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A Survey on Contamination of Iranian Pistachio Cultivars to *Aspergillus* Section *Flavi* and Aflatoxin

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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

Population density of *Aspergillus* section *Flavi* in pistachio kernels of Iranian cultivars was studied in main pistachio growing areas of Iran during 2015 to 2017. In order to investigate the rate of contamination of pistachio cultivars to *Aspergillus* section *Flavi* and aflatoxins, 13 pistachio cultivars, including Akbari, Kaleh-ghouchi, Ovhadi, Ahmad-aghaee, Momtaz, Italian pistachio, Shahpasand, Pesteh-e-ghermes, Pesteh-e-garmeh, Ghazvini, Abbasali and Khanjari were selected from different parts of Semnan, Khorosan Razavi and Kerman provinces. A total of 125 samples of pistachio kernels were collected from different cultivars of pistachio trees in orchards. The samples were cultured on AFPA using serial dilution method. After 3 to 7 days, the plates were examined and isolates of *Aspergillus* section *Flavi* were identified and relative densities of them were recorded. The values ranged from 1.6×10^3 to 1.6×10^4 CFU/g in pistachio samples.The statistical analysis showed that the population density of *Aspergillus* section *Flavi* was varied with the type of cultivars. The results indicated that among tested cultivars, Akbari showed the least and Shahpasand the most population density of *Aspergillus* section *Flavi* among pistachio cultivars, respectively. The aflatoxins content of pistachio samples were extracted and analysed through Thin Layer Chromatography (TLC) and flurodensitometer. There were Significant differences among different cultivars in the contamination of pistachio kernels to *Aspergillus* section *Flavi* and aflatoxin. (α =5%). It was observed that Akbari had the least whereas Shahpasand had the greatest amount of aflatoxin production, respectively.

Introduction

Pistachio, belonging to the family *Anacardiaceae*, is a broad-leaved, dioecious and wind-pollinated species. Iran is one of the most important pistachio genetic diversity centers, which include a large number of wild varieties as important components of Iranian forests (Sharifkhah *et al*., 2020). Pistachio is an economically important product since it is exported to other countries (Alipour, 2018).

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Pistacia vera is the only cultivar of this species (Zohary, 1952). The history of planting pistachios is very old and has most probably been started from the areas adjacent to the wild masses in low-altitude and slopes of south and central Asian semi-desert regions. Distribution range of pistachios is from northeastern Iran and northern Afghanistan to Tien-shen and Karatan mountains through Turkmenistan, Uzbekistan, Tajikistan, Kazakhstan and Kyrgyzstan (Kayimov *et al*., 2001). It is believed that the cultivation of pistachios has firstly become widespread in the empires of Iran, and from there expanded to the west, so that, the name of the pistachio is derived from the ancient Persian language (Hormoza and Wunsch, 2007). Incidentally, Iran has the greatest *Pistacia vera* genotypic diversity in species in the world (Sheibani, 1995). Esmail-pour *et al*. (2005) have recognized, evaluated and collected many cultivars, genotypes and varieties of pistachio orchards in Iran. About 200 new genotypes and varieties have been recognized. The native cultivars (Badami-sefid, Pesteh-gharmez and Pesteh-garmeh) and commercial cultivars (Akbari, Kalleh-ghouchi and Ovhadi), are the most important cultivars in Khorasan province. Whereas in Semnan province the Shahpasand, Abbasali and Khanjari (native cultivars) and Akbari, Ahmad-aghayee and Ovhadi (commercial cultivars) are cultivated mainly. Ovhadi, Akbari, Kalleh-ghouchi, and Ahmad-aghayee cultivars, have the greatest pistachio cultivation area in Kerman province (Sherafati, 2005).

Iran is the most important pistachio producing and exporting country throughout the world. Recently, the main and the most important problem encountered with the pistachio exportation of the country is the contamination of pistachio kernels with aflatoxins that continuously threatens Iranian pistachio exportations. In several cases during past 46 years, Iranian pistachio has been re-submitted back to the country or sold for very low prices.

Aflatoxins are a group of secondary metabolites mainly produced by the fungi such as *A. flavus*, *A. nomius*, and *A. parasiticus*. These compounds are highly toxic and carcinogenic, and regarded as mutagenic agents in the world (Abbas *et al*., 2005), therefore, consumption of food stuff contaminated with aflatoxins could lead to highly deleterious effects on human and livestock health and safety (Cole *et al*., 1982), and today it is considered as an economic problem and a serious threat for human health (Klich *et al*., 1995; Abbas *et al*., 2005).

