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ORIGINAL ARTICLE

Effect of Packaging and Storage Temperature on the Population of *Aspergillus* section *Flavi* and Aflatoxin Production in Fresh Pistachios

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ABSTRACT

The study was performed to investigate the *Aspergillus* growth and aflatoxin production in fresh pistachios stored in different packagings. Forty kg of fresh pistachios of the Abbasali cultivar were sampled and kept in different temperature conditions including 4°C and ambient conventional temperatures in plastic baskets or polyethylene plastic bags. Sub-samples were taken at 4 days intervals for 32 days to measure aflatoxin and evaluate the population of *Aspergillus* section *Flavi*. Aflatoxins were quantified by HPLC and the fungal population was monitored by dilution series method and AFPA medium. The results showed that on the first day of storage in all conditions the amount of aflatoxin was undetectable. Aflatoxin infection started on the fourth day after storage in the ambient conventional temperature and increased over time. The lowest production of aflatoxin B_1 was in the 4°C treatment. Pistachios stored at ambient conventional temperature and packed in plastic baskets showed the highest amount of aflatoxin B_1 production. The effect of the treatments on the production of aflatoxin B_1 showed that the samples in polyethylene plastic bags at ambient temperature were the highest. The results showed that in the 4°C treatment, the fungal population was significantly less than in ambient temperature treatments. The lowest fungal population was in the 4°C plastic baskets packaged treatment. This treatment was not significantly different from the 4°C polyethylene bag treatment but was significantly different from other treatments ($P \leq 0.01$). In general, keeping fresh pistachio at 4°C and suitable plastic is trustworthy. Storage for more than 8 days in the ambient conventional temperature greatly increases the risk of fungal and aflatoxin contamination.

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Introduction

Considering the massive growth in the human population until 2050, food preservation and preventing food loss are among the strategies to improve food security (Mohammadi-Benaruiyeh and Sharifi-Sirchi, 2021; Habibie *et al.*, 2019). Twenty percent of the world's food products are contaminated by mycotoxins annually, with aflatoxins being the most common. The damage caused by the destruction of food and crops by this toxin is estimated to be over $ 100 million per year (Ehrlich *et al*., 2003). In Africa, more than $ 221 million is the annual cost of aflatoxin contamination (Gbashi *et al*., 2018). Pistachio is one of the most delicious nuts in the world. Its annual production in the world is about 1,375,770 tonnes. Iran, the United States, and Turkey are among the major pistachio-producing countries (FAO 2020), therefore are of great economic importance (Shamshiri and Hasani, 2015; Sharifkhah *et al*., 2020). Contamination with aflatoxin has been a major challenge for consumer and international trade. Various methods have been recommended for the management of *Aspergillus* or Aflatoxin contamination of different crops such as agronomic, mechanical, physical, and biological strategies, each with its disadvantages and advantages depending on location, time, product type, usability, and efficiency (Barkai-Golan and Paster 2011, Brans 2011, Moradi and Hokmabadi 2011).

One possible strategy to reduce postharvest contamination and crop loss is the development of appropriate packaging (Abbaszadeh *et al*., 2018; Hadadinejad *et al*., 2018; Talukder *et al*., 2020)

