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ORIGINAL ARTICLE

Reaction of Iranian Almond Cultivars to Toxigenic *Aspergillus flavus*

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KEYWORDS

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ABSTRACT: Aflatoxins are fungal secondary metabolites, mutagenic and carcinogenic substances that are often produced by *Aspergillus* section *Flavi* on food and feed under certain environmental conditions. Dried fruit has always been exposed to contamination by toxigenic fungi and aflatoxin, and the health of the produced product is a challenge for the consumer and trading. Susceptibility of five almond cultivar kernels including Sefid (Kaghazi), Mamaei, Aliakbar Rabi from Isfahan province and Shahrood 12 and Shahrood 13 cultivars from Semnan province to *Aspergillus flavus* and aflatoxin production were evaluated. A toxigenic *A. flavus* strain was used for the artificial inoculation of almond kernel. Ten grams of almond kernels samples were surface disinfected in three replications in a completely randomized design. The samples were inoculated with one milliliter of *A. flavus* spore suspension (2×10^6 ml L^{-1}). The growth rate and colonization of the fungus on the kernels as well as the fungal sporulation rate on kernels were evaluated after 5, 7, and 12 days of incubation at 98±2% humidity, 28ºC, and dark conditions. Aflatoxin B_1were measured in inoculated samples using thin-layer chromatography (TLC). Assays were performed in three replications in a completely randomized experimental design ($P\leq0.05$). The results showed that Mamaei and Kaghazi cultivars with 20.36 and 95.7% kernel colonization, 2.65×10^7 and 3.3×10^8 spore/ml, 9495.7 and 14057 µg/Kg have the lowest and highest sensitivity to *A. flavus* colonization, sporulation, and aflatoxin B_1 respectively. The results of this study can be used in the selection of aflatoxin resistant cultivars.

INTRODUCTION

Almond, *Prunus amygdalus* Batsch, 1801 Syn. *Prunus dulcis* (Mill.) D. A. Webb is one of the native species of dry and semi-arid mountainous regions of Central and Western Asia. Many wild almond species and commercial cultivars are also found in these areas. The growth of 19 types of almonds in the country. The cultivation and production of almonds in Iran have a very long history, so this country is considered one of the oldest areas of almond production [1]. Almonds have a very high nutritional value and are very important in the food basket. Due to its ability to be transformed into many products, this product is also of considerable importance in the food, pharmaceutical and, health industries. The characteristics of the almond tree such as

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adaptation to the climatic conditions of arid and semi-arid regions, the ability to grow in weak and calcareous soils, high water consumption efficiency, long-term storage in normal conditions, ease of transportation and packaging, and have enough information of with almond cultivation, has proposed the tree as one of the best options in many areas of Iran [1]. The area under almond cultivation in Iran is 177737 hectares, the amount of production is 128323 tons, and the average yield is 245 and 832 kg per hectare in dryland and irrigated agriculture respectively [2].

Mycotoxins are secondary metabolites produced by some filamentous fungi in sensitive hosts such as grains, dried fruits and, nuts under certain environmental conditions. Contamination of food products with mycotoxins causes global health and economic concerns [3]. Every year, 20% of food products produced in the world are contaminated by mycotoxins, of which aflatoxins have a greater share. The damage caused by the destruction of food and agricultural products by aflatoxin in the United States has been estimated at more than 100 million dollars annually [4]. In Africa, more than $750 million is the cost of aflatoxin contamination of crops per year [5]. Among the fungi that produce mycotoxins, *Aspergillus* is one of the largest and most important genera in this field, with more than 50 mycotoxin-producing species [6]. The most important producers of aflatoxins are *Aspergillus* species, especially *A. flavus*. More than 200 have been identified in the *Aspergillus* genus. These species have wide geographic distribution and are found in Polar Regions to tropical regions. *Aspergillus* species can produce different enzymes and grow on numerous kinds of foodstuffs. If there is a food environment containing some organic matter and a little moisture, it is likely that *Aspergillus* species will be observed on it. Human life is affected by *Aspergillus* species in many ways. [7]. Different methods have been introduced to mitigate aflatoxin in agricultural products. These methods, including cultural, mechanical, physical and biological methods, may aim the toxin-producing fungus or aflatoxin. These methods have advantages and disadvantages depending on the place, time, type of product, application, and efficay [8 - 11]. One of the most effective ways to prevent aflatoxin contamination is to select cultivars resistant to *Aspergillus* and aflatoxin production. By selecting these cultivars in plant breeding programs, aflatoxin contamination is naturally reduced [12]. Unfortunately, there is no effective and practical strategy to control aflatoxin-producing fungi, so sometimes almonds are contaminated with aflatoxins. Aflatoxin contamination can cause product rejection, which causes severe economic losses to producers and related industries. Aflatoxin contamination of almonds can be considered a serious concern. Aflatoxin contamination of almonds may be reduced using pre- and post-harvest strategies. Methods such as agricultural practices, use of insect-resistant cultivars, insect control, mechanized harvesting and sorting, proper storage and, transportation [13- 15]. For the integrated management of aflatoxin control, the use of resistant cultivars can be considered in nuts such as pistachios and almonds [16, 17]. This cultural method is sustainable and compatible with the environment and reduces the amount of fungal contamination and mycotoxin contents. The purpose of this study was to compare common commercial almond cultivars in Iran to identify the most resistant cultivar to *A. flavus* and aflatoxin.

