The Population Density Effects of Aspergillus Spores on Pistachio Seeds in Aflatoxin Production

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Abstract

Introduction: Nuts are from among the most popular snacks worldwide and constitute a major part of Iranians' food habits. The natural contamination of nuts with aflatoxins is unavoidable and poses a special challenge to food safety. Aflatoxins are carcinogenic secondary metabolites that are mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus* and frequently contaminate food stuffs, such as pistachios. To find a suitable method for tracing and predicting the production of aflatoxins by fungal species in pistachios, especially in the field of exportation, this study was conducted to determine the ratio of the number of fungal spores to the sample value of pistachios.

Materials and Methods: Firstly, some suspensions containing 5 to 5×10^6 spores of *A. flavus* were prepared and then used for the inoculation of 10 grams of pistachio nuts. After inoculation, the samples were kept under optimal conditions. Sampling was done 24 hours after inoculation and performed in six days. Pistachio nut slurries were extracted using methanol, water, and hexane (30ml, 7.5ml, and 10ml), and aflatoxins were analyzed using the high performance liquid chromatography (HPLC) method.

Results: The results showed that the amount of aflatoxins inoculated at the concentration of the 10^6 spore suspension was more than the standard values after day 5.

Conclusion: It can be concluded that the use of mechanized pistachio processing lines accompanied with the proper use of pistachio washing methods can contribute to the reduction of the fungal sporulation of inoculums, thereby reducing the production of aflatoxins. Findings of this paper can support decision making at transport and storage levels for producers and processors to predict the time for AFB1 production by *A. flavus* in pistachio nuts in the postharvest phase.

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Introduction

Aflatoxins (AFs) are potent, carcinogenic, mutagenic, and teratogenic metabolites produced primarily by some fungal species, such as *Aspergillus flavus* and *Aspergillus parasiticus*. Foodstuffs, especially in warm climates, are prone to the invasion by the species of aflatoxigenic fungi and the subsequent production of aflatoxins at harvesting, processing, transportation, and storage stages. The four major naturally produced AFs are Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), Aflatoxin G1 (AFG1), and Aflatoxin G2 (AFG2). Letters "B" and "G" refer to blue and green fluorescent colors produced by these compounds under UV light on thin-layer chromatography plates, while subscript numbers 1 and 2 indicate major and minor compounds, respectively [1]. *A. flavus* is more adapted to the aerial parts of plants (leaves, flowers, and fruit) and produces only B AFs, while *A. parasiticus* produces both B and G AFs and is well adapted to the soil environment [2]. However, the mold infestation level and the identification of common species are from among the major parameters that may show the quality of the material and the future potential for the presence of mycotoxins. The Institute of Standards and Industrial Research of Iran (ISIRI) has set an MTL of 5 and 15 ng/g for AfB1 and total aflatoxins, respectively. The European Union has set limits for AFB1 and total AFs at 2- 4 and 10 ng/g respectively, in pistachios [3- 5]. Therefore, mold counts are included in the quality control assurance of many foods. The current methods used in assessing the mold presence are time-consuming, labor-intensive, and costly; besides, they require mycological facilities and expertise. Because of the carcinogenic potential of aflatoxins, there is an urgent need for developing detection methods that are rapid, repeatable, and highly specific.

The fungal contamination of pistachios may occur at three times. Firstly, it may occur when the nuts are on trees. In this case, ripe pistachios are more susceptible to contamination by the windborne and insect-borne spores of fungal species [5]. Secondly, at the postharvest stage, when nuts are de-hulled, washed, and sorted, they are also susceptible to contamination. The washing water can be a source of contamination, and if the nuts are left wet, they will be contaminated. Thirdly, contamination can occur during storage periods, especially when nuts are stored under adverse conditions of temperature and relative humidity [6]. In brief, when nuts are on trees, the concentration of aflatoxins is generally low; however, the high levels of humidity and temperature in bulk bins provide ideal conditions for the contamination of cracked pistachio fruits [7]. In general, nuts with thick shells, like macadamia nuts, are better protected against the intrusion of molds. Other nuts, like pistachios, are most susceptible to mold infestation due to shell splitting at the end of the maturation stage [8]. The sorting and elimination of split nuts can decrease significantly the contamination of mycotoxins [9]. Studies conducted on hazelnuts and pistachios suggested that the optimum temperature and RH for the production of AF

were 25– 30ºC and 97– 99%, respectively. Hence, the high levels of humidity and temperature are more suitable for the production of toxins [10, 11]. As a result, the climate of Mediterranean countries is most favorable for mold infestation and mycotoxin synthesis [1, 12].

