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ORIGINAL ARTICLE

Efficacy of some Yeast Strains for Preventing Infection of Pistachio with *Aspergillus flavus* and Aflatoxin

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K E Y W O R D S

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A B S T R A C T

Aspergillus flavus and aflatoxin on pistachio are the most important hazards to human health, which start in the orchard and will continue to the storage. This research selected 16 most effective yeast strains through dual culture (DC) assay according to the interaction between 376 yeast strains and *A. flavus* isolate P1684 in an initial screening. The antifungal ability of the strains was determined to be 94.5%, 72.08 %, and 91.31% in DC, volatile organic compounds (VOCs), and extracellular secretions (ESs) assays, respectively. In DC and VOCs assays, the YE 43-6 yeast strain showed maximum inhibition of growth, whereas powder and liquid formulation of the YE 43-10 yeast strain showed better performance. Ammonia vapor assay revealed that yeast strains significantly reduced aflatoxin production in *A. flavus*. Under in-situ conditions, trees were sprayed with cell/spore populations of both effective yeasts and *A. flavus* to determine the ability of yeast strains to compete with the pathogen. After 45 days, the fruits were harvested, and the average number of colonies per pistachio fruit unit (CFU/Nut) was determined. The highest and lowest reduction of populations were observed in YE 43-10 (85.71%) and YE 36-9 (35.18%), respectively. In both YE 43-6 and YE 43-10 strains, powder formulation was slightly more effective than liquid, and VOCs effectively prevented pistachio contamination with *A. flavus* and aflatoxin.

Introduction

Pistachio trees produce commercially valuable edible seeds that are mainly traded as dry nuts (Hosseini *et al*., 2022; Nazoori *et al*., 2022a,b). Contamination of pistachio with *Aspergillus flavus* and aflatoxin has greatly influenced the export of this product. Most contamination with aflatoxin occurs in the orchard and will continue until consumption (Mahbobinejhad *et al*., 2019). Environmental factors (e.g., relative humidity and temperature), the amount of water available in pistachio kernels, variety, harvesting time, irrigation, processing and storage, pests, and mechanical damage are the factors leading to pistachio contamination with aflatoxin (Ren *et al*., 2020).

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Bio-control agents can be considered an effective alternative and eco-friendly method for inhibiting fungi and mycotoxin in food products instead of chemical pesticides, which are harmful to humans and the environment. Ren *et al*. (2020) reported that yeasts are involved in 12% of projects regarding bio-control agents against aflatoxin-producing fungi and ranked 3 after bacteria (61%) and Fungi (27%). Fiori *et al*. (2014) and Farbo *et al*. (2018) showed that some yeasts prevented growth rate and aflatoxin production of *A. flavus* pre-harvest and/or post-harvest. It is demonstrated that pre-harvest application of yeast can prevent aflatoxin contamination (Hua, 2008). Fiori *et al*. (2008) reported that *Pichia angusta* was an effective yeast preventing apple decay caused by *Botrytis cinerea* and *Monilia fructicola*. Zhang *et al*. (2011) showed the high potential of *Meyerozyma guilliermondii* for bio-control of the grey mold of apples. Tayel *et al*. (2013) investigated the use of *Pichia anomala* as a feed supplement because of its potential to reduce aflatoxin production of *A. flavus*.

Competition, enzyme secretion, toxin production, volatiles, parasitism, and resistance induction are possible mechanisms in yeast activity as potent antagonists (Freimoser *et al*., 2019). Niche and competition for nutrients, such as iron, are considered primary modes of action of bio-control yeasts (Li et al. 2008). Secretion of different enzymes, e.g., chitinase (Zajc *et al*., 2019) and glucanase (Lopes *et al*., 2015), are effective in degrading cell wall components of aflatoxin-producing fungi. Lipase and protease activities have also been reported in some yeast strains (Sommer *et al*., 2016; Pretscher *et al*., 2018). Production of toxins and parasitism are the next mode of action, reported in yeast-fungus interaction. Toxins produced by many yeast strains are proteins in nature, originally identified in *Saccharomyces cerevisiae* (Luksa *et al*., 2015). Hyphal collapse, in parasitism, was observed in *Penicillium* species when influenced by *Saccharomycopsis* (Junker *et al*., 2019).

