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ORIGINAL ARTICLE

Correlation between Testa Thickness of Damghan Pistachio Cultivars with *Aspergillus flavus* Growth and Aflatoxin Production

Mehdi Mohammadi-Moghadam[*1], Mohammad Ali Khaleghi[2], Mahdi Naeimi[3], Amir Hossein Mohammadi[4], Seyedhamidreza Ziaolhagh[5]

[1]*Crop and Horticultural Sciences Research Department, Agricultural and Natural Resources Research and Education Center of Semnan Province (Shahrood), AREEO, Shahrood, Iran*

[2]*Graduate of Plant Pathology, Damghan Branch, Islamic Azad University, Damghan, Iran*

[3]*Department of Plant Protection, Damghan Branch, Islamic Azad University, Damghan, Iran*

[4]*Pistachio Research Center, Horticultural Sciences Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Rafsanjan, Iran*

[5]*Agricultural Engineering Research Department, Agricultural and Natural Resources Research and Education Center of Semnan Province (Shahrood), AREEO, Shahrood, Iran*

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ABSTRACT: The issue of contamination of pistachios with *Aspergillus flavus* and aflatoxin is a serious and important threatto the production, export and consumption of pistachios worldwide. Investigating the resistance of pistachio cultivars to *A. flavus* and its relationship with the physicochemical characteristics of pistachio kernels can be considered as one of the main management strategies to deal address aflatoxin contamination. In this research, the resistance of six important pistachio cultivars to *A. flavus* and aflatoxin was first determined. The relationship between the thickness of the testa (the thin skin on the kernels) and the growth of *A. flavus* and aflatoxin production was then investigated. The testa of pistachio cultivars was inoculated using a toxin-producing isolate of *A. flavus*. Eight days after inoculation, the growth and colonization rate of the fungus was measured and evaluated using the Kernel Screening Assay (KSA) method and the amount of aflatoxin determined by the High-Performance Liquid Chromatography (HPLC) method. The thickness of testa was measured using a micrometer. To investigate the possible relationship between the thickness of testa and the growth of *A. flavus* and aflatoxin production, the correlation coefficient (r) was calculated. According to the results of statistical analysis, the cultivars Shahpasand and Ghaniabadi exhibited the lowest resistance to the growth of *A. flavus*. The research results indicated that there was no significant relationship between the amount of fungus growth and aflatoxin and the thickness of the pistachio kernel skin in different cultivars.It appears that the chemical characteristics of the pistachio kernel and kernel skin play a more important role in resistance to fungal growth and aflatoxin production.

INTRODUCTION

Pistachio is a strategic and economically important product, but in the last 40 years, Iranian pistachios have

*Corresponding authormm.moghadam52@gmail.com (M. Mohammadi-Moghadam)
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been exported or returned at a much cheaper price due to the high amount of aflatoxin in them. Mycotoxins have an adverse effect on 25% of the world's agricultural and food products every year. Among them, aflatoxins have a special position in the economy and health of humans and animals due to their various biochemical and biological effects. The annual damage caused by the destruction of food and agricultural products by these toxins is estimated to be more than 100 million dollars [1, 2]. Aflatoxins are a large group of mycotoxins which are secondary metabolites of fungi and are produced by species such as *Aspergillus flavus*, *A. parasiticus*, *A. tamarii*, *A. bombycis* and *A. nomius* [3].

Today, food contamination with fungal spores is considered as one of the most important health problems [4]. The potential risks of aflatoxin contamination of agricultural and horticultural products such as corn, peanuts, cotton seeds and pistachios have caused concern in food hygiene in different countries of the world, and for this reason, there are special regulations for the import and export of agricultural products in many countries [5]. Since 1971, when the United States of America stopped some of Iran's exported pistachios due to contamination with aflatoxin, the issue of contamination of Iranian pistachios with this toxin was taken into consideration [6].