So far 16 *Aspergillus* species are capable of producing aflatoxins B_1, B_2, G_1, and G_2. The most important of these is *Aspergillus flavus* (Frisvad *et al*., 2018). This species has well adapted to harsh environmental conditions, is globally dispersed, and is more important than other species. The fungus spends most of its life cycle in the soil, in the form of saprophytic in animal and plant organic matter (Amaike and Keller, 2011). Mycelium is the main structure of the fungus in the soil and its long-term survival is made by sclerotium production. In addition to aflatoxin, this fungus can produce more than 14 other types of mycotoxins (Amaike and Keller, 2011; Frisvald *et al*., 2018). Aflatoxin biosynthesis is subject to various environmental conditions including light, temperature, acidity (pH), nitrogen source, carbon source, and metals (Mahbobinejhad *et al.*, 2019) Understanding the association of these agents with aflatoxin biosynthesis is crucial in determining the role of aflatoxin in the fungal ecology. This can help identify target sites for controlling aflatoxin production. Among environmental factors, light plays a role in regulating the expression of aflatoxin biosynthetic genes and forming resistant structures such as sclerotium (Mirabolfathy *et al*., 2005). Acidic conditions are more favorable for aflatoxin production than alkaline conditions, and aflatoxin gene transcripts in alkaline conditions are negatively regulated compared to acidic conditions. Metals, especially zinc, are important and indispensable factors for aflatoxin production (Keller *et al*., 1997). A mixture of copper, iron, and zinc enhances gene expression and aflatoxin production. The optimum temperature for aflatoxin production is 28-30°C and when the temperature approaches the optimum temperature for the growth of the fungus (37°C), aflatoxin production decreases. Decreased aflatoxin production also occurs at temperatures below 18°C (Cuero *et al*., 2003). Studies show that when temperatures rise above or below the optimum temperature for fungal growth, aflatoxin production decreases linearly. This is due to reduced transcription and expression of some regulatory genes such as *afl*R (Liu and Chu, 1998).

According to research, spores of aflatoxin-producing fungi are found in soil, air, orchards, around the processing site, and pistachio storage. Therefore, the contamination of pistachio to *Aspergillus* and aflatoxin

is primarily pre-harvest and under field conditions (Moradi *et al*., 2010). Green skin as a natural barrier protects the pistachio nut against external factors, especially *Aspergillus*, but due to various factors, this green skin may be cracked and exposed to the air, causing contamination and producing aflatoxin. The cracking of pistachio is affected by various factors such as irrigation stress, cultivar, harvest time, bird damage, soil texture and structure, nutrient balance in the soil (Moradi and Hokmabadi, 2011). The presence of cracked pistachios, delays in harvesting, or any damage that causes airborne spores to build up in the pistachio nut are the basis of early infections. Improper processing, storage, and transportation can lead to increased fungal growth and increased aflatoxin production. Research on the ecology of *Aspergillus* species in pistachio gardens has shown that *Aspergillus* species populations throughout the year are affected by factors such as the number of pistachios thrown on the ground, male pistachio inflorescences, plant residues such as pistachio fruits, Irrigation intervals, horticultural operations and animal fertilizers (Moradi *et al*., 2004). The presence of aflatoxin in a pistachio mass is correlated with the number of pistachio fruits abandoned, the rate of pest infestation, the appearance, and the damage to the pistachio hull (Ghahdarijani and Javanshah, 2006). Factors that contribute to the development of post-harvest fungal contamination include temperature, pest infestation, rodents, microbial interaction, atmospheric gas composition, environmental humidity, and moisture content of pistachio fruit during processing, storage, and transportation (Moradi *et al*., 2015).

Materials and Methods

Sampling

Abbasali cultivar, which is one of the most important commercial pistachio cultivars in the Damghan region, was sampled for assays. Samples were obtained from 20-year-old trees in Damghan Pistachio Research Station, Semnan/Iran, and transferred to the laboratory immediately. The rootstock of the trees was Badami Rize Zarand. Samples taken in late September that about 70 to 80% of the upper skin of the pistachio is easily separated from the inner hard skin and the green skin of the pistachio changes color to red, pink, or cream at this time. Sampling was done randomly from all parts of the trees. 40 kg of freshly prepared pistachios divided into 4 parts. Then these 4 parts were placed in 4 different conditions; (1) refrigerator conditions (3.5±0.5°C) inside the conventional plastic baskets, (2) refrigerator conditions inside the polyethylene plastic bags, (3) ambient temperature conditions (25±2°C) inside conventional plastic baskets, and (4) ambient temperature conditions inside the polyethylene plastic bags. Every four days until 32 days, 1 kg subsamples were taken and dried for aflatoxin quantification. Also, 30 grams of pistachios were sampled to monitor *A. flavus* population.