Some projects focused on volatile organic compounds (VOCs) of yeasts, which are effective against many fungal pathogens (Fialho *et al*., 2010; Huang *et al*., 2011; Ando *et al*., 2012; Pie-Hua *et al*., 2018). For instance, Masoud *et al*. (2005) revealed that VOCs of some yeast strains inhibited the growth and ochratoxin production of *A. ochraceus* during the processing of *Coffea arabica*. Arrarte *et al*. (2017) pointed out the efficacy of VOCs by *Candida sake* for preventing post-harvest disease. Moreover, 2-phenylethanol produced by *P. anomala* is reported as the major VOC, inhibiting the growth and aflatoxin production of *A. flavus* (Hua *et al*., 2014). Ando *et al*. (2012) reported that *Candida maltosa is* able to produce isoamyl acetate and isoamyl alcohol, which inhibit the conidial germination of *A. brasiliensis*. GC-MS analysis of extracellular secondary metabolites of *S. cerevisiae* revealed the existence of 4-Hydroxyphenethyl alcohol, 4,4-Dimethyloxazole, and 1,2-Benzenedicarboxylic acid dioctyl ester as VOCs against *A. flavus* (Abdel-Karim *et al*., 2019). Induction of systemic resistance, pointed out by some researchers, is another mode of action. Hadwiger *et al*. (2015) reported that yeasts can induce systemic resistance in potatoes against *Phytophthora infestans*. Applying yeast strains with salicylic acid (as a systemic resistance inducer) enhanced the biocontrol efficacy of *Cryptococcus laurentii* in apple fruit (Yu and Zheng, 2006).

In this research, 376 yeast strains were initially screened to evaluate their ability against *A. flavus*. Then, the powder and liquid formulations of superior yeast strains were prepared to assess their potential to reduce Aspergillus's growth and aflatoxin production under both in vitro and in vivo conditions.

Materials and Methods

Preparation and purification of yeast strains and A. flavus

In this research, 376 yeast strains already isolated from soil and nuts of several pistachio orchards in Kerman province, and *A. flavus* isolate P1684 with a high ability to produce aflatoxin B1, were obtained

from the Technology and Production Management Department of Pistachio Research Institute (Rafsanjan, Iran). Yeast strains were purified on Yeast Malt Extract Agar (YMA: 0.3% yeast extract, 0.5% peptone, 2% glucose, and 2% agar) and divided according to their colony appearance and growth pattern on the culture medium. For long-time storage, glycerin (30 mL) and Nutrient Broth (70 mL) were mixed, and 1 mL of the sterilized mixture and 2 blocks of fresh yeast colony were added to the vials. After shaking, vials were kept at -20°C.

Initial screening by dual culture (DC) assay

The dual culture (DC) assay was used for initial screening to investigate the inhibitory effects of yeast isolates against *A. flavus*. YMA culture medium was prepared, and 50 µL of the suspension of 10^8 cells mL^{-1} of each yeast strain was placed 1 cm away from the edge of the plate and incubated for 24 h at 25°C. Afterward, 10^6 spores mL^{-1} of *A. flavus* were placed at the opposite end of the plate and kept at 25°C under dark conditions. Plates inoculated with the pathogen alone served as the control. The average mycelium growth of *A. flavus* was recorded every 24 h for 5 days, and the inhibition of growth rate was calculated using the following formula (La-Penna *et al*., 2004).

$$\text{Inhibition of growth (\%)} = [(C - T)/C] \times 100$$

where C is the growth of the pathogen without yeast (mm), and T shows the growth of the pathogen with yeast (mm) (Whipps, 1997).

Volatile organic compounds (VOCs) assay

In this study, 16 yeast strains that showed the best results against *A. flavus* in the DC assay were sub-cultured on YMA and kept at 25°C for 48 h. Next, 10^6 spore m L^{-1} of *A. flavus* was cultured on potato dextrose agar (PDA) medium. Then, a 3-day-old culture of *A. flavus* was placed on the plate containing yeast strain and sealed with Parafilm®. Plates were kept at 25°C for 5 days in the control treatment. *A. flavus* was cultured in the center of the plates and placed upside the plates containing YMA without yeast. When the control plates showed full growth, inhibition of mycelia growth was calculated using the mentioned formula (Farbo *et al*., 2018).

Extracellular secretions (ESs) assay

A 24-hour-old culture of each yeast strain (100 µL of 10^8 cells mL^{-1}) was added to a flask containing 50 mL of liquid medium. The flasks were placed on a shaker (150 rpm) at room temperature for 4 days. Then, the culture medium containing the yeast was passed through filter paper (Whatman No. 1) and centrifuged for 20 min (500 rpm). The sediment was passed through a micropore filter (0.2 µ) and mixed with YMA. A 5 mm disc (plug) of *A. flavus* was placed in the center of each plate. When the control plates showed full growth, growth rate inhibition was calculated using the mentioned formula (Bar-Shimon *et al*., 2004).

