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Contamination of Stored Pistachios to Aspergillus Section Flavi and Aflatoxins

Younes Salehian; MSc[1], Adeleh Sobhanipour; PhD [1], Mehdi Mohammadi-Moghadam; PhD [2] & Seyed Reza Fani; PhD *[3]

[1]*Department of Plant Protection, Damghan Branch, Islamic Azad University, Damghan, Iran.*
[2] *Crop and Horticultural Sciences Research Department, Agricultural and Natural Resources and Education Center of Semnan Province (Shahrood), AREEO, Shahrood, Iran.*
[3] *Plant Protection Research Department, Yazd Agricultural and Natural Resources Research and Education Center, AREEO, Yazd, Iran.*

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Corresponding author:
rezafani52@gmail.com
Plant Protection Research Department, Yazd Agricultural and Natural Resources Research and Education Center, AREEO, Yazd, Iran.

Postal code: 8915813155
Tel: +98 3538249901

ABSTRACT

Background: Contamination of stored pistachio nuts to *Aspergillus* section *Flavi* and aflatoxins (AFs) as well as effective parameters on fungal growth and AF production during storage was assayed. **Methods:** Twenty one pistachio nut samples were taken from some warehouse of Damghan county. Different parameters of storage, including temperature, relative humidity, and pest infestation were recorded. Contamination to aflatoxigenic fungi was investigated by serial dilution method and aspergillus flavus and parasiticus agar (AFPA) medium with three repetitions. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) method were used to measure quality and quantity of the produced AFs, respectively. **Results:** There was a significant difference in contamination to *Aspergillus* section *Flavi* between different samples on the basis of statistical analysis. There was no significant difference between samples contamination to *Aspergillus* section *Flavi* and storage parameters. The ability of AF production of isolates was variable. More than 88% of isolates were able to produce one or several types of AFs and less than 12% of isolates produced no AF. HPLC assay showed that out of 21 pistachio nut samples, 3 samples were contaminated with various types of AFs, but were below the allowable levels ($P \leq 0.05$). **Conclusion:** Despite the population of aflatoxigenic strains in the mycoflora of warehouse, AF contamination of pistachios was estimated to be about 14%, which was also less than the allowable level. The results showed that the environmental parameters required for the production of AF in the warehouse of Semnan province do not exist and the storage conditions in this province are suitable.

Keywords: *Mycotoxin; Food safety; Contamination; Environmental factors; Semnan*

Introduction

The area under pistachio cultivation of Iran as one of the biggest pistachio producers of pistachio around the world in 2018 was 498693 hectares with a production rate of 172614 tons. Semnan province has 20835 hectare pistachio orchards consist of 7839 hectare non-fertile and

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12996 fertile trees. On the basis of fertile trees, Semnan ranks fifth among other provinces in Iran (3.31% of total fertile area). In 2018, Semnan produced about 15288 tons and yield was about 1176 kg per hectare also, main pistachio growth area belongs to Damghan county (Azizi *et al.*, 2020).

Aflatoxins (AFs) are toxic secondary metabolites often produced by some *Aspergillus* species, although all the strains of one species do not produce AF (Fani *et al.*, 2014a, Frisvad *et al.*, 2019). The discovery and detection of AF in the early 1960s in England occurred by the prevalence of an unknown disease, named "Turkey-X" disease that killed farm animals, including more than 100000 turkeys fed with contaminated feed (Benkerroum, 2019, Pitt and Miller, 2017). According to Benkerroum, at least 18 different types of AFs and metabolites have been identified. However, the four main types of AF with high toxicity and widespread contamination include B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2). The four AF types are separated by light colors (green or blue) emitted under UV and with relative movement in thin-layer chromatography (Bennett *et al.*, 2007). Different crops, including cereals, oil seeds, tuber crops, spices, dried fruits, also animal feed are affected by AFs (Fani *et al.*, 2014a, Frisvad *et al.*, 2019)

Species of *Aspergillus* which produce AFs according to Benkerroum, are classified into three sections, including section *Flavi*, section *Ochraceorosei*, and section *Nidulantes (Benkerroum, 2019)*. There are also significant variation in section *Flavi* on the basis of strain abilities for colonization, crop contamination, and AFs production (Frisvad *et al.*, 2019, Moghadam *et al.*, 2020, Moradi *et al.*, 2014). Although some *A. flavus* strains do not have the potential of AFs production (atoxigenic strains) (Cotty, 1990, Fani *et al.*, 2014a, Moradi *et al.*, 2011), the others are so toxic and have the potential to produce large amount of AFs more than 10000µg per kilogram (Bandyopadhyay *et al.*, 2016).