Experimental design

The assay was performed as a factorial experiment in a completely randomized design with 4 replications. The first factor in this study was temperature, which was done at the two levels: ambient (25±2°C) and refrigeration (3.5±0.5°C) temperatures. The second factor was the type of packaging, which included the treatments in 2 levels: storing fresh pistachios in conventional plastic baskets and polyethylene plastic bags. The aflatoxin production and the fungus growth were measured in 9 steps during pistachio storage time. The first stage of the evaluation was on the first day of storage and the next 8 stages were performed 4 days apart. Thus, the last samples were assayed 32 days after the first day of storage.

Aflatoxins measurement of contaminated pistachio kernels

Pistachio kernels were dried in the oven to prevent further growth of *A. flavus* and aflatoxin production. Quantification of aflatoxin was measured using Waters e2695 (USA) high-performance liquid chromatography (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. 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 a filter paper No. 4. Filtrates (8ml) were mixed with phosphate buffer (42ml). Immunoaffinity columns (VICAM; Milford, MA 01757 USA) were used for the purification of samples. Cleanup was performed according to the factory's instructions. Finally, 200 µl of the preparation was injected into the HPLC apparatus (Fani *et al*., 2014). Aflatoxins $B_1$and B_2 was 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) was used for the derivation of potassium bromide, nitric acid, and Kobra cell. The chromolite column (10cm) with an internal diameter of 4.6 mm (Partisil 5 ODS3, USA) was used. The column temperature was set to 35ºC with a moving phase of 2.5 ml min^{-1}. The fluorescence detector was set at wavelengths ex=365 nm and em=355 nm (Iranian National Standard, No. 6872). The detection limit was 0.4 ng g^{-1}, based on a signal-to-noise ratio of 3:1. Aflatoxin standard chromatogram and AFB_1 and AFB_2 chromatogram of contaminated pistachio samples are shown in Fig. 1.

