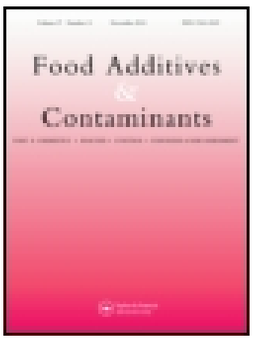

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Biocontrol potential of native yeast strains against *Aspergillus flavus* and aflatoxin production in pistachio

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ABSTRACT

Aspergillus flavus: is the main aflatoxin producer in food and feed and has wide ecological niches. Contamination of food products such as pistachio nuts and aflatoxin secretion directly affects food safety and international food product trades. Abilities of 13 yeast strains isolated from 200 soil and pistachio nut samples collected in Iranian orchards to reduce the growth of *A. flavus* as well as aflatoxin production were assessed in dual culture, volatile and non-volatile compounds tests. The growth of *A. flavus* was reduced by 32–60%, 13–31% and 40–61% in dual culture, volatile and non-volatile compounds, respectively, while aflatoxin B1 production was diminished by 90.6–98.3%. Based on these assays, five yeast strains were selected for co-inoculation experiments using soil, pistachio hulls and leaf. A significant reduction in colony-forming units (CFU) ranging from 23% to 110% ($p < .05$) was observed. Molecular, physiological and morphological identification revealed these were strains of *Pichia kudriavzevii* and *Lachansea thermotolerans*. Aflatoxin biocontrol with yeast strains possesses many advantages including the ease of commercial production and organic application which is an environmental approach. More investigation is required to understand the efficiency of selective strains to inhibit *A. flavus* and aflatoxin production as well as withstand predominant abiotic stress in pistachio orchards and mass production in field application.

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Biological control; food safety; interaction

Introduction

Pistachio is one of the most valued food product in Iran and one of the most important agricultural export items of the country. These nuts are susceptible to aflatoxin contamination, endangering food security and global markets for Iranian pistachios (Bui-Klimke et al. 2014). Biological control of mycotoxins through different microorganisms such as bacteria, atoxigenic *Aspergillus flavus* strains, and yeasts are considered efficient and environmentally friendly approaches in reducing the risk of food mycotoxins content (Dorner 2004; Nguyen et al. 2017; Mwakinyali et al. 2019). Commercial atoxigenic *A. flavus* strains are commonly applied in different crops to mitigate aflatoxins in field conditions (Dorner 2004).

Many bacterial strains such as *Bacillus subtilis*, *Pseudomonas* spp., *Lactobacillus* spp., *Ralstonia* spp. and *Burkholderia* spp. are able to inhibit the growth and aflatoxin production *in vitro* (Nesci et al. 2005; Palumbo et al. 2006). However, because *Aspergillus* strains outcompete bacteria they are not able to mitigate aflatoxin production under field conditions (Dorner 2004).

Previous studies revealed that different saprophytic yeasts strains are able to inhibit growth and aflatoxin production of *A. flavus* both *in vitro* and *in vivo* (Masoud and Kaltoft 2006). Yeast strains have a high potential as biological control agents for plant diseases due to their ability to colonise ecological sites under dry conditions, the production of extracellular polysaccharides and their general low sensitivity to pesticides (Wilson and Wisniewski 1989). Saprophytic yeasts isolated from fruits of almond, pistachio, and walnut trees have been shown to inhibit the spore density of *A. flavus* and aflatoxin production in field application (Dorner 2004; Hua 2013). The advantages of

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yeasts are manifold, for example, they are easily cultivated, do not produce allergenic conidia, have a natural saprophytic lifestyle and are highly competitive microorganisms which spread rapidly in orchards. Especially their fast growth rate enables yeasts to be excellent, natural competitors for food sources and ecological niches (Hua 2013). Therefore, saprophytic yeasts are frequently studied as potential biological control agents for fungal pathogens such as *Botrytis cinerea* and *Penicillium* species (Gramisci et al. 2018) and are already commercially applied to reduce post-harvest damage to these pathogens (Hua 2013). Yeast-based formulations include Shermer (active ingredient *Metschnikowia fructicola)*, BioNext (*Candida oleophila*) and Candifruit (*Candida sake*), which are commonly applied for post-harvest diseases control of fruits and vegetables. An added benefit of applying yeast-based biocontrol products is the ability to combine these products with a reduced amount of fungicide sprays which both maximise disease control and limits fungicide use on food products.