Additionally, section *Flavi* includes members which produce AF, ochratoxin, and cyclopiazonic acid (Ostry *et al.*, 2018, Pourhosseini *et al.*, 2020).

Normally, production of AFs occurs before crop harvesting and under orchard condition (Ghadarijani and Javanshah, 2005). Therefore, harvest time delay, improper process of the crop and storage conditions lead to fungal growth severity and AFs production (Gupta and Gopal, 2002, Moradi *et al.*, 2011) . The growth of *A. flavus* and the production of AFs in storage conditions on food substrates, including nuts, are affected by the gaseous composition of the environment, water activity, acidity, microbial interaction, and storage duration. For example, in the case of peanuts, storage at 0.94 water activity, 25 °C, and 10% oxygen (60:40 equilibrium ratio of carbon dioxide: nitrogen) produced the highest amount of AF in 21 days (Ellis *et al.*, 1994) .

It has been reported that the minimum relative humidity for fungal growth and AF production is provided during short time (25 hours). As the temperature of stores is low at that time in the year, the potential of producing AFs is zero. Studies have proven that in spite of the proper conditions for contamination and AFs production in the orchards, the process and storage conditions for AFs production are not suitable (Danesh *et al.*, 1979, Mehrnezhad and Panahi, 2006, Moradi *et al.*, 2004, Moradi *et al.*, 2015). For latent contamination to aflatoxigenic strains, proper humidity and temperature in next storage steps or transportation could lead to inclusive fungal development and increasing AFs concentration which is affected by contaminated pistachios in orchards, storage pests, and storage time (Bensassi *et al.*, 2010, Ellis *et al.*, 1994, Set and Erkmen, 2010). Storing pistachios in 5-7 °C and relative humidity 45-60% result in improper condition for contamination to AFs (Georgiadou *et al.*, 2012). However, drying pistachio during process leads to the reduction of kernel humidity from 50 to less than 5% that is a proper condition for pistachio storage (Set and Erkmen, 2010). This finding has been achieved in suggested models of *A. flavus* fangal growth and AFs production in pistachios, experimentally (Marín *et al.*, 2012). Another study (Moradi *et al.*, 2015), investigated the factors affecting fungal growth and AFs production in 47 stores in 4 towns of Kerman. They found that pistachio storage condition and environment affect pistachio contamination to *Aspergillus* and AFs. As a result, kernel humidity variation, water activity,

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relative humidity, and temperature during storage time in Kerman condition per a year were investigated. The data of kernel humidity and water activity in estimated stores showed that humidity equation does not affect these important agents in growth and production of AFs by *A. flavus* in kernel with short or long time storage. Pistachio warehouse in Kerman province shows proper condition. Variation range of kernel humidity amount during the storage from 1.3 to 5.3 and water activity from 0.12 to 0.46 varied in all the estimated stores. Variation range of the two mentioned agents was less than critical levels for growth and AFs production. It could be due to proper pistachio drying during pistachio process in terminals and dry weather of Kerman province (Moradi *et al.*, 2015).

This study aims to investigate the contamination of stored pistachios with *Aspergillus* section *Flavi* and AF. During this study, other effective parameters in pistachio contamination, such as temperature and relative humidity of the warehouse, percentage of pests, and length of storage period were also studied in Semnan province.

Materials and Methods

Sampling: Twenty one samples were taken from 21 pistachio warehouse in Damghan on 18-19 February 2019 based on Iranian National Standard sampling method (No. 13534 and 1036) (Fallah *et al.*, 2013). Storage types were chosen completely random and with various conditions and proper distribution. Then, 6-8 pockets with 50 kg of pistachio (include 3-4 pockets at the surface of pallet and 3-4 pockets in the center part) were chosen. For final sampling of every storage types, 1 kg/50 kg were selected and then the samples were mixed and 500 g of the samples were chosen and labeled. The prepared samples were transferred to the laboratory and estimated for existence of pest on kernels by eliminating shell and evaluating under the binocular (**Table 1**).