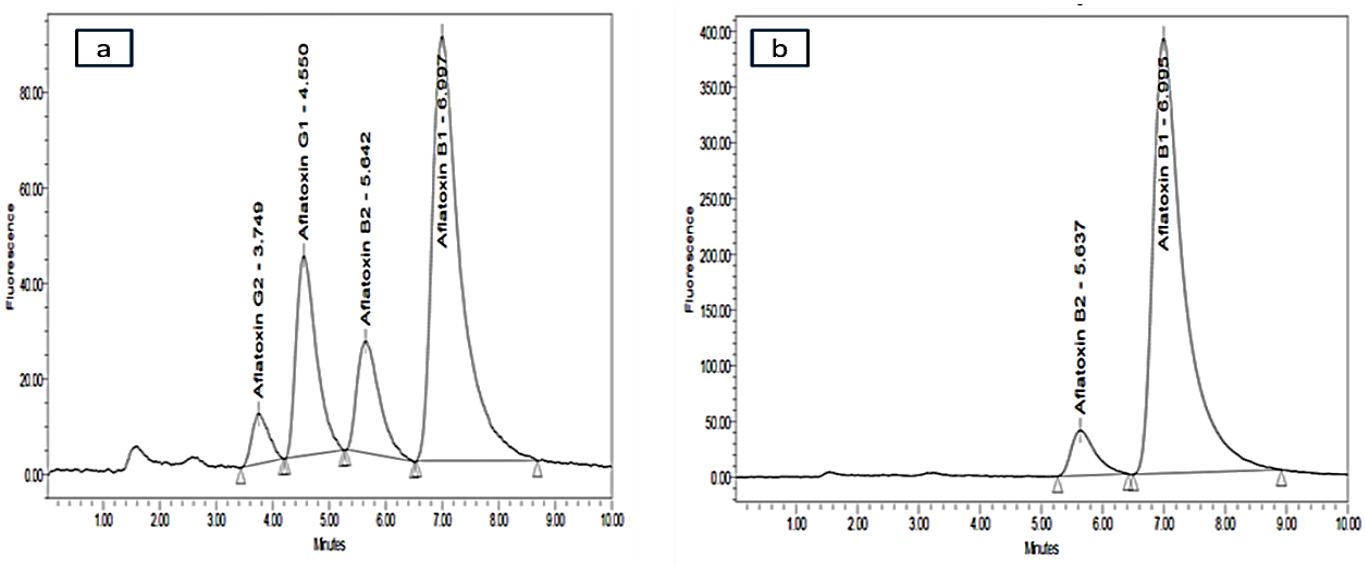

Fig. 1. Aflatoxins standard Chromatogram (a) and AFB_1 and AFB_2 Chromatogram of contaminated pistachio samples (b).

Fungal isolation

In this study, AFPA selected medium was used to enumerate and evaluate *A. flavus* and *A. parasiticus*

fungi. These fungal species produce orange-yellow reverse colony pigmentation after growing on this medium (Gourama and Bullerman, 1995). Laboratory-grade chemicals and ultra-pure water were used for media preparation. The media contained 10 g of peptone, 20g of yeast extract, 0.5g of ammonium ferric citrate, 200 mg of pentachloro nitrobenzene, and 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 121°C for 15 to 20 minutes (Gourama and Bullerman, 1995). To determine the surface contamination of *Aspergillus*, 100 fruits were poured into an Erlenmeyer flask containing 500 ml of distilled water and placed in a shaker for 30 minutes at 150 rpm. One milliliter of suspension was then cultured on an AFPA culture medium, and *A.* section *Flavi* population was determined after five to seven days at 25ºC and dark.

Results

The results showed that the effective methods of storage (basket and plastic) and temperature (refrigerator (4°C) and ambient (25±2°C)), as well as the interaction of these treatments on pistachio contamination with aflatoxins type B_1, B_2, and total, was significant during 32 days of storage ($P < 0.01$).

The population of fungi of Aspergillus section Flavi

The results of statistical analysis showed that the type of packaging did not have a significant effect on the growth rate of the fungus (Fig. 2), while the population of *A.* section *Flavi* significantly increased at ambient temperature ($P < 0.01$). Fig. 3 showed that storing the pistachios at temperatures of 3.5±0.5°C reduces the fungal population of the *A.* section *Flavi* by 67.5 percent, compared with storing at ambient (25±2°C) temperatures.

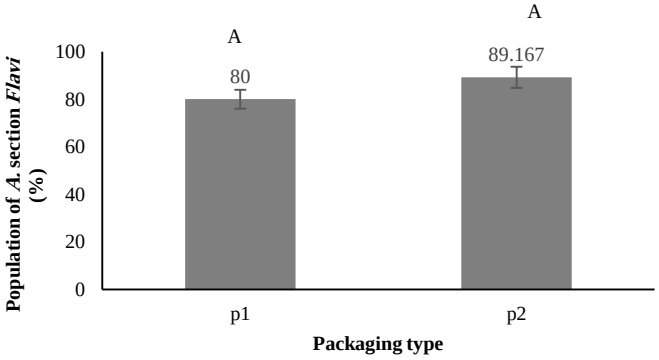

Fig. 2. The effect of packaging type on the population of *Aspergillus* section *Flavi*. p1: plastic baskets, p2: polyethylene bags. Similar letters have no significant effect

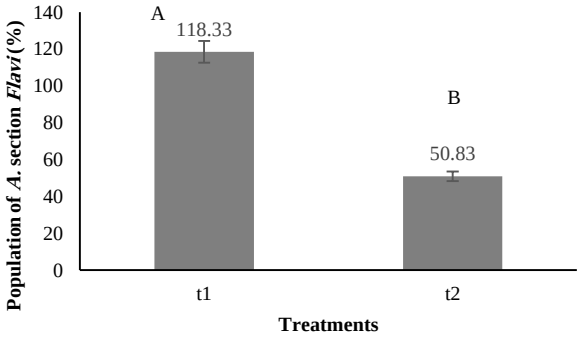

Fig. 3. The effect of storage temperature on the population of *Aspergillus* section *Flavi*. t1: 25±2 °C, t2: 3.5±0.5 °C. Similar letters have no significant effect.