Ammonia assay

The *A. flavus* colonies were exposed to ammonia vapor by a few drops of ammonia on the plate. The ability of yeast strains against aflatoxin production is evaluated according to the colony color change. A decrease in red color indicates less aflatoxin production (Fani *et al*., 2014).

Preparation of liquid formulation of yeast strain

A liquid formulation of yeast strain was performed using sugar beet molasses (40 gr of sugar beet molasses, 1.2 gr L^{-1} of urea, deionized water) by adding an isotonic preservative solution containing glycerol (92.0 gr L^{-1}), glucose (187.3 gr L^{-1}), sorbitol (98.2 gr L^{-1}), and trihalose (0.98 mol L^{-1}). Then, 50 mL of autoclaved culture medium with sugar beet molasses and 1 mL of 10^8 cells mL^{-1} suspension of each yeast strain were mixed in an Erlenmeyer flask and kept for 48 h at 28°C on a shaker (150 rpm). The content was centrifuged (4000 rpm) for 20 min, and phosphate buffer was added to each Erlenmeyer and kept at 4°C (Abadias *et al*., 2003).

Preparation of powder formulation of yeast strain

In an Erlenmeyer flask, 50 mL of autoclaved culture medium with sugar beet molasses and one mL of 10^8 cells mL^{-1} suspension of each strain were mixed and kept for 48 h at 28°C on a shaker (150 rpm). The content of each Erlenmeyer was centrifuged at 4000 rpm for 20 min. For each strain, 1 mL of 0.1 mol (w/v) of MgSo4 was added to the falcons, and a suspension was prepared. Next, 10% (v/v) of glycerol was added to the suspensions and mixed with an equal volume of autoclaved sodium alginate (1.5). Afterward, 8% whey, rice bran protein (v/v), 1% sucrose (w/v), were mixed with 1:4 v/v% of wheat bran (ground and passed through mesh 500) in the suspension. About 7% (w/w) of calcium lingo-sulfate was added as a moisture-retaining and spreader. The mixture was placed under the biological safety cabinet for 24 h until humidity reached 0.5%. The final formulation was kept at 4°C (Revillion *et al*., 2003).

Competitive ability of yeast strains in fermenting infected pistachios in vitro

Healthy pistachio fruits were selected and immersed in 0.1% sodium hypochlorite for surface disinfection, followed by washing twice with sterile water and immersing in 70 % ethyl alcohol. After evaporating the alcohol, 1 g of the formulation was mixed in 9 mL of sterile distilled water, and then each fruit was treated with 40 µL of the resulting suspension. After 24 h, 20 µL of the 10^6 spore mL^{-1} suspension of *A. flavus* were inoculated on each fruit. Treated pistachio fruits were kept in plates containing sterile filter paper without touching each other. Treatment of powder formulation and yeast fungus alone served as healthy control and treatment of A. flavus as infected control. After 15 days, the number of *A. flavus* colonies per fruit unit (CFU/Nut) was checked and compared to those of the infected and healthy controls. Each treatment was performed five times, and each repetition included one fruit (Vero *et al*., 2002).

Competitive ability of yeast strains in fermenting infected pistachio fruit in vivo

A commercial pistachio orchard was selected for experiments in Kerman Province, Iran. The temperature was 22-40°C in the orchard during experiments. Pistachio fruits were inoculated on the trees with yeast isolates with a concentration of 10^8 cells mL^{-1} of each yeast strain and 10^6 spores mL^{-1} of *A. flavus*. Control treatments were as follows: 1) *A. flavus* alone, 2) Yeast strain alone, 3) Sterile distilled water, and 4) Control without applying any treatment. Six weeks after inoculation, 200 pistachio fruits were randomly harvested from several trees in the row. The samples were transported to the laboratory in sterile zipped plastic bags and kept in a refrigerator (4°C). AFPA culture medium was used to determine the population (CFU/Nut) of *A. flavus* on fruit (Pitt *et al*., 1983). A total of 7 dilution series were prepared, each with one repetition. From each replicate, 100 µL were spread on plates and kept at 28℃ for 8 days. After 48 h, the plates were reviewed, and the colonies were counted. The average population was estimated based on the total number of colonies per pistachio fruit unit (CFU/Nut).

