0.2 μg/g

[b] Aflatoxin B1 less than≤0.2 μg/g

[c] Aflatoxin B1 was quantified directly on TLC plates with a scanning densitometer. The limit of detection was 0.5 ng/g

vapour assays using CAM. In addition to triggering aflatoxin production in most isolates, CAM using Ammonium vapour also showed the highest intensity of colour changes compared to PDA and YES. Intensity of colour changes and aflatoxin values as determined by TLC were in 100 % agreement confirming reliability and precision of the technique (Table 3, Fig. 2). TLC assays showed a positive relationship between colour intensity and aflatoxin production (Table 4).

Molecular detection of atoxigenic isolates

PCR assays using primers specific for five genes in the aflatoxin biosynthesis gene cluster were used to assess genetic deletions explaining atoxigenicity in 63 isolates. In contrast to the tested atoxigenic isolates, no deletions were detected in aflatoxin-producing isolates. Results varied from no detected deletion (3 %) to deletions in all five genes (22.2 %). Most isolates (30 %) had major

Table 2 Frequency of toxigenic (+) and atoxigenic (−) *Aspergillus flavus* isolates using cultural, analytical and molecular assays

Group	No. of Isolates	Source	FD on		AV on			TLC	HPLC
			YES-MβC	CAM	PDA	YES	CAM		
1	308/72	Nut/Soil	+	+	+	+	+	+	+
2	116/28	Nut/Soil	−	+	+	+	+	+	+
3	108/26	Nut/soil	−	−	+	+	+	+	+
4	91/20	Nut/soil	−	−	−	+	+	+	+
5	62/15	Nut/soil	−	−	−	−	+	+	+
6	52/11	Nut/soil	−	−	−	−	−	−	−

FD fluorescence detection assay, *AV* ammonium vapor assay, *YES-MβC* yeast extract sucrose medium amended with 0.3 % methyl β-cyclodextrin, *CAM* coconut agar medium, *PDA* potato dextrose agar, *YES* yeast extract sucrose medium, *TLC* thin-layer chromatography, *HPLC* high performance liquid chromatography

Table 3 Intensity of color changes after exposure to ammonia vapor on coconut agar medium and corresponding aflatoxin B1 levels in rice flour after inoculations with *Aspergillus flavus* isolates

AFB_1[a] production (ng/g)	Color changing after AV response				
	Dark red >1000	Red Pink 200–1000	Pink 20–200	Pink Pale <20	No color 0
Number of isolates (Nut/soil)	13/3	234/55	84/20	43/9	52/11
Percentage (%)	3	55	20	10	12

[a] Aflatoxin B1 was quantified directly on TLC plates with a scanning densitometer; The limit of detection was 0.5 ng/g

deletions in three genetic regions (Table 5). The absence of detectable deletion in 3 % of isolates is currently under investigation.

Discussion

Atoxigenic isolates of *A. flavus* exist naturally and can be found on any susceptible crop anywhere around the globe where environmental conditions favour growth of *Aspergillus* species. Thus, the diversity of native *Aspergillus* communities associated with crops of interest can be exploited to develop substrate specific, local biological control strategies for long-term reduction of aflatoxin contamination (Mehl et al. 2012).

This is the first report identifying community structures of *A. flavus* in pistachio producing areas in Iran. *A. flavus* resided in all sampled pistachio orchards; displaying high variability in aflatoxin production across regions and provinces. For example, the most aflatoxigenic isolates (≥0.2 μg/g) were detected in Kerman province. Here incidences of atoxigenic isolates were low which might be explained by the intensive cultivation practices in the area with more than 55 % of orchards in a monoculture cropping, a long history of pistachio cultivation, dry and high temperature conditions, the highest area (56 %) under Ohadi cultivation (high frequency of early splitting and cracking among cultivars) (Tajabadipour and Panahi, 2005, reports of Iranian Pistachio Research Institute), (Tajabadipour et al., 2005, reports of Iranian Pistachio Research Institute) and (Abdolahi-Ezzatabadi et al., 2008, reports of Iranian Pistachio Research Institute), higher salinity in soil and water as well as longer irrigation intervals. Several theories regarding the influence of climate and climate change on aflatoxin production have been being developed. For example, Moradi and Javanshah (2006) demonstrated major differences in the quantity of aflatoxin contamination in small, deformed pistachios with yellow shell discolouration (either sinker or floater pistachios) depending on where samples were obtained from. Pistachios collected in Yazd province had less aflatoxin contamination than pistachios obtained from Kerman province. Jaime-Garcia and Cotty (2006) showed a direct impact of climate on the fungal community structure, quantity of aflatoxin producer, and aflatoxin contamination in different crops. Here contamination process undergoes two major phases; primary infection via pistachio hull cracking during nut developing (first phase) and secondary increases in aflatoxin levels during nut maturation until final consumption (second