Fig. 4 shows the mutual effects of packaging type and storage temperature on the population of *A.* section *Flavi*. The lowest percentage of population of *A.* section *Flavi* was observed in the samples packed in baskets and at r temperature ($p<0.01$). The samples stored at ambient temperature showed the highest thepopulation of *A.* section *Flavi*, regardless of the packaging type.

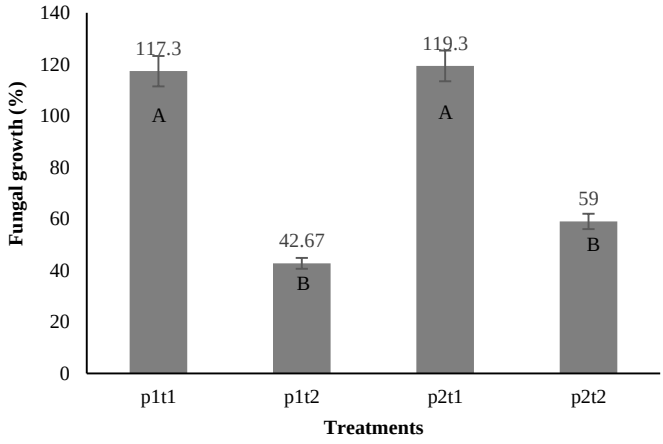

Fig. 4. The effect of packaging type and storage temperature on the population of *Aspergillus* section *Flavi*. p1: plastic baskets, p2: polyethylene bags, t1: 25±2°C, t2: 3.5±0.5°C. Similar letters have no significant effect.

Aflatoxin production (B_1, B_2, and total)

A comparison of means by the Duncan multiple tests showed that storing fresh pistachios in the basket compared to plastic significantly increased the production of B_1, B_2, and total aflatoxins. B_1, B_2, and total aflatoxins of the stored samples in plastic packages increased 71.6, 33.68, and 66.49%, respectively, compared with stored samples in baskets (Fig. 5.).

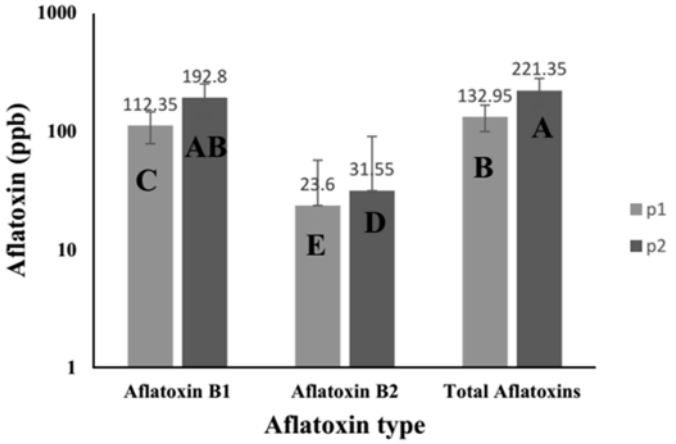

Fig. 5. The effect of packaging type on B_1, B_2, and total aflatoxins contamination during 32 days of fresh pistachio storage. The numbers on the bars are the means on an arithmetic scale. p1: plastic baskets, p2: polyethylene bags. Similar letters have no significant effect.

Storage of the samples at ambient temperature greatly increased the amount of B_1, B_2, and total aflatoxins, so that they increased from 5.4 to 299.75, 3.55 to 51.6, and 5.95 to 348.35 ppb, respectively (Fig. 6).