Aspergillus Flavus and Parasiticus Agar (AFPA) medum: Laboratory grade chemicals and ultra-pure water were used for media preparation. Aspergillus flavus and parasiticus agar (AFPA) is a selective media for the enumeration in foods of the mycotoxin producing fungi *Aspergillus flavus* and *Aspergillus parasiticus* that produce orange-yellow reverse colony pigmentation. The media contained 10 g of peptone, 20 g of yeast extract, 0.5 g of ammonium ferric citrate, 200 mg of pentachloro nitro benzene along with 15 g of agar per liter of sterile distilled water. The pH of the medium was adjusted to 6.5 after mixing and volume up to one liter, then 50 mg of chloramphenicol was added and then autoclaved at 1 atmospheric pressure and 121 °C for 15 to 20 min (Gourama and Bullerman, 1995).

Potato Dextrose Agar (PDA) medium: 39 g of PDA (Merck, Germany) was dissolved in 1 liter of water and after raising the pH to 6.5, it was sterilized using an autoclave and transferred to Petri dishes.

Fungal isolation: 100 g of kernels per every prepared sample was picked for detection of samples contamination to *Aspergillus* section *Flavi* and achieving uniform sample. The samples were grinded for 2 min. Grinded samples were cultured by serial dilution method. Therefore, 10 g of grinded kernels was mixed with 90 ml peptone water 0.1% and shake for 20-40 min, gently. Finally, 0.1 ml of the mixture cultivated on AFPA medium in completely randomized design with 3 repetitions of treatment. The plates were incubated at 25 °C for 5-7 days until the colonies of *Aspergillus* section *Flavi* were detected, counted, and isolated. By counting colonies on media, contamination rate in various samples were compared. After purification by single spore method, fungal isolates were stored on PDA slant at 4 °C for long-term and also in sterile distilled water for work.

TLC assay: TLC assay used for AF production ability of *Aspergillus* section *Flavi* isolates. Fungal isolates were cultured on rice flour to test aflatoxigenic trait. After providing fungal spore suspension of every isolate, 1 ml of spores (spore concentration of 10^3 / ml) was cultured on 22 g sterile rice flour with 25% moisture in 250 ml flasks and incubated at 28 °C for one week (Wei and Jong, 1986). To extract AF from the samples, 3 g of sodium chloride and 125 ml of 55% methanol were added to the content of each flask. The flasks were shaken for 30 min and then the contents were passed

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through filter paper (Wattman No. 4). In the next step, 25 ml of the filtered methanolic extract was transferred to the decanter funnel and 25 ml of chloroform was added to it. The contents of each decanter were stirred manually and vigorously for at least 3 min to completely transfer AF to the chloroform phase. The decanter funnels were held in place until complete separation and the chloroform phase layer was separated. After evaporating the chloroform at 40 °C by the rotary system and under the fume hood, the remaining amount of chloroform was transferred to a small tube and completely evaporated by passing nitrogen gas made from concentrated solution. In order to analyze the AF in the samples, 400 µl of puncturing solvent (including a mixture of hexane, chloroform, and acetone with a volume ratio of 90: 5: 5) was first added to each vial and their contents were shaken vigorously with the help of a tube shaker. Then, with the help of a capillary dispenser, the sample step was performed on the TLC silica gel plate (F254). To identify AFs, a mixture of group B and G AF standards was scored on each plate. In order to isolate AFs, the plates were placed in tanks containing separation and emergence solvents containing a mixture of chloroform and acetone in a volume ratio of 90:10 and in the dark. After separating the sample components (when the mobile phase surface reached about 1-2 cm at the upper end of the plate), the plates were kept in the dark for a short time to evaporate the mobile phase and then were examined under a wavelength of 366 nm UV in the device (UV Cabinet, CAMAG, Switzerland) (Sweeney and Rexroad, 1987).

HPLC assay: Assessment of AF production in pistachio were 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. The excitation and emission wavelengths for detection were 365 nm and 435 nm, respectively. To this end, pistachio samples were slurred up with water in a ratio of 1/3 for 15 min, and then slurred samples were extracted (30 g) with 90 ml of pure methanol on the blender (Waring, USA) for 3 min and filtered through filter paper No. 4. Filtered liquid (8 ml) were mixed with phosphate buffer (42 ml). Immunoaffinity columns (VICAM; Milford, MA 01757 USA) were used for purification of the samples. Clean up was performed according to the factory instruction. Finally, 200 µl of the preparation was injected into the HPLC apparatus (Fani *et al.*, 2014b). AFs B1 and B2 was measured by comparing the peak areas with a calibration curves obtained by AF 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 0.4–10 ng/ml AFs B1. In the case of mobile phase HPLC, the methanol/water (40/60) was used for the derivation of potassium bromide, nitric acid, and Kobra cell. The chromolite column (10 cm) with an internal diameter of 4.6 mm (Partisil 5 ODS3, USA) was used. The column temperature was set to 35 ºC with a moving phase of 2.5 mL/min. Fluorescence detector was set at wavelengths ex=365 nm and em=355 nm.

