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ORIGINAL ARTICLE

Relationship between *Aspergillus* section *Flavi* Growth and Aflatoxin Production with Early Splitting in Pistachio Cultivars

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KEYWORDS

Aspergillus flavus;
Aflatoxin;
Pistachio;
Cultivar;
Early splitting

ABSTRACT: In this research, we evaluated the relationship of early splitting with the growth of *Aspergillus* section *Flavi* and aflatoxin production in Iranian pistachio cultivars. From each cultivar, six trees and from each tree, six fruit clusters were randomly harvested at maturity time and their fruits mixed. Finally, healthy fruits, and early-splitted fruits with irregular cracks of each cultivar were separated. The population density of *Aspergillus* section *Flavi* fungi in the nut kernel was measured using AFPA and DRBC culture media suspension preparation and serial dilution methods. The amount of aflatoxin in different pistachio samples was measured by high-performance liquid chromatography (HPLC). The results showed a significant difference in *Aspergillus* section *Flavi* contamination among different pistachio cultivars. The population density of *Aspergillus* section *Flavi* colonies in pistachio cultivars of Shahpasand (74.6667 CFU g^{-1}) and Kal-Khandan (73.6667 CFU g^{-1}) was much higher than other cultivars. The percentage of early-splitting was higher in the Shahpasand cultivar (13.66%) than in other cultivars, followed by the Kal-Khandan cultivar (5.66%). There was a positive and significant relationship between the growth rate of *Aspergillus* section *Flavi* and the fruit's early splitting percentage (r = 81%). In other words, with an increase in the early splitting percentage of pistachio fruit, the growth rate of *Aspergillus* section *Flavi* increased. It can be said that early-splitting is an unfavorable trait and causes an increase in aflatoxin contamination, and early-splitted fruits of Shahpasand and Kal-Khandan cultivars showed more contamination. Therefore, cultivars with less early-splitted nuts should be considered more in export.

INTRODUCTION

Pistachios were produced 1026802.86 tons worldwide in 2022. In world pistachio production in 2022, the US

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ranks first with 400070 tons and Iran ranks second with 241668 tons of production. In terms of cultivated area, Iran ranks first with 497484 hectares and the US ranks second with 173207 hectares [1]. Aflatoxins are fungal secondary metabolites produced in many crops, mainly in corn and dried crops in the pre-harvest stages. However, delay in harvesting, lack of proper processing, presence of contaminated fruits, and unfavorable storage conditions can also cause contamination in the post-harvest stages [2- 5]. Crop management in the field and ensuring optimal storage conditions for products greatly influence the contamination of crops by *Aspergillus* spp. at the post-harvest stage [6]. In certain temperature and humidity conditions, pistachio fruits can be infected by aflatoxin-producing isolates belonging to *Aspergillus* section *Flavi*, which results in the biosynthesis and accumulation of aflatoxin [7]. Among the 33 species in the *Flavi* section of the *Aspergillus* genus, 18 species are capable of producing aflatoxin or 3-O-methylsterimatocystin as a precursor of aflatoxin; the most important ones are *A. flavus*, *A. sergii*, *A. parasiticus*, *A. nomius*, *A. novoparasiticus*, *A. pseudotamari* [8].