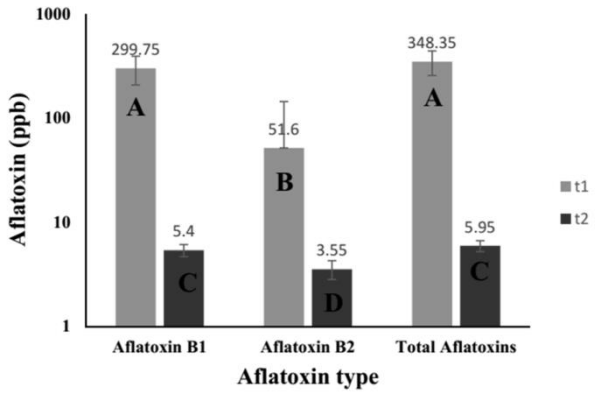

Fig. 6. The effect of storage temperature on B_1, B_2, and total aflatoxins contamination during 32 days of fresh pistachio storage. For better representation, the vertical axis is plotted on a logarithmic scale. The numbers on the bars are the means on an arithmetic scale. t1: ambient temperature, t2: refrigerator temperature. Similar letters have no significant effect.

The interaction of storage in polyethylene bags and ambient temperature compared to other treatments showed the highest production of aflatoxin B_1 and had a significant effect compared to other treatment samples (both in plastic baskets and in polyethylene bags) had the lowest aflatoxin B_1 levels. The sample stored in the plastic basket at room temperature had the highest production of aflatoxin B_1, which was significantly different from other treatments. The highest B_2 aflatoxin was observed in the samples stored in the polyethylene bags at ambient temperature. In this case, the samples stored at temperature (both in the plastic basket and in polyethylene bags) had the lowest production of aflatoxin B_2, but the difference between the method of storage in the plastic baskets and the method of storage in polyethylene bags was not significant. The pattern of changes related to total aflatoxin was similar to the changes related to aflatoxin B_1 (Fig. 7).

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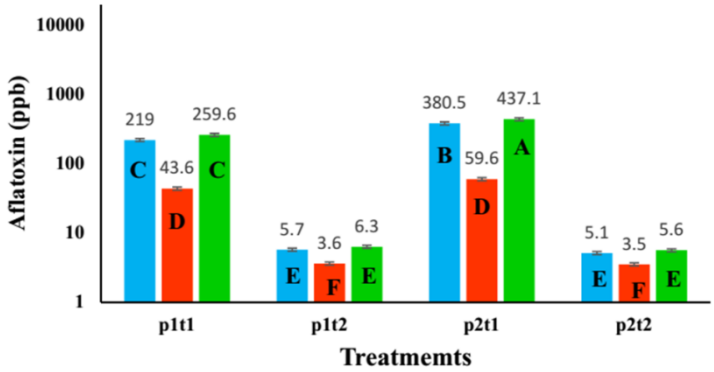

Fig. 7. Effect of storage temperature and packaging type on B_1, B_2, and total aflatoxins contamination during 32 days of fresh pistachio storage. p1: plastic baskets, p2: polyethylene bags, t1: 25±2°C, t2: 3.5±0.5°C. Similar letters have no significant effect.

Changes in the population of A. section Flavi and aflatoxin production during storage

As it is shown in Fig. 8, the population of *A.* section *Flavi* has not changed significantly during the 32 days of storage. The production of B_2 aflatoxin was less than other aflatoxins and it showed a somehow sinusoidal trend. Total and B_1 aflatoxins were changing similarly and the curves overlay each other. In the first 4 days of storage, all aflatoxins increased sharply, but they remained constant up to the end of the storage time.