Table 4 Agreement between cultural and analytical aflatoxin detection methods for reliable identification of atoxigenic *Aspergillus flavus* isolates

	Fluorescence detection		Ammonium vapor			TLC	HPLC
	YES-MβC	CAM	PDA	YES	CAM		
Agreement%[a]	85	87	92.8	97.4	100	100	100
False negative%	15	13	7.2	2.6	0	0	0

YES-MβC yeast extract sucrose medium amended with 0.3 % methyl β-cyclodextrin, *CAM* coconut agar medium, *PDA* potato dextrose agar, *YES* yeast extract sucrose medium, *TLC* thin-layer chromatography, *HPLC* high performance liquid chromatography

[a] Agreement was calculated for fluorescence, ammonium vapor and TLC assays with HPLC in the identification of atoxigenic isolates

Table 5 Deletion of genes in the aflatoxin biosynthesis gene cluster (*nor*B, *afl*R, *est*A, *avn*A) or flanking region (C3) among 63 atoxigenic *A. flavus* isolates

N	Code	Source	Origin	Genes involved in aflatoxin biosynthesis				
				C3	*nor*1	*afl*R	*est*A	*avn*A
1	16481	Nut	Khorasan Razavi	●	●	●	●	●
2	16488	Soil	Khorasan Razavi	●	●	●	●	●
3	16457	Nut	Esfahan	o	●	●	●	●
4	16471	Nut	Khorasan Razavi	○	●	●	●	●
5	16447	Nut	Kerman	●	○	●	●	●
6	16464	Nut	Esfahan	●	●	○	●	●
7	16478	Nut	Khorasan Razavi	●	●	○	●	●
8	16448	Nut	Yazd	○	○	●	●	●
9	16452	Nut	Kerman	○	○	●	●	●
10	16482	Nut	Khorasan Razavi	○	○	●	●	●
11	16485	Nut	Khorasan Razavi	○	○	●	●	●
12	16486	Nut	Khorasan Razavi	○	○	●	●	●
13	16472	Soil	Semnan	●	○	○	●	●
14	16445	Nut	Kerman	○	○	○	●	●
15	16468	Soil	Kerman	○	○	○	●	●
16	16469	Nut	Esfahan	○	○	○	●	●
17	16470	Nut	Esfahan	○	○	○	●	●
18	16483	Nut	Khorasan Razavi	○	○	○	●	●
19	16487	Nut	Khorasan Razavi	○	○	○	●	●
20	T2181	Nut	Kerman	○	○	○	●	●
21	16443	Nut	Kerman	○	○	●	○	●
22	16455	Nut	Esfahan	○	○	●	○	●
23	16456	Nut	Esfahan	○	○	●	○	●
24	T1681	Nut	Esfahan	○	○	●	○	●
25	16441	Nut	Khorasan Razavi	○	○	●	●	○
26	16458	Soil	Esfahan	○	○	●	●	○
27	16466	Nut	Khorasan Razavi	○	○	●	●	○
28	16475	Nut	Khorasan Razavi	○	○	●	●	○
29	16480	Nut	Khorasan Razavi	○	○	●	●	○
30	16492	Nut	Kerman	○	○	●	●	○
31	T2245	Nut	Kerman	○	○	●	●	○
32	16494	Nut	Kerman	●	○	○	○	●
33	16444	Nut	Kerman	○	○	○	○	●
34	16449	Soil	Yazd	○	○	○	○	●
35	16451	Soil	Kerman	○	○	○	○	●
36	16454	Nut	Kerman	○	○	○	○	●
37	16461	Nut	Qom	○	○	○	○	●
38	16498	Nut	Kerman	○	○	○	○	●
39	16459	Soil	Esfahan	○	○	○	●	○
40	16460	Nut	Esfahan	○	○	○	●	○
41	16489	Nut	Khorasan Razavi	○	○	○	●	○
42	16484	Nut	Khorasan Razavi	○	○	○	●	○