Statistics

The data obtained from different experiments were subjected to statistical analysis in a completely random design. Duncan's multiple range test was used to compare the means at the 5% probability level. SAS 9.4 software was used to analyze the data and compare the means.

Results

After initial screening, effective yeast strains were selected, and a code was assigned to each strain (Fig.1).

The aflatoxin production of *A. flavus* was evaluated using the following index to differentiate the color changes of colonies (Fani *et al*., 2014) (Fig. 2).

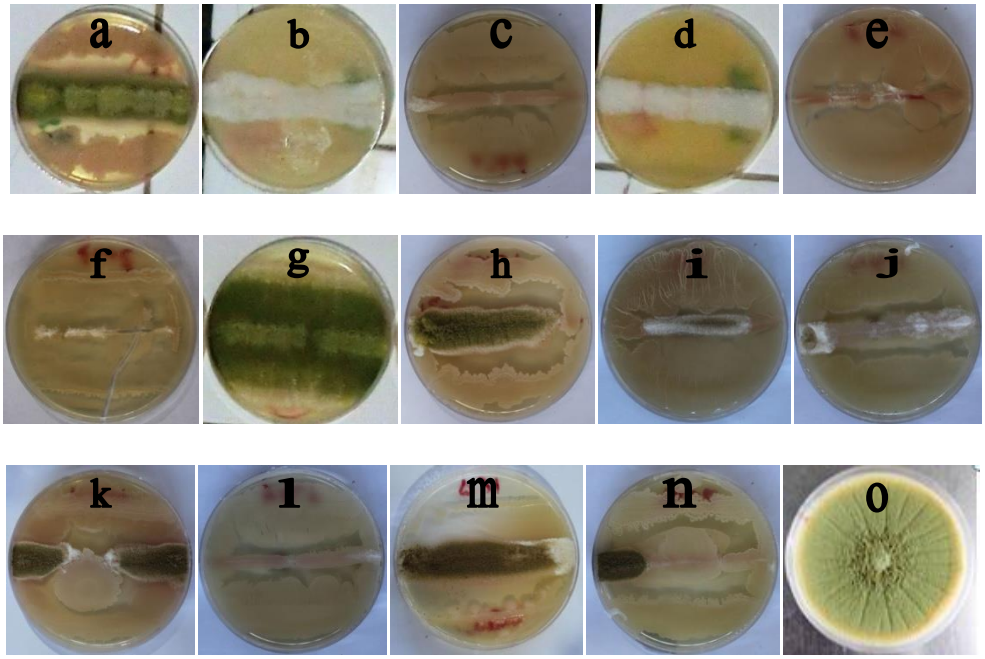

Fig. 1. Initial screening of yeast strains against *A. flavus* in dual culture assay compared to the control: (a) YE36-9, (b) YE43-6, (c) YE43-9, (d) YE43-10, (e) YE45-7, (f) YE45-9, (g) YE80-9-16, (h) YE83-8-7, (i) YE104, (j) YE109, (k) YE112-7, (l) YE127, (m) YE150, (n) YEyp9-16, and (o) Control

Fig. 2. Color intensity index after exposing the colony of *A. flavus* to ammonium hydroxide vapor: 1) no production of aflatoxin, 2) very low production of aflatoxin, 3) low production of aflatoxin, 4) moderate production of aflatoxin, 5) semi-high production of aflatoxin, 6) high production of aflatoxin, and 7) very high production of aflatoxin

Table 1 presents the results of different treatments for inhibiting growth and aflatoxin production. The highest and lowest growth inhibition in the DC assay was observed in YE 43-6 (95%) and YE 80-9-16 (63%), respectively. Also, in the VOCs assay, the highest and lowest growth inhibition was observed in YE 43-6 (72.08%) and YE 45-7 (31%), respectively. Finally, the highest and lowest growth inhibition in the ES assay was observed in YEyp 9-16 (91.31%) and YE 80-9-16 (59.55%), respectively. Almost in all treatments, yeast strains significantly reduced aflatoxin production in ammonia assay.

