Tolerance of Yeast Antagonists Isolated from Pistachios in Environmental Stresses

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Abstract

Background: Aflatoxins are the most contaminant of various agricultural products, especially pistachios. Nowadays, biological control is one of the successful methods in aflatoxin mitigation. Yeasts can be used as a safe and efficient strategy to mitigate aflatoxin in pistachio. The present study screens the high tolerant yeast isolates to environmental stresses.

Materials and methods: Soil and pistachio nut samples were collected from commercial pistachio orchards. A dilution series method and GPYA medium were used to isolate yeast species. The ability of yeast isolates to inhibit the growth of *Aspergillus flavus* was investigated through dual culture, volatile compound, and non-volatile compound assays. The ammonia vapor method was used to evaluate the effectiveness of yeast isolates on aflatoxin production in cultural assays. The tolerance of isolates to non-living stresses was measured in optical density (OD) using spectrophotometry assays at 600 nm in a TSB medium.

Results: The highest inhibitions of mycelial growth in volatile and non-volatile compounds, as well as dual culture, belonged to 78-3-2, YP4-11, 58-15, and 47-8 isolates by 79%, 88%, and 75%, respectively. The highest tolerance to salinity, drought, and heat stresses in the TSB medium was measured for 9, 6-5, and 181yeast isolates by 1.84, 0.75, and 2.18 (OD), respectively.

Conclusion: Selected isolates can be suitable candidates for field testing after further assays and accurate identification.

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1. Introduction

The domestic pistachio tree (*Pistacia vera* L.) is a dicotyledonous and deciduous plant mainly cultivated in subtropical regions [1]. Pistachio is one of the most important agricultural products and one of Iran's main non-oil export products. Iran is the second-largest producer of pistachios globally, with a high export volume. At present, the area under pistachio cultivation in Iran is higher than 425,286 hectares, the yield is about 668 kg per hectare, and the production is 551,307 tons [2]. Due to the toxicity and carcinogenicity of aflatoxins (AFs), their amounts in food are carefully monitored; in most countries, the acceptable limit is determined between 5 and 15 ng/g [3, 4]. About 20% of the food production in the world is contaminated with mycotoxins annually, among which aflatoxin is more important. More than 13 species of *Aspergillus*species can produce AF, and the most important species in the pistachio planting areas is *Aspergillus flavus* [5]. This species has a global distribution because it adapts well to adverse environmental conditions and contains soilborne and airborne spores. Also, its metabolites can cause liver cancer if they enter the human and animal bodies through food [6]. The highest contamination of pistachio nuts with AFs is in the pre-harvest stage and under orchard conditions, and a key factor in the occurrence of AF infection is the exposure of pistachio kernels to fungal infection. Different cultural, physical, chemical, and biological methods are recommended for AF mitigation, which can be time-consuming and costly. Accordingly, the results have shown that with an increase in the concentration of Shirazi thyme in this type of coating, aflatoxin production decreases. Besides, the sage extract has the highest potential for preventing aflatoxin production compared to the thyme extract and cumin [7]. Research has shown that antagonist microorganisms can significantly reduce AFs without using harmful chemicals due to their compatibility with the environment. It was found that yeasts and non-toxigenic *A. flavus* are suitable candidates for the biological control of AFs in the pre- and post-harvest stages [3, 8]. Among the biocontrol agents, yeast isolates have a high biological control capability due to colonization (occupation) of environmental sites in dry conditions (suitable for pistachio growing environment), production of extracellular polysaccharides, production of volatile and non-volatile compounds, and their low sensitivity to pesticides [9. 10, 11]. Multiple mechanisms are involved in preventing plant diseases by the yeast biological control agents, including enzyme exudation, toxin and volatile organic compounds (VOCs) production, direct parasitism, plant resistance induction, competition for food and habitat [9].