Fruit early splitting is one of the main causes of pistachio contamination with aflatoxin, which has been an important challenge in healthy pistachios for export in the last few years. Early-splitted pistachios are one of the main sources of pistachio contamination with aflatoxin. Studies have shown that the cracking of the green skin (hull) causes the pistachio kernel to lose moisture. This creates suitable conditions for the growth and development of different species of *Aspergillus* fungus on pistachio nuts compared to other fungi [9]. Therefore, it is expected that early-splitted pistachios, which are formed at a longer time interval than harvest date (early-splitted pistachios with dry and wrinkled hull), are more contaminated with different types of *Aspergillus* and aflatoxin. These pistachios which set earlier absorb more than 99% of aflatoxin [10]. Sorting can reduce the amount of aflatoxin in processed pistachios by 2 to 4 times compared to unsorted pistachios. The results of Sherafati *et al*. (2012) on 12 pistachio cultivars showed that the lowest percentage of early-splitting disorder was found in the Garmeh cultivar (0) followed by Badami-Sefid (0.1 percent), Kaleh-Qouchi (0.1 percent), and Abbasali (0.2 percent), Barg-Siah (0.6 percent) and Momtaz (0.8 percent) cultivars. The highest early splitting was also observed in Shahpasand (14.8 percent), Owhadi (2.7 percent), Akbari (1.7 percent), Khanjari (1.6 percent), Daneshmandi (1.2 percent) and Qermez-pesteh (1.1 percent) respectively [11].

Tajabadipour (1998) reported that early-ripening and medium-ripening Qazvini cultivars did not have early-splitting. The percentage of early-splitted nuts in Italian, Ahmad Aghaei, Akbari, Kaleh Ghochi, Owhadi, and late-ripening Qazvini cultivars was less than 1%. The highest early-splitting percentage was related to LakSirizi, Momtaze-Tajabadi and Ibrahimabadi cultivars [12].

Early cracking of nut hulls, which is one of the sources of contamination, occurs in pistachios that are not in a normal state, outer hull splits along the groove of the wooden shell, this causes the pistachio kernel to be exposed to microorganisms such as *A. flavus*. One of the factors that facilitate contamination in the product is the regular and irregular cracks created in the upper skin (hull) of pistachio fruit, through which the fungal spores penetrate around the fruit and grow in suitable conditions and produce the toxin and even fungal growth [13, 14]. The moisture content of pistachio kernels at harvest time varies from 30 to 40%, at which level different species of *Aspergillus* can grow and produce aflatoxins and compete with other microorganisms. Early-splitted pistachios with dry and semi-dry hulls are more sensitive to *Aspergillus* contamination compared to soft-hull pistachios [15].

However, shell early splitting and hull cracking are also related to the rootstock, cultivar, irrigation management, and harvest date. It has been reported that any delay in fruit harvesting can lead to an increase in aflatoxin contamination [16, 17]. Also, insect damage such as carob moth, which causes physical damage and transfer of fungal spores to the fruit, can lead to aflatoxin contamination [18- 20]. In the post-harvest stage, factors such as temperature and sufficient humidity of the environment, type of pistachio processing, transportation [21- 23, 13] and during the storage stage, water activity (wa), storage temperature, and moisture level of stored nuts are highly effective on fungi growth and aflatoxin production [24]. Good Agricultural Practices, control of

storage pests including carob moth, and separation of misshapen, discolored, and moldy fruits can be applied to decrease aflatoxin contamination [25-27]. The use of resistant cultivars to *A. flavus* and the production of aflatoxins is a requirement [28]. Because so far there has been no research on the relationship between early-splitting with the growth of *Aspergillus* section *Flavi* fungus and aflatoxin production in Iranian pistachio cultivars, therefore, in the present study, an attempt has been made to determine the relationship between early-splitting with the population and density of *Aspergillus* section *Flavi* and the amount of aflatoxin production in important cultivars of pistachio.

MATERIALS AND METHODS

Fruit Sampling

This research was carried out in two collections of pistachio cultivars of Damghan pistachio research station and Qazvin pistachio research station. This experiment was conducted as a completely randomized design with three replications. Fruit harvest was done at time of pistachio ripening in August and September. From each cultivar, 6 trees, and from each tree, six fruit clusters were randomly selected and mixed. Finally, three samples of healthy fruits and three samples of early-splitting pistachios with irregular cracks were separated from each cultivar.

