JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2012. Vol. **6**(4), p. 1811-1816

Study on the Amount and Type of Aflatoxin Produced by *Aspergillus flavus* in Some Pistachio Cultivars of Khorasan-e-Razavi Province

Leila Jalali[1]*, Hossein Afshari[2], Shahram Ashraf[3] and Abdol Ghaffar Ebadi[4]

[1]Department of Plant Protection, [2]Department of Horticulture, [3]Department of Soil Science, Damghan Branch, Islamic Azad University, Damghan, Iran.
[4]Department of Agriculture, Jouybar Branch, Islamic Azad University, Damghan, Iran.

(Received: 25 September 2012; accepted: 12 November 2012)

In order to measure the amount and type of aflatoxin produced in various cultivars of pistachio from Khorasan e Razavi Province, 4 cultivars of popular pistachio of Khorasan-e-Razavi were selected and collected. For this research, an isolate of aflatoxigenic *Aspergillus flavus* separated from the pistachio was used. Initially, 60 grams of pistachio kernels in 3 consecutive 20-gram sampling were selected and placed on Petri-dishes separately. 1 ml of the spore suspension of aflatoxigenic *Aspergillus flavus* added to each Petri-dish (spore suspension adjusted to contain of 2×10^6 spore/ml). The plates placed over water in plastic boxes and then placed inside an incubator at 26°C. After 5 and 8 days of inoculation, growth rate and colonization of *A. flavus* on pistachio kernels measured in different cultivars. For the purpose of extraction of aflatoxin from pistachios; initially, contaminated pistachio nuts are dried inside the oven with a temperature of 60 ° C for 48 hours to prevent the fungi from growing and further production of aflatoxin. Then, the amount and type of aflatoxin in pistachios were analyzed with HPLC method. The results of the study demonstrated that among the examined cultivars, this isolate is able to produce both aflatoxin $B_1$and B_2. It is noteworthy that the isolate is not able to produce aflatoxins G_1 and G_2.The highest amount of aflatoxins B_1 and B_2 produced was for Daneshmandi cultivar and the lowest figure was for Garmeh.

Key words: Khorasan-e-Razavi Province, *Aspergillus flavus*, Pistachio, Aflatoxin, HPLC.

Aflatoxins, a group of fouranocomarines derived from polyketids, are secondary metabolites. Produced by some species of *Aspergillus*, particularly *Aspergillus flavus* and *A.parasiticus*. Aflatoxins B_1, B_2, G_1, and G_2 are the most toxic and carcinogenic recognized compounds. Among mycotoxins that contaminate agricultural products (Rahimi *et al.,* 2007). Among this four major aflatoxins, B_1 possesses the highest toxic level and after which G_1, B_2, and G_2 aflatoxins have lower toxicities respectively (Wogan, 1966). Consumption of foodstuffs contaminated with aflatoxin may create severe side effects in human or the animal (Yabe *et al.,* 1993).

Due to the importance of the issue, much research was conducted in order to identify the disease agent and the way it behaves and it was found out that *Aspergillus* fungi plays an important role in the production of Aflatoxin. Consequently, a wide range of studies have been carried out regarding the lifestyle of fungus, aflatoxin formation conditions and the way prevention and detoxification are done (Castegnaro and McGregor, 1998). Not all species of *Aspergillus*

* To whom all correspondence should be addressed.
E-mail: L.jalali79@yahoo.com

flavus produce aflatoxin and the amount of aflatoxin production by aflatoxin producing species is not the same across different food environments (Moradi and Javanshah, 2006). Aspergillus fungus, which produces Aflatoxin, lives and survives in a wide range of materials and in different climatic conditions. In pistachio groves, these fungi live as growing organs (sclerot) on crop residue or other organic matter and in the beginning and throughout the season of growth, favorable environment for fungal growth and reproduction are prepared. Surveys carried out have shown that the resistant organs of (sclerot) fungi remain alive in soil for several years and in case of proper conditions are germinated, grow and spores are produced (Bayman and Cotty, 1991). Numerous studies demonstrate that the main cause of contamination of Pistachio yield is lack of proper management of grove and amongst the major reasons for contamination in pistachio groves; one can refer to existence of early-opened pistachios, pest attacks and delayed harvest. Therefore, meeting grove establishment principles and practices of proper grove management can play a significant role in controlling contamination. Awareness of the climate of the region in terms of providing heat, no spring and autumn cold, having hot and a long summer, absence of rainfall and strong winds at flowering and harvest season are among the necessities of economic production of healthy pistachios (Guo *et al.,,* 2005). It should be noted that existence of rain in harvest season results in a rise in relative humidity which is likely to increase Aflatoxin contamination of the yield. In a study by Ghewande and others (1993), the resistance level of peanut cultivars to fungus growth and aflatoxin formation were analyzed and they fond out that there are significant differences between various cultivars of peanut regarding growth and colonization level of fungi and aflatoxin levels.