Application of yeasts in biocontrol formulations for use in pistachio orchards is mainly studied in California but warrants further investigation in Iranian pistachio orchards where it is necessary to select native strains of high potential ability compatible with local environmental conditions. In California, application of *Pichia anomala* reduced the spore density of *A. flavus* in male inflorescences and pistachio fruit by more than 80% (Hua 2013). Hua (2009) also introduced a strain of *P. anomala* for protection of nut and corn to inhibit the infection by *Aspergillus* species and aflatoxin production (Hua 2009). Application of yeast strains in pistachio orchards in California indicated a reduction in *A. flavus* outbreaks by more than 97% compared to the control treatment (Hua 2013). This strain was registered as a biological pesticide in the United States in 2009 (No. US 7579183 B1) (Hua et al. 1999; Hua 2009), and can also be applied on harvested or stored products (Hua 2013). *Pichia anomala* is currently used as a biological control agent to reduce aflatoxin contamination in pistachio nuts through different mechanisms such as production of 2-phenylethanol which affects spore germination, fungal growth, aflatoxin production and gene expression in *A. flavus* (Hua et al. 2014). The antifungal activity of 2-phenylethanol produced by different species of yeasts through different mechanisms has been well documented (Liu et al. 2014; Chang et al. 2015). Inactivation and removal of regulatory genes in the aflatoxin biosynthesis pathway and gene transcription prohibition may lead to inhibition of growth and of aflatoxin production by otherwise toxigenic strains of *A. flavus* subjected to biocontrol conditions by applying bacteria and yeast species (Kong et al. 2014; Al-Saad et al. 2016; Hassan et al. 2017). Inactivation of the ATP-generating systems, which damage hyphal cell walls and decrease the membrane fraction potential in *A. flavus* in co-culture with *P. anomala* was demonstrated for potential use in biocontrol to reduce mycotoxin contamination (Hua et al. 2011). According to Hua et al. (2015), maintaining the viability of *P. anomala* is a great challenge for commercial application. Viability is significantly affected by the composition of media and their supplements.

There is scarce information on competitive abilities of yeast strains and *A. flavus* and aflatoxin production in Iranian pistachio. Therefore, the present study was undertaken to assess the potential ability of native yeast strains to reduce the population density of *A. flavus* on soil, pistachio hulls and leaf and aflatoxin production under *in vitro* experiments.

Materials and methods

Yeast strains

Thirteen yeast isolates from soil and pistachio nuts saved in the Iranian Pistachio Centre (IPC) were used in all experiments.

Aspergillus flavus *isolate*

A pure culture of an aflatoxin-producing strain of *A. flavus* originally isolated from pistachio nuts in the Kerman region (ITEM code 16499) maintained on potato-dextrose agar (PDA, 39 g/L, Merck, Germany), was used for experiments (Fani et al. 2014). The ability of the strain to produce aflatoxin B1 was already assessed through culture and analytical approaches in Iranian Pistachio Research Centre (IPRC, Rafsanjan, Iran) and Institute of

Sciences and of Food Production (Bari, Italy) as described by (Fani et al. 2014).

In vitro screening of yeast antagonistic activity

Dual culture

The ability of yeast isolates to reduce mycelial growth of *A. flavus* was determined in dual culture assays. Here, 15 ml of YPGA medium (0.3% yeast extract, 0.5% peptone, 2% of glucose and 2% of agar) (Melo et al. 2007) was poured into a 9 cm petri plate. In dual culture assays, 50 µl of an actively growing suspension of yeast strains (10^8 cells mL^{-1}) was streaked 3.5 cm away from the centre of the plates and subsequently incubated at 25°C for 24 h. Post-incubation, an *A. flavus* spore suspension (10^6 cells mL^{-1}) was streaked in the centre of each petri dish and incubated at 28°C in the dark. The inhibition of radial fungal growth was recorded every 24 hours for 5 days. Fungal growth with no yeast inoculum was used as a control. The ability of strains to inhibit *A. flavus* growth was calculated with the following equation:

$$I = C\text{-}T/C \times 100$$

where I is the inhibition of mycelial growth (%), C is the growth of *A. flavus* in control petri-dishes, and T is the growth in the interaction assays. The yeast strains with high potential in reducing mycelial *A. flavus* growth were further subjected to volatile and non-volatile compound tests.

Volatile compounds

To assess the effects of volatile compounds on mycelial *A. flavus growth*, yeast strains were streaked out on YPGA plates and incubated for 2 days at 27° C. A 5 mm agar plug of an actively (3 days old) growing *A. flavus* strain was placed in the centre of the plate and placed upside down on a petri dish containing a 48-h inoculated yeast strain. The construct plates were sealed with paraffin film (Parafilm, Sigma-Aldrich, Germany) and incubated for 7 days at 27°C in the dark. Mycelial growth of *A. flavus* was measured every 48 h. Construct plates with no yeast applied were used as a control.

Non-volatile compounds

For this test, 24 h old cultures of yeast strains were cultured in individual flasks containing 50 ml sterilised 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 growth of yeast strains. Post-incubation, the suspensions were passed through No. 1 filter paper (Whatman, Sigma-Aldrich, Germany) and autoclaved. The sterilised suspension was mixed with YPGA at various ratios (1:19, 3:17, and 5:15), and poured into petri dishes at 45° C. Small discs of 3-day old growing *A. flavus* were cultured on the centre of the plates. The mycelial growth of *A. flavus* was monitored every 48 hours for 5 days as described by Singh and Deverall (1984).