Table 5 (continued)

N	Code	Source	Origin	Genes involved in aflatoxin biosynthesis				
				C3	*nor*1	*afl*R	*est*A	*avn*A
43	16467	Nut	Qom	○	○	○	●	○
44	T5412	Nut	Esfahan	○	○	○	●	○
45	16497	Nut	Khorasan Razavi	○	○	●	○	○
46	16442	Nut	Khorasan Razavi	○	○	●	○	○
47	16491	Nut	Khorasan Razavi	○	○	●	○	○
48	16474	Nut	Khorasan Razavi	○	○	●	○	○
49	T4578	Nut	Esfahan	○	○	●	○	○
50	16446	Nut	Kerman	○	○	○	○	○
51	16450	Soil	Yazd	○	○	○	○	○
52	16453	Nut	Kerman	○	○	○	○	○
53	16462	Nut	Esfahan	○	○	○	○	○
54	16463	Nut	Esfahan	○	○	○	○	○
55	16465	Soil	Qom	○	○	○	○	○
56	16473	Nut	Markazi	○	○	○	○	○
57	16476	Nut	Khorasan Razavi	○	○	○	○	○
58	16477	Soil	Khorasan Razavi	○	○	○	○	○
59	16479	Nut	Khorasan Razavi	○	○	○	○	○
60	16490	Nut	Khorasan Razavi	○	○	○	○	○
61	16493	Soil	Kerman	○	○	○	○	○
62	16495	Nut	Khorasan Razavi	○	○	○	○	○
63	16496	Nut	Khorasan Razavi	○	○	○	○	○
64	16499 (toxigenic)	Soil	Kerman	●	●	●	●	●

Absence (empty circle) or presence (filled circle) of specified genetic regions in the aflatoxin biosynthesis gene cluster or flanking region of *Aspergillus flavus* isolates obtained from pistachio nuts and soil in Iran

phase). They have also mentioned aflatoxin contamination is prevalent both in warm humid climates and in irrigated hot deserts. In maize, Okoth et al. (2012) reported that the occurrences of *Aspergillus* spp. in two-agroecological zones (Makueni compared to Nandi) in Kenya was the same regardless of the differences in temperature and rainfall. Other studies have been shown that high temperatures, incidences of highly toxigenic strains and isolates, crop rotation and history of crop cultivation promote the occurrence of *A. flavus* and subsequently aflatoxin production (Cotty 1997; Donner et al. 2009; Jaime-Garcia and Cotty 2006; O'Brian et al. 2007; Zablotowicz et al. 2007; Atehnkeng et al. 2008). The underlying mechanisms explaining the varying aflatoxin levels in pistachio orchards still has to be investigated. Monitoring frequencies of atoxigenic isolates in the future will help to determine if Kerman is indeed favoured by toxigenic isolates or if low frequencies can be explained by yearly variations.

During the search for native atoxigenic *A. flavus* isolates associated with pistachio nuts in Iran, the present study evaluated the relative value of several cultural methods for reliable identification of atoxigenic *A. flavus* isolates. Most cultural methods overestimated the amount of atoxigenic isolates of *A. flavus* which can cause delays during the development of biological control strategies and, if undetected, also may jeopardize the success of the project. Coconut agar medium has been developed as a visual tool for aflatoxin detection more than two decades ago (Davis et al. 1987) and has been claimed both reliable and unreliable throughout the years (Ritter et al. 2009; Yazdani et al. 2010). However, visual detection of aflatoxins using coconut agar medium can be conducted multiple ways, e.g. using either ammonium vapour or fluorescence detection, and

