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ORIGINAL ARTICLE

A Simple Procedure to Evaluate Competitiveness of Toxigenic and Atoxigenic Isolates of *Aspergillus flavus* in Solid and Liquid Media

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ABSTRACT: Application of atoxigenic strains of *Aspergillus flavus* to reduce aflatoxin levels is th successful strategy applied in some agricultural crops. The role of ammonium hydroxide for preli screening of the competitiveness of atoxigenic *A. flavus* isolates to interfere with aflatoxin produc highly toxigenic isolates were evaluated. Out of 270 *A. flavus* isolates, 17 were detected as true ato using cultural methods and confirmed by analytical assays from different pistachio agro-ecologica during 2013. For assessment competitive ability among atoxigenic isolates of *A. flavus* with highly t ic one, rice flour, coconut agar and coconut broth medium substrates were inoculated with mixtures ing combinations of toxigenic and atoxigenic isolates, simultaneously. The rice flour substrate was quantify the content of aflatoxin in either co-inoculations or toxigenic isolate alone on thin layer chro raphy plates with a scanning densitometer. While the culture media were used to determine the inte color change on exposing to ammonium hydroxide vapor. The reduction rates of aflatoxin B_1 inoculations were varied and ranged from 2%-82%. Based on the intensity of colony color chan competitiveness of the isolates was classified into five groups. Atoxigenic isolates with high comp ness have shown low color changes in culture media and high aflatoxin reduction in TLC assays with of higher than 78%. The method will facilitate preliminary screening of efficient atoxigenic isolates

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igation of aflatoxins in food and feed as a cheap, simple and quick method.

INTRODUCTION

Aflatoxins are toxic secondary metabolites produced by some species of *Aspergillus* on different crops that have adverse effects causing disease and death in humans and other animals. Several approaches have been proposed to reduce the risks of aflatoxin contamination in different crops through physical, chemical, cultural, and biological methods [1, 2]. Among these methods, biological control has been shown the most promising for long-term management of aflatoxin-producing molds under field conditions [1, 3]. A number of biological control agents including bacteria, yeasts, and atoxigenic (non-toxigenic) strains of *A. flavus* have been evaluated for ability to reduce densities of natural *A. flavus* populations, to interfere with aflatoxin biosynthesis, or to competitively exclude aflatoxin producers from crops. Atoxigenic strains of *A. flavus* are the most approaches to reduce aflatoxin contents in food and feed through competitive exclusions [4-8]. To achieve sustainable biological control it is necessary to identify native, well adapted atoxigenic strains [9]. Identification of atoxigenic isolates of *A. flavus* native to Iran might provide an environmentally sound, ecologically adapted, native, biological resource useful in mitigating aflatoxin contamination of pistachio produced in Iran.

In particular, analytical methods including thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) or immunoassay method such as enzyme-linked immune sorbent assay (ELISA) are proven for aflatoxins detection. These methods are accurate and reliable but require expensive laboratory equipment and supplies [10]. Availability of these methods may be limited especially in developing countries. Here, qualitative, cultural methods provide a cheaper alternative to screen large volumes of fungal isolates for aflatoxin production. Culture-based methods for detection of a contaminated sample with aflatoxins may classify as blue fluorescence of aflatoxin B_1 which enhanced by iodine or β-cyclodextrin in medium [11, 12], yellow pigment production [13] and color change as exposure to ammonium hydroxide [14, 15]. Cotty [16] described a simple method for detection of aflatoxins in solid media by measuring fluorescence using scanning densitometer. Hybrid cultural/analytical methods have also been developed. Cultural methods are inexpensive, time effective, no advanced facilities and skills required, fast and cost effective especially in developing countries.