In situ assays

Substrate preparation

The hull and leaves of pistachio nuts (cultivar Ohadi) were collected, manually separated and stored at −20°C. Fifty grams of either hull or leaves were sterilised in 0.5% hypochlorite sodium-containing 0.2% Tween® 20 for 30 seconds and triple rinsed with sterilised distilled water. The sterilised substrates were dried in a laminar air flow hood for 30 minutes under sterile conditions. Also, 50 g of moistened soil samples collected from a depth of 20 cm were sterilised by autoclaving.

Inoculum production

A. flavus was grown on PDA for 2 weeks. Conidia were harvested by adding sterile-distilled water containing 0.1% peptone. Mycelium was lightly scraped off with a spatula to dislodge the conidia. The suspension was passed through double-layered cheesecloth. The conidial concentration was adjusted to 10^4 conidia/ml. The yeast strains were cultured in potato-dextrose broth (PDB, 24 g/l) using 1000-ml Erlenmeyer flasks for 24 h on a shaker (200 rpm) at room temperature. The yeast concentrations were adjusted to ^{7} cells/ml. Densities were determined with a haemocytometer.

Inoculation

The soil, leaf and hull substrates were inoculated with either single isolates of yeast or *A. flavus* or in combinations of both yeast and *A. flavus* Table 1. Fifty grams of soil, hull and leaves were inoculated with conidia and cells of either *A. flavus* or yeast

Table 1. Experimental design by applying conidia concentration approach (total amount of inoculum 120 ml).

Inoculum	Concentration (ml^{-1}) Yeast	*A. flavus*	Suspension volume (ml) Yeast	*A. flavus*	Water volume (ml)	Total volume (ml)
YB39 + F[a]	10^7	10^4	5	5	-	10
YB41 + F	10^7	10^4	5	5	-	10
YB43 + F	10^7	10^4	5	5	-	10
YB9 + F	10^7	10^4	5	5	-	10
YB10 + F	10^7	10^4	5	5	-	10
YB39	10^7	-	5	-	-	10
YB41	10^7	-	5	-	-	10
YB43	10^7	-	5	-	-	10
YB9	10^7	-	5	-	-	10
YB10	10^7	-	5	-	-	10
F	-	10^4	-	5	5	10
Control	-	-	-	-	10	10

aF: Fungi, *Aspergillus flavus*

strains or a combination of both with a hand sprayer Table 1. After inoculation, the substrates were incubated for 7 days at 28°C in dark. The experiments were conducted in a completely randomised design with five replications. The substrates were kept in sterilised vegetable boxes to assure high relative humidity.

Microbiological detection of interaction between yeast strains and A. flavus

Substrates were poured into one-litre flasks containing 450 ml sterilised distilled water containing 0.2% Tween®20. The flasks were shaken at 300 rpm for 45 minutes. Using Aspergillus Flavus and Parasiticus Agar (AFPA) (Gourama and Bullerman 1995) and Nutrient Agar (NA) (Sinclair and Dhingra 1995) a serial dilution was applied to determine population density of yeast strains and *A. flavus*. For this purpose, 100 µl of the suspension in each dilution were spread on the culture media through rotator plates. Plates were incubated at 30°C for up to 3 days after which the colony of *A. flavus* and yeast strains were counted on each plate individually.

Identification

Molecular identification yeast strains

Genomic DNA was extracted using a CTAB method as described previously (Moradi et al. 2010). Yeast strains were identified based on the partial sequence of 26S rDNA as described by Kurtzman et al. (2003). The quantity and quality of the amplified PCR products were determined by spectrophotometry and electrophoresis assays. Sequencing was carried out using the Automate DNA Sequencer (3100-Avant Genetic Analyser). Sequences were aligned and compared with the NCBIN option of the NCBI nucleotide database (http://database blast.ncbi nlm.nih.gov/Blast.cgi).

The morphological and physiological features

Morphological features of selected yeast strains were revealed by observation of cell and colony patterns based on methods introduced by Kurtzman et al. (2011). The yeast cells of different isolates were cultured in 5% malt extract broth at 25°C for 48–72 h. Yeast cells were observed to be single, coupled or aggregated in a mass. The average length and the width of 20 cells of each strain were measured. The colony morphology of each yeast strain was assessed in cultures grown in 5% malt extract agar at 25°C. The yeast cultures' colour and texture were recorded after 3–7 days incubation period. The physiological features of these yeast antagonists were determined such as their capabilities to ferment essential sugar types in a semi-anaerobic manner while absorbing carbon compounds as the main carbon source in an aerobic state. These tests were repeated three times per strain. The obtained morphological and physiological features were applied to verify the yeast species following the rDNA sequence determination.