The area under pistachio cultivation in Iran is more than 498.7 thousand hectares, of which 106.3 thousand hectares are fertile and 392.4 thousand hectares are non-fertile. Pistachio ranks first among the horticultural products of Iran with a fertile level of 15.7% in 1996 and 15.9% in 1997. Pistachio also covers 57.2% of the total area of dry fruit orchards, both non-fertile and fertile, and has taken the first place in the country's dry fruit production. The cultivated area of pistachio increased by 4.6% from 2015 to 2016, while many horticultural products have experienced a decrease in the area under cultivation in recent years [7, 8]. The reason for this can be the increase in the intensity of environmental stresses due to climate changes along with global warming and its effect on the quantity and quality of the country's water resources, and it shows the unique characteristics of pistachio in tolerating environmental stresses compared to other horticultural products and its economic production in Inadequate water and soil conditions.

In recent years, the main and important problem of the country in the field of pistachio export is its contamination with *A. flavus* and aflatoxin, which can threaten this source of foreign exchange income and prevent us from competing in the world market. Therefore, various aspects of pistachio contamination with this fungus should be seriously studied. The thin kernel skin (testa) of the pistachio nut can be one of the important barriers against the growth of toxin-producing fungi and the production of aflatoxin, and its physical and chemical properties are very important in this regard [5]. In this research, the relationship between the thickness of pistachio kernel skin in different cultivars with the growth rate of *A. flavus* and aflatoxin production has been investigated.

MATERIALS AND METHODS

Selection and collection of Damghan pistachio cultivars

In order to carry out this research, six local and important pistachio cultivars in Damghan city, including Shahpasand, Ghaniabadi, Khanjari, Khani, Abbas Ali and FAS-13-73, which occupy a large area under cultivation, were selected and collected.

In order to prevent wounding and damage to the pistachio kernel skin (Testa), and also to minimize the possibility of contamination of pistachios with *Aspergillus* section Flavi, sampling was done from the tree. After harvesting fresh pistachios, pest-infected pistachios and pistachios with possible aflatoxin contamination (distorted, yellow and discolored pistachios) were removed. Then, hull (outer soft skin of nuts) was manually separated from its horny shell, so as not to damage the thin skin of the kernel. After that, the nuts were dried under favorable conditions and used for in vitro experiments.

Isolation of Aspergillus flavus

To carry out this research, an isolate of *A. flavus* from the infected pistachios of the region was used. Czapek Yeast Extract Agar (CYA) and Malt Extract Agar (MEA) were used in all stages of the work, for isolation culture, subculture or to prepare Slant.

Investigating the aflatoxinogenic property of Aspergillus flavus isolate

Sucrose Low Salts medium (SLS) was used to investigate the aflatoxin-producing properties of the fungus isolate and the type of aflatoxin produced by this isolate (G2, G1, B2, B1) [9]. To prepare the fungus inoculum, first the fungal isolate was cultured in the tubes containing the MEA culture medium for 7 to 10 days, and then by adding some sterile distilled water along with a few drops of Tween 20 to the tubes and shaking them, a suspension of fungi spores was prepared. Using a hemocytometer, the number of spores present in each milliliter of the obtained suspension was counted, and then by dilution, the number of spores was adjusted to 7×10^6 per milliliter. In the flasks containing 50 ml of SLS liquid culture medium, the amount of 7×10^6 spores per milliliter of spore suspension was added and the flasks were kept at 28 degrees Celsius in an incubator for one week. After this period, the production of aflatoxin by *A. flavus* isolate was investigated in liquid culture medium [10]

Aflatoxin extraction from SLS liquid culture medium

As shown in Figure 1, the color of the SLS medium turns yellow after the growth of *A.flavus* due to the production of fungal metabolites such as aflatoxin. To extract the aflatoxin from the SLS culture medium, the medium containing aflatoxin was mixed with chloroform in a decanter funnel. After shaking continuously for 5 minutes, the aflatoxin transferred to the chloroform phase. To remove the water from the chloroform phase, it was filtered through anhydrous sodium sulfate (Na2SO4). For better extraction of aflatoxin from the liquid culture medium, the extraction with chloroform was repeated 3 times. The chloroform containing aflatoxin was then evaporated and dried in a bain-marie at 50 degrees Celsius. The remaining materials were mixed with one milliliter of chloroform. Following this step, the type of aflatoxin produced in the culture medium was determined using the thin layer chromatography (TLC) method [9].