A new, sensitive and rapid method was developed for differentiation of aflatoxin-producer and non-producer strains of *A. flavus*, *A. parasiticus*, *A. oryzae*, and *A. sojae* [15]. In this method, undersides of aflatoxin-producing colonies quickly turn plum red as exposure to ammonium hydroxide vapor. The intensity of color change was affected by culture media that was the highest for colonies grown on yeast extract sucrose and coconut media. The method was validated by HPLC and UV fluorescence assays in 120 strains [15]. Toxigenicity of isolates affected on color change intensity that was the highest for high toxigenic isolates [14]. The mechanism based on the production of yellow anthraquinone biosynthetic intermediates in the aflatoxin pathway. These compounds are as pH indicator, which at alkaline pH are colored red [17, 18]. Application of cultural based methods for detecting aflatoxin in fungal culture has been more pronounced to screen a candidate for successful of biological control aflatoxigenic fungi which required to screen numerous isolates of *A. flavus*. The present study was undertaken to assess the role of ammonium hydroxide vapor for preliminary screening of the competitiveness of atoxigenic *A. flavus* isolates

for their ability to interfere with aflatoxin production by highly toxigenic isolates.

MATERIALS AND METHODS

Fungal isolates

To obtain *Aspergillus* section *flavi* isolates, pistachio nuts and soil samples were collected from Kerman, Esfahan, Semnan, Khorasan Razavi, Qom, Markazi and Yazd Provinces, Iran, during 2010-2013. An *A. flavus* and Parasiticus agar (AFPA) medium and dilution plate technique were used for isolation [19]. *A. flavus* isolates were identified based on morphological features [20] and molecular assay [21].

Spore preparation

Single spore *A. flavus* isolates (n=9; 8 atoxigenic, 1 highly toxigenic) were cultured on potato dextrose agar (PDA) slant for 10 d to produce conidia. The conidia suspension were prepared by adding 10 ml sterile distilled water with 0.05 tween 80 to each slant and scrapping the mycelia with a loop. The conidia suspensions were filtered through sterile cheesecloth filter paper and filter paper to remove the hyphae. The concentrations of conidia were determined using a turbiditimeter (Hach, 2100Q, Loveland, Colorado, USA) [22].

Assessment of aflatoxin production

A. flavus isolates (n=270) were cultured on yeast extract- sucrose + methylated beta- cyclodextrin (YES+MβC) [11] and incubated at 30 ºC for 3-5 d in the dark. Presence (positive= aflatoxin producer) or absence (negative= non-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). For ammonium hydroxide vapor analyses, *A. flavus* isolates (n=270) were cultured on the coconut agar medium (CAM) [23] and incubated at 30 ºC for 3 d in the dark. The toxigenicity of the isolates was also confirmed by TLC [21].

Competitiveness tests

TLC assays

Among 17 atoxigenic, isolates screened from pistachio orchards using cultural and analytical methods, eight isolates were used to assess their ability to interfere with aflatoxin production by a highly toxigenic *A. flavus* strain (Ker30) isolated from highly infected pistachio nuts. For this, rice flour was distributed into glass flasks (10g of rice flour per 250-ml flask) and sterilized. Equal amounts inocula (2×10^5 conidia/isolate/flask) of toxigenic and atoxigenic isolates were mixed immediately before inoculations of rice flour by gently agitated to coat the flour surface with the inoculum. Rice moisture was set to 25%. Each *A. flavus* isolate was tested using three replicates. Inoculated rice flour was incubated at 30 °C for 7 d in the dark. To extract aflatoxins, 50 ml of 80% methanol were added to the colonized rice in each flask and evenly homogenized. Then the mixture was filtered through Whatman® no. 4 filter paper. Culture filtrates were spotted directly onto thin-layer chromatography (TLC) plates (Silica gel 60; EMD, Darmstadt, Germany) adjacent to aflatoxin standards. The plates were developed in ethyl ether/methanol/water (96:3:1), air-dried, and the aflatoxins were visualized under 365 nm UV light [24]. Aflatoxins were quantified directly on TLC plates with a scanning densitometer (TLC Scanner 3; Camag Scientific Inc., Wilmington, NC). Aflatoxin production was measured in ng/g of rice flour 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 five concentrations over the range 2.5-12.5 (2. 5, 7.5, 10 and 12.5) ng/mL aflatoxins B1 (Figure 1). The limit of detection was 4 ng/g for aflatoxins B1 based on a signal to noise ratio of 3:1.