Aflatoxin B_1 detection

The ability of yeast isolates to reduce aflatoxin production by a toxigenic strain of *A. flavus* was assessed in co-inoculations on maize meal extract agar (MMEA) (La Penna and Etcheverry 2006). Briefly, 10 µl of yeast strain suspensions (10^8 cells mL^{-1}) were inoculated 3.5 cm away from the centre of the plates and subsequently an *A. flavus* spore suspension (10^6 cells mL^{-1}) was inoculated in the centre of each petri dish and incubated at 28°C in the dark for 10 days. To analysis aflatoxin, from each plate 2 mycelial blocks with a diameter of 1 cm were obtained and placed into in a sterile bag and crushed well. Then, 5 ml of 80% methanol was added and shaken for 30 minutes. The extract was passed through Whatman filter paper No. 1 and 100 µl of the extract was used for analytical assays.

A high-performance liquid chromatography was used to measure the aflatoxin content of MMEA as describe by Fani et al. (2014).

Statistical analysis

The average population density of *A. flavus* and the yeast strains was determined for each replication. Significant differences were analysed by applying the Proc GLM procedure (SAS 9.0, SAS Institute, Inc., Cary, NC) followed by Duncan's new multiple range tests at 5% probability. The data was log-transferred prior to analysis, when applicable.

Results

Aspergillus flavus *and yeast isolates interaction*

The potential of yeast strains to reduce growth and aflatoxin production of *A. flavus* were assessed in dual culture, volatile and non-volatile compound tests and on different substrates such as soil, pistachio hulls and leaf.

Mycelial growth reduction

Yeast strains reduced *A. flavus* mycelial growth, significantly, although to different degrees Table 2. Mycelial growth reduction ranged from 32–60%, 13–31% and 40–61% in dual culture, volatile and non-volatile assays, respectively (Table 2). In dual culture and non-volatile compounds tests, yeast strain YB39 had the highest ability to inhibit mycelial growth of *A. flavus*, by 60.4% and 61.1%, respectively, with lower ratios achieved by YB10 and YB24. In volatile assays, YB43 and YB15 showed the highest and the lowest inhibitory activities against *A. flavus*, respectively. Although isolates YB36 and YB41 showed high degrees of inhibition, no significant differences were found compared to other isolates like YB43 and YB39.

Aflatoxin production

Aflatoxin production was reduced by the presence of yeast isolates. For example, the *A. flavus* control produced 53 to 135 ng/g aflatoxin B1, compared to 1.4 to 7.7 ng/g in the presence of yeast. The reduction rates of aflatoxin B1 in co-cultures were in the range of 90.6% to 98.3%. The highest and lowest reduction rates were due to YB9 and YB39, respectively (Table 2). The obtained data indicate interactions between yeast isolates and *A. flavus* in growth and aflatoxin production. In this interaction, the effect on *A. flavus* is negative. Among these 13 strains, isolates YB9, YB10, YB43, YB39 and YB41 have the highest inhibitory activity on *A. flavus* mycelial growth and aflatoxin reduction rates compared to others, therefore, their ability was further assessed on different substrates such as soil, leaf and hulls.

Interactions of yeast strains and Aspergillus flavus

In general, interactions between *A. flavus* and yeast strains were assessed in mixed inoculations on soil, pistachio hulls and leaves by measuring colony-forming units (CFU).

The presence of yeast isolates reduced the inoculum density of *A. flavus* to different degrees. In soil,

Table 2. Effects of yeast isolates on the mycelium growth of *Aspergillus flavus* in volatile, non-volatile and dual culture assays and aflatoxin B1 reduction.

		Inhibition (%)	Non-volatile compounds*			
Yeast isolate	Dual culture	Volatile compounds	5	15	25	Aflatoxin B1 Reduction %
YB39	60.4 A	24.0 ABC	49.7 A	57.1 A	61.1 A	90.6
YB43	58 AB	30.6 A	44.8 B	52.4 B	58.2 A	97.4
YB36	53.5 ABC	22.0 ABC	41.1 CD	47.6 C	53.4 B	97.0
YB41	53.5 ABC	24.0ABC	44 BC	50.5 BC	53.4 B	97.8
YB25	51.1 BC	30.6 A	42.8 BC	48.6 C	54.2 B	91.2
YB32	48.8 BCD	16.3 BC	35.4 EFG	41.0 DEF	45.7 D	94.6
YB40	48.8 BCD	16.3 BC	38.2 DE	43.8 D	49.7 C	96.3
YB34	48.8 BCD	25.7 AB	36.2 EF	42.9 DE	47.7 CD	97.0
YB15	48.8 BCD	12.6 C	32.5 GH	40.0 EF	44.8 DE	94.5
YB11	44.3 CD	24.0 ABC	34.2 FG	41.0 DEF	47.7 CD	94.4
YB9	44.3 CD	16.3 BC	28.5 I	36.2 GH	42.8 DE	98.3
YB24	40.9 C	18.3 ABC	26.8 I	33.3 H	40 F	94.0
YB10	31.7 E	24.0 ABC	29.7 HI	37.1 FG	44.8 DE	97.8

*Means followed by the same letter in each column are not significantly different ($p < 0.05$) by Duncan's new multiple range test.

the highest effects were caused by isolates YB43 and YB39, which reduced the density of *A. flavus* by 4.75 * 10^5 and 4.25 * 10^5 CFU in soil, respectively, compared to inoculations of *A. flavus* alone (mean CFU = 7.8 * 10^6) (Figure 1). Overall, in soil, the presence of yeast isolates in the mixtures reduced the CFU of *A. flavus* in the range 38.4–94.6%.