2. Materials and methods

Yeast isolates

Seven hundred yeast isolates belonging to the Department of Technology and Production Management-Pistachio Research Center (PRC) were used in all assays. The abilities of isolated yeast to inhibit the mycelial growth of *A. flavus* were assessed in a dual culture test. Yeast isolates with the greatest inhibitory potential were further selected and analyzed in dual culture, non-volatile, and volatile assays, as described below.

Effect of yeast isolates on mycelial growth of *Aspergillus flavus*

Dual culture

A.flavus isolate PRC106 with a high ability to produce AFB_1 belonging to the Department of Technology and Production Management-Pistachio Research Center (PRC) was used in all

assays. For spore suspension preparation, the *A. flavus* isolate was cultured on a PDA medium for 7 days, and spores were collected in sterile distilled water containing 0.2% peptone. A dual culture assay was used for preliminary screening of yeast isolates on *A. flavus* inhibition. Isolates with a high potential ability to inhibit *A. flavus* mycelial growth were selected for further assays. For this purpose, 24-hold-yeast colonies were spotted on one side of Petri plates on a GPYA medium, and after 48 hours, the fungal isolates were inoculated on the opposite side. The plates were incubated at 28 ° C in the dark for 7 days. Fungal growth was measured using a ruler with millimeter accuracy, and the inhibition rate was calculated in comparison with control plates (yeast-free control) [13].

Volatile compounds

Yeast isolates were cultured in a GPYA medium and incubated for 48h at 28 ° C in the dark [14]. Then, after 48 hours, *A. flavus* isolates were inoculated on coconut agar medium (CAM), placed upside down on yeast Petri dishes, and sealed with paraffin film (Parafilm, Sigma-Aldrich, Germany) [15]. The mycelial growth was measured using a ruler, and the inhibition percentage was calculated using the following formula.

Inhibition percentage = ((Treatment colony growth diameter - Control colony growth diameter) /Control colony growth diameter) × 100

Non-volatile compounds

The selected yeast isolates were inoculated (50 µl, 10^4) in 50 ml of a GPY broth medium and shaken at 150 rpm and 27 ° C for one week [16]. The contents of the flasks were then centrifuged at 4000 rpm for 1 hour, and supernatants passed through a 0.2 µl syringe filter under sterile conditions. Concentrations of 5, 15, and 25% of the extract were prepared. They were added to the CAM.10 µl of *A. flavus* suspension with a concentration of 50,000 spores per ml at the center of the Petri dish and incubated at 28 ° C in the dark for 5 days. Fungal growth inhibition of *A. flavus* compared to the control was calculated using the formula of Section 2.3.3. The experiment was repeated twice, and 2 replicates were considered for each isolate.

Evaluation of tolerance of yeast isolates to drought stress

To evaluate the tolerance of yeast isolates to drought stress, 30 g of tryptic soy broth (TSB) (Himedia, Pvt. Ltd., India) and 326 g of polyethylene glycol (PEG 6000, Sigma-Aldrich, Germany) were dissolved in 644 ml of distilled water; then, its volume was increased to one liter [15]. Next, 50 ml solution was added to each flask and autoclaved at a temperature of 121 ° C and a pressure of 1.5 atmospheres. Each flask was inoculated separately with a loop of a 24-hour culture of yeast isolates and then shaken for 24 to 72 hours at 150. The amount of light absorption of the suspension was measured at 600 nm using a spectrophotometer (T60 Spectrophotometer, PG Instruments Limited, UK). Yeast-free culture medium was used for the blank sample.

Evaluation of growth ability of yeast isolates with high antagonistic in drought, salinity, and high-temperature conditions

The tolerance of yeast isolates to abiotic stresses in the TSB (Tryptone Soy Broth) medium was investigated *in vitro*, according to the method used by Praveen Kumar et al. [17]. In order to measure the ability of the isolates, the adsorption rate of culture media in comparison with the control (culture medium alone) at a wavelength of 600 nm was measured.

Effect of isolated yeasts on aflatoxin production

Here, the intensity of color change in colony reverses on exposure to ammonium hydroxide vapor was used to detect yeast isolates with a high ability to inhibit AF production in dual culture, volatile compounds, and non-volatile compounds on CAM [18]. Then, the intensity of color change in the reverse colony was grouped from indices 1 to 7, as described in figure 1.