Population density of Aspergillus flavus in the nut kernel of pistachio cultivars

The population density of *Aspergillus flavus* (*A. flavus* clade) fungi in the kernel of harvested fruits of different pistachio cultivars was measured using AFPA (Aspergillus flavus/parasiticus agar) and DRBC (Dichloran rosebengal chloramphenicol agar) culture media and suspension preparation and the dilution serial methods. Petri dishes containing the above media were prepared four days before use and kept in the dark until their surface was completely dried. Each sample obtained from healthy and cracked fruits was individually poured into 450 ml of water- sterile peptone 0.1% and placed on a shaker at a speed of 80 r.p.m. for one hour. To prepare dilution series, 1 ml of prepared suspension was added to 9 ml of 0.1% water-peptone to prepare 10^{-2} dilution. The dilution of 10^{-3} and 10^{-4} were also prepared in the same way. From each dilution, 100 µl of prepared suspension was poured on the surface of Petri dishes and then spread uniformly using a sterile L-shaped glass rod. Petri dishes were kept at 25˚ C and in the dark, and three to four days later, they were examined for the colony growth of *Aspergillus flavus* fungi. By culturing the isolates in three spots on CYA (Czapek yeast autolysate agar), CZA (Czapek Dox agar), CYA20S (CYA+20% sucrose), MEA 2% (Malt extract agar) culture media at 25˚ C and CYA culture medium at 37˚ C for 7 days and kept in the dark [29, 30], the macromorphological characteristics of fungal colonies were evaluated based on valid keys [31].

Aflatoxins measurement of pistachio nuts by HPLC

The pistachio fruit kernels were dried by oven to prevent further growth of *A. flavus* and production of aflatoxin. Assessment of aflatoxin production in pistachio cultivars was measured using waters e2695 (USA) HPLC, consisting of a chromolith C18, 250 mm × 4.6 mm, column (Phenomenex, USA) equipped with a fluorescence detector (Waters 2475, USA). The mobile phase was water/methanol/ acetonitrile (60:20:20) with a flow rate of 2.5 ml min^{-1}. The excitation and emission wavelengths for detection were 365 nm and 435 nm, respectively. The chromatogram of AFB1, AFB2, AFG1, and AFG2 can be seen in Figure 1. For this purpose, pistachio samples were slurred up with water in a ratio of 1/3 for 15 minutes, and then slurred samples were extracted (30 g) with 90 ml of pure methanol in the blender (Waring, USA) for 3 minutes and filtered through filter paper No. 4. Filtrates (8 ml) were mixed with phosphate buffer (42 ml). Immunoaffinity columns (VICAM; Milford, MA 01757 USA) were used for the purification of samples. Clean-up was performed according to the factory's instruction. Finally 200 µl of the preparation was injected into the HPLC apparatus. Aflatoxins B1 and B2 were measured by comparing the peak areas with calibration curves obtained by aflatoxin pure standard solutions (Sigma-Aldrich, Milan, Italy). The linearity of the analytical response was checked by analyzing the calibration standards and using seven concentrations over the range of 0.4–10 ng ml^{-1} aflatoxins B1. In the case of mobile phase HPLC, the

methanol/water (40/60) is used for the derivation of potassium bromide, nitric acid, and Kobra cell. The chromolite column (10cm) with an internal diameter of 4.6mm (Partisil 5 ODS3, USA) was used. The column temperature was set to 35 ºC with a moving phase of 2.5 Ml min^{-1}. The fluorescence detector was set at wavelengths ex=365 nm and em=355 nm [32].