In leaves, mixed inoculations reduced the CFU of *A. flavus* from 53.3% to 98.1%. The greatest effects were observed in mixtures including YB43 and YB39 strains with a mean density of *A. flavus* by 3.75 * 10^5 and 1.5 * 10^5 compared to 7.9 * 10^6 CFU/g in inoculation alone Figure 2.

Similar to soil and leaf the presence of yeast strains in mixtures caused a significant reduction ranging from 40.4% to 99.7% of *A. flavus* CFU/g in hulls. YB43 and YB39 strains had the highest potential ability to reduce the density of *A. flavus*, by means of 2.5 * 10^4 and 1.75 * 10^5 CFU in hulls Figure 3 compared to inoculations of *A. flavus* alone (1.1 * 10^7).

Combined inoculations increased the population density of yeast to different degrees in comparison with that of inoculation alone on all substrates (Figure 2). This effect is more pronounced in isolates YB43 and YB39, where the density increases 3 to 4 times compared to single isolate inoculations. Other yeast isolates revealed lower effects on *A. flavus* population density. The inhibitory effects of yeast isolates on *A. flavus* population density are not affected by the substrate type. The data may indicate the presences of interactions between yeast isolates and *A. flavus*, where the *A. flavus* population density is negatively affected while the CFU of yeast isolates increase in a positive manner.

Identification of the yeast antagonist

The sequences of the large subunit (26S) ribosomal DNA gene of yeast strains YB10 and YB11 are identical (100%) with those of *Pichia kudriavzevii*,

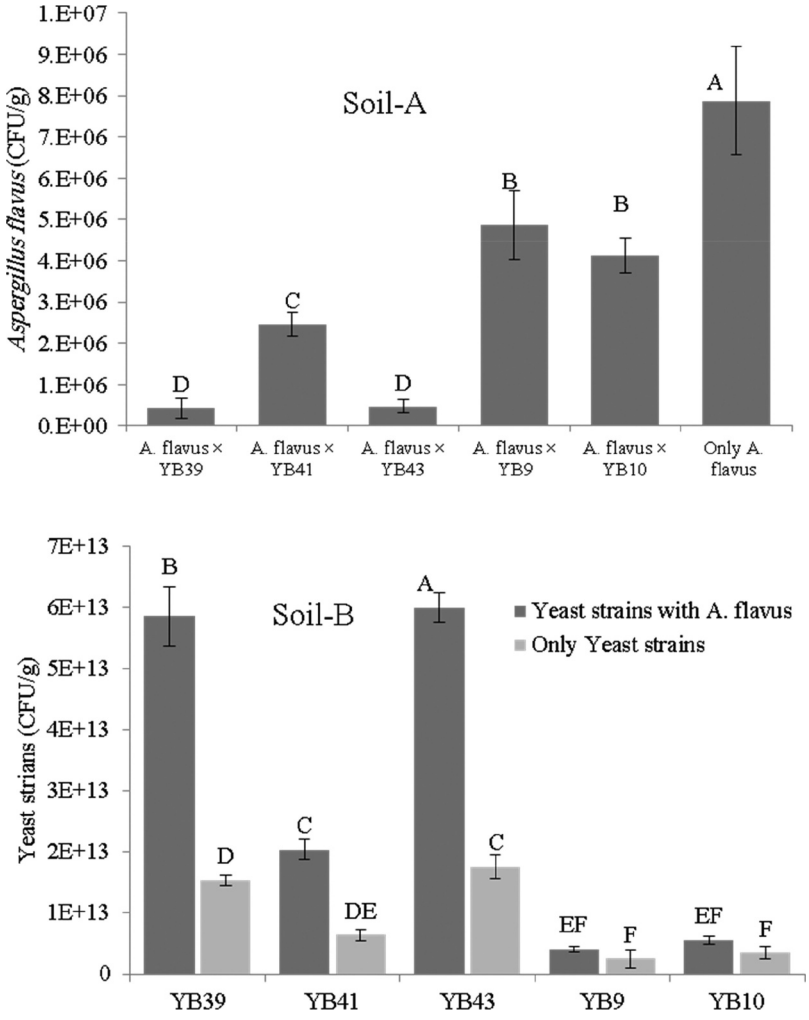

Figure 1. Colony-forming units of *Aspergillus flavus* (A) and yeast strains (B) after inoculations of soil with *Aspergillus flavus* alone or in mixture with yeast strains*Means followed by the same letter in each column are not significantly different ($p < .05$) by Duncan's new multiple range test.