Table 1. Percentage of growth inhibition and prevention of aflatoxin production in different treatments; Values with different small letters in the columns are significantly different by Duncan's test at $p < 0.05$ (DC: Dual culture; VOCs: Volatile organic compounds; ESs: Extracellular secretion)

Yeast strains	DC	VOCs	ESs	Ammonia (DC)	Ammonia (VOCs)	Ammonia (ESs)
YE 36-9	87.25 ± 0.23^{c}	60.83 ± 0.53^{b}	77.82 ± 0.60^{cd}	1.75 ± 0.23^{b}	1.75 ± 0.23^{bc}	1 ± 0.00^{c}
YE 43-6	94.5 ± 0.14^{a}	72.08 ± 0.33^{a}	76.11 ± 0.54^{d}	1.25 ± 0.12^{b}	1.25 ± 0.12^{d}	1 ± 0.00^{c}
YE 43-10	84.25 ± 0.23^{cd}	62.45 ± 0.25^{b}	74.55 ± 0.64^{d}	1 ± 0.00^{b}	1 ± 0.00^{d}	1 ± 0.00^{c}
YE 45-7	83.25 ± 0.23^{de}	31.37 ± 0.27^{h}	86.22 ± 0.37^{b}	1 ± 0.00^{b}	1.75 ± 0.23^{bc}	1 ± 0.00^{c}
YE 45-9	90 ± 0.20^{b}	56.62 ± 0.27^{c}	78.02 ± 0.65^{c}	1.25 ± 0.12^{b}	2.25 ± 0.23^{b}	1 ± 0.00^{c}
YE 53-14	94 ± 0.20^{a}	52.04 ± 0.18^{d}	83.63 ± 1.00^{b}	1.25 ± 0.12^{b}	1.25 ± 0.12^{d}	1 ± 0.00^{c}
YE 57-6	82.25 ± 0.23^{de}	62.12 ± 0.11^{b}	76.5 ± 0.41^{cd}	1 ± 0.00^{b}	1.5 ± 0.14^{bc}	1 ± 0.00^{c}
YE 80-9-16	62.25 ± 0.23^{g}	56.75 ± 0.16^{c}	59.55 ± 0.75^{f}	1.5 ± 0.25^{b}	1.75 ± 0.23^{bc}	1 ± 0.00^{c}
YE 83-8-7	75 ± 0.35^{f}	57 ± 0.10^{c}	79.21 ± 0.76^{cd}	1 ± 0.00^{b}	1 ± 0.00^{d}	1 ± 0.00^{c}
YE 104	87.25 ± 0.23^{e}	41.95 ± 0.12^{g}	69.66 ± 0.38^{e}	1.25 ± 0.12^{b}	1.25 ± 0.12^{d}	1.5 ± 0.14^{c}
YE 109-6	82.25 ± 0.23^{e}	32 ± 0.10^{h}	86.20 ± 0.42^{b}	1 ± 0.00^{b}	1.75 ± 0.23^{bd}	1 ± 0.00^{c}

YE 112-7	81.75±0.23^{de}	49.5±0.14^{e}	89.69±0.45^{a}	1.25±0.12^{b}	2±0.20^{d}	1.5±0.14^{c}
YE 127	92.5±0.32^{a}	56.75±0.16^{c}	75.37±0.22^{cd}	1±0.00^{b}	1.25±0.12^{d}	1±0.00^{c}
YE 150	84.25±0.23^{d}	44.5±0.14^{f}	76.18±0.32^{cd}	1.25±0.12^{b}	1.75±0.23^{bc}	1.5±0.14^{b}
YE 183	84.5±0.14^{d}	32.25±0.07^{h}	69.88±0.33^{e}	1±0.00^{b}	1.25±0.12^{d}	1±0.00^{c}
YE yp-9-16	83.25±0.31^{de}	55±0.10^{c}	91.30±0.45^{a}	1.25±0.12^{b}	1.75±0.23^{bc}	1±0.00^{c}
Control	-	-	-	7±0.00^{a}	7±0.00^{a}	7±0.00^{a}

Efficacy of liquid and powder formulation of yeasts against A. flavus on pistachio fruit in vitro and decrease in CFU/Nut in vivo conditions

As shown in Table 2, liquid formulation of yeast strains led to a significant decrease in the population of *A. flavus*. The highest inhibition belonged to YE 43-10 (98.75%) and YE 43-6 (97.45%). Furthermore, powder formulation of yeast strains led to a significant decrease in the fungus population. The highest inhibition belonged to YE 43-10 (98.75%) and YE 43-6 (97.45%). However, powder formulation was slightly more effective than liquid formulation. In vivo, a significant decrease in the number of colonies per pistachio fruit unit (CFU/Nut) was observed in the treatment of fruits by YE 43-10 (85.71%). Results showed that yeast strains at least 35% effectively reduce *A. flavus* and aflatoxin contamination under orchard conditions.