Since the discovery of aflatoxin (1960s), the *A. flavus* and *A. parasiticus* fungi have been known as the most common fungi which contaminate food products, and their names are frequently mentioned in scientific sources which indicates their economic significance (Carlile *et al*., 2001; frisvad and Samson, 2004). These fungi are spread throughout the world and is considered as a permanent microflora of air and soil, being found in connection with all sorts of live or dead animals and plants throughout the world (Abbas *et al*., 2005). These fungi have a special tendency to contaminate nuts, oil seeds, and grains. Peanuts, corn, wheat, rice, pistachio, and almond are the most important products which are invaded by such fungi. As a matter of fact, pistachio is one of the most important source of income for country, in such a way that, in recent years it has ranked second after oil. Therefore, there is increasing demand for high quality product (Eslami *et al*., 2019). Nevertheless, pistachio export is facing the issue of aflatoxin contamination which is produced by isolates of *A. flavus*. Therefore, the issue should be seriously studied. In this study, we compare the contamination of pistachio cultivars to *Aspergillus* section *Flavi* and aflatoxin and recommend more resistant cultivars, in addition we report isolating and identifying *Aspergillus* section *Flavi* in contaminated pistachios in pistachio orchards of Iran.

Materials and Methods

In order to evaluate the rate of contamination of pistachio cultivars to *Aspergillus* section *Flavi* and aflatoxin, pistachio kernels of 13 cultivars were collected from different parts of pistachio belt ie Semnan, Khorosan Razavi and Kerman provinces of Iran.

Pistachio cultivars

13 pistachio cultivars were used in these studies including: Momtaz, Abbasali, Ghazvini, Khanjari, Shahpasand, Ahmad-aghayee, Kalleh-ghouchi, Akbari, Pesteh-e-ghermes, Pesteh-e-garmeh,and Ovhadi. Akbari, Kalleh-ghouchi, Momtaz, Italian pistachio, Ghazvini, Ovhadi and Ahmad-aghayee cultivars were collected from Rafsanjan area (Kerman province); while Shahpasand, Abbasali, Akbari, Ahmad-aghayee, Kalleh-ghouchi, Ovhadi and Khanjari cultivars were collected from Damghan (Semnan province), Seven cultivars, Pesteh-e-ghermes, Pesteh-e-garmeh, Shahpasand, Akbari, Kalleh-ghouchi, Ovhadi and Khanjari were collected from Feizabad (Khorosan Razavi province), Iran.

Culture media

Two culture media, AFPA (Aspergillus Flavus Parasiticus Agar) and MEA (Malt Extract Agar) were used in this research. AFPA as a selective medium applied for enumeration *Aspergillus* section *Flavi* (Ghaemmaghami *et al*., 2016). AFPA medium is contained peptone, 10 g; yeast extract, 20 g; ferric ammonium citrate, 0.5 g; chloramphenicol,100 mg; agar, 15 g; distilled water, 11; and dichloran, 2 mg. The final pH of the medium is ca. 6.2. Cultures on AFPA are incubated at 30°C for 42 to 48 h. Dichloran inhibits spreading of fungi, while chloramphenicol inhibits bacteria. *A. flavus* and *A. parasiticus* are identified on this medium by production of typical yellow to olive green spores and a bright orange reverse. Another advantage of this medium is that *A. flavus* and *A. parasiticus* grow rapidly because the medium is incubated at 30°C, permitting identification within 3 days in most cases. This medium was recommended for use in enumerating *A. flavus* species in nuts, com, spices and soil (Pitt and Hocking, 1985). Malt Extract Agar (MEA; malt extract 20g, peptone 1g, glucose 20g, agar 20g, distilled water to 1L). Culture media were autoclaved at 121°C for 15 minutes.

In order to survey the rate of contamination of pistachio kernels to *Aspergillus* section *Flavi* and determining the aflatoxin content of samples, a total of 125 pistachio samples belonging to native and foreign cultivars (*Pistacia vera*) were collected during 2015 to 2017 from different orchards in main pistachio growing areas of Iran (Semnan, Khorosan Razavi and Kerman provinces). The sampling was done in the time of ripening stage of pistachio in orchards. They were examined for the occurrence of *Aspergillus* section *Flavi* species. Likewise, the aflatoxins content of samples was determined. In order to prepare each sample, we took 10 subsamples of 200 grams from various places of the orchard. The 10 subsamples were totally mixed and then 500 grams of it was taken as the main sample, thus 125 main samples were prepared from all provinces.