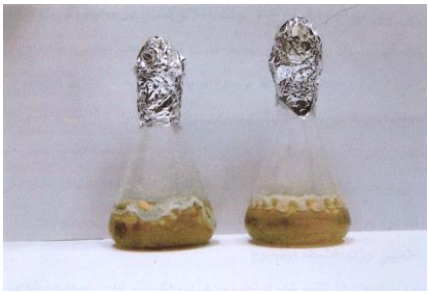

Figure 1. *Aspergillus flavus* mycelial mass on the surface of SLS medium and color change of culture medium from white to yellow (one week after inoculation)

Measuring and evaluating resistance of pistachio cultivars to Aspergillus flavus fungus in laboratory conditions (KSA)

In order to investigate and study the resistance of important Damghan pistachio cultivars to the growth of aflatoxin-producing fungi, the conventional KSA method was used. The basis of using this method is to investigate the resistance of kernels or seeds of different cultivars of agricultural and horticultural crops such as corn, peanuts or pistachios in laboratory conditions to the growth and colonization of *A. flavus* fungus. This method is a fast laboratory screening method for different cultivars of *A. flavus*. Kernel or seed is the most important tissue attacked by *A. flavus* [11, 12].

To ensure that the kernels are not contaminated with aflatoxin, 50 grams of pistachio kernels were selected from each of the collected cultivars and aflatoxin was extracted from it by the BF method. Then, using thin layer chromatography (TLC), the possibility of contamination of pistachios with aflatoxin was investigated.

After this stage and to make sure that pistachios are not contaminated with aflatoxin, *A. flavus* isolate was first cultured on MEA culture medium inside Slant tube. 8 to 10 days later, after fungi growth and sporulation, some sterile distilled water along with a few drops of Tween 20 was added to the slants. Fungi spore suspension with a concentration of 2×10^6 spores per ml was prepared using a Toma slide (hemocytometer). A replicate and a control were considered for each cultivar. After surface disinfection and soaking pistachios in sterile distilled water, one milliliter of spore suspension was added to each petri dish containing 20 grams of pistachio kernels. To ensure sufficient humidity, the petri dishes containing pistachio kernels were placed in plastic containers with lids, where some sterile distilled water was poured at the bottom, and kept at a temperature of 26 degrees Celsius. After fungi growth and pistachio surface colonization, the amount of fungi colonization on the pistachio surface was calculated on the eighth day [5].

Measuring the amount of aflatoxin using thin layer chromatography (TLC)

After extracting aflatoxin from SLS medium, TLC method was used to determine the type of aflatoxin produced in the extracts. The types of aflatoxins present in the extract are determined on the gel (TLC) with high wavelength ultraviolet (UV) radiation in terms of fluorescence and RF (the ratio of the movement of the sample to the movement of the emerging solvent). Aflatoxin B1 and B2 present in the spots on the gel (TLC) emit relatively strong blue fluorescent light under UV radiation. After observing the plates under ultraviolet rays, it was found that the fungus isolate is capable of producing both types of aflatoxin B1 and B2.

Investigating the effect of pistachio kernel thin skin (Testa) on the colonization of Aspergillus flavus on pistachio kernel

The thin skin of the pistachio kernel (Testa) can act as a physical barrier against the penetration of *A. flavus* into the pistachio kernel and reduce the amount of aflatoxin caused by the growth and activity of the fungus (Figure 2). To evaluate the physical effect of this thin skin in reducing the colonization rate of the fungus on the pistachio kernel, its thickness was measured using a micrometer.

Figure 2. Comparison of *A. flavus* growth rate on healthy (right) and injured (left) pistachio kernels of Shahpasand cultivar

Measuring contamination of samples with aflatoxin by high performance liquid chromatography (HPLC)

To determine the amount of aflatoxin, first the kernel samples were dried in an oven at 60 degrees Celsius for 24 hours and then ground. High-performance liquid chromatography (HPLC) was used to test and measure the amount of aflatoxin contamination of the samples. The most important advantage of HPLC method compared to TLC is to be quick, automatic control, accuracy and precision of this method. The HPLC system creates a liquid chromatography system with high pressure (5800 psi). This system is used in cases where high separation power is needed in the final stages of purification. HPLC involves the separation of substances between solid and liquid phases.