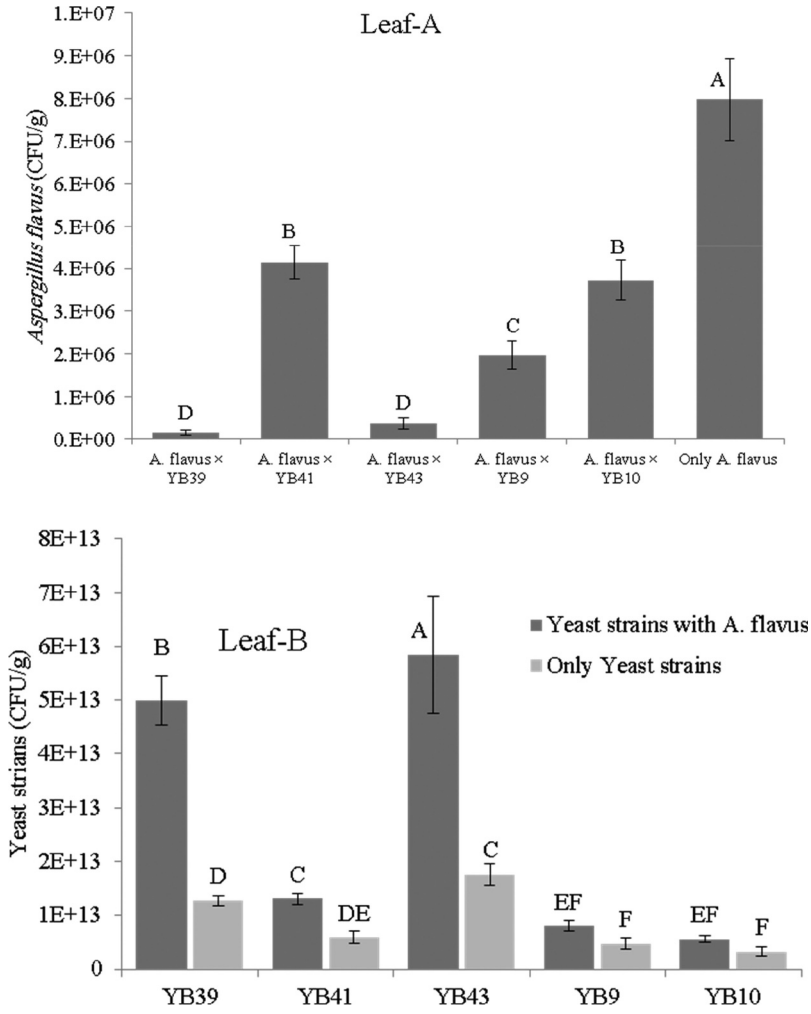

Figure 2. Colony-forming units of *Aspergillus flavus* (A) and yeast strains (B) after inoculations of leaf with *Aspergillus flavus* alone or in mixture with yeast strains*Means followed by the same letter in each column are not significantly different ($p < .05$) by Duncan's new multiple range test.

while YB39, YB40 and YB41 revealed 99%, 100% and 100% identity with *Lachancea thermotolerans*, Table 4 (Kurtzman 1992; Martorell et al. 2005).

The results of morphological characteristics and physiological features such as fermentation, assimilation of different carbon and nitrogen sources, can be seen in Table 3. In all cases, the biochemical and morphological tests confirmed the results obtained by the rDNA sequencing technique Table 4.

Discussion

Identification, analysis and application of native microbial isolates withstanding abiotic stresses in ecological niches is one of the essential processes in implementing biological control agents to mitigate plant disease. For this purpose, overall screening must be conducted according to real conditions prevailing in ecological niches. The 13 yeast isolates identified in this study originated from different pistachio-growing areas and showed great potential to reduce *A. flavus* population densities in natural substrates.

In general, information on interactions among *A. flavus* and yeast isolates are lacking and the interaction of isolates in culture media or on different natural substrates both in mixed inoculations and alone is rarely assessed. The present study revealed that native yeast isolates isolated from soil and nuts in different pistachio-growing regions were able to inhibit *A. flavus* growth under *in vitro* conditions and on different natural substrates like leaf, soil and hulls.