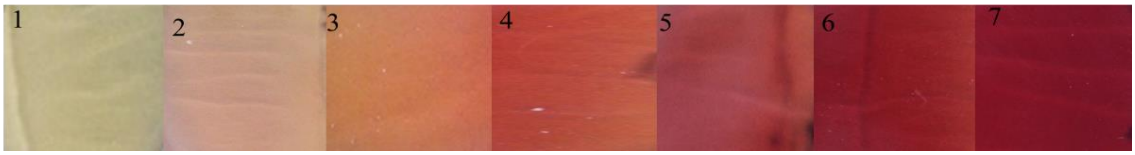

Figure 1. Color intensity indices after exposing the colony of *Aspergillusflavus* to ammonium hydroxide vapor; 1, non-producing aflatoxin 7, high-producing aflatoxin.

Statistical analysis

The data obtained from different experiments were statistically analyzed in a completely randomized design. Duncan's multiple range tests at a 5% probability level were used to compare the means. SPSS 16 statistical software was used to analyze the data and compare the means.

3. Results

Mycelial growth and aflatoxin production

Dual culture

The average rate of mycelial growth inhibition by yeast isolates varied from 48% to 75%. The highest and lowest effects were related to 47-8 and 75-4-11 isolates, respectively. The highest color intensity was related to isolate 9 by 5.5. The greatest impact was observed in the 33-1 isolate with a high ability to reduce the mycelial growth.

Volatile compounds

The highest and lowest inhibitions of mycelial growth were related to 78-3-2 and 63-2 yeast isolates.

Yeast isolates reduced the color intensity of colony reverse by 1.5 to 7 times lower compared to *A. flavus* alone, indicating a decrease in aflatoxin production in the culture medium. The highest effect was observed in the 28-4-1 isolate.

Non-volatile compounds

The lowest inhibitory of mycelial growth in non-volatile assays at concentrations of 5%, 15%, and 25% were related to 29-5-13, 40-3, and 38-4 yeast isolates, being increased by the 25% concentration. The highest reduction rates were observed in the YP 4-11 isolate, followed by some other isolates such as 90, 58-15, and 122, although to insignificant degrees. The results also showed that isolates such as YP 3-5, YP 4-11, 90, and 122 could reduce the mycelial growth in both dual culture and non-volatile compounds but not in volatile compounds, indicating the possibility of different mechanisms involved in inhibiting the mycelial growth of *A, flavus.* The color intensity in the reverse colony showed that the yeast isolates reduced AF production in culture media by 1.5 to 7 times compared to *A. flavus* alone, the control treatment after exposure to ammonia. Isolation was 9-48 (Figure 4-9). Overall, 10 isolates reduced the color intensity by 2.0 in the 25% concentration.

Tolerance of screened yeast isolates to abiotic stresses

Most isolates showed the same tolerance to salinity stress. The highest tolerance was measured in 9, 122, and 90 yeast isolates, showing optical density (OD) greater than 1.6. The lowest ratios were related to 38-1, 6-5, 181, and 78-3-2 isolates (Table 1). Out of 25 isolates, 14 showed an optical density higher than 0.2 (drought tolerance index) using polyethylene glycol (6000). Among the yeast isolates, the highest measured ratios belonged to the 6-5 isolate with an optical density equal to 0.75, followed by YP 5-3 and 40-4 yeast isolates (Table 1). Overall, all isolates could withstand heat stress and a temperature of 50 ºC. The highest measured optical densities were related to yeast isolates 181 and 9, followed by 63-2, 60-3, 90, and 6-5.