Data analysis: The data were analyzed with the SPSS statistical analysis system. Mean scores were separated using Duncan's multiple range tests at P-value ≤ 0.05. The Excel software was used for chart drawing.

Results

Storage condition: In the current study, some of environmental parameters affecting *A.* section *Flavi* growth and AF production in 21 selected warehouses were estimated. Investigating the warehouses ventilation conditions showed that 38% of the warehouses had fan and the others had windows or some valves. No damaging agents were found, including mouse, bird, and insect in any warehouse. The pistachio nuts in total warehouses varied from 4 bags (70 kg) to more than 400 bags. In more than 52% of the warehouse, pistachio nuts were kept on the pallets not on the surface. The good transfer period from storage to market ranged between 4-5 months. Analysis of two important factors in this research showed that the range of warehouses

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temperature varied 12-17 °C and relative humidity was 29-40%. Results of two parameters with mean counted colonies of *A.* section *Flavi* are shown in **Table 1**.

Contamination to A. section Flavi: Out of 21 stored pistachio nut samples, *A.* section *Flavi* was isolated from 17 samples and no contamination was observed in 4 samples. In some samples, *A. niger* also grew and in most cases grew faster (**Table 1**, **Figure 1**). A statistically significant difference was detected between *Aspergillus* section *Flavi* colony and warehouses ($P \leq 0.05$, **Table 2**).

The results showed that, the most number of colonies belonged to U warehouse and the least belonged to M, I, E, and R warehouses. No fungal colonies were isolated from pistachio nut samples from warehouses B, D, P, and T (**Figure 2**).

According to very low numbers R^2 square in linear graphs of degree 2 and 3 and logarithmic chart, no significant relationship was observed between warehouses temperature and humidity with colony numbers of *A.* section *Flavi* (**Figures 3**, **Figures 4**).

The results of aflatoxigenic potential of *A.* section *Flavi* isolates showed that the potential of toxin production by isolates are different. Out of 45 isolates, 40 isolates (88.88%) produced one or several AFs (B_1, B_2, G_1 and G_2) and 5 isolates (11.11%) produced any type of AF. Among fungal isolates, 17.5% isolates (7 isolates) produced four types of AFs (B_1, B_2, G_1 and G_2), 12.5% of the isolates (5 isolates) AFs (B_1, B_2 and G_1), 37.5% isolates (15 isolates) AFs (B_1, B_2) and 32.5% isolates (13 isolates) produced only AF B_1.

By evaluating the possibility of AF rate in pistachio nut samples using HPLC method, out of 21 tested samples, 3 samples were contaminated to all types of AFs. The results showed that 3 samples were less than the maximum permitted level (total AF less than 15 ng/g and B_1 AF less than 4 ng/g). Therefore, 14.28% collected samples were contaminated by AF. In 3 contaminated samples, 2 samples were contaminated to B_1 and B_2 and one sample to B_1 AF.

Table 1. Warehouse status and mean colonies of *Aspergillus* section *Flavi*

Warehouse No.	Code	Cultivar	Temperature (°C)	Relative humidity %	Storage period (month)	Pest contamination %	*Aspergillus* section *Flavi* (CFU/g)
1	A	Shahpasand	13	37	5	6	323.33
2	B	Mixed	15	35	5	1	0
3	C	Akbari	16	35	4	1	263.3
4	D	Abasali	16	37	5	0	0
5	E	Akbari	16	29	5	1	170
6	F	Akbari	17	38	5	3	290
7	G	Kalleghoochi	12	40	4	1	460
8	H	Shahpasand	14	39	4	4	470
9	I	Khanjari	13	33	4	0	190
10	J	Abasali	12	34	4	0	223.3
11	K	Khanjari	13	36	4	3	653.3
12	L	Khanjari	14	39	4	2	563.3
13	M	Shahpasand	12	34	5	0	176.7
14	N	Mixed	13	36	5	0	610
15	O	Khanjari	14	37	5	1	263.3
16	P	Akbari	14	31	5	0	0
17	Q	Mixed	13	38	5	2	466.7
18	R	Akbari	14	33	5	2	143.3
19	S	Khanjari	12	31	5	2	623.3
20	T	Akbari	13	32	5	1	0
21	U	Shahpasand	14	38	5	2	843.3