Based on the capabilities of the selected yeast isolates to inhibit *A. flavus* growth and aflatoxin

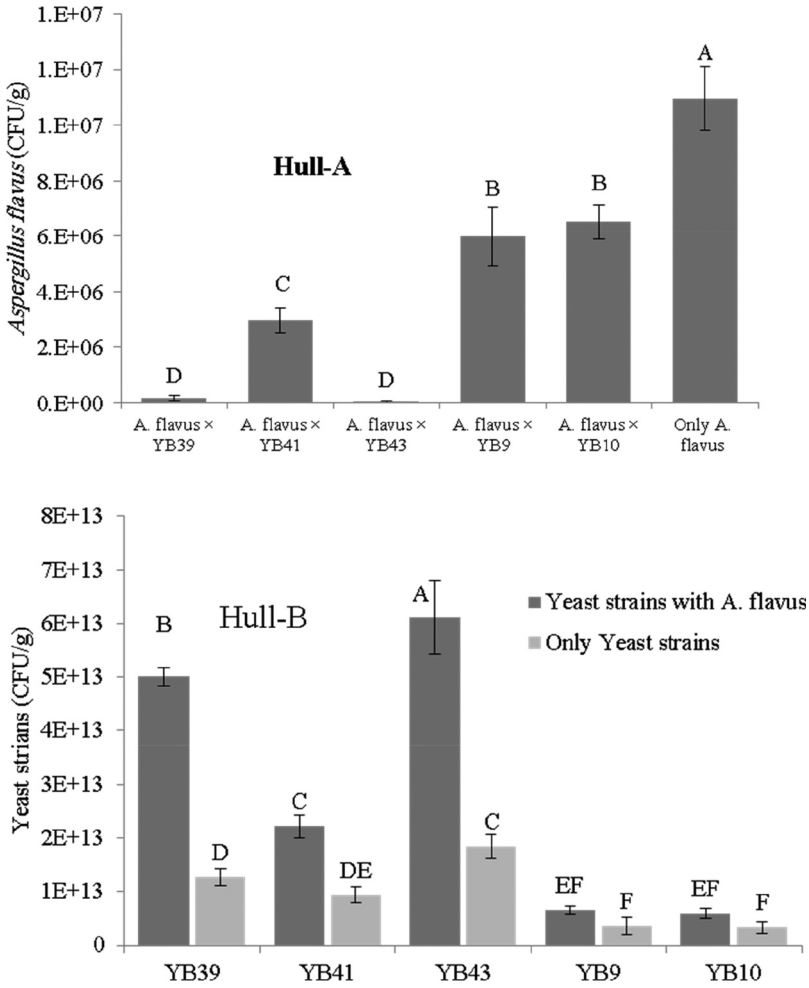

Figure 3. Colony-forming units of *Aspergillus flavus* (A) and yeast strains (B) after inoculations of hulls with *Aspergillus flavus* alone or in mixture with yeast strains*Means followed by the same letter in each column are not significantly different ($p < .05$) by Duncan's new multiple range test.

production in different assays, five of the 13 isolates were selected as candidates for further tests. These isolates showed different inhibitions of *A. flavus* mycelium growth in dual culture, volatile compound, and non-volatile compound tests as well as aflatoxin production in culture media. This effect can be related to different mechanisms of competitive interactions between the yeast isolates and *A. flavus*. Hua et al. (2011) assessed the influence of *Pischia anomala* on metabolic activity of atoxigenic *A. flavus* in simultaneous co-culture assays which revealed that the ATP-generating system of *A. flavus* became deactivated on increasing the yeast to fungi ratio. They found that in co-culture the cellular wall membrane became damaged which prevented growth and biomass production of *A. flavus*. In 2015, the same researchers, while comparing four different formulations to assess *Pichia anomala* spore durability, found that media containing trehalose and sorbitol can significantly increase spore durability by more than 83% after 12 months (Hua et al. 2015). Hua et al. (2014) assessed the effect of volatile compounds of *Pichia anomala* on *A. flavus* spore germination and aflatoxin production and revealed that 2-phenylethanol is a volatile compound which caused a 10000-fold decrease in gene expression in the aflatoxin gene cluster. Moreover, they found that the produced 2-phenylethanol effected spore germination, toxin production and gene expression in *A. flavus*. Hassan et al. (2017) assessed the molecular effects of bacteria and yeasts on the aflatoxin gene cluster and found that *Bacillus subtilis* and *Candida albicans*, both biological control agents, are capable of deactivating or removing the regulatory genes (*afl*O and *afl*D) in biosynthesis pathway. The

Table 3. The phenotypic characterisation of selected yeast strains.