a direct comparison of these techniques is lacking but may explain the differences experienced among research groups. The results of our study suggest coconut agar medium in combination with Ammonium vapour maybe the best assay to employ to reliably and quickly differentiate between toxigenic and atoxigenic isolates of *A. flavus*. Indeed this assay is in total agreement with findings from analytical methods (HPLC and TLC). This is in contrast to results obtained with coconut agar medium in combination with fluorescence detection where a high number of false negatives were experienced. In addition, the color response using ammonium vapour was positively correlated with the amount of aflatoxin produced by the tested isolates. In the future, color intensity may be used as qualitative indicator for estimating aflatoxin-producing potential of species and isolates helping to identify causal agents of contamination.

Reliable identification of atoxigenic isolates is a crucial step during the development of management strategies employing biological control (Mehl et al. 2012). *A. flavus* was the most prevalent aflatoxin-producing species in pistachio orchards across different agro-ecological zones. As expected, aflatoxin-producing potential ranged from highly toxigenic to completely atoxigenic. The latter ones might provide biological resources to competitively exclude aflatoxin producers and, thereby, reduce aflatoxin concentration in Iranian pistachio. The pool of identified atoxigenic isolates will be further investigated and tested for their potential as biological control isolates.

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References

Abbas, H. K., Shier, W. T., Horn, B. W., & Weaver, M. A. (2004a). Cultural methods for aflatoxin detection. *Journal of Toxicology, 23*, 295–319.

Abbas, H. K., Zablotowicz, R. M., Weaver, M. A., Horn, B. W., Xie, W., & Shier, W. T. (2004b). Comparison of cultural and analytical methods for determination of aflatoxin production by Mississippi Delta Aspergillus isolates. *Canadian Journal of Microbiology, 50*(3), 193–199.

Amaike, S., & Keller, N. P. (2011). *Aspergillus flavus. Annual Review Phytopathology, 49*, 107–133.

AOAC. (2000). Section 49.2.02 (AOAC Method 971.22) Preparation of standards. In Official methods of analysis, Seventeenth Edition. Gaithersburg, MD, USA, AOAC International.

Atehnkeng, J., Ojiambo, P. S., Donner, M., Ikotun, T., Sikora, R. A., Cotty, P. J., & Bandyopadhyay, R. (2008). Distribution and toxigenicity of *Aspergillus* species isolated from maize kernels from three agro-ecological zones in Nigeria. *International Journal of Food Microbiology, 122*(1), 74–84.

Bhatnagar, D., Ehrlich, K. C., & Cleveland, T. E. (2003). Molecular genetic analysis and regulation of aflatoxin biosynthesis. *Applied Microbiology and Biotechnology, 61*, 83–93.

Chang, P. K., Horn, B. W., & Dorner, J. W. (2005). Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. *Fungal Genetics and Biology, 42*(11), 914–923.

Cheraghali, A. M., Yazdanpanah, H., Doraki, N., Abouhossain, G., Hassibi, M., Ali-Abadi, S., & Zamanian, F. (2007). Incidence of aflatoxins in Iran pistachio nuts. *Food and Chemical Toxicology, 45*(5), 812–816.

Cotty, P. J. (1990). Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cotton seed. *Plant Disease, 74*, 233–235.

Cotty, P. J. (1997). Aflatoxin-producing potential of communities of *Aspergillus* section *Flavi* from cotton producing areas in the United States. *Mycological Research, 101*, 698–704.

Cotty, P. J., & Mellon, J. E. (2006). Ecology of aflatoxin producing fungi and biocontrol of aflatoxin contamination. *Mycotoxin Research, 22*, 110–117.

Cotty, P. J., Probst, C., & Jaime-Garcia, R. (2008). Etiology and management of aflatoxin contamination. In J. F. Leslie, R. Bandyopadhyay, & A. Visconti (Eds.), *Mycotoxins: Detection methods, management, public health and agricultural trade* (pp. 287–299). Oxfordshire: CAB International.