The mycotoxin contamination of foodstuffs and feedstuffs has been studied in Iran [11, 13-15]. To investigate the importance of the inoculum source in pistachio contamination, the quantitative analysis of the relationship between the density of the pathogen or propagules in nuts and the aflatoxin levels of nuts is critical in epidemiological studies.

2. Materials and Methods

2.1. Fungal isolates and culture conditions

Aspergillus flavus isolates used in this study were obtained from the Mycology Culture Collections of our laboratory. The isolates were maintained by sub-culturing on Potato Dextrose Agar (PDA) (Sigma Aldrich) at 28±1°C for 3- 4 days. Fungal spore suspensions were obtained by suspending a slice (0.5×0.5 cm) of the fungal culture in 5ml of sterile distilled water, centrifuged for one minute, and taken from Whatman paper No. 4 to remove additional components, such as media and mycelia, and were then stored as spore suspensions in 15% glycerol, at −80°C.

2.2. Preparation of pistachio nut samples

Pistachio nuts, of the Kalehquchi cultivar, were selected for the experiment. To ensure the non-contamination of the samples with aflatoxins, the healthy and non-split pistachio nut samples were used. Before inoculation with aspergillus spore concentrations, the samples were disinfected with sodium hypochloride (10%).

2.3. Preparation of aspergillus spore suspensions

Aspergillus spore suspensions at the concentration of 5 to 5×10^6 spores/mL were produced by suspending a plug of 7-day old culture of aspergillus grown on PDA in 5ml of sterile distilled water, centrifuged for one minute, and then taken from Whatman paper No. 4 to remove additional components, such as media and mycelia. The spores were quantified using a haemocytometer and then diluted serially in Milli Q water with 0.01% Tween 20. Next, the suspensions containing 5 to 5×10^6 spores were used.

2.4. Sample inoculation

For the inoculation of the samples, two milliliters of the dilution series with a specific spore number were used to inoculate 10 grams of pistachios seeds.

2.5. Aflatoxin analysis

2.5.1. Preparation of the aflatoxin standard solution

The standard mix was diluted with methanol to obtain the concentration of 10 ng ml^{-1} for AFB1, AFB2, AFG1, and AFG2. From this intermediate solution, a series of working standard solutions were prepared at the concentrations of 0.1, 0.2, 0.4, 1.2, 2, 4, and 5 ng ml^{-1}, freshly in the LC mobile phase.

2.5.2. Aflatoxin extraction

The samples were analyzed by the high performance liquid chromatography (HPLC) method (AOAC official method 999.07) [16]. Regarding the HPLC analysis, pistachio nut saps were extracted with methanol, water, and

hexane (30ml, 7.5ml, and 10ml). They were then filtered, with the extract diluted with water and filtered through Whatman filter paper no 2. For the cleanup of the samples, Aflatest IACs were used. Firstly, 10ml of the phosphate buffer saline (PBS) was passed through the IAC. Next, 5ml of the filtrate was passed through it at the flow rate of ca. 1 drop/s. The column was washed with 10ml of water and dried by vacuum drying application. In the end, AFs were eluted with methanol by the following procedure, i.e., firstly, 0.5ml of methanol was applied to the column, which was passed through by gravity. Secondly, after one minute, the second portion of the 0.75ml of methanol was applied and collected. The elute was then diluted with water and analyzed by HPLC.

2.5.3. HPLC conditions

The limit of detection (LOD) and the limit of quantitation (LOQ) were defined as the concentration obtained by calculating the standard deviation of the calibration curves of aflatoxins, multiplied several times. Chromatographic analyses were performed using a Hewlett-Packard 1090 series II L instrument (Agilent Technologies Palo Alto, CA, USA), equipped with an auto-injector and an HP FLD series 1100 fluorescence detector. Excitation and emission wavelengths were 365 and 455nm, respectively. Agilent SUPELCOSIL LC-18 and the HPLC Column (Agilent Technologies, 150- 406 mm, 5µm particles) were used. The mobile phase consisted of water, methanol, and acetonitrile (63:26:11), and the flow rate was 1.0ml min^{-1}, with a volume of 20ml injected. Under these conditions, retention times were approximately 2 minutes and 2.4 minutes for AFB2 and AFB1, respectively.

