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Reactions of Various Cultivars of Almond to Toxin-Producing *Aspergillus flavus* Isolates

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A R T I C L E I N F O

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A B S T R A C T

Samples were collected from different regions of two provinces, Markazi and Semnan, their contamination by *Aspergillus flavus* was examined. The results of *A. flavus* colony counts in different samples of almond showed that among 20 experimented samples, 11 samples were contaminated by fungus; therefore, 8 cultivars of almond, Shahroud 6, Shahroud 21, Shahroud 12, Shahroud 8, Shahroud 17, Rabie, Mengha, and Sangi Shireen, were collected in order to evaluate their sensitivity to fungal colonization and the sporulation rate of fungus on them. The results of statistical analysis showed that on the third, fifth, and seventh days, Shahroud 12 was the least resistant cultivar [average pollution (99.5%)], whereas Shahroud 6 was the most resistant [average contamination (7.36%)] at a level of 5 % for growth of *A. flavus* after 7 days. Experiments related to the testa of almond show that the testa can be a barrier against penetration of fungus into the inner part of the almond, decreaseing fungal growth, and thus reducing the weight of mycelium and sporulation in almonds.

Introduction

Almond (*Prunus amygdalus*) belongs to the family Rosaceae.Almonds are traditionally planted and cultivated in Iran and is compatible with the climatic conditions found there (Afshari and Abbaspour, 2010) . Iran, according to statistics published by the FAO in 2012, ranks third in the world as a producer of almonds, generating 158 050 tons per year (FAO STAT, 2012). Almonds are a source of various mineral elements and vitamins, including B vitamins (riboflavin and niacin), vitamin E,calcium,iron, magnesium, manganese, phosphorus, and zinc. Almonds are rich in fiber and can reduce blood cholesterol (Berryman *et al*., 2011). Without a doubt, the country's main problem in exporting dried fruits is fungal contamination with *Aspergillus flavus* and the presence of aflatoxin in these products. Nowadays, aflatoxin contamination in agricultural products is a serious health problem in the international community, and different countries have set rules governing the production, consumption, and import of nutritional material to address the serious danger of mycotoxins. In the US, nutritious materials and pharmaceuticals that have more than 20 parts- per- billion (ppb) total aflatoxin and foods containing 15 ppb aflatoxin B1 cannot be exported (Gourama and Bullerman, 1995; Trial *et al*., 1995). Toxins come from moldy food in the animal and human food chains. *Aspergillus* fungus, especially species of *A. flavus*, can attack agricultural products and make them infectious. After infecting food products, the fungus proliferates and produces toxin. If there is environmental stress, the production of toxin is increased(Almasian *et al*., 2008). One of the main mycotoxins is aflatoxin produced by different species of *Aspergillus*, such as

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Aspergillus flavus and A. parasiticus. Aflatoxin is found in most herbal products, especially in such oily seeds as almond, pistachio, coconut, soybean, corn, rice, and wheat (D'Mello and Macdonald, 1997; Amiri dumari *et al*., 2013). Of 18 known aflatoxins, aflatoxins B1, B2, G1, and G2 are included in the group of *A. flavus* toxins known as carcinogens by the International Agency for Research on Cancer. Meanwhile the, toxicity and carcinogenicity of aflatoxin B are reported more often than those of other varieties. Aflatoxins B1, B2, G1, and G2 are toxic metabolites of dihydrofuran tetrahydrofuran bonded to a coumarin ring, while having a deterrent effect on acute toxicity, mutagenicity, teratogenicity, and carcinogenesis (Arrus *et al*., 2005; Ricordy *et al*., 2002 and Fani Makki *et al*., 2014). Regarding its importance in terms of the food supply, this paper aims to assess the resistance of almond cultivars to contamination by *A. flavus* and the protective effect of the outer epidermis against penetration and colonization of fungus in the kernel.

Materials and Methods

Assessing the Degree of Contamination of Almond Cultivars with A. flavus

In order to study the prevalence of almond contamination with *A. flavus*, samples were taken from different regions of two provinces, Markazi Province and Semnan Province (without regard to the cultivars of almond). For this purpose, five samples were selected from each site, and each sample weighed about approximately 1000 g. All samples were combined to generate a 500 g pooled sample. Twenty such pooled samples were taken.