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Table 2. AFs contamination rate (G_2, G_1, B_2, B_1) in pistachio samples

Sample code	AF content (ng/g)				
	B_1	B_2	G_1	G_2	Total
F	3.11	1.27	ND	ND	4.38
K	2.93	1.03	ND	ND	3.96
L	3.39	ND	ND	ND	3.39

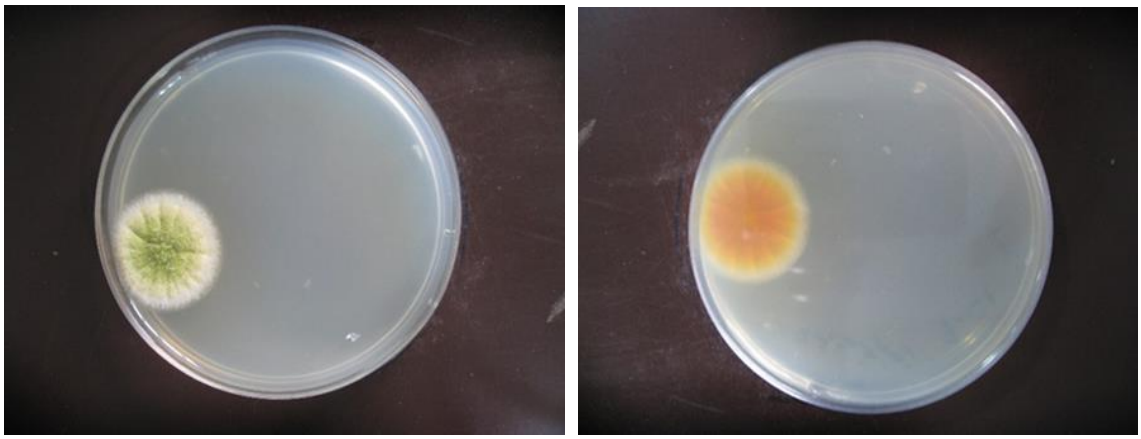

Figure 1. Growth of *Aspergillus* section *Flavi* on AFPA medium

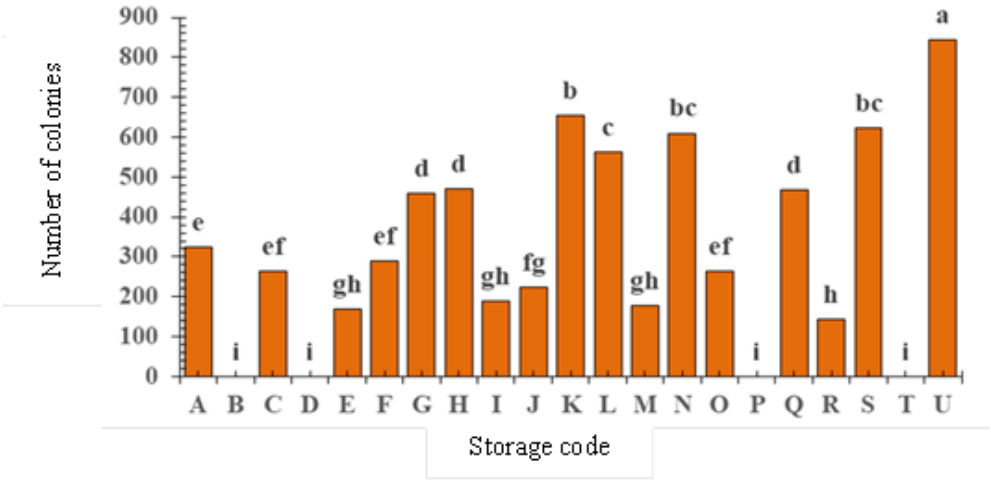

Figure 2. Number of *Aspergillus* section *Flavi* colonies isolates of pistachio nut samples from different warehouses
* Different letters indicate significant differences according to Duncan's multiple range test at P=0.05)