2.5.4. Calibration curves and standard solutions

Calibration curves were determined for the analysis of aflatoxins, using standard solutions. Standard solutions were used at four concentration levels (from 20 to 1000 Ag ml^{-1}), with three concentrations applied to each level. Linear calibration graphs were obtained within the range studied by plotting the peak areas against the aflatoxin amount injected under the conditions mentioned above. Aflatoxin quantification was conducted by comparing the peak areas with the calibration curves (Fig 1).

3. Results

The results implied that the number of *A. flavus* spores in pistachio samples played an important role in the contamination and increase of the aflatoxin concentration. Regarding the spore numbers of 10^0 to 10^5 from each 10 grams of pistachio samples, after six days, the aflatoxin concentration was less than the detectable concentration, with the aflatoxin content being detectable only at the concentration of 10^6. The results are shown in diagram 1. Fungal colonies were identifiable on the samples of the inoculated pistachios at different concentrations after 15 days of incubation, under optimal environmental conditions (Fig 2).

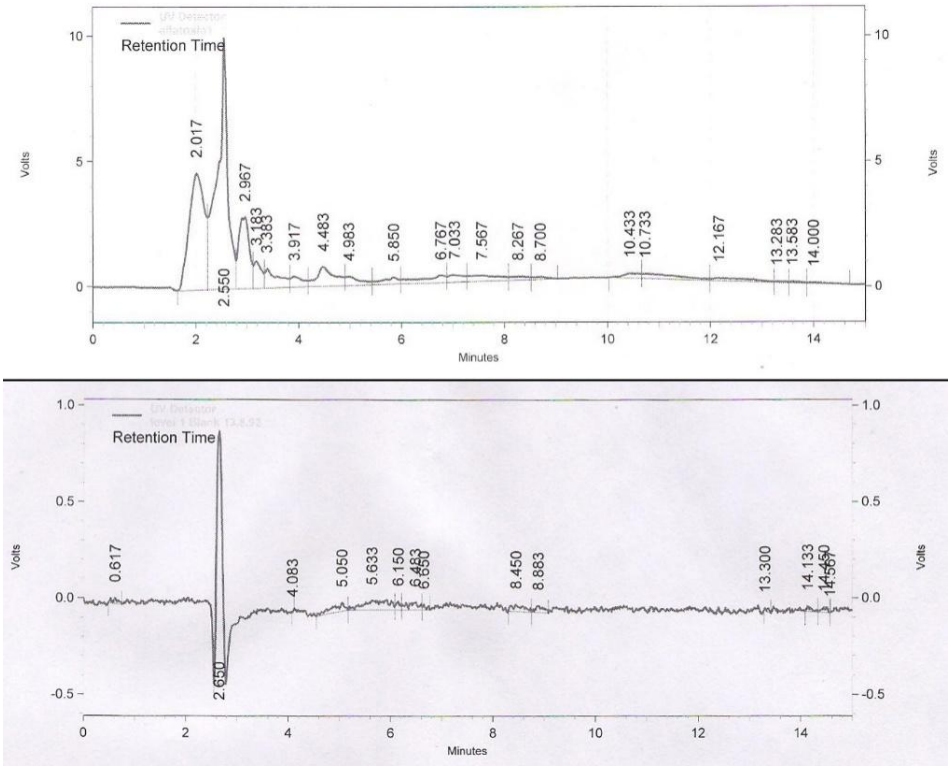

Fig1. Standard sample (bottom) along with a sample extracted (above) from pistachio nuts to determine retention time.