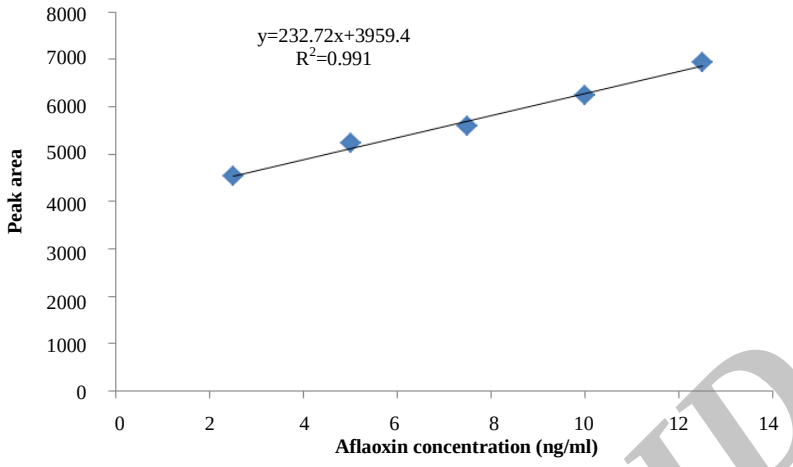

Figure 1. Standard curves for detection of aflatoxin B1 in rice flour

Ammonia vapor (AV) assays

Coconut agar medium (CAM) as a solid substrate [21] and coconut broth (CB) as a liquid medium were used to assess the competitiveness between toxigenic and atoxigenic isolates using ammonium vapor analyses. A mixture of two parties included toxigenic and atoxigenic isolates (4×10^3 conidia/isolate/plate) were prepared and inoculated at center of plates in three replicates. The plates were incubated at 30 ºC for 3 d in the dark. Color development (yellow pigmentation) (Figure 2A) and intensity of ammonia vapor exposure of the colonies (Figure 2B) were used to assess the interaction between toxigenic and atoxigenic isolates which is an indicative for aflatoxin synthesis. 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 vapor [14, 15]. Based on the color development and intensity, isolates were classified into five groups- from strongest to no color changes (Figure 2). In another set of experiments, coconut broth was used to assess interactions between toxigenic and atoxigenic isolates. For this, 200 g desiccated or shredded coconut homogenized for 5 min in one liter of boiled water and filter through 4 layers of cheesecloth. Five ml aliquots of the suspensions were aseptically transferred to glass-tube and sterilized. The coconut broth was inoculated with equal amounts of toxigenic and non-toxigenic isolates of *A. flavus* in two party mixtures with concentration of 5×10^5 conidia/ml. The glass-tubes were incubated at 30 ºC for 3 d in the dark. After incubation, a drop (0.2 ml) of 25% ammonia solution (Merck, Germany) was placed into each media tubes to release ammonium color development (Figure 2. C). Each isolate was tested three times and experiments were replicated twice.

The Pearson correlation coefficient was used to measure the strength of a linear association between aflatoxin production in rice flour (ng/g) and intensity of color development in culture medium on exposing to ammonium vapor.