Samples were first ground, then diluted 10^{-1} and 10^{-2} in cultivation medium and re-cultivated three times in a completely randomized model on the surface of AFPA medium in petri dishes containing peptone 10 g, ferric ammonium citrate 5.0 g, yeast extract 20 g, chloramphenicol 100 mg, agar 15 g, dicloran, 2 mg, and distilled water to 1 L (Gourama and Bullerman, 1995). In this method, 10 g of almond from each sample were added to 90 ml of peptone water 1% and diluted to 10^{-1} and 10^{-2}, 0.1 ml of each provided dilution is distributed on the surface of petri dish containing AFPA medium and kept at 25°C for 3-7 days. After this period, colonies of *A. flavus* that grew on the surface of the agar were counted and separated, and then contamination in various samples was compared.

Evaluating the Sensitivity of Almond Cultivars to the Fungus A. flavus

In order to evaluate the sensitivity of almond cultivars to aflatoxin- producing *A. flavus*, five almond cultivars from Semnan Province called Shahroud 17, 12, 8, 6, and 21 and three almond cultivars of Markazi Province called Mengha, Sangi Shireen and Rabie were collected for testing. To conduct this search, a fungal isolate of *A. flavus* that was separated from almond and whose aflatoxin production was demonstrated by high-performance liquid chromatography was used. Three replications and a control were in cluded for every cultivar; instead of a spore suspension, sterilized distilled water was added to the control petri dish. After surface disinfection and soaking the almond in sterilized distilled water, 10 g amounts of each almond sample were separately weighed on a digital scale and placed in a 10 cm petri dish,and 1 ml of spore suspension of fungus was poured into petri dish. Then, the petri dish was kept in an incubator at 26°C. After fungal growth and colonization of the almond, the degree of colonization was calculated after 3, 5, and 7 days (Ghewande *et al*., 1993).

Calculating the Weight of Mycelium and Sporulation of A. flavus

In order to calculate the weight of mycelium and sporulation of fungus, on the seventh day, colonized almonds from every petri dish were combined with 100 mL of distilled water, poured into an Erlenmeyer flask, and incubated with shaking for24 hours. First, a piece of filter paper was weighed on a digital scale. Next, the filter paper was placed on a Buchner funnel. Almond kernels and spores that had been shaken in an

Erlenmeyer flask were poured on the Buchner funnel, and then the thallus of the fungus was separated from the almond kernels with forceps. Finally, the kernels were removed from the surface of the filter. The filter paper containing the thallus of fungus and its inner spore was dried,the filter paper was weighed, and the spores present in distilled water (100 ml) were counted in a hemocytometer; then, the spores produced due to fungal growth were calculated per 10 mg of almond kernels for each petri dish. Finally,the thickness of the almond's testa was measured with a micrometer in order to assess the effect of almond testa on reducing colonization by the fungus, thus its role in decreasing the growth of fungus in kernels of different almond cultivars was assessed.

In this research, a factorial experiment in a completely randomized design was used to assess simple and interaction effects. In this design, the main factors included eight cultivars expos ed to fungus in for different times (3, 5, and 7 days after inoculation). SAS software and Duncan's multiple range tests were used to analyze variance and classification averages.

Results

Colony counts of *A. flavus* were obtained in various samples of almond Of the 20 tested samples, 11 samples were contaminated by *A. flavus*, while contamination was not seen in 9 samples (Fig.1). The results of the study of growth of *A. flavus* in kernels of different almond cultivars showed that growth of fungus (colonization) on the kernels was significantly different between cultivars after 3, 5, and 7 days of inoculation. Of the experimented cultivars, Shahroud 12 was the least resistant, and Shahroud 6 was the most resistant to *A. flavus*, showing growth at a level of 5% (Table 1 and Fig. 2). The results for amount of sporulation and weight of mycelium for *A. flavus* fungus in kernels of different almond cultivars showed that, of the experimental cultivars, Shahroud 6 had the lowest weight of mycelium and sporulation whereas Shahroud 21 has the most mycelium and sporulation at the level of 5% on all almond kernels (Table 2). The results assessing the effect of the almond testa on reducing colonization of fungus on almond kernels showed a correlation coefficient of r = -0.063 between rates of fungal colonization on the seventh 7 day on the seeds of different almond cultivars with the thickness of the seed coat. Therefore, this correlation evaluated at the probability level of 1% and 5% is significant according to statistical analysis (Table 3).