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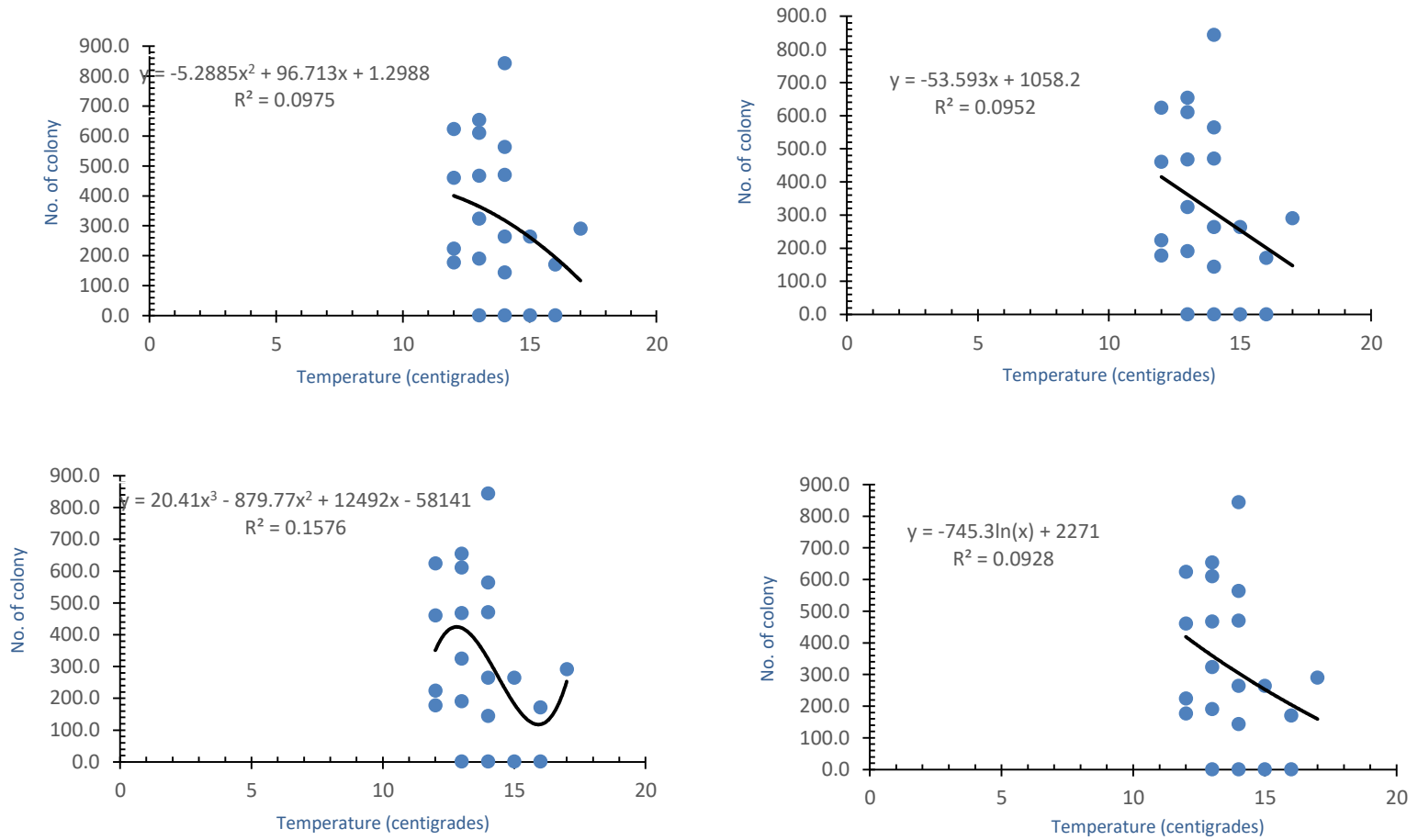

Figure 3. Evaluating the relationship between warehouse temperature and colony number of *Aspergillus* section *Flavi* using various degrees of regression chart