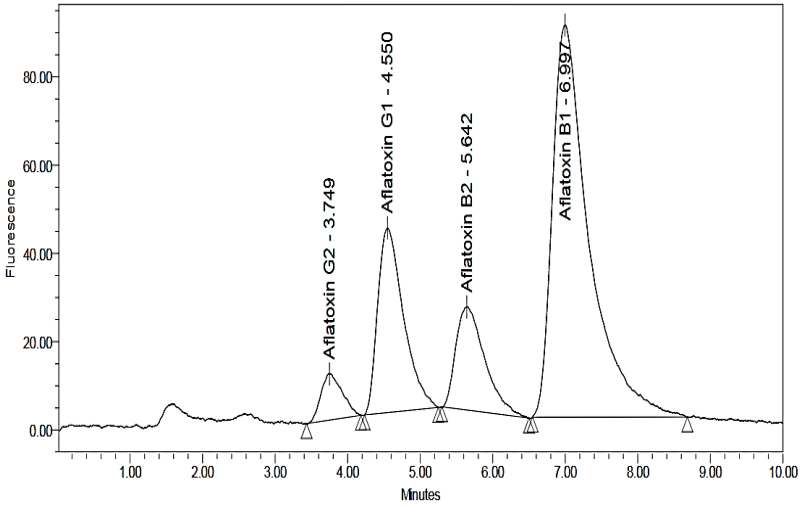

Figure 1. Chromatogram of standard of AFB1, AFB2, AFG1 and AFG2

RESULTS

The results of the analysis of the variance of the data showed that the studied cultivars showed a significant difference in the amount of contamination with *Aspergillus* section *Flavi* growth and the percentage of nut-early splitting ($p \leq 0.01$) (Table 1).

Table 1. Variance analysis of the data on the amount of aflatoxin infection and the early splitting percentage in 6 pistachio cultivars

Variation source	Degree of Freedom	Contamination	Early splitting
Cultivar	5	367.689**	83.514**
Error	12	1.333	0.113

** Data are significant at the 1% level

Comparison of Aspergillus section Flavi contamination of nut kernel and nut early-splitting in pistachio cultivars

The results of the statistical analysis obtained from counting and checking the population density of *Aspergillus* section *Flavi* colonies in the kernel of pistachio cultivars showed a significant difference in terms of *Aspergillus* section *Flavi* contamination among different pistachio cultivars. Among the cultivars tested, the population density of *Aspergillus* section *Flavi* colonies in pistachio cultivars of Shahpasand (74.6667 CFU g^{-1}) and Kal-Khandan (73.6667 CFU g^{-1}) was much higher than other cultivars. The lowest density of the fungi population (47.6667 CFU g^{-1}) was also observed in the Kaleh-bozi cultivar (Table 2).

Comparison of nut early-splitting in pistachio cultivars

Pistachio cultivars also showed a significant difference in terms of nut early-splitting. Among the cultivars tested, the percentage of early-splitting was higher in the Shahpasand pistachio cultivar (13.66%) than in other cultivars, followed by the Kal-Khandan cultivar (5.66%). The lowest nut early-splitting (0.1%) was also observed in the Kaleh-Bozi variety (Table 2).

Table 2. Comparison of Aspergillus section Flavi contamination of nut kernel and nut early-splitting in pistachio cultivars

Pistachio cultivar	The average number of colony *Aspergillus* section *Flavi*(CFU g^{-1})	Statistical grouping (α=%5)	Early splitting percentage	Statistical grouping (α=%5)
Shahpasand	74.6667	a	13.6667	a
Kal-Khandan	73.6667	a	5.6667	b
Abbasali	61.3333	b	0.3333	de
Khanjari	59.6667	b	1.8333	c
Kaleh-bozi	47.6667	d	0.1000	e
Ghermez	51.6667	c	0.8333	d

The means with the same letters in each column do not have statistically significant differences at the five percent level (Duncan's multiple range test method).

Relationship between early–splitting and the growth of

Aspergillus section Flavi in pistachio cultivars

To investigate the possible relationship between the growth rate of *Aspergillus* section *Flavi* and the early-splitting percentage of pistachio cultivars, the correlation coefficient (r) was calculated (Table 3). The results of the statistical analysis showed that there was a positive and significant relationship between the growth rate of *Aspergillus* section *Flavi* and the fruit early splitting percentage of different cultivars (r = 81%). In other words, with an increase in the early splitting percentage of pistachio fruit, the growth rate of *Aspergillus* section *Flavi* in pistachio fruit also increases. Therefore, the correlation analysis showed that early-splitting is an unfavorable trait and causes an increase in contamination, and early-splitted fruits such as Shahpasand and Kal-Khandan showed more contamination as shown in Table 2.