Isolation of fungi

Ten grams of each sample were added to 90 ml of peptone water 0.1% (wt/v) and this sample was then diluted to final concentrations of 10^{-2} and 10^{-3}. From each dilution, 0.1 ml of inoculum was spread (completely randomized design with 5 replications) on the surface of AFPA as a selective medium. AFPA was used for the differential detection of the *Aspergillus* section *Flavi* by production of typically yellow to olive green spore and a bright orange reverse coloration

(Magnoli *et al*., 1998). All samples were incubated for 3 to 7 days at 25°C. Then the plates were examined and isolates of *Aspergillus* section *Flavi* were identified and relative densities of them were recorded. Taxonomic identification of the fungi was made according to microscopic criteria in accordance with appropriate keys (Klich, 2002). The mean value counts ranged from 1.6×10^3 to 1.6×10^4 CFU/g for the *Aspergillus* section *Flavi* in pistachio samples. After isolation, identification and enumeration of *Aspergillus* section *Flavi*, the aflatoxins contents of samples were measured.

Extraction of Aflatoxins

In order to Extract and calculate the aflatoxin content of different pistachio samples of different cultivars, the prevalent method of BF was followed. After desiccation of pistachio kernels in oven, the dried kernels were completely grinded. To extract aflatoxins, the resulted pistachio meal was completely mixed with 55% methanol. To remove and omit the lipids and pigments from this mixture, n-hexane was added. Then, to separate methanol-water phase (aflatoxin containing phase) from hexane phase (lipid containing phase) and pistachio meal particles, the mixture was centrifuged at 2000 rpm for 5 minutes. To optimize the extraction of aflatoxins, chloroform was added and completely mixed with methanol-water phase after its separation. Aflatoxins left methanol-water phase and were transferred into chloroform phase. After the complete removal of chloroform through its evaporation in the water bath (60-70°C), the obtained mixture of aflatoxins was dissolved in the given volume of chloroform. The resulted solution was stored at 4°C under dark conditions for next steps of aflatoxins detection and quantification.

Detection and Quantification of aflatoxins in infected pistachio kernels

After extraction of aflatoxins from the infected kernels of pistachio, Thin Layer Chromatography (TLC) and flurodensitometer were applied. An aliquot of each of the stored aflatoxin solution was separately blotted on TLC plates and the blotted plates were placed inside a tanker of developing solution of chloroform: methanol (97: 3). The comparison of unknown spots was performed with that of the standard aflatoxins under ultra violet light beams, and the spots related to Aflatoxins were detected. The plates were placed inside the densitometer and the information related to the position of the spots were transferred into the instrument, and the ratio of the amount of aflatoxin of each sample to that of aflatoxins standard spot was calculated.

Results

Mycological studies of the survey on 125 pistachio samples indicated the presence of *Aspergillus* section *Flavi* in pistachio kernels of different cultivars. The *Aspergillus* species has been recorded as the most prevalent one in pistachio kernel samples in previous research. Different species of *Aspergillus* have been reported in the pistachios of Iran (Mojtahedi *et al*, 1979), Turkey (Denizel *et al*., 1979), and USA (Thomson and Mehdi, 1979). Mojtahedi and Danesh (1979) successfully isolated 13 species of *Aspergillus* from the pistachio orchards of Iran. Doster and Michailides (1994) isolated 14 species of *Aspergillus* from the pistachio orchards of California. As the fungi population density of different climatic conditions of the studied provinces may vary, the results of the rate of contamination of the samples were statistically analysed separately for each province.

In Table 1, the results of *Aspergillus* section *Flavi* colony counting in different pistachio cultivars are shown. As shown in Table 1, the rate of contamination

of pistachio kennels of different cultivars to *Aspergillus* section *Flavi* is different. In some samples had less contamination to *Aspergillus* section *Flavi*, while in other samples, there was serious contamination to this fungus.

The statistical findings (Duncan's multiple range test) showed that the average difference of colony number of *Aspergillus* section *Flavi* in different pistachio cultivars of different provinces was meaningful at statistical level of %5. The statistical analysis of the results of the pistachio samples of Semnan province indicated that the lowest population density of *Aspergillus* section *Flavi* belong to Akbari cultivar and the highest density was observed in Shahpasand cultivar.