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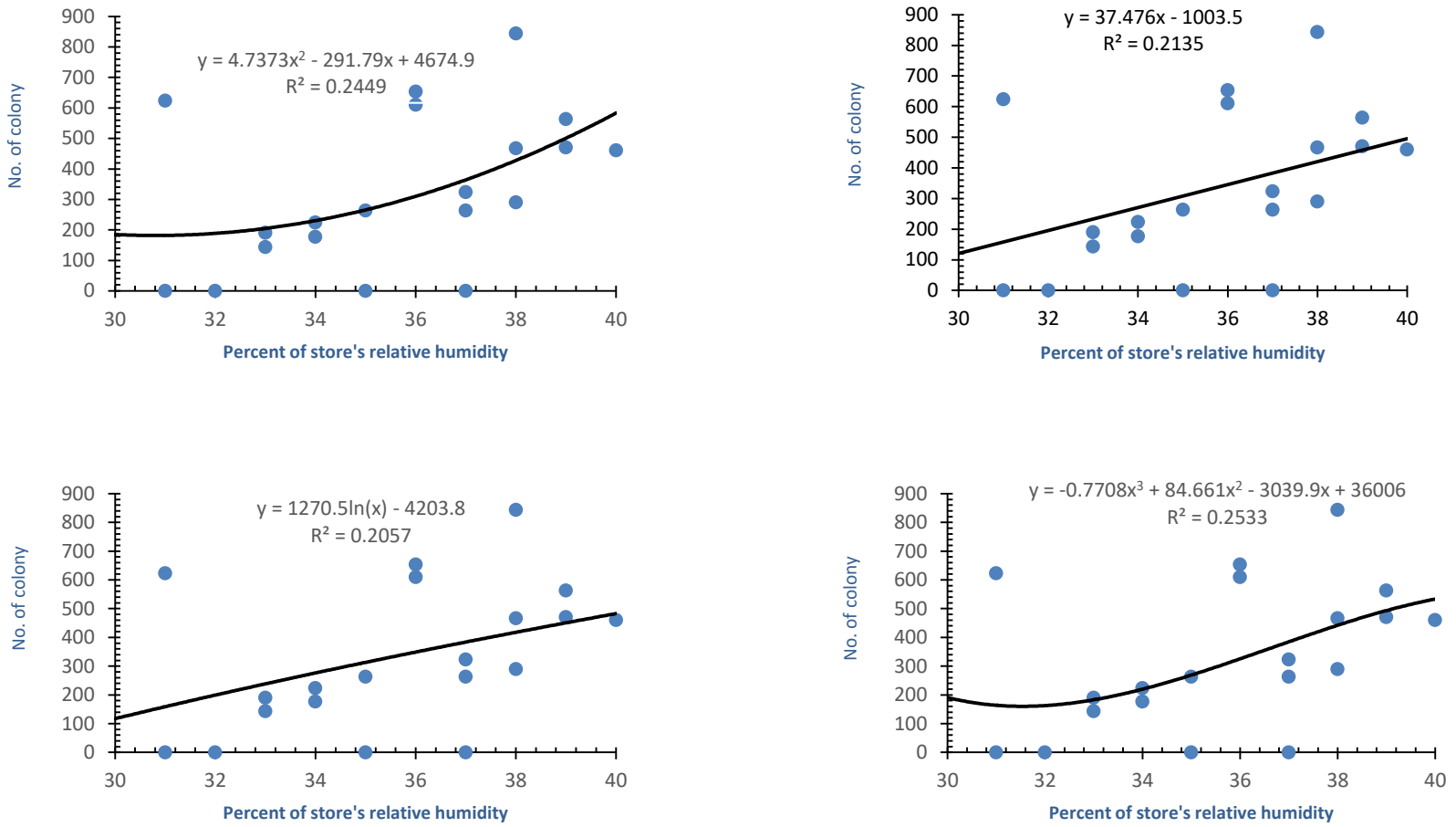

Figure 4. The relationship between relative humidity percentage of warehouses and colony number of *Aspergillus* section *Flavi* using various degrees of regression chart

