Relative susceptibility of pistachio kernel to Aspergillus flavus among different commercial cultivars and wild species

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Abstract

Background: Aflatoxins are carcinogenic secondary metabolites that are mainly produced by several different species of *Aspergillus* in a variety of foods. The problem of pistachio contamination with *A. flavus* and aflatoxin is a serious health threat that affects the export and trade of pistachios in the world. This study assessed the susceptibility of 12 commercial pistachio cultivars (*Pistacia vera*) and 2 wild pistachio species (*Pistacia atlantica*) to the colonization of *A. flavus* in vitro.

Materials and methods: In the present study, 40 pistachio kernels samples were prepared from each cultivar and inoculated in four replications with spore suspension of *A. flavus* IPRC30 isolate. After one week, the percentage of fungal colonization on the surface of pistachio kernels and its penetration into pistachio kernel tissue were measured. The experiments were performed with four replications in a completely randomized experimental design. The data were analyzed by SAS software and the means were compared using Duncan's new multiple range test at the significance level of 0.05.

Results: The results indicated that the fungal colonization on most cultivars was equally 60-80%. The lowest rate of colonization was related to wild *Pistacia atlantica* species with less than 15%. The percentage of colonization of internal tissue of pistachio kernel was variable. The highest percentage of fungal penetration was found in Ohadi cultivar (70%) and no penetration was observed in Sarakhs cultivar.

Conclusion: The results of this study can have some implications for selecting resistant cultivars in seed breeding programs.

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1. Introduction

Pistachio is a subtropical, dicotyledonous, and deciduous plant of the Anacardiaceae family and the *Pistacia* genus, which was named in 1737 by Carl Linnaeus. There are more than 15 different species in the genus *Pistacia*. Among these species, *P. vera* is known as a commercial species and *P. atlantica* as a wild pistachio species. Only commercial pistachios have economic value and the other species are used as a rootstock or as an ornamental plant [1].

There are more than 80 commercial pistachio cultivars in Iran and Mumtaz, Koleghoochi, and Ohadi cultivars are among the first-grade pistachios, and Qazvini, Ebrahimi, and Vahedi cultivars are of secondary importance [1]. So far, more than 70 pistachio cultivars have been identified in Kerman province, some of which are more important than others, such as Akbari, Ohadi, Koleghoochi, Ahmad Aghaei, Badami Zarand, and Rezaei. Ohadi and Koleghoochi cultivars are the most frequent pistachio cultivars in Rafsanjan and Kerman, so these two cultivars are grown in more than 60-70% of pistachio orchards in this region [2]. This species can be considered as the mother of commercial pistachios in more than 95% of Iranian pistachio orchards. It has also been used as a rootstock. This species has a great genetic diversity in different parts of Iran and is well adapted to all cultivars [2].

Pistachio is one of the most important horticultural products in Iran, and Kerman province has the highest area under cultivation and production [3]. Pistachio accounts for a major part of Iran's non-oil exports, generating significant foreign exchange revenues [4]. However, contamination of pistachios with *Aspergillus* fungi and mycotoxins produced from them has made pistachio exports difficult [5]. The most important factor underlying the production of aflatoxins are strains belonging to several species of *Aspergillus* fungi, especially *A. flavus*. The genus *Aspergillus* has more than 200 species, with a wide range of distribution from the Polar Regions to the tropical warm regions. Species of the genus *Aspergillus* can secrete several enzymes and, therefore, can grow on numerous types of food. In fact, it is difficult to find a food environment that contains some organic matter and a small amount of moisture where *Aspergillus* species cannot grow on, and thus the Aspergillus species can affect humans in different ways [6].

