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ORIGINAL ARTICLE

Efficacy of saprophytic yeasts on growth of Aspergillus flavus and aflatoxin production

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Background: Contamination of pistachio nuts with aflatoxin, which produces strains of *Aspergillus* section Flavi, has been a concern for the human and animal health and food safety.

Materials and Methods: Competitive ability of saprophytic yeasts in interfering with toxigenic strain of *Aspergillus flavus* was assessed through different experiments. 186 soil samples were collected from different agro-ecological zones of pistachio growing areas of Kerman province. A serial dilution method and YMA media were used to isolate yeast strains. Overall, 60 yeast isolates were screened for their ability to reduce the biomass of *Aspergillus flavus* via dual culture assays.

Results: The highest inhibition was achieved by five yeast isolates in the ranges 38 to 82%. Other investigations have demonstrated the presence of significant interactions between saprophytic yeasts and *A. flavus* on biomass and aflatoxin production. The highest rate of reduction belonged to the IPRIY-43 and IPRIY-39 which reduced the growth of *A. flavus*in culture media.

Conclusions: Quantitative assessments showed the ability of five selected yeast isolates to reduce aflatoxin production by 85-98% in dual culture assays. More investigations are required to understand the ability of yeast isolates to reduce aflatoxin production with toxigenic strains of *A. flavus* and withstand predominant abiotic stresses.

Keywords: biological control; food safety; health; pistachio; yeast

1. Introduction

Contamination of pistachio nut by *Aspergillus* species is the most serious challenge to pistachio production, consumption, and exportation in Iran. As a result, pistachios are commonly infected with aflatoxins, which are highly carcinogenic secondary fungal metabolites [1]. Aflatoxins diminish crop value and impose health risks to both humans and animals [2]. 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 [3, 4].

Physical, chemical, cultural, and biological control strategies have been developed to mitigate aflatoxin [5-8]. Applicability of these strategies depends on state legislation, agricultural commodity, and financial and practical capabilities [9, 10]. However, most methods have not widely been applied due to state legislation, high cost, or difficulties in applications [11]. Biological control has been used as the most promising approach to control aflatoxins [12]. Extensive research has been conducted on using biological control to interfere or modify natural *A. flavus* populations and the resultant aflatoxin biosynthesis in different crops using different species of bacteria, yeasts, and atoxigenic strains of *A. flavus* [9, 13-16]. Saprophytic yeast species such as *Candida krusei* and *Pichia anomala* have been promising as a biological control to mitigate mold population and aflatoxin in pistachios [17]. Several studied have shown the ability of saprophytic yeasts to manage many plant diseases before and after harvest [9]. High potential ability to colonize various ecological niches under abiotic and biotic stresses, production of extracellular polysaccharides, and low sensitivity to pesticides could make them candidates with high potential for biological control of mycotoxigenic fungi [18, 19]. The present study was conducted to screen a high number of yeast strains isolated from commercial pistachio orchards of Kerman province for their ability to reduce the growth of *Aspergillus flavus* and aflatoxin production in different assays.

2. Material and methods

2.1. Sampling and Isolation of yeasts

Soil samples were collected from commercial pistachio orchards in Kerman province during 2012-2015 using a diagonal pattern. In each orchard, 15 sub-samples were taken from the top 5 cm of the soil surface and thoroughly homogenized and sieved. Ten grams of each sample was

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mixed with 90 ml of 0.1% sterile peptone water and shaken vigorously for 60 min. Nuts were randomly picked up from 8 pistachio trees in each orchard (50 nuts per tree) and transported in paper bags to the laboratory for yeast isolation. One hundred pistachio nuts were submerged in 500 ml of 0.1% peptone water and shaken vigorously for 60 min. A serial dilution of 10^{-1} to 10^{-6} in 100 µl aliquots were spread on YMA medium in two replications using a rotator plate [20]. Petri-dishes were incubated at 28°C in darkness for 2 to 3 days. The yeast colonies were purified based on their shapes and colors [20] and stored in sterile distilled water for a short period of time.

2.2. *A. flavus* isolate

A strain of *A. flavus* originally isolated from pistachio nuts in the Kerman region was obtained from the Iranian Pistachio Research Center culture collection and maintained on Potato Dextrose Agar (PDA, Merck, Germany).