The stationary phase is supported in a column with a length of 25 cm and an inner diameter of 4 mm. The mobile phase is pressurized along the separating column

and then pushed towards the detector. The time it takes for each material to reach a fixed position in the column is called the survival time. Qualitative analyzes for each sample are performed by comparing this time with existing standards. In the case of aflatoxins, due to their fluorescence characteristics, a fluorescent detector can be used that detects less than one µg of aflatoxin per kilogram of sample. In this method, Reversed Phase (C18) is used. The solvent used is a combination of acetonyril: water: methanol (10: 60: 30), which actually forms the mobile phase [13, 14].

RESULTS

Aflatoxinogenic property of Aspergillus flavus isolate

Investigating the aflatoxin production ability of *A. flavus* isolate showed that this isolate was able to produce aflatoxins B1 and B2, but could not produce aflatoxins G1 and G2.

The growth rate of A. flavus on the kernels of different pistachio cultivars

As can be seen in Table 1, eight days after inoculation, the amount of fungal growth (colonization) on pistachio nuts of different cultivars had a significant difference at the level of 5%. Among the tested cultivars, Shahpand and Ghaniabadi cultivars showed the least resistance and Khanjari, Khani and FAS-13-73 cultivars showed the highest resistance to the growth of *A. flavus*. The percentage range of *A. flavus* colonization on pistachio kernels of different cultivars varied between 75.40 and 51.91%.

Table 1. Comparison of the average percentage of *A. flavus* colonization and aflatoxin production and the average skin thickness (testa) of pistachio kernels of different cultivars.

Pistachio cultivar	Mean shell thickness (Testa) of pistachio kernels (mm)	Mean aflatoxin production in pistachio kernel (ppb)	Mean percentage of colonization in pistachio kernels (%)
Shahpasand	42.66a	9391a	75.40a
Ghaniabadi	42.33a	8407b	69.14a
Abasali	41.33a	6418c	59.81b
Khanjari	46.33a	5654d	52.28c
Khani	43.66a	5350d	48.69c
FAS-13-73	40.66a	5098d	51.91c

* Numbers with common letters are not significantly different from each other, based on Duncan's test at the level of 5%.

The amount of aflatoxin production in the kernels of different pistachio cultivars

As can be seen in Table 2, there was a significant difference in the amount of aflatoxin production on pistachio cultivars at the level of 1%. Among the tested cultivars, Shahpasand cultivar showed the least resistance and compared to Khanjiri, Khani and FAS-13-73 cultivars. These later three genotypes showed the highest resistance to aflatoxin production and were placed in one group (Table 1). The range of aflatoxin production in the studied cultivars varied between 5098 and 9391 ppb.

Table 2. The effect of pistachio cultivar on growth and colonization by *A. flavus* and aflatoxin production.

Sources	Degree of freedom	Means of squares	
		Production of aflatoxins	Growth and colonization
Pistachio cultivar	5	9429339.5**	346.56**
Test error	12	180217.8	13.1
CV	-	6.14	6.08

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

The Thin skin thickness (Testa) in pistachio nuts of different cultivars

As shown in Table 2, the thickness of the thin skin of the kernel in different cultivars did not show a statistically

significant difference, and all the studied cultivars were placed in the same statistical group (Table 1).

The relationship between Aspergillus flavus growth rate and aflatoxin production with pistachio kernel thin skin thickness

The results of the regression relationship between pistachio kernel skin thickness and fungi growth rate and aflatoxin production are shown in Table 3. There was a correlation of 0.004 and 0.083 between the thickness of the kernel skin and the fungal growth rate and aflatoxin production, respectively, which was not statistically significant. Therefore, in general, no significant relationship and positive correlation was observed between the thickness of the skin of pistachio kernels with the growth rate of *A. flavus* and the production of aflatoxin in different cultivars.