Table 2. Efficacy of liquid and powder formulation of yeast strains against *A. flavus* on pistachio fruit in vitro and vivo conditions; values with different small letters in the columns are significantly different by Duncan's test at $p < 0.05$

Yeast strains	Liquid formulation	Powder formulation	CFU/Nut
YE 36-9	72.11±0.53^{m}	76.39±0.36^{i}	35.18±0.96^{k}
YE 43-6	97.45±0.28^{ab}	97.14±0.43^{bc}	81.63±0.61^{b}
YE 43-10	98.75±0.11^{a}	99.96±0.01^{a}	85.71±0.65^{a}
YE 45-7	78.32±0.44^{kl}	82.66±0.46^{g}	48.28±0.69^{i}
YE 45-9	76.93±0.47^{l}	80.42±0.29^{gh}	37.67±0.76^{jk}
YE 53-14	90.22±0.39^{g}	92.73±0.51^{de}	64.55±0.57^{f}
YE 57-6	96.7±0.53^{abc}	98.59±0.18^{ab}	79.58±0.74^{bc}
YE 80-9-16	81.52±0.53^{j}	86.87±0.38^{f}	58.18±0.38^{h}
YE 83-8-7	94.4±0.47^{cde}	95.15±0.48^{cd}	82.03±0.37^{b}
YE 104	91.65±0.59^{efg}	90.7±0.45^{e}	63.32±0.60^{fg}
YE 109-6	91.31±0.43^{efg}	95.84±0.52^{c}	78.17±0.53^{bc}
YE 112-7	95.38±0.40^{bcd}	94.86±0.26^{cd}	73.51±0.74^{de}
YE 127	93.76±0.42^{def}	96.21±0.35^{bc}	76.34±0.47^{cd}
YE 150	80.77±0.54^{jk}	78.5±0.55^{hi}	38.96±0.39^{j}
YE 183	87.38±0.38^{i}	88.24±0.35^{f}	60.11±0.47^{gh}
YE yp-9-16	92.64±0.64^{defg}	91.09±0.40^{e}	70.24±0.77^{e}

Discussion

Contamination of pistachio with different species of *Aspergillus* and aflatoxin is considered among the serious risks to human health. Using economical and environmentally friendly methods, such as yeast strains, is important in protecting products pre-harvest and post-harvest. The safety of using some yeast strains in food products has been approved. A few yeast strains, including *S. cerevisiae*, are registered as safety plant protection agents (European Food Safety Authority 2005; 2015).

Among yeast strains used in this study, 16 strains showed a higher ability to prevent mycelia growth and

reduce toxin production of A. flavus, especially in DC and VOCs assays, and used to prepare powder and liquid formulations. The efficiency of yeast strains to control *A. flavus* and reduction of aflatoxin production has been confirmed in different studies (Zhang *et al*., 2011; Armando *et al*., 2012; Lopes *et al*., 2015; Abdel-Kareem *et al*., 2019), which produced results consistent with those of the present research. Most studies have demonstrated the role of VOCs in reducing mycelia growth and aflatoxin production. According to the results, VOCs of YE 43-6 (72.8%) and YE 43-10 (62.5%) play an essential role in the reduction of mycelia growth of *A. flavus* and aflatoxin production. This finding is consistent with the results of some other studies (Fialho *et al*., 2010; Farbo *et al*., 2018; Ren *et al*., 2020). The results of DC, VOCs, and ES assays indicated that different yeast strains have different control abilities in reducing mycelia growth and aflatoxin production. In line with the results of the present study, the better ability of VOCs is approved in several projects (Hua *et al*., 2014; Farbo *et al*., 2019).

Results of the ammonia assay revealed that all 16 yeast strains were able to reduce the amount of aflatoxin compared to the control, which was in agreement with the results of Fani *et al*. (2014).

Application of powder and liquid formulations of yeast strains in the orchard showed a significant reduction in CFU/Nut, and powder formulation was a little more effective than liquid. These findings are in agreement with the results of previous projects (Melin *et al*., 2007; Liu *et al*., 2009). The formulation can protect the yeast strains from high/or low temperatures and oxidative stress and cover the fruit as a mechanical barrier. Furthermore, compounds produced by yeast strains prevented settling *Aspergillus* on the fruit and reduced aflatoxin contamination. Finally, powder formulation is recommended as a useful strategy for improving the viability and performance of yeast strains and enhancing biocontrol efficacy against *Aspergillus flavus* on pistachio.

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Conflict of interests

The authors declare no conflict of interest.

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