Davis, N. D., Diener, U. L., & Eldridgeand, D. W. (1966). Production of aflatoxins B_1 and G_1 by *Aspergillus flavus* in a semisynthetic medium. *Applied Microbiology, 14*, 378–380.

Davis, N. D., Iyer, S. K., & Diener, U. L. (1987). Improved method of screening for aflatoxin with a coconut agar medium. *Applied and Environmental Microbiology, 53*(7), 1593–1595.

Dini, A., Khazaeli, P., Roohbakhsh, A., Madadlou, A., Pourenamdari, M., Setoodeh, L., & Khodadadi, E. (2012). Aflatoxin contamination level in Iran's pistachio nut during years 2009–2011. *Food Control, 30*(2), 540–542.

Donner, M., Atehnkeng, J., Sikora, R. A., Bandyopadhyay, R., & Cotty, P. J. (2009). Distribution of Aspergillus section Flavi in soils of maize fields in three agroecological zones of Nigeria. *Soil Biology and Biochemistry, 41*(1), 37–44.

Dorner, J. W. (2004). Biological control of aflatoxin contamination of crops. *Journal of Toxicology, 23*, 425–450.

Dorner, J. W. (2008). Management and prevention of mycotoxins in peanuts. *Food Additives and Contaminants: Part A, Chemistry, Analysis, Control, Exposure and Risk Assessment, 25*(2), 203–208.

Doster, M. A., & Michailides, T. J. (1994). The development of early split pistachio nuts and their contamination by molds, aflatoxins, and insects. *Acta Horticulturae, 419*, 359–364.

Fente, C. A., Ordaz, J. J., Vazquez, B. I., Franco, C. M., & Cepeda, A. (2001). New additive for culture media for rapid

identification of aflatoxin-producing *Aspergillus* strains. *Applied and Environmental Microbiology, 67*(10), 4858–4862.

Gourama, H., & Bullerman, L. B. (1995). Aspergillus flavus and Aspergillus parasiticus: aflatoxigenic fungi of concern in foods and feeds: a review. *Journal of Food Protection, 58*(12), 1395–1404.

Hara, S., Fennel, D. I., & Hesseltine, C. W. (1974). Aflatoxin-producing strains of *Aspergillus flavus* detected by fluorescence of agar medium under ultraviolet light. *Applied Environmental Microbiology, 27*(6), 1118–1123.

Jaime-Garcia, R., & Cotty, P. J. (2006). Spatial relationships of soil texture and crop rotation to *Aspergillus flavus* community structure in south Texas. *Phytopathology, 96*, 599–607.

Klich, M. A. (2002). *Identification of common Aspergillus species*. Utrecht: Centraalbureau voor Schimmelcultures.

Mehl, H. L., Jaime, R., Callicott, K. A., Probst, C., Garber, N. P., Ortega-Beltran, A., Grubisha, L. C., & Cotty, P. J. (2012). *Aspergillus flavus* diversity on crops and in the environment can be exploited to reduce aflatoxin exposure and improve health. *Annals of the New York Academy of Sciences, 1273*, 7–17.

Michailides, T. J., Doster, M., Cotty, P. J., Morgan, D., Bockler, L. Felt, D. & Reyes, H. (2007). Aflatoxin control in pistachio: Biocontrol using the atoxigenic strain AF36, survival of AF36 and EUP status. Resource document. Proceeding of the 2007 annual multicrop aflatoxin/fumonisin elimination and fungal genomics workshop. http://www.ars.usda.gov/SP2UserFiles/Place/53254100/AEW/AEW2007.pdf. Accessed 3 Aug 2014.

Moradi, M., & Hokmabadi, H. (2011). Control of mycotoxin Bioactives in nuts: Farm to fork. In O. Tokusoglu & C. Hal III (Eds.), *Fruit and cereal bioactives, sources, chemistry, and applications* (pp. 291–315). Turkey: CRC Press.

Moradi, M., & Javanshah, A. (2006). Distribution of aflatoxin in processed pistachio nut terminals. *Acta Horticulturae (ISHS), 726*, 431–436.

Moradi, M., Oerke, E.-C., Steiner, U., Tesfaye, D., Schellander, K., & Dehne, H. W. (2010). Microbiological and Sybr®Green Real-Time PCR detection of major Fusarium Head Blight pathogens on wheat ears. *Microbiology, 79*(5), 646–654.