Table 3. The correlation coefficient between the growth rate of *A. flavus* and the early-splitting percentage of pistachio cultivars

Variables	N	r	a	b	r^2
The growth rate of *A. flavus* × Early splitting percentage	18	0.810**	55.071	1.7046	0.6564

** a significant difference at the level of 0.01%.

Regression coefficient (r^2), which is the square of the correlation coefficient showed the linear regression between early-splitting and fungal contamination, and its value is equal to 0.6564. According to the obtained relationship, we can predict the amount of fungus contamination by 65% according to the percentage of early-splitting and the formula obtained in the graph (Figure 2).

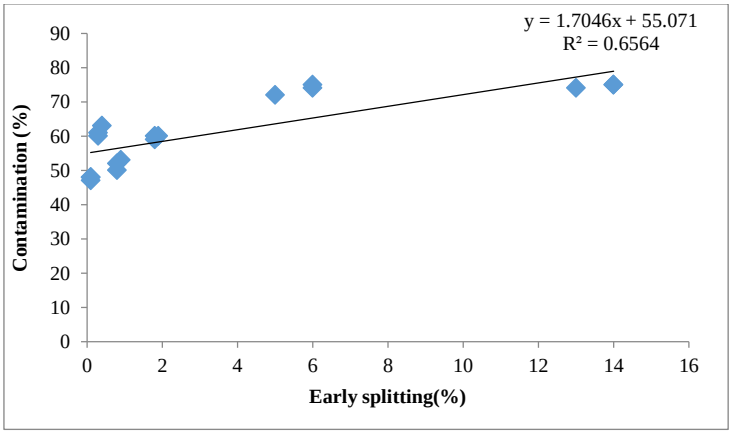

Figure 2. Regression coefficient between the growth rate of *A. flavus* and the early-splitting percentage of pistachio cultivars

Contamination of pistachio varieties by aflatoxin types

Assaying aflatoxin production level in pistachio kernels is shown in Table 4. Out of 18 samples of pistachio nut kernels of different cultivars that were evaluated for aflatoxin by HPLC method, it was found that only 2 samples obtained from Shahpasand and Kal-Khandan cultivars were contaminated with B2 and B1 aflatoxins. However, the contamination of all cultivars was below the threshold limit. The samples did not show contamination with G1 and G2 aflatoxins.

Table 4. The amount of aflatoxin contamination (B1, B2, G1, G2) in pistachio samples

Pistachio cultivars	Repeat	Amount of aflatoxin contamination(ppb)				
		B1	B2	G1	G2	TOTAL
Shahpasand	1	ND	ND	ND	ND	NA
Shahpasand	2	3.37	1.05	ND	ND	4/42
Shahpasand	3	ND	ND	ND	ND	NA
Abbasali	1	ND	ND	ND	ND	NA
Abbasali	2	ND	ND	ND	ND	NA
Abbasali	3	ND	ND	ND	ND	NA
Khanjari	1	ND	ND	ND	ND	NA
Khanjari	2	ND	ND	ND	ND	NA
Khanjari	3	ND	ND	ND	ND	NA
Kal- khandan	1	ND	ND	ND	ND	NA
Kal- khandan	2	3/15	1/11	ND	ND	4/26
Kal- khandan	3	ND	ND	ND	ND	NA
Kaleh- bozi	1	ND	ND	ND	ND	NA
Kaleh- bozi	2	ND	ND	ND	ND	NA
Kaleh- bozi	3	ND	ND	ND	ND	NA
Ghermez	1	ND	ND	ND	ND	NA
Ghermez	2	ND	ND	ND	ND	NA
Ghermez	3	ND	ND	ND	ND	NA