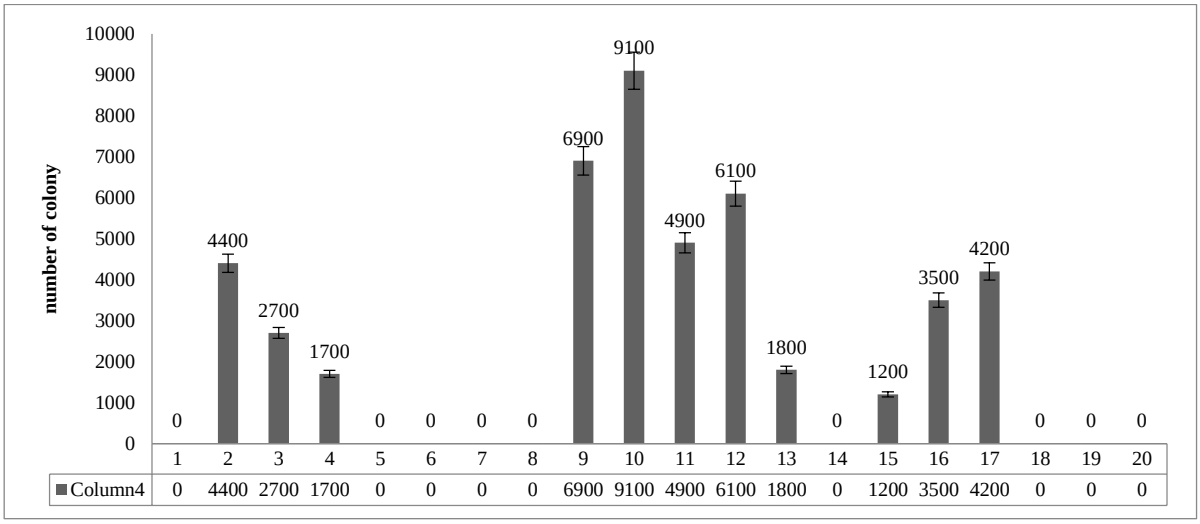

Fig.1. Comparison of the number by colony of *A. flavus* in 20 samples of almond grown in Markazi and Semnan Provinces.

Table 1. Comparison of the percentage by colonization of *A. flavus* on kernels of different almond cultivars 3, 5, and 7 days after inoculation.

	Average percentage of colonization					
Almond cultivar	After 3 days of inoculation		After 5 days of inoculation		After 7 days of inoculation	
Shahroud 12	13.46	ghi	47.74	c	99.05	a
Shahroud 21	16.13	fg	45.97	c	80.44	b
Sangi Shireen	2.18	l	4.45	kl	8.38	hijkl
Mengha	2.79	l	5.77	jkl	10.08	ghijkl
Rabie	7.05	ijkl	10.16	ghijk	14.23	fgh
Shahroud 6	1.88	l	4.72	kl	7.36	ijkl
Shahroud 8	10.24	ghik	22.48	e	35.24	d
Shahroud 17	5.29	kl	12.13	ghij	20.00	ef

The same letters after the average percentage in every column indicate the absence of a significant difference at a significance level of 5% (Duncan's multiple range test).