In a study by Gradziel and Wang)1994(, the sensitivity level of different cultivars of almonds were analyzed from California America towards aflatoxigenic *Aspergillus flavus* and figured out that the sensitivity kernel in prevention and reduction of penetration of fungus in the core and found out that it plays the role of a resistant barrier. "Kamimura and others (1990) did" extensive research in regards with contaminated crops to aflatoxin. They reported the most contamination to B_1 aflatoxin in pistachios as 1382ppb. "Mojtahedi and others (1980) stated that" the minimum relative humidity required for infection to aflatoxin of pistachio in warehouses is %85 and the least interval needed for the creation of poison in this relative humidity is between 7 to 10 days depending on temperature from 20 to 27 degrees centigrade. studied aflatoxigenic *Aspergillus* molds in infected native Iranian Pistachio and reviewed the capability of aflatoxigenic properties. In his studies, he observed that most of the examined samples were contaminated with *A. flavus* and *A. parasiticus* molds and aflatoxin. Moradi and Javanshah based on the importance of the contamination of Iranian pistachio nuts to aflatoxin carried out research in the Iranian Pistachio Research Institute and presented very useful findings in reduction of this toxin. Study of climate and its effect on drying time of pistachio nut showed that pistachio drying time in the sun, especially if along rainfall increases up to 120 hours which would be desirable for rapid growth and germination of fungal spores and production of aflatoxin. Therefore, the use of new methods and machinery for rapid drying of pistachio nuts seizes the opportunities for growth and germination of spores. The aim of this study was to measure the amount and type of aflatoxin produced in contaminated pistachios by aflatoxigenic *Aspergillus flavus* and identification various crops's resistance level to this mold.

MATERIALS AND METHODS

Select and Collect Different Cultivars of Pistachio for the Purpose of measuring the amount and type of aflatoxin produced in contaminated pistachios by aflatoxigenic *Aspergillus flavus*

At first, 4 various cultivars of Khorasan-e-Razavi Province were selected and collected. While experimenting, it was tried to use cultivars which were among the most important and commercially available ones of the regions that possessed a great deal of cultivation. Therefore, 4 cultivars of pistachio named Daneshmandi, Ghermez, Sefid Badamy, and Garmeh were collected in the time of harvest for the purpose of the experiment. In order to minimize possible contamination of pistachios to *Aspergillus flavus* mold and aflatoxin, they were collected from trees at the time of sampling. After collecting the fresh

pistachios, the pest-stricken ones and those with a potential to be contaminated were removed. Then, the outer soft layer of the pistachio was separated from the horny skin by hand to avoid any damage to the inner shell. After that, pistachios were dried under proper conditions and were used for laboratory purpose in vitro.

Fungus Isolate

For this research, an isolate of aflatoxigenic *Aspergillus flavus* mold separated from the pistachio was used and throughout all stages of isolate cultivation, subculture, or for the production of slant, the two medium MEA (Malt Extract Agar) and PDA (Potato Dextrose Agar) were used.

Before the experiment, in order to ensure no *Aspergillus flavus* mold contamination for the nuts, initially, 60 grams of pistachio kernels in 3 consecutive 20-gram sampling were collected (completely randomized design in 3 replications). These 20 grams were sterilized by the help of %0.5 Sodium hypochlorite solution.Then, they were thoroughly rinsed in sterile distilled water. After that, in order to absorb the primary moisture of kernels, they were soaked in sterile distilled water for 10 minutes. In the next stage, kernels were taken out from the sterile distilled water and were put in sterile Petri and 1 milliliter of sterile distilled water was added to it.To perform this experiment, 2×10^6 spore per milliliter is needed. Hemacytometer was used for the purpose of counting spores (Pitt and Hocking 1985(. For every cultivar, 3 repetitions alongside with an observant were considered and in control Petri, instead of adding spore suspension, sterile distilled water was added. After each surface disinfection and soaking pistachios in the sterile distilled water, one milliliter of the fungal spore suspension was added to each Petri including 20 grams of kernels.By shaking the Petri, fungal spore suspension was thoroughly spread throughout the Petri until every surface was impregnated. To provide adequate moisture (up to saturation level), Petri containing the moist kernels were put inside plastic containers with lids at the bottom of which a little sterile distilled water was poured and the plastic container lid was firmly closed and these dishes were incubated inside the incubator for a period of one week at 26 degrees centigrade. After growth of fungus and covering all the surfaces by the fungus, the amount of fungal colonization throughout the surfaces on the fifth and eighth days was calculated (Ghewande *et al.,,* 1993) For the purpose of extraction of aflatoxin from pistachios; initially, contaminated pistachio nuts are dried inside the oven with a temperature of 60 ° C for 48 hours to prevent the fungi from growing and further production of aflatoxin. Then, the amount and type of aflatoxin in pistachios were analyzed with HPLC method.