Test	YB10 *Pichia kudriavzevii*	YB11 *Pichia kudriavzevii*	YB39 *Lachancea thermotolerans*	YB40 *Lachancea thermotolerans*	YB41 *Lachancea thermotolerans*
Fermentation					
D-Glucose	+	+	+	+	+
D-Galactose	-	-	+	+	+
Sucrose	-	-	+	+	+
Maltose	-	-	-	-	-
Lactose	-	-	-	-	-
Raffinose	-	-	-	-	-
Trehalose	-	-	+	+	+
Assimilation					
D-Glucose	+	+	+	+	+
D-Galactose	-	-	-	-	-
D-lucosamine	+	+	-	-	-
Saccharose	-	-	+	+	+
N-acetyl-D-glucosamine	+	+	-	-	-
DL-Lactate	-	-	-	-	-
L-Arabinose	-	-	-	-	-
Cellobiose	-	-	-	-	-
Raffinose	-	-	-	-	-
Maltose	-	-	+	+	+
Trehalose	-	-	+	+	+
2-Keto-D-gluconate	-	-	-	-	-
a-Methyl-D-glucoside	-	-	+	+	+
D-Glucitol	-	-	-	-	-
D-Xylose	-	-	-	-	-
D-Ribose	+	+	-	-	-
Glycerol	-	-	+	+	+
L-Rhamnose	-	-	-	-	-
Erythritol	-	-	-	-	-
Melibiose	-	-	-	-	-
Melezitose	-	-	+	+	+
D-Gluconate	-	-	+	+	+
D-Mannitol	-	-	+	+	+
Lactose	-	-	-	-	-
myo-Inositol			-	-	-

Table 4. Identification of the five strains of yeast isolates by rDNA sequencing and morphological characteristics (colony, cell, and spore morphologies).

Yeast isolate	GenBank accession No.	Identity[a] (%)	Species	Colony Morphology on YPGA	Cell morphology	Spore morphology
YB10	MK403670	100	*Pichia kudriavzevii*	Light cream, butyrous	Ovoidal to elongate, (1.4–5.8) × (3.1–13.7) µm, singles, pairs	Spherical, 1 spore per ascus
YB11	MK403671	100	*Pichia kudriavzevii*	Light cream, butyrous	Ovoidal to elongate, (1.3–5.7) × (3.2–14) µm, singles, pairs	Spherical, 1 spore per ascus
YB39	MK403667	99	*Lachancea thermotolerans*	Cream, butyrous	Spheroidal to ellipsoidal, (3.3–4.5)× (6.2–7.7) µm, single or in short clusters,	Spherical, 2 spore per ascus
YB40	MK403668	100	*Lachancea thermotolerans*	Cream, butyrous	Spheroidal to ellipsoidal, (3.5–5.4)× (6.7–7.7) µm, single or in short clusters,	Spherical, 2 spore per ascus
YB41	MK403669	100	*Lachancea thermotolerans*	Cream, butyrous	Spheroidal to ellipsoidal, (4.3–5.5)× (6.5–7.9) µm, single or in short clusters,	Spherical, 2 spore per ascus

aThe percentage identity among DNA fragments is calculated with BLAST program and the sequences are compared with those from NCBI database.
bRegion of the rDNA gene applied in identification.

2-phenylethanol effect on aflatoxin biosynthesis was also reported by Chang et al. (2015).

Our results indicate that the competitive abilities of the five selected yeast isolates on the leave, hulls and soil in mixed inoculations reduced the *A. flavus* population density by 0.5–438 times. The most effective isolates were YB39, YB43 and YB41 in soil and hulls and YB9 in soil. In mixed inoculations, the population density of the yeast isolates increased by four-fold compared to single isolate inoculations. The results obtained from the selected yeast isolates and *A. flavus* interactions revealed inhibitory effects on *A. flavus*, while the yeast isolate population subjected to the same interactions followed an increasing trend.

The results may indicate different direct and indirect mechanisms involved such as rapid growth, sporulation, germination and substrate colonisation, consummation of available resources without

reducing access of the other organisms to the same resource pool, chemical substances production, parasitism, and ability to neutralise chemical by-products.

Although the yeast isolates collected from soil and nuts of pistachio orchards are able to inhibit *A. flavus* growth and aflatoxin production and could be candidates for biological control strategies to reduce the risks of pistachio nuts being contaminated with toxigenic *A. flavus* in the orchards, the isolates require more investigation.

Based on spraying trails of *P. anomala* on pistachio and almond trees, it was found that this yeast species is able to compete with other microorganisms like *A. flavus* for occupying ecological niches. Hua (2009) suggested application of this yeast as an environment-friendly approach to protect nuts and corn which are frequently contaminated with aflatoxins. Experiments in Californian pistachio orchards revealed that spraying *P. anomala* on trees prevented *A. flavus* occurrence by more than 97% in relation to the control treatments. According to Hua (2013), *P. anomala* could be sprayed on harvested or stored crops, instead of the trees. The selected yeast isolates could rapidly stop the expansion and the growth of microorganisms which compete for nutrition and space on the leaf, nuts, blossom, soil and the hulls. Additionally, the yeasts are easily grown and present in a variety of ecological niches.

Conclusion

The use of yeasts can be considered as an effective approach for biocontrol of toxin-producing fungi, especially in edible food such as pistachios with high nutritional and economic values. Some yeast strains have been applied commercially for biocontrol of plant diseases. They are available and have the necessary efficacy. The present assays have shown the high potential ability of YB39 and YB43 strains against *A. flavus* growth and aflatoxin production in different substrates. The commercial applications of the strains under field conditions require further studies.

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