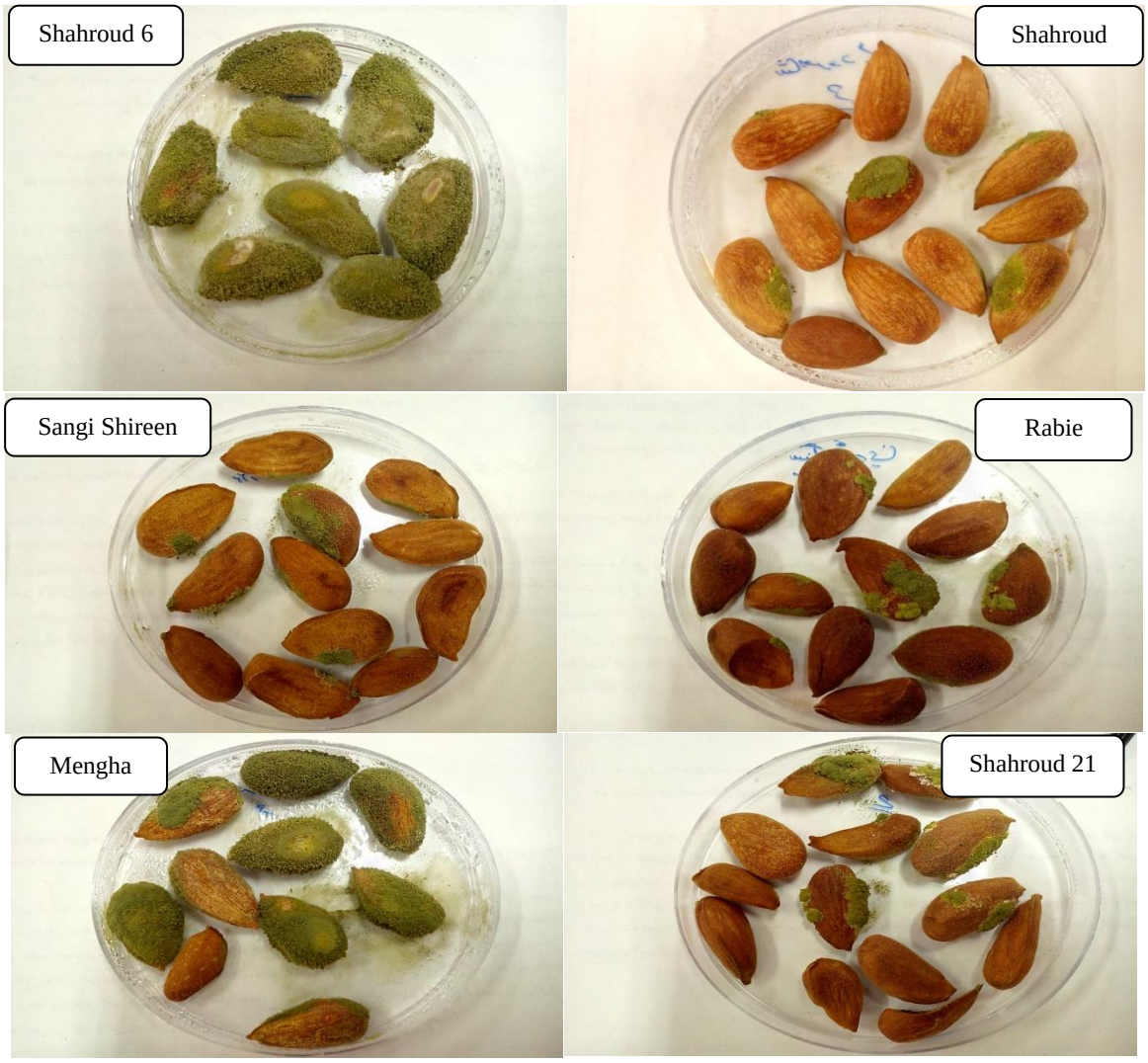

Fig. 2. Overview of growth and colonization of *A. flavus* on the almond kernel after 7 days of inoculation.

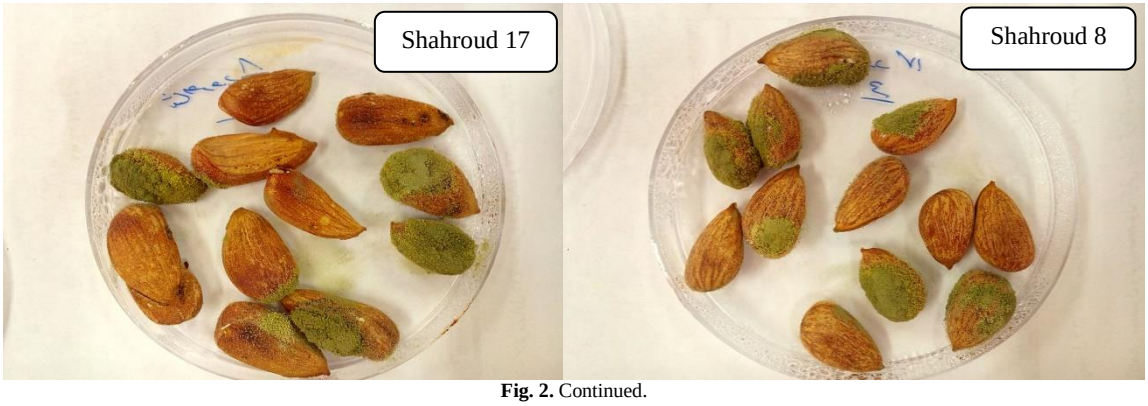

Fig. 2. Continued.