Table 3. Correlation coefficient between *A. flavus* growth and pistachio kernel skin thickness (testa) of different cultivars.

Pairs of variables	a	b	r^2	r
***A. flavus* growth rate× Skin thickness of pistachio**	59.98	-0.01	0.0006	0.004
Production of aflatoxins× Skin thickness of pistachio	5277.92	33.93	0.007	0.083

DISCUSSION

Pistachio is one of the important horticultural products of the country and is considered one of the important non-oil export items and a source of foreign exchange for the country, therefore, protecting this great national wealth and using it optimally is the basis for the prosperity of the national economy and is very important.

Different species of fungi have been isolated and identified from pistachios in different stages of pistachio production from the garden to the storage. Some of them, by growing on pistachio fruit, reduce the quality and marketability of this product. Many studies have been done in this field. The genera of *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, *Cladosporium*, *Rhizopus*, *Ulocladium* and *Helminthosporium* are among the most known of them. Among them, Aspergillus is dominant. The production of secondary metabolites, especially mycotoxins, by some of these fungi, especially Aspergillus species, and product contamination are among the most important challenges in the production and supply of agricultural products, especially pistachios [15, 16, and 5].

The turning point in the study of mycotoxins dates back to 1959, when thousands of turkeys died in a short period of time on a farm in England. The cause of death was found to be the presence of toxins in ground peanuts used in the production of protein supplement for poultry feed. Various tests proved the presence of A. flavus mycelium on peanuts and thin layer chromatography showed the presence of several new compounds that fluoresced under UV light. These very toxic metabolites were called aflatoxin [17].

Aflatoxins, which are a group of furanocoumarins derived from polyketides, are the most toxic and carcinogenic compounds known among mycotoxins, and for this reason, they have been studied a lot. Polyketides are made in a similar way to fatty acid synthesis and are the most abundant secondary metabolites produced by fungi [18]. There are about 20 types of aflatoxins. Among known aflatoxins, only four main aflatoxins AFB1, AFB2, AFG1 and AFG2 contaminate agricultural products and are considered a serious threat to human and animal health [19]. It should be mentioned that the production of this toxin is not necessary for pathogenicity, growth and development, and it is produced only under special food and fungal growth conditions [2].

Today, the contamination of agricultural products with aflatoxins is one of the most important health problems of the world community, and different countries have established special laws and regulations for the production, consumption and import of food due to the serious dangers of mycotoxins [15]. In the United States, food and medicine containing more than 20 ppb of aflatoxin cannot be bought, sold, imported or exported. In recent years, the main and important problem of the country in the field of pistachio export is the problem of

its contamination with *A. flavus* fungus and aflatoxin caused by it, which can threaten this source of foreign exchange income and prevent us from competing in the world market. So that in the last 49 years, Iran's exported pistachios have been returned or sold at a very low price due to the presence of aflatoxin. Therefore, various aspects of pistachio contamination with this fungus should be seriously studied. Pistachio kernel shell can be one of the most important physicochemical factors affecting the growth of fungi and aflatoxin production, and the difference in the chemical composition and thickness of the kernel thin skin of different varieties of a product can be the basis of the sensitivity or resistance of that product to the growth of *A. flavus*. According to the above reason, in this research, the possible relationship between the thickness of pistachio kernel thin skin of different cultivars and the growth rate of *A. flavus* was investigated.

In general, the production of aflatoxin is affected by factors such as the genetic characteristics of the fungus and the physicochemical environment in which they grow [20]. One of the most effective, least costly and most harmless solutions to fight and control this fungus is to plant the most resistant cultivars to the growth of the fungus and of course to the aflatoxin caused by it. In most parts of the world, extensive research is being done to identify resistant cultivars of various products to the aflatoxin-producing *A. flavus*, and good and successful results have been reported. During the stages of this research, the resistance of pistachio cultivars to aflatoxin-producing *A. flavus* was evaluated and the amount of aflatoxin production in these cultivars was investigated. The results of the experiments showed that among the pistachio cultivars, there was a significant difference in the amount of fungal growth on the pistachio kernel of different cultivars. By measuring the aflatoxin produced in different cultivars, a significant difference was observed in the amount of aflatoxin production between different cultivars.