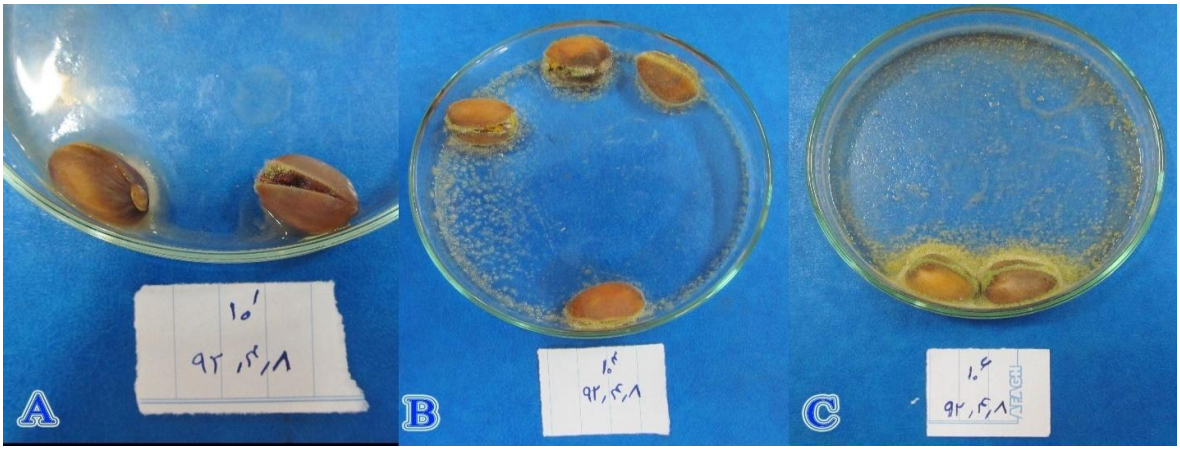

Fig 2. Infected pistachio samples with three spore concentrations of 10^1 (A), 10^2 (B) and 10^6 (C) growth results of *A. flavus* after 15 days under optimal environmental conditions.

4. Discussion

The proportion of the spore concentration to AFB1 produced can be shown by reducing the fungal inoculum and using appropriate tools during the pistachio processing phase; besides, the use of the proper structure of the industrial processing of pistachios can reduce the population of fungi and the amount of aflatoxins, especially during the storage and export phases of this product. Regarding groundnuts, the roasted groundnuts in shells also showed a lower contamination level than the raw shelled groundnuts. This observation indicates that roasting and heating processes may reduce the level of the initial inoculum, and that the presence of the shells can act as a protective layer to shield the nuts from contamination [17]. AF contamination has been reported in different types of nuts. Chun et al. (2007) reported that nut samples in South Korea were contaminated with AFs (10.6% of the incidence) within the range of 0.20–28.2 lg/kg. In China, peanuts were found to be contaminated with AFs, with the average level and highest level being 80.3 lg/kg and 437 lg/kg, respectively [19]. In Turkey, Yentur, Er, Ozkan, and Oktem (2006) reported that the levels of AFB1 and total AFs in peanuts were within the range of 2.06–63.7 ng/g and 8.16–75.7 ng/g, respectively. According to Cheraghali et al. (2007), 11.8% and 7.5% of 10,068 Iran's pistachio nut samples contained AFB1 and total AFs, respectively, over the maximum tolerated level (MTL). The incidence and levels of AFs found in this study under optimal conditions were relatively high compared to the values quoted in the literature. Considering the amount of the spore contamination of the fungi producing aflatoxins, it is possible to create conditions that prevent the production of secondary metabolites, such as aflatoxins, by tracing and identifying them at the early stages of contamination. The incidence of AFs in foods is relatively high in tropical and subtropical regions, where warm and humid climates provide optimal conditions for the growth of the molds [17]. Therefore, the higher incidence rates and levels of AFs found in the sample inoculated with 10^6 spores in this study are probably associated with the high temperature and humidity in the sample conditions.

The fact these fungi are often found in substrates, such as corn, nuts and wheat, emphasizes the importance of the identification of these species. This will let institutions check the amount of the fungal inoculum to predict the production of AFB1 as well as AFG1, AFG2, and AFB2.

5. Conclusion

This study helps estimate the relative occurrence of aflatoxins in crops and predict the toxin profiles that may be produced. This will improve the diagnosis at an early stage and integrates all critical control points in HAPPC strategies. In addition, these assays help identify the proportion of the number of spores in the initial substrate to the amount of aflatoxins, which can be used at quarantine stations to forecast contamination. The results of the present paper will also help gain some knowledge about these species in terms of eco-physiological factors as well as distribution or

host/matrix preferences to improve the strategies of preventing and controlling fungal colonization and the aflatoxin risk in food stuffs.

Conflicts of interest

The authors declare no conflicts of interest.

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