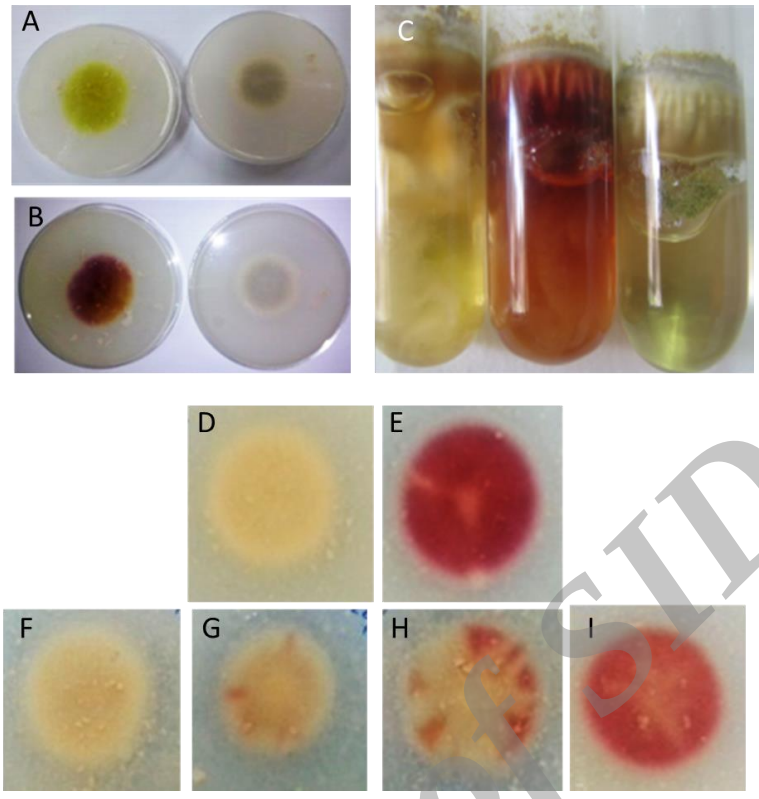

Figure 2. Intensity of color development (presence of aflatoxin development) on coconut agar medium of upon contact with ammonium hydroxide in competition tests.

A. Yellow pigments in mycelium of toxigenic strain (Ker30) of *Aspergillus flavus* (left) and absence in the atoxigenic isolate (2621), B. Color change of colony exposure ammonium hydroxide (left to right, Ker30 and 2621), C. left to right two party combinations of atoxigenic + toxigenic isolates, inoculation of CB medium with toxigenic and atoxigenic isolates alone, D. No color change (atoxigenic isolate), E. Dark red (toxigenic isolate); F. No color (78%-82% aflatoxin reduction, atoxigenic dominancy), G. Pink pale or weak pink streak (56%-61% aflatoxin reduction), H. Pink or strong pink streak (25%-38% aflatoxin reduction), I. Red Pink (20% aflatoxin reduction, toxigenic strain dominancy).

RESULTS AND DISCUSSION

Overall, 270 isolates of *A. flavus* were isolated from pistachio producing areas such as Kerman, Isfahan, Semnan, Khorasan Razavi, Yazd and Qom provinces, Iran. Applications of cultural and analytical methods showed that only 17 isolates are not able to produce aflatoxins named as true atoxigenic isolates. Inoculation of rice flour with toxigenic isolate resulted in aflatoxin production ranged from 1309 to 1521ng/g.

All eight atoxigenic *A. flavus* isolates were associated with lower content of aflatoxin in rice flour when co-inoculated with a highly toxigenic compared with inoculations of toxigenic strain alone. The reduction rate of aflatoxin contents varied from 1.9±1.2% to 80±2.0% in co-inoculations compared to inoculations with toxigenic alone (Table 1).

Based on the color changes the competitiveness of the isolates was classified into five groups as shown in Table 1 and Figure 2 (D-I). Atoxigenic isolates with high competitiveness such as 2221, 1921 and 64222 shown low color changes assessing with cultural methods and high aflatoxin reduction in TLC experiments by a reduction of higher than 78%. Similar results also were obtained with low competitive atoxigenic isolates such as

502, where TLC assays shown low aflatoxin reduction and the presence of color changes in colonies on ammonia vapor exposure. The both analytical and cultural methods produce the same results for all atoxigenic isolates in interactions with toxigenic strains to reduce aflatoxin biosynthesis. The ability of atoxigenic isolates to reduce aflatoxin production by highly toxigenic reduce in the order of by cultural and TLC assays 2221, 1921, 64222, respectively. Coconut broth produced the same results (Figure 2-C) as coconut agar medium; however, more investigation is required to produce quantitative using the intensity of color change.