MATERIALS AND METHODS

Sampling

Five cultivars of commercial almond kernels were sampled from Isfahan and Semnan provinces. Almond cultivars, including Sefid (Kaghazi), Aliakbar Rabi and, Mamaee cultivars from the Isfahan province and Shahrood 12, and Shahrood 13 cultivars from the Shahrood County (Semnan province) were collected and stored in the refrigerator (5ºC) for assays.

Colonization of almond kernels by A. flavus

Ten- gram samples of almond kernels with intact testa were used with 3 replications. Kernels were sterilized in 0.5% NaOCl for 1 min and then washed three times with sterile distilled water under a sterile laminar flow cabinet. The sterilized kernels were placed in a sterile Petri dish. The toxigenic *A. flavus* strain originated from the soil of the pistachio orchard of Kerman (ITEM 16499) [19, 20] and was used for kernels' artificial

inoculation. The seven days old culture of *A. flavus* was floated with sterile distilled water containing 5% tween 20 (v/v) and the spore was detached gently from the cultures and removed in the suspension which was adjusted to contain 2×10^6 conidia per ml. Haemocytimeter counting slide used for counting the fungal spore. One ml of spore suspension was spread over the kernels by gently whirling them around within the dish. Incubated Petri dishes were incubated at 98±2% humidity and 28°C temperature for 5, 7 and, 12 days. After the incubation period, the percentage of fungal colonization on the surface of the kernels was recorded [16].

Sporulation of A. flavus on almond kernels

The sporulation rate of the *A. flavus* on kernels was measured 12 days after inoculation. The colonized almonds were mixed with 100 ml of sterile distilled water and shaken at 15 rpm for 24 hours. Number of spores were counted by the hemocytometer counting slide [19].

Aflatoxin B_1 quantification

Aflatoxin B_1 of almond kernel samples was extracted by Best Food (BF) method and aflatoxin B_1 contents were evaluated by thin layer chromatography (TLC) and scanning densitometer (TLC Scanner 3; Camag Scientific Inc., Wilmington, NC). Extraction of aflatoxin in the samples was done as follows. 12 days after inoculation of almond kernels with *A. flavus*, inoculated kernels were dried in an oven at 50°C. 20 g of powdered samples, with 100 ml of methanol: water (55/45 V/V) was shaken for 30 minutes. In the next step, 40 ml of hexane was added and shaken for 15 minutes, then centrifuged at 2000 rpm for 3 minutes. The methanol layer was extracted with chloroform (50 ml) thrice. The chloroform phase was filtered through cheesecloth and anhydrous Na_2SO_4 to remove fungal tissue and dehydrate the extracts respectively. The extracts were evaporated in Bain-marie. The residue was dissolved in chloroform and analyzed for the presence of aflatoxin on silica gel 60 TLC plates. The developing solvent was chloroform: methanol (97:3). The separated aflatoxin was quantified by fluorodensitometric measurement of extracts spots with Rf value and fluorescence similar to the aflatoxin standard. The detection limits of the technique were 2-3 ng g^{-1} of the aflatoxins reference standard. The stock solutions of aflatoxins were prepared in chloroform and stored in darkness at 4°C [21].

Statistical analysis

The experiments were performed in three replications in a completely randomized experimental design. Data analysis was performed using SPSS 17.01 software. Duncan's multiple range tests were used to compare the means and test the significance of the means.

RESULTS

The results showed that there is a significant difference in the fungal colonization of the almond kernels of different cultivars at 5, 7, and 12 days after inoculation ($P\leq0.05$). The Sefid and Mamaei cultivar showed the lowest and highest resistance to the growth of *A. flavus* 12 days after inoculation respectively (Table 1, Figure 1).

Table 1. Comparison of kernel colonization of different almond cultivars with *Aspergillus flavus* on the 5, 7, and 12th days.

Almond cultivar	Kernel colonization (%)		
	Day 5	Day 7	Day 12
Sefid	18.27* a	45.36 a	95.7 a
Shahrood 12	11.56 b	25.41 b	79.53 b
Aliakbar Rabbi	10.43 b	19.26 b	41.83 c
Shahrood 13	9.26 b	18.75 b	36.33 c
Mamaei	8.53 b	14.26 b	20.36 d

*Significant differences are denoted by different letters within each column at $P\leq0.05$ according to Duncan's Multiple ranges Test.