RESULTS

Aflatoxin analysis

To examine the mutagenicity of aflatoxin as well as analyzing the production of aflatoxin by fungal isolates according to the described methods in section Materials and Methods, aflatoxin contained in four cultivars of pistachio of Khorasan Razawi Province including Ghermez, Garmeh, Daneshmandi and Sefid Badamy, were extracted; then, aflatoxin produced by HPLC technique were studied. As it is illustrated in the tables, these isolates are able to produce both aflatoxins $B_1$and B_2. It is noteworthy that the isolate is not able to produce aflatoxins G1 and G2.

Among the cultivars, the fungus causing toxin varied according to strength and intensity of toxin produced, so that the range of aflatoxin B_1 was 1106.36 to 1759.68 ng/gr and the aflatoxin B_2 varied between 0 to 21.52 ng/gr. The highest

Table 1. Variance analysis of aflatoxins $B_1$and B_2 rate produced by *Aspergillus flavus* amongst various cultivars of pistachio

Source	DF Sum of Squares	Mean Square F	Value Pr > F
Level of Aflatoxin B1 produced by 3the fungus *A. flavus* on various cultivars of pistachio	285093.05	95031	4.1ns 0.0520
Level of Aflatoxin B2 produced by 3the fungus *A. flavus* on various cultivars of pistachio	339.4	113.13	9.27ns 0.0056

Table 2. Comparison of average production rate of aflatoxin B_1 by *Aspergillus flavus* amongst various cultivars of pistachio

Type of pistachio production	Average aflatoxin B_1	Dunken Statistical
1-Garmeh	1268.84	b
2-Sefid Badamy	1376.38	ab
3- Ghermez	1609.14	a
4- Daneshmandi	1632.64	a

Table 3. Comparison of average production rate of aflatoxin B_2 by *Aspergillus flavus* amongst various cultivars of pistachio

Type of pistachio production	Average aflatoxin B_2	Dunken Statistical
1-Garmeh	2.12	b
2-Sefid Badamy	11.64	a
3- Ghermez	14.76	a
4- Daneshmandi	15.43	a

Sefid Badamy

Daneshmandi

Ghermez

Garmeh

Fig. 1. The percentage of colonization and sporulation levels of *Aspergillus flavus* mold on 4 Cultivars of Pistachio

amount of aflatoxins $B_1$and B_2 produced was for Daneshmandi cultivar and the lowest figure was for Garmeh.

DISCUSSION

Given the fact that *A. flavus* and aflatoxin contamination process is too complex and requires total destruction or serious control of toxin contamination, there is need for several approaches to the problem. Thus research on identification of resistant cultivars to *A. flavus* and aflatoxin production, may be good strategies to create a suitable knowledge base for controlling aflatoxin contamination of agricultural products, in this case pistachio(Moghaddam and Hokmabadi 2010). Applying crops that are sensible to the contamination of Aspergillus, pests or other microbial agents increases the potential to be contaminated by aflatoxin. Therefore, resistance of the chosen cultivar should be considered and farmers need to consult with plant breeding professional and agricultural promotion experts to find the most suitable cultivar (Jalali *et al.,* 2011a).The amount of fat and sugar and elements such as zinc, manganese, magnesium, iron, etc are different for various cultivars of pistachio which may address the amount of sporulation of *Aspergillus flavus* for every genotype and naturally, the aflatoxin resulted from its growth (Jalali *et al.,* 2011b).In most regions of the world, extensive research are being done for the purpose of identifying various crops's resistance level to aflatoxigenic *Aspergillus flavus* whose reports imply success. "Mohammadi Moghaddam and others)2006) studied" the sensitivity level of 10 cultivars of pistachios cultivated in Kerman, Semnan, and Ghazvin regions to *Aspergillus flavus* and aflatoxin. The findings suggested a significant difference in fungal growth and toxin production in different studied cultivars which is aligned with the findings of ours concerning pistachio cultivars in Khorasan-e- Razavi. "Ghewande and others (1993) stated that" the resistance of host as one of the most critical and important aspects for lowering contamination levels based on the genetic diversity of different peanut cultivars. They also performed studies regarding the resistance level of peanut cultivars relative to fungual growth of *Aspergillus flavus* and the consequent aflatoxin resulted from its growth. Their findings suggested a prominent correlation between resistance of variant cultivars and fungal growth. "Magnoli and others (1998) studied" on the aflotoxin producing property of different isolates after identification and isolation of *A. flavus* from foods and observed that the property and power of producing toxin in these isolates were very different from each other so that only %74 of *A. flavus* isolates produced toxin. "Gradziel and Wang (1994) analyzed" the sensitivity level of various cultivars of almonds from California America towards aflatoxigenic *Aspergillus flavus* and figured out that the sensitivity level of various cultivars to this fungus are significantly different. They also studied the rate of fungal penetration to the core as a result of damage to the cover of almonds and could demonstrate its role in reducing fungal growth. Throughout stages of performing this research, sensitivity levels to aflatoxigenic *Aspergillus flavus* were also studied and the rate of B_1 produced was analyzed. The findings represent a significant correlation between fungal growth on the surface of nuts and show that various cultivars react differently based on their sensitivity level towards this fungus.