ND=not detected NA=not applicable

In this research, a total of 127 fungal isolates belonging to the *Aspergillus flavus* division, whose colony color was green on the DRBC culture medium and could be easily distinguished from other fungi, especially *A. niger*, were isolated and identified (Figure 3). Among these isolates, based on macromorphological characteristics, 120 and 7 isolates belonging to *Aspergillus flavus* and *A. parasiticus* were identified respectively. The colonies of these two species are green in color with a relatively fast growth, and the diameter of the colonies reaches about 65-70 mm in 7 days at a temperature of 25°C. Morphologically, the most important difference between *A. flavus* with *A. parasiticus* is the darker green color of the colonies and the Rough-walled conidium (Figure 4) in *A. parasiticus* [33].

Figure 3. Green colonies of *Aspergillus* section *Flavi* on DRBC medium

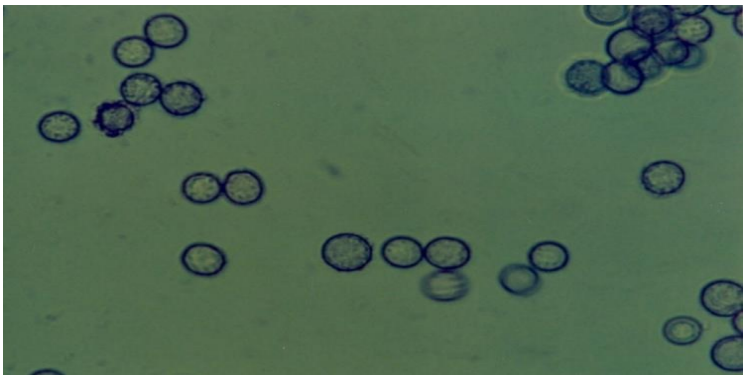

Figure 4. Rough-walled conidium of *Aspergillus parasiticus* (×1000).

DISCUSSION

Pistachio contamination with *Aspergillus* Section *Flavi* fungi and aflatoxin is one of the most important problems in the production and export of this valuable product. Contamination of pistachio fruit with mold and aflatoxin is always influenced by several factors such as the cracking of the green skin of the fruit, the frequency and time of irrigation, the environment, fruit moisture, plant residues, organic matter, and animal manure in the orchard, pests and birds and harvest time [34, 2]. Investigating various references shows the role of early splitting and cracking of the green skin of pistachio fruit in contamination with *Aspergillus* fungi and aflatoxin production [35, 3]. The present research also confirmed that early splitting in pistachio fruit plays an important role in contamination with *Aspergillus* fungi. Cracking of the green skin of the fruit in pistachios (hull) creates suitable conditions for the growth and development of different species of *Aspergillus* fungus on fruits [9].

It is expected that early-splitted pistachios, which are formed at a longer time interval than harvest date (early-splitted pistachios with dry and wrinkled hull), are more contaminated with different types of *Aspergillus* and aflatoxin. It means that early-splitted pistachios with dry and wrinkled hulls set earlier absorb more than 99% of aflatoxin (10) and sorting can reduce the amount of aflatoxin in processed pistachios by 2 to 4 times compared to unsorted pistachios. Doster and Michaelides (1993) determined the percentage of early-splitted pistachios in Kerman cultivar pistachios from 0.6 to 8.2% and 0.8 to 5.8% in 1992 and 1993, respectively [36].