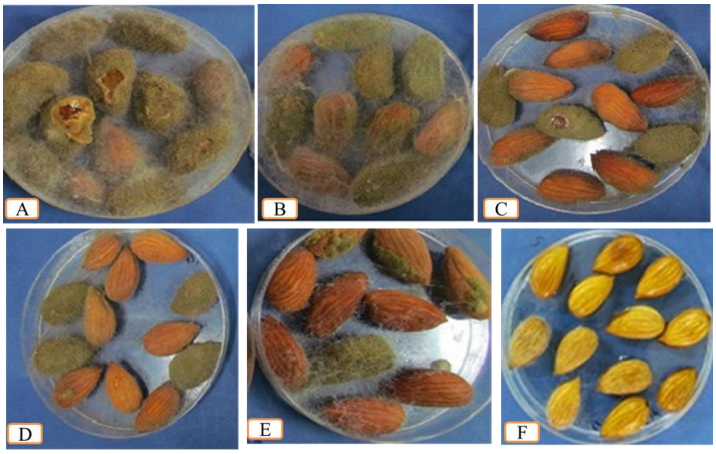

Figure 1. Colonization of different almond cultivars kernels with *Aspergillus flavus* after 12 days of incubation A) Sefid B) Shahrood12, C) Rabbi, D)Shahrood13, E) Mamaee, F) Control

Fungal sporulation on kernels

The results showed that there is a significant difference in the fungal sporulation on kernels of different cultivars at 12 days after inoculation (P≤0.05). The Sefid and Mamaee cultivars had the highest and lowest spore production on pistachio kernels with the production of 3.3×10^8 and 2.65×10^7 spores per milliliter, respectively. The average spore production and fungal growth in Sefid and Shahrood 12 cultivars were higher than other cultivars (Table 2).

Table 2. Comparison of means of *Aspergillus flavus* sporulation on almond kernel on the 12th day

Almond cultivar	Fungal sporulation (spore/ml)
Sefid	3.3×10^8 * a
Shahrood12	2.9×10^8 b
Aliakbar Rabbi	2.5×10^8 b
Shahrood13	3.99×10^7 c
Mamaee	2.65×10^7 d

*Significant differences are denoted by different letters within each column at P≤0.05 according to Duncan's Multiple ranges Test

Aflatoxin content

The results showed that there is a significant difference between aflatoxin B_1 production on kernels of almond cultivars 12 days after inoculation (P≤0.05). Mamaee had the least aflatoxin production among other cultivars whereas Sefid had the greatest rate of aflatoxin B_1 production, respectively (Table 3).

Table 3. Aflatoxin production on kernels of almond cultivars on the 12th day

Almond cultivar	Aflatoxin content (µg kg^{-1})
Sefid	14057 *a
Shahrood 12	11804 b
Aliakbar Rabbi	10198 c
Shahrood13	9628.7 cd
Mamaee	9495.7 d

*Significant differences are denoted by different letters within each column at P≤0.05 according to Duncan's Multiple ranges Test.

DISCUSSION

A. flavus has been introduced as an important pathogen in agriculture and medicine [7]. Since this fungus infects a wide range of food and feed with aflatoxin production and endangers human and livestock health, its role as a plant pathogen is more prominent [22]. In areas with tropical and subtropical climates, this contamination is

more common [23]. Placing sensitive products such as nuts in hot and humid environments can also contribute to their contamination with aflatoxin. In many regions, the stress caused by drought has led to an increase in the incidence of aflatoxigenic fungi [24, 25]. Contamination of almonds with aflatoxin can be one of the important restrictions for consumers and national and international trade. The importance of aflatoxin in almonds has a lot to do with the navel orange worm (*Amyelois transitella*) [15]. Fortunately, this pest has not been reported in Iran so far, so the beginning of aflatoxin contamination in the country's almond orchards is not very important. Nevertheless, having sufficient information about the sensitivity of different cultivars is useful in choosing the right cultivar and establishing new orchards. The use of resistant cultivars has always been one of the most appropriate ways to control many pests and diseases. This study was conducted to find out the resistance level of common Iranian almond cultivars to *A. flavus* as the most important species producing aflatoxin and to introduce the most resistant cultivar.

In this study, the colonization and sporulation of *A. flavus* on the kernel of 5 dominant commercial cultivars of almonds in Iran have been described. In addition to these two parameters, the sensitivity of these cultivars to the production of aflatoxin B_1 was also compared. This is the first study on the sensitivity of almond cultivars to *A. flavus* and aflatoxin production in Iran. The results showed that the amount of colonization, sporulation, and aflatoxin B_1 production is lower in the Mamaee cultivar and higher in the Sefid cultivar than in other cultivars. In other words, Mamaee and Sefid (Kaghazi) cultivars are the most resistant and sensitive cultivars to aflatoxin B_1, respectively. The difference in the sensitivity of almond cultivars can be related to the chemical composition of the fungal growth substrate. Any increase or decrease in the amount of some genetic or chemical compounds may decrease the production of aflatoxin [26]. This was a preliminary study there is a need to complete the information regarding the mechanisms of resistance in different cultivars and the relationship of microelements in almond kernels in this case. The results obtained in other studies have shown that aflatoxin B_1 was positively correlated with moisture, fat, nitrogen-free extract (NFE), zinc, magnesium, and calcium. A negative correlation of B_1 with ash percentage was observed, in which iron, copper, manganese, cadmium, Fe, sodium, and potassium were significant [26].

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Conflict of interests

All the authors declare that there is no conflict of interest in the study.

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