REFERENCES

1. Bayman, P. and P.J. Cotty, Vegetative Compatibility and genetic diversity in the *flavus* population of a single field. *Can. Bot.,* 1991; **69**: 1707-1711.
2. Castegnaro, M. and McGregor, D., Carcinogenic risk assessment of mycotoxins. *Rev. Med. Vet.* 1998; **149**: 671-678.
3. Ghewande, M. P., G. Nagaraj, S. Desai and P.Narayan, Screening of groundnut bold seeded genotypes for resistance to *Aspergillus flavus* seed colonization and less aflatoxin production. *Seed Science and Technology,* 1993; **21**: 45-51.
4. Gradziel, T.M. and D. Wang, Susceptibility of California almond cultivars to aflatoxigenic *Aspergillus flavus*. *Hort science,* 1994; **29**: 33-35.
5. Guo, B.Z., Widstrom, N.W., L.ee, DR., Wilson, D.M. and Coy, A.E., Prevention of preharvest aflatoxin contamination: integration of crop management and genetics in corn, *in:* Abbas, H.K. [Ed.] Afiatoxin and Food Safety. CRC Press, New York, NY. 2005; 437-457.
6. Jalali, L., H. Afshari, Sh. Ashraf and M.M.

Moghadam, Study on the sensitivity level of some pistachio cultivars of Khorasan-e-Razavi province to *Aspergillus flavus. Adv. Environ. Biol*., 2011b; **5**: 3535-3539.

7. Kamimura, H., M. Nishijima., K. Yasuda., H. Ushiyama., S. Tabata., S. Matsumoto and T. Nishima, Simple clean up method for analysis of aflatoxins and comparison with various methods. *J. Assoc. Off. Anual. Chem*., 1990; **68**(3): 458-461.
8. Magnoli, C., A.M. Saenz, S.M. Chiacchiera, R. Miazzo and M.A. Saenz, Enumeration and identification of *Aspergillus* group and *Penecillium* species in poultry feeds from Argentina. *Mycopathologia.,*1998; **142**: 27-32.
9. Mohammadi Moghaddam, M., E. Mohammadi Goltapeh, H. Hokmabadi and A.H. Mortazavi, Evaluation of susceptibility of pistachio cultivars to aflatoxigenic *Aspergillus flavus* and aflatoxin B1 production. *Acta Hort*., 2006; **726**: 655-657.
10. Mohammadi Moghaddam, M. and H. Hokmabadi, Study on the effect of pistachio testa on the reduction of Aspergillus flavus growth and aflatoxin B1 production in kernels of different pistachio cultivars. *AJCS*. 2010; **4**(9): 744-749.
11. Mojtahedi, H., D. Danesh., B. Haghighi and S.Fathi, Storagerelative humidity in Rafsanjan and impossibility of pistachio aflatoxicosis after nut processing. *Iran. J. Plant Path*., 1980; **16**: 1-4.
12. Moradi, M. and A. Javanshah, Distribution of aflatoxin in processed pistachio nut terminals. Acta Hort., (ISHS) 2006; **726**: 431-436.
13. Pitt, J.I. and A.D. Hocking, Aspergillus and its telemorphs. In "fungi and food spoilagespoilage decomposition; said of meat, milk, animal feeds especially ensilage. (B.S.Schweigert, G.F. Stewart, eds), CSIRO. Academic press Australia, 1985; 259-313.
14. Rahimi, P., B. Sharifnabi, and M. Bahar, Detection of aflatoxin in Aspergillus species isolated from pistachio in Iran. Journal of Phytopathology., 2007; **156**(1): 15–20.
15. Wogan, G.N., Chemical nature and biological effects of the aflatoxin. *Bacteriol. Rev.,* 1966; **30**(2): 460.
16. Yabe, K., Y. Matsuyama, Y. Ando, H. Nakajima and T. Hamasaki, Sterochemistry during aflatoxin biosynthesis: Conversion of norsolorinic Acid to Averufin. *Applied Environ. Microbial*., 1993; **59**: 2486-2492.