2.3. *In vitro* screening of yeast antagonistic activity

The ability of isolated yeast to reduce mycelial growth of *A. flavus*was determined in dual culture assays. Fifteen ml of YMA medium was poured into a 9 cm petri plate. In dual culture assays, 5 µl of an actively growing suspension of yeast strains was streaked 3.5 cm apart from the center of the plates and incubated at 25°C for 24 hours. Then *A. flavus* was streaked in the center of each petri dish. Petri dishes were incubated at 28˚C in the darkness, and inhibition of radial fungal growth was noted every 24 hours for 5 days [21]. Fungal growth with no yeast inoculum was used as control plates. The yeast strains with high potential ability to reduce mycelial growth of *A. flavus* growth were further assessed via volatile and non-volatile compounds. For this purpose, 24 h old cultures of yeast strains were cultured into individual flasks containing 50 ml sterilized potato dextrose broth (PDB) (Himedia, Pvt. Ltd., India). Flasks were placed on a rotary shaker (150 rpm) at room temperature for 4 days to promote the growth of yeast strains. The suspensions were passed through Whatman no. 1 filter paper (Whatman®, Sigma-Aldrich, Germany) and autoclaved. The autoclaved suspension was mixed with YMA in ratios of 1:19, 3:17, and 5:15, and poured into petri dishes at 45°C. Then small discs of actively growing of 3 days *A. flavus* old were cultured on the center of the plates. The mycelial growth of *A. flavus* was monitored every 48 hours for 5 days [22]. To assess the effects of volatile compounds on mycelial growth of *A. flavus*, yeast strains were streaked out on YMA plates and incubated for 2 days at 27˚C. A 5 mm agar plug of an actively growing *A. flavus* strain was placed in the center plates and placed upside down on a petri dish containing a yeast strain. The construct plates were sealed with paraffin film (Parafilm, Sigma-Aldrcih, Germany) and incubated for 7 days at 27˚C in the dark, and mycelial growth of *A. flavus* was monitored every 48 hr. The ability of strains to inhibit growth of *A. flavus* was calculated using the following formula:

$I = \frac{C-T}{C} \times 100$, where I is the inhibition of mycelial growth (%), C is the growth in control petri-dishes, and T is growth in the interaction assays.

Five yeast strains with the most promising biocontrol features were used to assess their ability to interfere with aflatoxin production by a toxigenic strain of *A. flavus.* For this purpose, yeast strains and *A. flavus* were streaked out on MMEA as described by La-penna *et al.* [14]. Petri-dishes were incubated at 28°C for 10 days in darkness. The ability of yeast strains to reduce aflatoxin in dual culture was assessed using high performance liquid chromatography, as describe by Fani *et al.* [3].

2.4. Statistical analysis

The average values of inhibition of mycelial growth of *A. flavus* and aflatoxin production were separately determined for each replication. Mean comparisons were made using Duncan's new multiple range test at 5% probability. If needed, the data were log-transferred prior to analysis.

3. Results

Preliminary screenings were carried out to identify the yeast isolates with high ability to reduce the growth of *A. flavus*. Overall, 60 isolates were selected for further assays to reduce the biomass of *A. flavus* via the dual culture method. According to the results, all 60 yeast strains were able to reduce the growth of *A. flavus*, ranging from 25 to 61%. Five yeast isolates with the highest antagonistic ability to reduce the growth of *A. flavus* and aflatoxin production were used in further experiments. The PRIY-39, PRIY-41 and PRIY43 isolates showed the highest antagonistic abilities to reduce the biomass of *A. flavus* in all experiments. More evaluations of the five yeast isolates showed different degrees of inhibitory effects on *A. flavus*, reducing mycelial growth from 28-63, 57-81 and 41-61% in non-volatile, volatile, and dual culture assays, respectively (Fig. 1).

Of the five isolates, PRIY-39 and PRIY-43 isolates exhibited the greatest inhibitory effect on mycelial growth of *A. flavus* compared to the other isolates for non-volatile, volatile, and dual culture assays. There were no significant differences between these two isolates in most estimated parameters to reduce the biomass of *A. flavus*.

The effects of selected yeast strains on aflatoxin production were more pronounced, which showed the greatest potential to inhibit aflatoxin production by more than 85% for all tested isolates. PRIY-41 had the highest effects to inhibit aflatoxin production by 98% compared to the other isolates as well as *A. flavus* alone.

Based on the morphological and physiological features PRIY-9 and PRI-10, and PRIY-41, PRIY-39 and PRIY-43 strains were identified as *Issatchenkia* sp. and *Kluyveromyces* sp., respectively.