Twenty percent of the world's food products are contaminated with mycotoxins annually, with the latter accounting for a higher percentage of contaminations. Losses from food and crops contaminated by this toxin in the United States is estimated at more than $100 million per year [7] followed by Africa with more than $750 million paying for the annual cost of aflatoxin contamination [8]. Different techniques such as agronomic, mechanical, physical, and biological solutions have been recommended for managing the contamination of different products with *Aspergillus* or aflatoxin, each of which has its advantages and disadvantages depending on the place, time, type of product, applicability, and efficiency [9, 10, 11, 12, 13]. In the recent years, a major problem faced by the country in the field of pistachio export is the issue of pistachio contamination with *A. flavus* and subsequently aflatoxin contamination. Pistachio contamination with aflatoxins can threaten this source of foreign exchange earnings and prevent us from competing in the global market [10].

Ghewande *et al.* [14].investigated the resistance of peanut cultivars to *A. flavus* growth and aflatoxin production and reported that there were significant differences between different

peanut cultivars in terms of fungal growth, colonization, and aflatoxin production. Gradziel and Wang [15] examined the susceptibility of California almond cultivars to *A. flavus* aflatoxicosis and showed that the susceptibility of different cultivars to this fungus varies significantly. They also investigated the effect of almond kernel coating on preventing and reducing the penetration of fungi into the almond kernel and highlighted the role of this coating as a barrier resistant to fungal intrusion.

Delayed harvest time, inadequate crop processing, and poor storage conditions can intensify fungal growth and aflatoxin production. The production of aflatoxins by different species of Aspergillus is extensively found in nature. Often, due to the continuation of environmental humidity conditions, environmental stresses such as drought or removal of barriers to the entry of the fungus, the hosts of this fungus become susceptible to contamination [2].

Given the multiplicity and diversity of Iranian commercial pistachio cultivars, as a unique product in the world, it was necessary to conduct a fundamental study on their resistance to aflatoxin-producing fungi. The present study aimed to assess the sensitivity of important pistachio cultivars to aflatoxigenic *A. flavus* and compare them with wild pistachio species.

2. Materials and methods

Sample selection and collection

The most important and commercial pistachio cultivars that have a high area under cultivation in Iran were selected for study. To prevent damage to the upper shell of the pistachio kernel (Testa) and also to minimize the possibility of contamination of pistachios with *Aspergillus* and aflatoxin due to its growth, sampling was performed on healthy un-cracked pistachios on trees at the time of pistachio harvest (September). After harvesting fresh pistachios, infected pistachios and those that were likely to be contaminated were removed. Then, the soft skin of the pistachio was separated from the bony skin (shell) by hand so that no damage was made to the pistachio kernel. The pistachios were then dried in the sunlight for three days and their humidity was increased to 6%. They were then kept in the refrigerator at 4° C until the experiments were performed and used for *in vitro* assay. Samples of common cultivars used in the study including Badami Zarand, Sarakhs, Ahmad Aghaei, Ohadi, Koleghoochi, Jandaghi, Fandoghi Zoodras, Badami Ravar, Badami Nishkalaghi, Italian, Fandoghi Riz, Fandoghi 48 and wild pistachio species included Bane (*Pistacia atlantica* subsp. *mutica*) and Atlantica (*Pistacia atlantica* subsp. *atlantica*) was collected from Rafsanjan.

Isolation of native *A. flavus* toxigenic strain from Rafsanjan pistachio growing areas

To analyze the resistance of different pistachio cultivars to the growth of *Aspergillus*, a native *A. flavus* toxigenic strain was isolated from the Rafsanjan region, as the most important pistachio cultivation area in the country. To isolate the fungi, 100 pistachio fruits were added to flasks containing 450 ml of sterile distilled water in 3 replications and placed in a shaker (Certomat, SII, UK) at 30 rpm for 30 minutes. The suspension was diluted. The dilutions of 10^{-1} and 10^{-2} were spread on Petri dishes containing Aspergillus Flavus and Parasiticus Agar (AFPA) in four replications. Each replicates containing 100 µl spore suspension and the Petri dishes were incubated at 28 °C in the dark for 3 days [16]. AFPA medium contained 10 g of peptone, 20 g of yeast extract, 0.5 g of ammonium ferric citrate, 200 mg of pentachloro nitrobenzene with

15 g of agar per liter of sterile distilled water. The pH of this medium was adjusted to 6.5 after mixing and the volume was increased up to one liter. Then, 50 mg of chloramphenicol was autoclave (Irantolid, Iran) at 1 atm and 121 °C for 15 minutes [17].