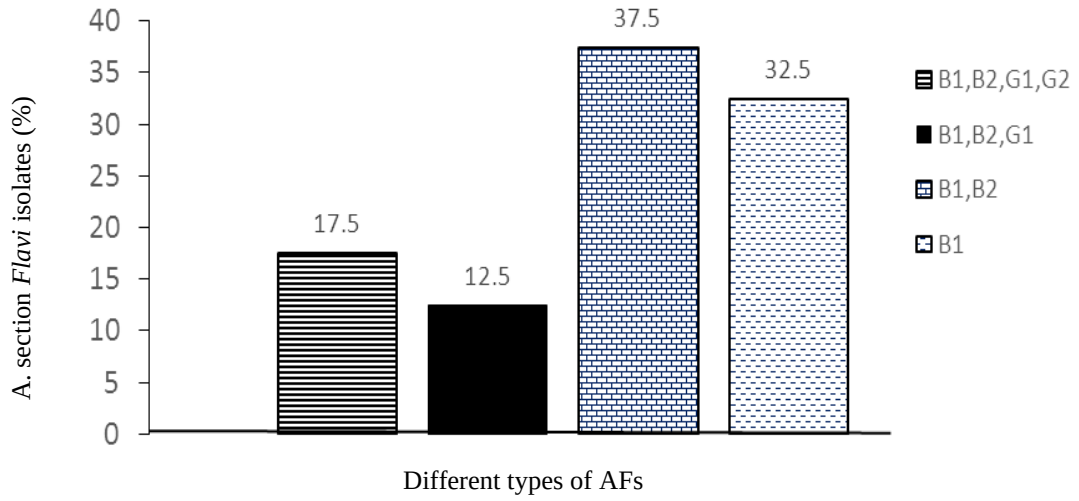

Figure 5. AF production potential of *Aspergillus* section *Flavi* isolates

Discussion

One of the effective stages in production to pistachio supply chain is storage period and storing until the arrival to market that its role on the basis of quality and quantity in any production zone should be investigated. Therefore, some warehouses in Damghan were chosen as main storage sources in Semnan province and during sampling, effective factors on storage were investigated, including temperature, relative humidity, storage time, etc. Generally, variation range of humidity of the warehouses varied from 29-40 % in 21 investigated warehouses. However, variation range was lower than effective critical values in growth and AF production that is due to low humidity of the region and totally cold and dry weather of Damghan in autumn and winter.

Moisture content of pistachio nut at harvest time varies from 30% to 45%, at which moisture levels of different species of *Aspergillus* are able to grow and produce AFs and compete with other microorganisms. The amount of pistachio nut moisture decreases in a short time during the processing. The moisture content of pistachio nuts in the samples collected from Kerman and Isfahan provinces was between 3% and 6%, respectively (Mojtahedi *et al.*, 1980, Moradi *et al.*, 2011). Storing pistachio nuts at a temperature of 5 ˚C and relative humidity of 32-33% or a temperature of 15 ˚C and relative humidity of 65-70% is considered appropriate and increases its shelf life (Tavakolipour *et al.*, 2009). The trend of changes in the amount of moisture in pistachios is decreasing, which continues with a faster speed by increasing temperature.

According to storage duration in the studied sites and estimating storage condition of pistachio that is traditional and without refrigerator equipment, with measurement of above parameters and calculating their association with sample contamination level to *A.* section *Flavi*, no significant relationship was found between the factors and contamination level. In other word, the numbers reveal that effective factors in fungal growth did not have the potential of contaminating. In addition, the significant relationship between repetitions proves uniform storage condition during storage time.

This indicates that despite the conditions for contamination and production of AFs in the orchard, processing and storage conditions are not suitable for the production of AFs. The distribution of AF in a pistachio mass is related to its sources and in the later stages of storage or transportation, if the conditions are suitable, contamination can occur due to dormant infection with AF-producing fungi (Georgiadou *et al.*, 2012, Mehrnezhad and Panahi, 2006, Moradi *et al.*, 2011, Moradi *et al.*,

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2015).

The results of this study show that belonging of isolates to *A.* section *Flavi* only could not be the reason of aflatoxigenicity. Out of 45 studied isolates, 5 isolates had the potential of no type of AF. Total HPLC test results also showed no type of contamination to total types of AFs among more than 85% of the samples and in 3 contaminated samples, the contamination was detected lower than the limited level. Other parameters and effective factors include relative humidity and temperature, late harvesting, unsuitable processing with delay, transformation condition, and etc. The parameters could have significant relationship with the collected samples and also AF contamination. However, the current study suggests more research on the mentioned subjects.

A study of the relative humidity of warehouses in Rafsanjan and the possibility of contamination of pistachios with AF after harvest showed that due to the low relative humidity of the warehouse, *A. flavus* spores, which are present on most warehouses, have the necessary opportunity to grow and do not cause AF production (Danesh *et al.*, 1979).

Conclusion

The current data of humidity and temperature in the estimated warehouses proved that with an average storage period of 140 days, moisture balance had no effect on growth and AF production by *A.* section *Flavi* in pistachio kernel. Storage of the crop in Semnan province is in suitable condition. Estimating the relative humidity in each warehouse indicates that it is low, which does not increase the humidity of pistachio kernel in the temperature range studied in this study (ie, 12 to 17 degrees Celsius). Considering the dry climate of Semnan province, the possibility of moisture exchange of pistachio kernel with the environment, which leads to an increase in its humidity, is very low. This issue is of particular importance. This is because stored pistachios may have dormant contamination with *A.* section *Flavi* or other microorganisms, and if the conditions are suitable, the contamination will appear in bulk, and if the pistachios are stored for export, the return of the consignment of pistachio nuts will not be surprising.

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Author's contributions

Salehi Y designed the study; Sobhanipour A and Mohammadi-Moghaddam M conducted the experimental work, Fani SR analyzed the data, Salehi Y and Fani SR wrote the manuscript. All authors revised and approved the final manuscript.

Conflicts of interest

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

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