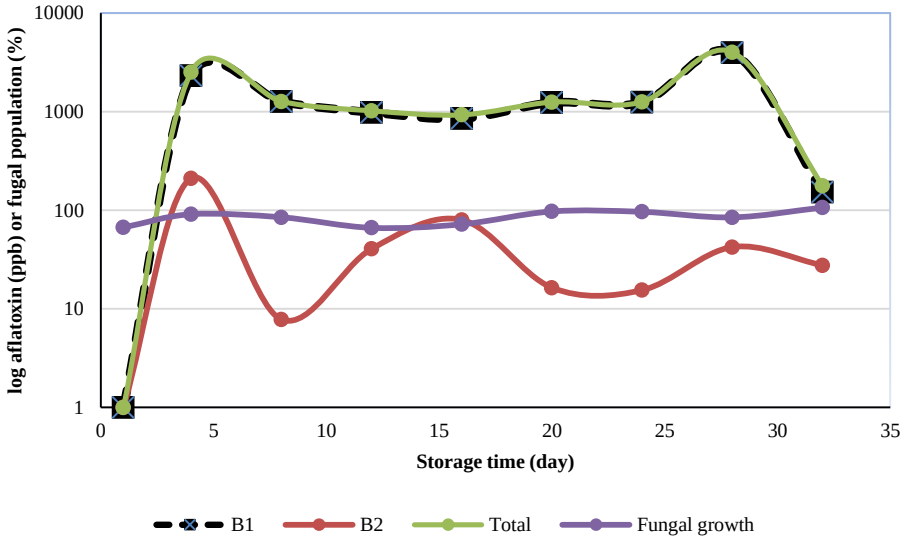

Fig. 8. The change of aflatoxin production and the population of *Aspergillus* section *Flavi* by storage time

Discussion

Many environmental factors are involved in the production of aflatoxins, but temperature and relative humidity are the most important of these factors. Other factors such as water activity, crop moisture, nutrient composition, storage time, insect damage, and the presence of soft skin also affect the growth of *A. flavus* and aflatoxin production (Eros *et al*., 2005).

Pistachio storage method and storage conditions are important factors affecting pistachio infection with *Aspergillus* fungus and aflatoxin production. The moisture content of pistachio kernels at harvest varies from 30 to 45%, at which level different species of *Aspergillus* can grow and produce aflatoxins and compete with other microorganisms (Kader, 1982). However, it should be noted that in pistachio processing, the amount of kernel moisture decreases to a reliable level in a short time (Moradi and Hokmabadi, 2011). Mojtahedi *et al*., (1980) reported that the moisture content of pistachio kernels in samples collected from Kerman and Isfahan provinces was between 3 and 6%. Tavakolipour *et al*. (2008) while investigating the effect of temperatures of 5, 15, 25, and 35°C in the relative humidity ranges of 11, 33-32, 73-62, and 87-82%, reported that increasing temperature in a certain relative humidity range, the trend of changes in the amount of moisture in pistachios is declining, which continues with increasing temperature. They also showed that in all the studied temperatures and humidity ranges up to 73%, the moisture content of pistachio kernels during 3 months of storage was less than 8%.

This point is especially important because stored pistachios have a dormant infection with *A. flavus* or other microorganisms and if the conditions are favorable, infection occurs (Moradi and Hokmabadi, 2011). This indicates that although the conditions for aflatoxin contamination and production in the orchards are appropriate, processing and storage conditions are not suitable for aflatoxin production. In storage or transportation, the infection can occur due to dormant contamination with productive fungi, if the conditions are appropriate.

The lack of a significant relationship between the studied factors can be due to the dry weather conditions and relatively high temperatures in Kerman province, which is why the moisture exchange between the environment and pistachio kernel is not enough to

increase the moisture content of the kernel and its water activity and the subsequent growth of microorganisms. It should be noted that in some cases the presence and lack of control of storage pests can provide the necessary conditions for the growth of A. flavus and aflatoxin production in storage (Doster and Michailides, 1999; Mehrnejad and Panahi, 2006; Mojthahedi *et al*., 1978 and 1980; Moradi and Javanshah, 2006; Moradi and Mirabolfathy, 2007; Moradi and Hokmabadi, 2011).

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