Morphological characteristics such as shape, color, and size of conidia, conidiophores, vesicles, and basal cells as well as the characteristics of strigmata, and growth rates in different culture media and temperatures were used for species identification [18]. To evaluate the toxinogenicity of the strains, and to select a toxinogenic strain, the initial screening of the strains was performed using ammonia vapor method and thin layer chromatography (TLC) used for results confirmation (Camag TLC Set, Switzerland) [19].

Resistance evaluation of pistachio kernel cultivars to *A. flavus* in vitro

Of 12 commercial pistachio cultivars and two wild pistachio species 40 intact pistachio kernels (without shells) were selected. Before performing this experiment, to ensure that pistachio kernels of different cultivars were not infected with *A. flavus*, four pistachio kernels from each variety were examined in four groups of 10. To absorb the initial moisture, the kernels were immersed in sterile distilled water for 10 minutes. After the kernels were removed from sterile distilled water, the pistachio kernel samples were first disinfected with 0.5% sodium hypochlorite solution and then thoroughly washed with sterile distilled water and placed in 70% ethanol for 60 seconds [20]. Afterward, to provide sufficient (saturated) moisture, the Petri dishes containing wet pistachio kernels were placed in plastic containers with some sterile distilled water contained in the bottom. The studied treatments (containing 40 pistachio kernels) were inoculated with one ml of *A. flavus* spore suspension (1×10^6 spores per ml). This propagule was obtained by culturing the fungus on malt extract agar (MEA) medium inside a tube for 10-8 days. For each pistachio cultivar, three replications and in the control treatment, only sterile distilled water was used instead of spore suspension. The Petri dishes were then placed in plastic containers and incubated (Sanyo MIR 152, Japan) at 28 °C. After fungal growth and colonization of pistachio surfaces by fungi, the rate of fungal colonization in pistachio surfaces was calculated on the seventh day [21, 22].

Assessing the percentage of fungal colonization

The pistachio kernel samples were examined with a stereomicroscope (Olympus SZX7, Japan) after seven days and the pistachio kernel colonization percentage was calculated based on the following indexes (kernel surface) in the four experiments. Index 1: 0-15%, index 2: 16-30%, index 3: 50-31%, and index 4: 51-51% [20]. The percentages were calculated visually for 40 pistachio kernels.

Investigation of fungal penetration in pistachio kernels

In the next step, the pistachio kernels were examined for the fungal penetration or non-penetration of the fungus into the tissue. For this purpose, pistachio kernels were cut with a scalpel and examined under a stereomicroscope. The fungal penetration into the tissue was scored 1 and non-penetration was scored 0. Therefore, the results were presented qualitatively and no statistical analysis was performed.

Statistical analysis

In this study, the experiments were performed in four replications in a completely randomized experimental design. The obtained data were

statistically analyzed by SAS software and the means of fungal growth and pistachio kernel colonization of different cultivars were compared by Duncan's multiple range test at the 5% probability level.

3. Results

Resistance of pistachio cultivars to *A. flavus*

Fungal growth and colonization of pistachio kernels was statistically different (P≤0.05) in the studied cultivars (Table 1). The relative sensitivity of the cultivars to *A. flavus* colonization was significantly different and varied from 60 to 80%. Among the cultivars, Ohadi, Jandaghi, Italian, and Fandoghi cultivars showed the highest sensitivity and *Pistacia atlantica* species showed the least sensitivity to the growth of *A. flavus*. The average percentage of colonization of *A. flavus* on the kernels of wild species and commercial pistachio cultivars varied between 13-75% (Figure 1).