Table 1. The effect of screened yeast isolates on growth inhibition of *Aspergillus flavus* in different tests

Isolates	Inhibition%																	Environmental stresses (OD)**		
	Dual culture			Volatile compounds			Non-volatile compounds											Drought	Salinity	Heat
							5%			15%			25%							
6-5	67	A-C	5*	43	D-I	4.5*	29	K	4.5*	42	I	5*	58	D-E	4.5*			0.7	0.6	1.9
YP4-11	60	BE	4.5	13	K-L	5.5	77	A	3	78	AB	3	88	A	2			0.4	1.4	1.1
63-2	64	A-D	3.5	8	L	5.5	37	I	2	48	FI	2	75	A-C	2			0.2	1.2	2.0
9	60	B-E	5.5	38	F-I	4.5	53	B-G	3	60	C-F	2.5	76	A-C	2			0.2	1.8	2.1
YP3-5	68	A-C	3.5	39	F-I	4	58	A-E	3	61	C-F	4	62	BCD	2.5			0.7	1.2	1.5
48-9	62	A-D	4	40	E-I	4	41	I-J	1	61	C-F	2	85	A	2			0.0	1.1	1.4
47-8	75	A	3	22	GK	5.5	44	GI	5.5	51	E-I	4.5	57	D-E	5.5			0.5	1.4	1.7
75-4-11	46	E	4	51	B-E	4.5	42	H-J	5.5	45	I	5.5	59	E	5.5			0.4	1.4	1.4
31-4	55	C-E	4.5	38	F-I	2.5	47	F-I	1.5	70	G	2	86	A	2			0.1	1.3	1.5
29-5-13	50	D-E	4	35	E-G	3.5	11	L	3	12	G	4	14	F	4			0.2	1.3	1.5
58-15	52	A-D	3	41	E-I	2.5	53	B-G	3	83	A	2	88	A	2			0.5	1.4	1.4
33-1	71	AB	1.5	45	C-F	5	50	C-G	5	68	B-D	3	78	AB	2			0.4	1.3	1.6
31-8	71	AB	3.5	46	B-F	2.5	54	B-F	3.5	58	C-F	3	65	B-D	4			0.1	1.1	1.2
17	65	A-C	4	30	I-G	5	41	IJ	5	43	I	5.5	63	B-D	4			0.3	1.4	1.9
28-4-1	64	A-C	2.5	60	B-D	1.5	54	B-F	3.5	70	BC	2	84	A	2			0.3	1.4	1.4
181	58	B-E	4.5	29	I-K	3	48	C-H	4	60	C-F	5	62	C-E	4			0.3	0.7	2.2
178	59	B-E	4	53	B-F	4	46	E-I	3	72	D-I	3	59	D-E	2.5			0.4	1.1	1.2
122	63	A-D	4	54	B-E	2	55	A-C	4.5	70	BC	3	75	A-C	2			0.3	1.7	1.8
90	63	A-D	3.5	29	G-I	5.5	57	AB	5	80	AB	4	86	A	3			0.2	1.6	2.0
58-8	63	A-D	3	58	D-F	4	53	B-G	4.5	55	C-I	4	57	D-E	3			0.2	1.0	1.3
38-1	59	B-E	3.5	43	D-I	4.5	54	B-F	2.5	64	B-E	3.5	75	A-C	2.5			0.1	0.0	1.7
78-3-2	68	AB	2.5	79	A	4	49	D-I	4.5	60	C-F	4.5	65	B-D	4			0.2	0.7	0.8
40-3	50	D-E	4	62	B	3.5	8	L	4	12	G	4	27	F	4			0.3	1.0	2.0
40-4	60	B-E	4.5	61	BC	4.5	56	A-D	3.5	70	BC	3	85	A	2			0.6	1.0	1.1
38-4	55	C-E	4	42	E-I	3.5	11	L	5	14	G	5	21	F	5			0.5	0.9	1.4
Control			7			7			7			7			7					