Nasir, M. S., & Jolley, M. E. (2002). Development of a fluorescence polarization assay for the determination of aflatoxins in grains. *Journal of Agricultural and Food Chemistry, 50*(11), 3116–3121.

O'Brian, G. R., Georgina, D. R., Wilkinson, J. R., Yu, J., Abbas, H. K., Bhatnagar, D., Cleveland, T. E., Nierman, W., & Payne, G. A. (2007). The effect of elevated temperature on gene transcription and aflatoxin biosynthesis. *Mycologia, 99*(2), 232–239.

Okoth, S., Nyongesa, B., Ayugi, V., Kang'ethe, E., Korhonen, H., & Joutsjoki, V. (2012). Toxigenic potential of *Aspergillus* species occurring on maize kernels from two agro-ecological zones in Kenya. *Toxins, 4*(11), 991–1007.

Park, D. L. (2002). Effect of processing on aflatoxin. *Advances in Experimental Medicine and Biology, 504*, 173–179.

Preacher, K. J. (2001). Calculation for the Chi-square test. An interactive calculation tool for chi-square tests of goodness of fit and independence. Ohio State University: http://www.quantpsy.org/-chisq/chisq.htm. Accessed 1 July 2014.

Probst, C., & Cotty, P. J. (2012). Relationship between in vitro and in vivo aflatoxin production. Reliable prediction of fungal ability to contaminate maize with aflatoxins. *Fungal Biology, 116*, 503–510.

Raper, K. B., & Fennell, D. I. (1965). *The genus Aspergillus*. New York: Springer.

Ritter, A. C., Hoeltz, M., & Noll, I. B. (2009). Toxigenic potential of *Aspergillus flavus* tested in different culture conditions. *Ciência e Tecnologia de Alimentos, 31*(3), 623–628.

Saito, M., & Machida, S. (1999). A rapid identification method for aflatoxin-producing strains of *Aspergillus flavus* and *A. parasiticus* by ammonia vapor. *Mycoscience, 40*, 205–208.

Sardiñas, N., Vázquez, C., Gil-Serna, J., González-Jaén, M. T., & Patiño, B. (2011). Specific detection and quantification of *Aspergillus flavus* and *Aspergillus parasiticus* in wheat flour by SYBR® Green quantitative PCR. *International Journal of Food Microbiology, 145*(1), 121–125.

Shier, W. T., Lao, Y., Steele, T. W., & Abbas, H. K. (2005). Yellow pigments used in rapid identification of aflatoxin-producing *Aspergillus* strain are anthraquinones associated with the aflatoxin biosynthetic pathway. *Biorganic Chemistry, 33*, 426–438.

Wei, D., & Jong, S. (1986). Production of aflatoxins by strains of the *Aspergillus flavus* group maintained in ATCC. *Mycopathologia, 93*, 19–24.

Williams, J. H., Phillips, T. D., Jolly, P. E., Stiles, J. K., Jolly, C. M., & Aggarwal, D. (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *American Journal of Clinical Nutrition, 80*, 1106–1122.

Wiseman, H. G., Jacobson, W. C., & Harmeyer, W. C. (1967). Note on removal of pigments from chloroform extracts of aflatoxin cultures with copper carbonate. *Journal of the Association of Official Analytical Chemists, 50*, 982–983.

Yazdani, D., Zainal-Abidin, M. A., Tan, Y. H., & Kamaruzaman, S. (2010). Evaluation of the detection techniques of toxigenic *Aspergillus* isolates. *African Journal of Biotechnology, 9*(45), 7654–7659.

Zablotowicz, R. M., Abbas, H. K., & Locke, M. A. (2007). Population ecology of *Aspergillus flavus* associated with Mississippi Delta soils. *Food Additives and Contaminants, 24*(10), 1102–1108.

Zheng, Z., Saghaian, S., & Reed, M. (2012). Factors affecting the export demand for U.S. pistachios. *Agribusiness Management Review, 15*(3), 139–154.