Table 1: ANOVA results for *Aspergillus flavus* colonization of different pistachio cultivars

Index	Source of changes	Sum of squares	df	Mean squares
Colonization (%)	Cultivar	15610.121	13	1200.779*
	Error	12514.125	42	297.955
	Total	28124.246	55	

*: Significant at p≤0.05

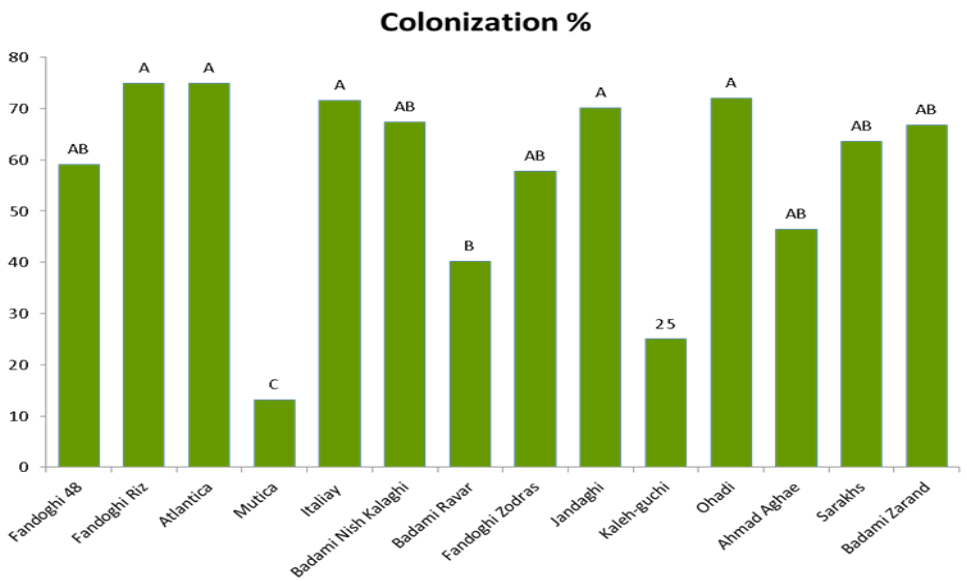

Figure 1: Colonization percentage of pistachio kernels by Aspergillus flavus in different cultivars

A. flavus penetration on the pistachio kernel tissue

The percentage of *A. flavus* penetration into pistachio kernel tissue was significantly different among cultivars. The highest and lowest percentages of *A. flavus* penetration in pistachio kernels found in Ohadi and Sarakhs cultivars were 70 and 0%, respectively (Figure 2).

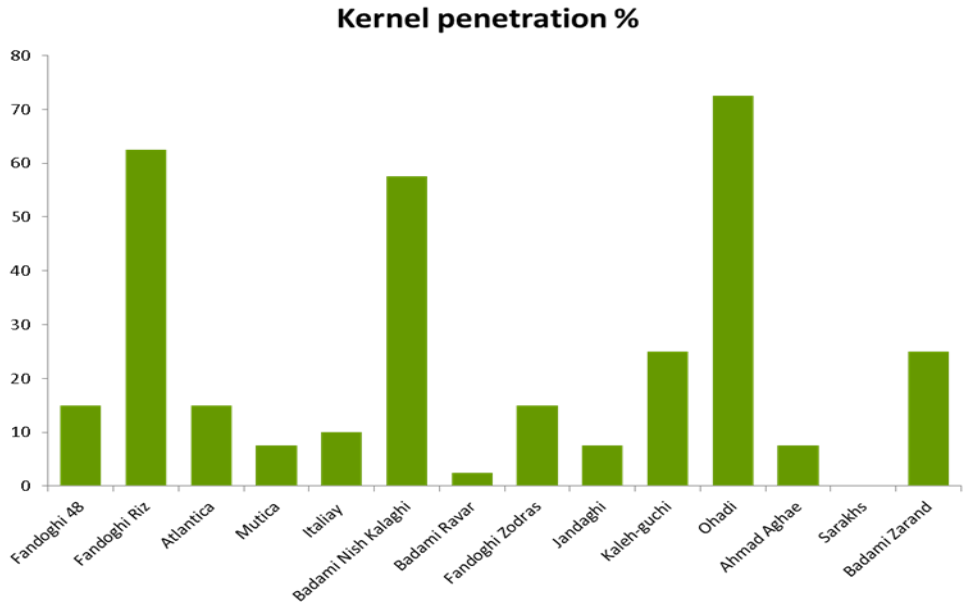

Figure 2: *Aspergillus flavus* internal tissue contamination of pistachio kernels of different cultivars