Table 1. comparison of total mould counts of *Aspergillus* section *Flavi* in different pistachio cultivars of Semnan province (compared based on Duncan's multiple range test)

Cultivar	Colony number of *Aspergillus* section *Flavi* (CFU/G)			Combined analysis
	2015	2016	2017	
Shahpasand	16380 a	16080 ab	15460 b	15970 a
Abbasali	5440 c	5140 cd	5580 c	5387 b
Ahmad-aghayee	5420 c	5040 cde	5300 cd	5253 bc
Kalleh-ghouchi	4460 def	4380 def	4500 def	4447 cd
Ovhadi	4160 ef	3940 f	4000 f	4033 d
Khanjari	3740 f	3900 f	2880 f	3840 d
Akbari	1700 g	1600 g	1800 g	1700 e

Similar letters after average percentages on each column indicate no significant difference at a 5% level (Duncan's multiple range test method).

The aflatoxin content of the kernels of different pistachio cultivars was determined for Semnan province, and statistically significant differences were found among various pistachio cultivars (α = 5%). The most abundant contamination to aflatoxin was observed in Shahpasand and the least contamination was determined in Akbari cultivar (Table 2). It must be mentioned that the contamination of pistachio samples to aflatoxins were less than permissive level in all cases.

Table 2. Aflatoxins contents of the kernels of the studied pistachio cultivars of Semnan province compared based on Duncan's multiple range test (µg/ kg)

Cultivar	Frequency of Aflatoxins production	Duncan's Group
Shahpasand	0.8	a
Abbasali	0.6	ab
Ovhadi	0.6	ab
Ahmad-aghayee	0.2	ab
	0.4	ab
Kalleh-ghouchi	0.2	ab
Khanjari	0	b
Akbari		

Similar letters after average percentages on each column indicate no significant difference at a 5% level (Duncan's multiple range test method).

the *Aspergillus* section *Flavi* contamination of the pistachio kernels of various cultivars of Khorosan Razavi provinces showed significant differences (α=5%). Pesteh-garmeh cultivar had the least and shahpasand had the most contamination to *Aspergillus* section *Flavi*, respectively (Table 3).

Table 3. comparison of total mould counts of *Aspergillus* section *Flavi* in different pistachio cultivars of Khorosan Razavi provinces (compared based on Duncan's multiple range test).

Cultivar	Colony number of *Aspergillus* section *Flavi*(CFU/G)			Combined analysis
	2015	2016	2017	
Shahpasand	93 14560 ab	14960 a	14100 b	14540 a
Ovhadi	8760 c	8540 c	9040 c	8780 b
Pesteh-ghermes	4520 de	4160 efg	4700 d	4460 c
Akbari	4100 efgh	4400 def	4540 de	4347 c
Kalleh-ghouchi	3980 fghi	3740 ghij	3540 ij	3753 d
Khanjari	3480 ij	3220 j	3600 hij	3433 d
Pesteh-garmeh	1770 k	1840 k	1900 k	1837 e

Similar letters after average percentages on each column indicate no significant difference at a 5% level (Duncan's multiple range test method).

The statistical analysis of the results of the pistachio samples of Kerman province indicated that the lowest population density of *Aspergillus* section *Flavi* belonged to Akbari cultivar and the highest density was observed in Ahmad-aghayee cultivar (Table 4).

Table 4. comparison of total mould counts of *Aspergillus* section *Flavi* in different pistachio cultivars of Kerman province (compared based on Duncan's multiple range test)

Cultivar	Colony number of *Aspergillus* section *Flavi* (CFU/G)			Combined analysis
	2015	2016	2017	
Ahmad-aghayee	11140 a	11640 a	11500 a	11430 a
Ovhadi	8600 bc	8160 c	8980 b	8580 b
Momtaz	6260 d	5940 de	6160 de	6120 c
Kalleh-ghouchi	5740 def	5580 efg	5880 de	5733 cd
Ghazvini	5240 fgh	5000 ghi	5580 efg	5273 de
Italian pistachio	4720 hi	4440 i	4960 ghi	4707 e
Akbari	2780 j	2640 j	2860 j	2760 f

Similar letters after average percentages on each column indicate no significant difference at a 5% level (Duncan's multiple range test method).