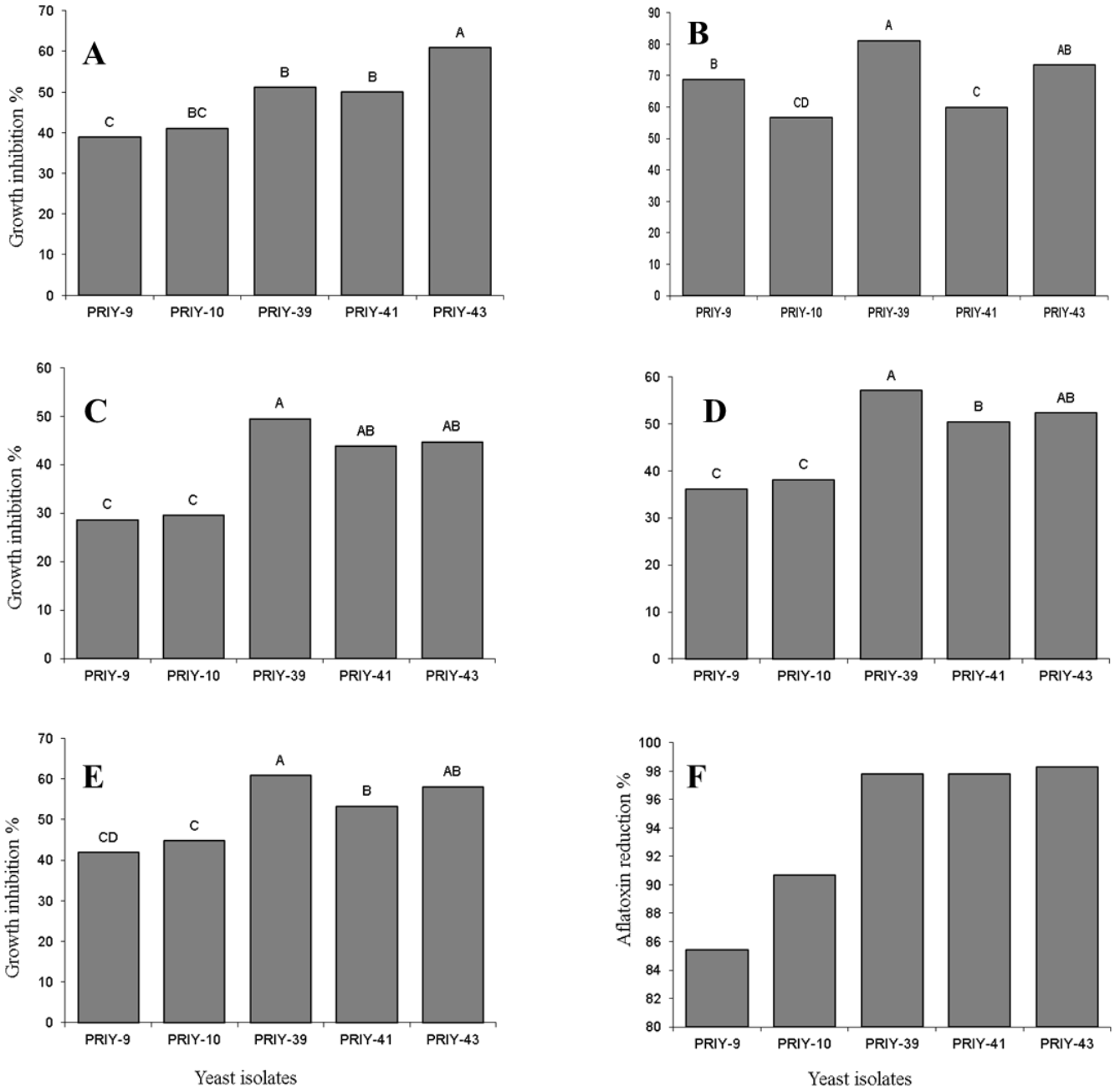

Fig. 1. Effects of yeast isolates on the biomass and aflatoxin production by *Aspergillus flavus*. A, B, C, D, E and F; Dual culture, volatiles compounds, culture filtrate 5, 15 and 25% and aflatoxin reduction, respectively

4. Discussion

Mold infection and its mycotoxins have been demonstrated in many food crops, and their commodities continue to remain as a challenge for food safety. Aflatoxins are the most dangerous mycotoxins which have been detected in food and feed. Yeast isolates may play an important role in reducing the risks of mycotoxins in various foods and feeds pre- and post-harvest.

The present study was carried out to select the potential yeast isolates that could reduce the colonization of pistachio nuts by toxigenic strains of *A. flavus*as well as aflatoxin production through different assays. Saprophytic yeasts were isolated from fruits of pistachio and soil of pistachio orchards. Overall, yeast isolates were found in different agro-ecological zones of pistachio producing areas of Kerman province with varying frequencies. Yeast isolates were identified as genus level based on physiological, biochemical, and morphological characterizations. The saprophytic yeast has shown a wide range ofinhibitory effects on mycelial growth and aflatoxin production. Our results showed that *Issatchenkia* sp. and *Kluyveromyces* sp. isolates may negatively affect the populations of toxigenic strains of *A. flavus* in the orchards and, as a consequence, aflatoxin production.

It has been reported that the production of 2-phenylethanol, trehalose, and sorbitol by *Pichia anomala* are major compounds which negatively affect spore germination, growth, toxin production, and gene expression in *A. flavus* [23, 24].

Of the isolates, PRIY-39 and PRIY-43 showed the greatest inhibitory effects on mycelial growth of *A. flavus* compared to the other isolates in different assays.

Hua *et al.* [25], using a visual agar plate assay, screened yeast strains for their abilities to inhibit aflatoxin production by nor mutants of *A. flavus*, which may be useful for reducing aflatoxin contamination in pistachios. It has been demonstrated that *Pichia anomala* WRL-076 could reduce the spore production of *A. flavusin vitro* and *in vivo* experiments [19, 26].

Further investigations are required to understand the competitive ability of native yeast isolates to reduce populations of *A. flavus* in soil, pistachio fruit, and plant debris and withstand predominant abiotic stresses in the orchards.

5. Conclusions

The present study showed that yeast isolates are common microflora of pistachio orchards and can be used to mitigate toxigenic molds in pistachio nuts, which require more investigations to develop biological control strategies.

Conflicts of interest

The authors declare no conflicts of interest.

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