4. Discussion

Since the discovery of aflatoxins, *A. flavus* has always been one of the most important food contaminating fungi. One of the most effective strategies that can overcome the aflatoxin contamination in different crops is using resistant cultivars. Extensive studies are being carried out in most parts of the world to identify cultivars resistant to different species of *A. flavus* [23, 24]. Ghewande *et al*. [25] pointed out that due to the genetic diversity of peanut cultivars, the most important and fundamental factor in reducing aflatoxin contamination of the crop was host resistance. The results indicated the differences in the resistance of various cultivars to the growth of this fungus. They also assessed the production of aflatoxin B1 in different cultivars and found that the most resistant cultivar and the most sensitive cultivar have 3900 ppb and 900000 ppb of aflatoxin, respectively. They also found that there is a relative correlation and a significant relationship between fungal growth and aflatoxin production. In other words, with increasing fungal growth in susceptible cultivars, toxin production also increases.

Gradziel and Wang [26] examined the susceptibility of California almond cultivars to the growth of *A. flavus* and found that the susceptibility of the cultivars to this fungus was significantly different. They also examined the effect of the almond kernel coating on reducing the penetration of the fungus into the almond kernel by injuring the coating. The results showed that almond kernel coating can act as a barrier against the penetration of fungi into the almond kernel. They also found that the kernel coating of all the studied almond varieties was highly resistant to A. flavus cloning. Cotyledon resistance was observed in some cultivars such as Ne Plus, Ruby, and Carrion.

Research has shown that damage to the testa increases the rapid and direct contamination of *A. flavus* on peanut seeds, which in turn increases the aflatoxin formation. Damage to the testa also contributes to the availability of nutrients necessary for the rapid growth of *A. flavus* [27].

Mahoney and Rodrigues [28] showed that pistachios with healthy cuticles are resistant to *A. flavus* colonization. However, it should be noted that damage to cuticles leads to rapid colonization of *A. flavus* in the green skin of the fruit. Mahoney and Rodrigues [28] could not detect any aflatoxin accumulation in the green skins of fruit despite the rapid colonization of A. flavus. In healthy pistachio seeds with coating, aflatoxin colonization and production were low. The cuticle layer may play a role in seed resistance to colonization [28]. Mohammadi Moghadam [28] evaluated the resistance of pistachio cultivars to *A. flavus* and aflatoxin production. The results showed that the colonization of fungus on pistachio kernels of cultivars was significantly different. Among the studied cultivars, Ahmad Aghaei and Ohadi cultivars showed the lowest resistance, and Akbari and Koleghoochi cultivars showed the highest resistance to the growth of *A. flavus*. Among the cultivars, Shahpasand and Abbas Ali cultivars had the highest aflatoxin B1 production and the lowest aflatoxin B1 cultivars were reported in Kal Khandan and Fakhri cultivars.

The present study also evaluated the resistance of commercial and wild pistachio cultivars to *A. flavus*. The experimental results showed a significant difference in the fungus growth rate on pistachio kernels between commercial and wild pistachio cultivars. Due to the importance of contamination of different pistachio cultivars with aflatoxigenic strains, further studies need to examine the response of important commercial cultivars to fungal growth and aflatoxin production and to determine the mechanism of resistance of some pistachio cultivars to growth-producing fungi. Considering the differences in resistance of different pistachio cultivars to *A. flavus* growth and aflatoxin production, it is recommended to use cultivars that have more resistance to fungi in breeding programs and construction of new orchards in different areas.

5. Conclusion

The data in this study indicated different levels of resistance to *A. flavus* fungal colonization that can be used in breeding programs.

Conflict of Interest

The authors of present researches declare that there is no conflict of interest.

Code of Ethics

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

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