Significant relationships (R^2>0.97; P≤0.001) were observed between qualitative and quantitative assessment of ability of atoxigenic isolates to interference with aflatoxin production by highly toxigenic isolate. A board agreement between TLC assays (aflatoxin B1 ng/g) and intensity of color change on exposing to ammonium hydroxide vapor to screen the competitiveness of atoxigenic isolates shown in Figure 3.

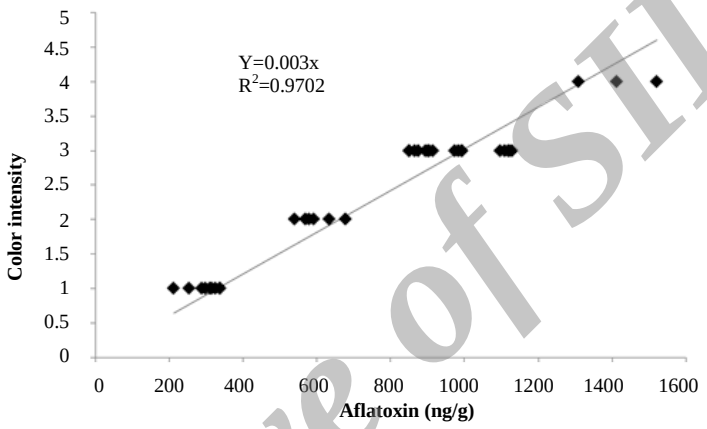

Figure 3. Pearson correlation coefficient between TLC assays (aflatoxin Con. ng/g) and intensity of color changes on exposing to ammonium vapor.

Table 1. Aflatoxin B_1 content and intensity of color changes after exposure to ammonium hydroxide vapor on coconut agar medium (CAM) and coconut broth (CB) in co-inoculation tests

No.	Inoculations	Aflatoxin B_1 (ng/g)	Reduction rate of aflatoxin B_1 (%)	Color change in CAM
1	2621×Ker30	894.8	38	+++
2	2622×Ker30	562.9	61	++
3	19121×Ker30	894.8	38	+++
4	19122×Ker30	1082.5	25	+++
5	64221×Ker30	635.1	56	++
6	64222×Ker30	317.5	78	+
7	1921×Ker30	317.5	78	+
8	2212×Ker30	995.9	31	+++
9	502×Ker30	1414.5	2	++++
10	K22×Ker30	981.5	32	+++
11	K85×Ker30	981.5	32	+++
12	2221×Ker30	259.7	82	+
13	Ker30	1443.5	0	+++++

+ No color, ++ Pink Pale (or weak pink streak), +++ Pink (or strong pink streak), ++++ Red Pink, +++++ Red

Aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2) produced by several species of *Aspergillus* in many of food and feed throughout world affecting global safety concern from farm to fork [25]. There are several highly specific and sensitive methods to detect aflatoxin concentrations in various commodities or substrates, such as HPLC, ELISA, TLC and fluorescence polarization assay [10, 26]. However, these methods are expensive laboratory analysis, technology based, operator skills, and time-consuming – difficult to use for developing countries and number of samples to be tested.

Here, we have developed a rapid, simple, low-cost and sensitive method to screen the competitive ability of atoxigenic isolates to interfere with aflatoxin productions by toxigenic strains.

Here, a new approach was developed used in laboratories with low facilities for screening of competitiveness as many as isolates/strains in a short period. The basic principle for detection of competitiveness of the atoxigenic isolates is color change and its intensity affected by the isolates. The visual color changes are the production of yellow anthraquinone pigments during aflatoxin biosynthesis by aflatoxigenic *A. flavus* isolates [27]. The yellow pigments turn to plum red in the presence of ammonia hydroxide at alkaline pH [15, 17, 18, 28 and 29].

CONCLUSIONS

A rapid, simple, low-cost and sensitive method to screen the competitive ability of a large number atoxigenic isolates to interfere with aflatoxin productions by toxigenic strains were developed. The method will facilitate studies on different aspects of atoxigenic isolates to mitigate of aflatoxins in food and feed.

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