The time of appearance and the amount of early-splitted and cracked pistachios in different years and regions are very different [37]. In addition, the pistachio cultivar, the type of rootstock, scion, the age of the trees, and even the irrigation program and system are also effective in the occurrence of early splitting and green skin cracking. Doster *et al*. (1998) reported that the rootstock, insufficient irrigation in late spring, and the application of Volk oil in winter has affect early splitting pistachios. Based on their results pistachio trees on the PGII rootstock had the highest percentage of early splitting (5.5%), followed by PGI, *atlantica*, and UCB-1 rootstocks, with 3.5, 2.9, and 2.6 %, respectively. Tajabadipour et al, (2011) investigated the effects of rootstock and scion on fruit early splitting of three

cultivars of Ahmad Aghaei, Kaleh Ghochi, and Owhadi on four rootstocks including Sarakhs, Baneh, and atlantica. The results showed that the total amount of early-spilited pistachios (with soft skin and wrinkled and dry hull) on the Atlantica and Beneh rootstocks was significantly higher than the *P. vera* rootstock. In commercial cultivars such as Kaleh-ghouchi, Owhadi, and Ahmad-Aghaei, the highest percentage of early splitted pistachios is observed 15 days before harvest [38]. Although different species of *Aspergillus* genus have been isolated and reported from pistachio nuts, soil, storage, and environment of pistachio orchards in Iran, America, and Turkey, two species, *A. flavus*, and *A. parasiticus*, as aflatoxin-producing molds, have always had a special position in the soil and pistachio fruit mycoflora [39-41].

This research showed that the percentage of early splitted fruits in Shahpasand and Kal-khandan cultivars was much higher than in the other studied cultivars. The growth and colonization of *Aspergillus* section *Flavi* were was also higher in pistachio cultivars with a higher percentage of early splitted fruits than other cultivars. In other words, fruit early splitting plays an effective role in contamination with *Aspergillus* section *Flavi* fungus. In this research, 127 fungal isolates were isolated and identified, of which 120 and 7 isolates belonged to *Aspergillus flavus* and *A. parasiticus*, respectively. The study of the changes in the population of *A. flavus* and *A. niger* in pistachio orchards showed that the population of these fungi increases in spring and reaches its maximum in September. Therefore, the maximum population density of these fungi coincides with the maturity of the fruit and cracking of the green skin [42]. The results of Sherafati *et al*. (2012) on 12 pistachio cultivars showed that the lowest percentage of early-splitting disorder was found in the Garmeh cultivar (0) followed by Badami-Sefid (0.1 percent), Kaleh-Qouchi (0.1 percent), and Abbasali (0.2 percent), Barg-Siah (0.6 percent) and Momtaz (0.8 percent) cultivars. The highest early splitting was also observed in Shahpasand (14.8 percent), Owhadi (2.7 percent), Akbari (1.7 percent), Khanjari (1.6 percent), Daneshmandi (1.2 percent) and Ghermez-pesteh (1.1 percent) respectively [11].

CONCLUSIONS

In general, the results showed a significant difference in *Aspergillus* Section *flavi* contamination among different pistachio cultivars. Among the cultivars tested, the population density of *Aspergillus* section *Flavi* colonies in pistachio cultivars of Shahpasand (74.6667 CFU g^{-1}) and Kal-Khandan(73.6667 CFU g^{-1}) was much higher than other cultivars. The lowest density of the fungi population (47.6667 CFU g^{-1}) was also observed in the Kaleh-bozi cultivar. The percentage of early-splitting was higher in the Shahpasand pistachio cultivar (13.66%) than in other cultivars, followed by the Kal-Khandan cultivar (5.66%). There was a positive and significant relationship between the growth rate of *Aspergillus* section *Flavi* and the fruit early splitting percentage of different cultivars (r = 81%). In other words, with an increase in the early-splitting percentage of pistachio fruit, the growth rate of *Aspergillus* section *Flavi* in pistachio fruit also increases. Based on the results, fruit early-splitting is an unfavorable trait and causes an increase in contamination, and early-splitted fruits of Shahpasand and Kal-Khandan cultivars showed more contamination. Therefore, we should pay attention to these cases in export, and cultivars with less early-splitted nuts should be considered.

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Conflict of interests

The authors declare that they have no conflicts of interest with respect to this work.

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