Table 2. Comparison average effect of almond kernel in different almond cultivars on examined traits.

	Diameter of testa		Weight of mycelium		Amount of The sporulation	
Effect of cultivar	average	grouping	average	grouping	average	grouping
Shahroud 12	138	g	0.412	a	15×10^7	a
Shahroud 21	136	g	0.381	a	10×10^7	b
Sangi Shireen	223	c	0.051	cd	52×10^5	e
Mengha	196	d	0.057	cd	16 × 106	e
Rabie	260	a	0.054	cd	11 × 106	d
Shahroud 6	228	b	0.023	d	42×10^5	e
Shahroud 8	177	e	0.152	b	48×10^6	c
Shahroud 17	173	f	0.099	c	34×10^6	d

Treatments with common letters based on Duncan multiple range test at probability level of 5% have no significant difference.

Table 3. Comparison coefficient between examined traits in the kernel of almond cultivars

Trait	Colonization	Diameter of testa	Weight of mycelium	Amount of sporulation
Colonization	1.000			
Diameter of testa	-0.63 **	1.000		
Weight of mycelium	0.70 **	-0.84 **	1.000	
Amount of sporulation	0.76 **	-0.83**	0.96**	1.000

ns,* and ** indicate insignificance and significance at the probability level of 5% and 1%

Discussion

Given that the contamination processes of *A. flavus* and aflatoxin are very complex, destroying or even controlling contamination with toxin requires the adoption of several approaches and different methods (Almasian *et al*., 2008). Therefore research that recognizes cultivars of different products resistant to *A. flavus* fungus and aflatoxin and that studies the physicochemical effects of almond testa on preventing fungal growth and toxin production can provide a suitable basis for controlling aflatoxin contamination. Therefore, many countries around the world have conducted a wide range of studies to recognize cultivars of agricultural and horticultural products resistant to *A. flavus* fungus and aflatoxin and to study their resistance mechanisms with promising results (Gradziel and Wang, 1994).

The results of studies on groundnut show different resistance levels of various groundnut cultivars to the growth of aflatoxin-producing *A. flavus*. Studies conducted on the production rate of aflatoxin B1 in different cultivars show that the production rate of aflatoxin varies among groundnut cultivars. While the

most resistant cultivar produces 3900µg/kg aflatoxin, the aflatoxin-production rate is approximately 90 000 µg/kg in the most susceptible cultivar. Furthermore, significant correlations and relationships between the rates of fungal growth and the production of aflatoxin have been observed (Ghewande *et al*., 1993).

In one study, researchers damaged the pericarp and assessed the effect of this layer on preventing permeation of *A. flavus* into the seed of thd kernel, observing that aflatoxin production was higher in damaged seeds than in intact seeds. Therefore,the pericarp and aleurone layer of the kernel are recognized as a resistant barrier to fungal penetration by *A. flavus* (Waliin, 1986). Damage to the seed's outer epidermis increases the rapid and direct attack of *A. flavus* on almond seeds. This in turn raises the chances of aflatoxin production. Wounding of the pod and sheath increases access to nutrients that are necessary for rapid growth of *A. flavus* (Ghewande *et al*., 1993).

In spite of rapid colonization of *A. flavus* on green skinned fruit, no accumulation of aflatoxin is detected in them. Moreover, colonization by fungus and production of aflatoxin are low in intact pistachios with seed coats, and the cuticle layer may play an effective role in resistance of the seed to colonization (Mahoney and Rodrigues, 1996).the sensitivity of Khorasan Razavi pistachio cultivars to *A. flavus* fungus was assessed in previously conducted research. The results showed that different cultivars vary in their sensitivity to the fungus, which can produce both aflatoxins B1 and B2 (Jalali *et al*., 2011). Phenolic compounds available in the pericarp of pistachios and almonds can reduce or inhibit the production of aflatoxin (Afshari and Hokmabadi, 2008).The use of a product sensitive to contamination by *Aspergillus*, pests, or other microbial agents can potentially increase their contamination rates by aflatoxin. Therefore, the resistance of the chosen cultivar should be considered, and it is necessary for the farmer to consult with plant breeders in order to find the best cultivars (Jalali *et al*., 2011).

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