4. Discussion

Based on the results, yeast isolates and predominant species of *Aspergillus* are present in pistachio orchards in common ecological sites. They may compete in these ecological sites, possibly effective in aflatoxin reduction in pistachio nuts. This factor in the mono-culture system of pistachios may determine the predominant microorganisms in the orchards. In the present study, 700 yeast strains were isolated from soil and pistachio nut, and their ability to reduce *A. flavus* mycelial growth and aflatoxin production was screened. Out of 700 screened isolates, 25 showed the greatest mycelial inhibition of *A. flavus*. Among the isolates, YP 4-11, 5-8, and 90 generally showed the greatest effect in all 3 concentrations of extracellular exudation, with more than 60% inhibitory at 5% concentration. This indicated a competitive interaction between yeast isolates and *A. flavus*, reducing the mycelial growth compared to the control treatment. The results obtained in the dual culture of isolates 47-8 and 78-3-2 showed the best effect in reducing the mycelial growth. Isolates 90 and 58-8 were the best in the extracellular exudation; however, in the dual culture, they were not statistically different from the 47-8 and 78-3-2 isolates. Based on the results obtained from the volatile culture, the greatest effect on reducing the mycelial growth was related to isolates 78-3-2, with a significantly higher inhibitory level than others. The interaction of yeast isolates with *A. flavus* needs further investigation to identify the mechanisms involved accurately. According to researches, various mechanisms may be involved, including faster growth, faster uptake of substrates in nutritional competition, production of inhibitors, neutralization of toxins, growth on a wide range of different food sources, and production of the enzyme. The ability of different yeasts to produce and synthesize different compounds has been studied by various researchers. *Pichia anomola*, for example, produces 2-phenyl ethanol, a volatile metabolite that reduces fungal growth, aflatoxin production, and gene expression [19]. Volatile compounds, such as aldehydes, acetate esters, and alcohols, can also affect the growth and production of aflatoxins by *A. flavus* [20]. The results of extracellular exudation, dual culture, and volatile compounds indicated the effect of different yeast isolates on the inhibition of mycelial growth and production of *A. flavus* spores, being varied depending on the different isolates. In the extracellular secretion assay, the inhibition of mycelial growth was increased by increasing the concentration of the extract. On the other hand, in the test of volatile metabolites, dual-culture as well as extracellular secretions, mycelial inhibition increased with increasing inocubation time and reaching a maximum of 85%, 80% and 84%, respectively. This indicates the production of different metabolites by yeast isolates differently with antagonistic properties and different mechanisms; this has been studied by different researchers in the competitive interaction of the pathogen with the biocontrol agent.

Toxigenicity and non-toxigenicity of *Aspergillus* isolate in culture alone or in contrast to other biocontrol agents is the research topic studied by several researchers in liquid or solid culture media. This property has been assayed by analytical and cultural medium-based methods [21, 22]. In the present study, the use of culture medium in combination with or without ammonia was well characterized by color differentiation of *A. flavus* alone or in interaction with yeast isolates. Accordingly, different isolates were divided into 6 separate groups by producing cream color in the back of the colony

and its intensity. The small amount of aflatoxin in the culture medium showed a positive relationship with the color intensity and increased with the amount of aflatoxin. The lowest amount of aflatoxin was observed in the samples in colonies with little cream color intensity. When the colonies were exposed to ammonia vapor, the colony color intensity also increased but followed a similar pattern (without ammonia). According to the results, ammonia caused the colony to change color from cream to orange or red, the intensity of which varied depending on the type of isolate. Based on the results, the CAM medium can be used to screen a large number of isolates without using the analytical method. This method can be used to screen the competitiveness of many isolates simultaneously with the study of mycelial growth inhibition, which is one of its advantages. Investigation of the effect of ammonia vapor on color intensity showed that exposure to *A. flavus* colonies could be used to detect competitiveness between toxigenic and non-toxigenic isolates [18].

5. Conclusion:

The compatibility of yeast isolates in the natural environment and their efficiency in biocontrol had a direct relationship with the tolerance of adverse environmental conditions. Therefore, the selection of isolates in field conditions should be done by considering this factor.

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Conflict of interest declaration

The authors state that there is no conflict of interest.

Code of ethics

In this research, no living entity has been used, and the research stages have been conducted in a laboratory.

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