Discussion

Contamination of agricultural products with mycotoxins is one of the most critical problems of the world health community. In view of the serious threat posed by mycotoxins, countries have made different rules and regulations for production, processing, and import of food and feed materials. Moreover, different packaging systems have been studied and applied for reducing secondary contamination of fruits during postharvest period (Hadadinejad *et al*., 2018; Mahbobinejhad *et al.*, 2019). Most countries are extraordinary sensitive to contamination of food products to *Aspergillus* section *Flavi* and the aflatoxin produced by them (Williams *et al*., 2004 ؛ Van Egmond *et al*., 2007).

In recent years the most important problem in the export of pistachio has been the issue of *Aspergillus* section *Flavi* and aflatoxin contamination which has served as a major threat to the export of this valuable product (Moradi and Hokmabadi, 2011). Hence, considering the economic value of pistachio and this fact that all aspects of pistachio contamination to *Aspergillus* section *Flavi* should be seriously evaluated, we will proceed to study and compare contamination of Iranian pistachio cultivars to *Aspergillus* section *Flavi*

and aflatoxin. Several factors that may affect the infection of nuts to the *Aspergillus* species and aflatoxin production include cracking the outer layer of nuts, environmental factors, cultural practices, insect damage, frequency and time of irrigation, plant litter, animal manures, frequency of toxigenic strains, type of cultivar and harvesting date (Moradi and Hokmabadi, 2011; Moghaddam *et al*., 2006). In artificial inoculations with *A. flavus*, the susceptibility cultivars differed in kernel colonization and aflatoxin concentrations. The highest kernel colonization belonged to the Ahmad-aghaee and Ovhadi cultivars, while the lowest ones belong to the Akbari and Kalleh-ghuchi cultivars (Moghaddam *et al*., 2006).

Tabata *etal*.(1993) studied the contamination of various products with aflatoxin and reported that the highest rate of contamination in pistachio was 1382 ppb with aflatoxin B1. Amin-shahidi (1996) has investigated aflatoxigenic *A. flavus* isolates in contaminated pistachios in Iran and studied about the capacity to produce aflatoxin by isolates and observed that most of the samples were contaminated with *A. flavus* and *A. parasiticus*. Magnoli *et al*. (1998) studied on the aflatoxin producing property of different isolates after identification and isolation of *A. flavus* from foods and observed that the mean value counts ranged from 1×10^3 to 9.5×10^4 CFU/g for the *Aspergillus* spp.

The results of this research indicated that a moderate frequency of *Aspergillus* section *Flavi* species was found. The average colony number of the *Aspergillus* section *Flavi* in pistachio samples of different cultivars ranged from 1.6×10^3 to 1.6×10^4 CFU/g.

Today, there is no method as magic bullet for solving the infection problem of crops like corn, cotton and peanut to *A. flavus* and we have to use several strategies simultaneously to ensure that a product is free of toxins (Cary *et al*., 2011). Different strategies of chemical, physical and biological control, before and after the harvest of crops were proposed to reduce toxin producing fungi. One of the most promising methods of pre-harvest control in the field that investigated throughout the world is applying non-toxigenic isolates of *A. flavus*. (Doner, 2004; Yin *et al*., 2008). Another effective method is cultivation of the varieties which have less susceptibility to fungal growth and toxin production, (Ghewande *et al*., 2000; Moghaddam *et al*., 2006).

The most economic and efficient strategy of aflatoxin infection reduction or removal is plant breeding and production of resistant cultivars to *A. flavus* growth and aflatoxin production. Much efforts were performed in plant breeding programs for diagnosis of resistant cultivars or even tolerance to pre-harvest *A. flavus* growth. Unfortunately, no cultivar was found with high resistance to the growth of aflatoxin-producing fungi in major crops such as corn, cotton and peanut; but cultivars with low or medium resistance in corn is under experiment and release. Some progresses have been made in identifying the genes resistant to aflatoxin-producing fungi in corn (Chen *et al*., 2010).

During this research, the rate of contamination of different pistachio cultivars with *A. flavus* was also evaluated and the amount of aflatoxin production in these cultivars was investigated. The results of this study also showed that there was a significant difference between the different pistachio cultivars for infection with *A. flavus*, so that the lowest infection rate was in Akbari cultivar and the highest infection rate was observed in Shahpasand cultivar. Therefore, due to the fact that the contamination rate of different pistachio cultivars to *A. flavus* varies, it is recommended that in breeding programs and construction of new orchards in different pistachio areas of the country, cultivars with less contamination to *A. flavus* and Aflatoxin production be used.

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