Among the effective factors in the production of aflatoxin in food, we can mention the characteristics of the fungi, the physicochemical composition of the food, heat, humidity and time, among which the type of physicochemical composition of the food is of particular importance. In most parts of the world, extensive research has been done to determine the role of chemical and physical factors of food in the rate of fungal growth and aflatoxin production in various products, and successful results have been reported. Most of the studies done in this field have been done on peanuts. The results of the research conducted on the relationship between the amount of sugar in the kernels of different peanut cultivars with the growth rate of *A. flavus* and the production of aflatoxin indicated that there was a significant relationship between the amount of aflatoxin production and the sugar percentage, and many other chemical and physical factors may be involved in this matter [21].

The results of Mohammadi Moghadam et al. (2020c) research showed that the difference in the average percentage of colonization, fungal sporulation and aflatoxin production on healthy pistachio kernels compared to injured pistachio kernels (whose pistachio kernel shell was damaged) was significant at the level of 1% [22]. In other words, in healthy pistachios, the shell can act as a physicochemical barrier resistant to the penetration of the fungus into the nut kernel and cause a decrease in the growth and sporulation of the fungus and the production of aflatoxin in comparison with the injured pistachios (Figure 4). Therefore, according to the importance of the subject, the physical and chemical role of Testa (thin skin around the kernels) in preventing the growth of fungi and toxin production should be investigated separately [22].

In a research conducted by Latha et al. (2007) on peanuts, it was found that among the 21 different genotypes of peanuts tested, four genotypes IC-48, J-11, ICGV 89104 and ICGS-76 had the lowest amount of aflatoxin and the highest amount of phenol. Aflatoxin production showed a negative relationship with the amount of phenol in kernels ($r2=-0.42$ $p<0.05$) and leaves ($r2=-0.37$ $p<0.05$) [23].

The results of this research showed that among the cultivars tested, Shahpandan cultivar had the least resistance to fungal growth. There was no significant difference (at the 5% level) in the thickness of the thin skin of different pistachio cultivars. In order to investigate the relationship between fungal growth and pistachio shell thickness, the correlation coefficient (r) was calculated with a confidence level of 95%. The

results of statistical studies showed that there was no significant relationship between the fungal growth rate and the thickness of pistachio kernel thin skin of different varieties. The results also confirmed that there was no significant relationship between the amount of aflatoxin production and the thickness of the thin skin of pistachio kernel.

Many other factors such as the chemical composition, including the type and amount of fats (saturated and unsaturated fatty acids), the type and amount of carbohydrates, phenolic compounds, vitamins and chemical compounds of pistachio kernel thin skin and many other physicochemical parameters can be involved in fungal growth and aflatoxin production. Therefore, before any general conclusions about the relationship between fungal growth rate and aflatoxin production with the physicochemical compounds of pistachio kernel and thin skin, the role of other compositions should also be checked in this case. In other words, with more comprehensive investigations on other physical and chemical parameters of the pistachio kernel and thin skin, it is possible to determine the logical relationship between the physicochemical composition of the kernel thin skin of different cultivars with the rate of fungal growth and aflatoxin production.

CONCLUSIONS

In the current study it was shown that Khanjari, Khani and FAS-13-73 cultivars were best in not supporting the growth of *A. flavus* and production of aflatoxin. Additionally, the thickness of the thin skin of the kernel in different cultivars had no effect on the growth rate of *A. flavus* and the production of aflatoxin. Considering the importance of contamination of pistachio cultivars with aflatoxin-producing fungi and aflatoxin production in them, it is necessary to carry out additional studies on the reaction of important commercial cultivars against fungal growth and aflatoxin production and the resistance mechanism of pistachio cultivars against fungal growth. Considering that many other factors such as the chemical composition of pistachio nuts, and many other physicochemical parameters can be involved in fungal growth and aflatoxin production, the role of other compounds should be investigated regarding the relationship between fungal growth and aflatoxin production with the physicochemical compounds of pistachio kernel thin skin.

Conflict of interests

NO conflict.

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