JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2012. Vol. **6**(4), p. 1623-1627

Study of Sporulation Levels of Aflatoxigenic *Aspergillus flavus* on 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: 18 September 2012; accepted: 06 November 2012)

In order to calculate the level of sporulation by aflatoxigenic *Aspergillus flavus* mold, 4 cultivars of popular pistachio of Khorasan-e-Razavi were selected and collected. Then 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 degrees centigrade. After 5 and 8 days of inoculation, growth rate and colonization of *A. flavus* on pistachio kernels measured in different cultivars. In order to calculate the level of sporulation, colonized pistachios of each Petri was mixed with 100 milliliter of sterile distilled water and were poured into an Erlenmeyer flask and were put on a shaker for 24 hours to ensure a thorough mixture of spores and water. In the next stage, the amount of spore in 100 milliliter of sterile distilled water was calculated by Hemacytometer and was reported as the amount of sporulation because of fugal growth in 20 grams of pistachio kernel in each Petri. The average difference in colonization levels of various cultivars of pistachio were analyzed statistically by the help of Duncan's multiple range test. As it was expected, in cultivars where the fungus had a higher rate of growth, its rate of sporulation was higher as well. The results of the study demonstrated that among the examined cultivars, Daneshmandi had the highest level of sporulation and in contrast, Garmeh addressed the lowest level of sporulation. Undoubtedly, the most effective and useful approach to reduce the contamination level of the crop to *A. flavus* and aflatoxin is to select the most persistent ones to fungal growth and consequently the aflatoxin resulted by its growth.

Key words: Khorasan-e-Razavi Province, *Aspergillus flavus*, sporulation, Pistachio and aflatoxin.

So far, various strains of *Aspergillus, Penecillium* and *Rhizopus* molds have been reported that produce aflatoxin; among which, *A. flavus* have been placed above all and is one of the major causes of aflatoxin (Bayman and Cotty, 1991). Since the discovery of aflatoxins in the 1960s, the *A. flavus* has been widely reported in scientific sources as the most common fungus affecting food products. This is more than sufficient to show its economic significance (Doster and Michailides, 1994). This fungus is common all over the world as an air and soil mycoflora found in live and dead animal and plant organisms. It is particularly interested in colonizing nut kernels and oily cereals. Peanut, corn, wheat, rice, pistachio and almond are the major products infected by this fungus (Geiser *et al*., 2000).The colony diameter of *A. flavus* on the Czapek Yeast extract Agar, CYA, is 50 to 70 mm. These colonies are flat, spread, or relatively dense and are velvet-like at least on edges. In most cases, the central area is convex-shaped and accumulated and floccose and in some others the

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

central areas are depressed. Mycelium is only observed in floccose regions and is white in color. Conidial heads usually cover the whole colony surface except those regions that are floccose or in case of secretion of sclerotinia become rare or extinct. Their color is olive green but was also identified as yellow which gradually changed their color to green (Pitt and Hocking, 1985). Among the four major aflatoxins, i.e. B_1, B_2, G_1, and G_2, B_1 possesses the highest toxic level. after which G_1, B_2, and G_2 aflatoxins have lower toxicities respectively (Wogan, 1966). Also, M_1 and Q_1 aflatoxins are somehow toxic and the reason for higher toxicity in B_1, G_1, M_1, and Q_1 rather than B_2 and G_2 is the existence of a double bind of 8 and 9 dihydrouridine (or in other words 2 and 3 Vinyl Ether) (Trail *et al*., 1995). In Iran the economic value of pistachio exports to 66 countries is about one billion dollars/year, ranking second among the nation' s sources of income after oil (FAO Stat, 2008). This alone is more than enough to show the strategic significance of this product and of course, the dire need to protect and optimize it to keep the edge in global commerce.Thus, Subject of foodstuffs contamination to Mycotoxins and especially aflatoxin were considered in our country regarding pistachio and broad range researches were started by State Research Institutes on the subject (Aminshahidi, 1997). Mojtahedi *et al.* (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. Aminshahidi (1997) 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. Ghewande *et al*. (1993) analyzed the resistance level of peanut cultivars to fungus growth and aflatoxin formation and found out that there are significant differences between various cultivars of peanut regarding growth and colonization level of fungi and aflatoxin levels. Gradziel and Wang (1994) studied the sensitivity level of different cultivars of almonds from California America towards aflatoxigenic *Aspergillus flavus* and figured out that the sensitivity levels of various cultivars are different. They also analyzed the impacts of coating of almond kernel in prevention and reduction of penetration of fungus in the core and found out that it plays the role of a resistant barrier.

MATERIALS AND METHODS

Select and Collect Different Cultivars of Pistachio for the Purpose of Calculating the Amount of Sporulation by *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. 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). Similarly, in order to calculate the level of sporulation by mold on the eighth day, colonized pistachios of each Petri was mixed with 100 milliliter of sterile distilled water and were poured into an Erlenmeyer flask and were put on a shaker for 24 hours to ensure a thorough mixture of spores and water. In the next stage, the amount of spore in 100 milliliter of sterile distilled water was calculated by Hemacytometer and was reported as the amount of sporulation because of fugal growth in 20 grams of pistachio kernel in each Petri.

RESULTS AND DISCUSSION

Results of the Study of Sporulation and Sensitivity Levels of *Aspergillus flavus* on 4 Cultivars of Pistachio

In order to evaluate the sensitivity level of cultivars of pistachio to the growth of *Aspergillus flavus*, after the growth of fungus on inoculated pistachios, the criterion for measuring fungal growth was considered as the fungal colonization on pistachio kernel. After recording the percentage of fungal colonization on kernels on the fifth day after inoculation, the average difference of various cultivars of pistachios were analyzed by the help of statistical method of Duncan's multiple range test. Table 1 presents the variance analysis of colonization rate of *Aspergillus flavus* mold amongst various cultivars of pistachios on the fifth and eighth day after inoculation and the amount of sporulation by *A. flavus* on various cultivars of pistachio. Results of the statistical analysis illustrate a significant

Table 1. Variance analysis of colonization rate of *Aspergillus flavus* mold amongst various cultivars of pistachios on the fifth and eighth day after inoculation and the amount of sporulation by *A. flavus* on various cultivars of pistachio

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
The percentage of fungal colonization on nuts on the fifth day after inoculation	3	149.46	49.82	9.92**	/ 0001
The percentage of fungal colonization on nuts on the eighth day after inoculation	3	500.07	166.69	19.55**	2826/0
The amount of sporulation by *Aspergillus flavus*	3	2.62	8.74	16.93 **	/ 0001

Table 2. Sporulation

Type of pistachio	Average sporulation rate	Dunken Statistical Classification (∞= 1%)
1-Daneshmandi	52416667	a
2-Ghermez	37083333	a
3-Sefid Badamy	25750000	b
4-Garmeh	12183333	c

difference in average difference of fungal colonization and sporulation rate in various cultivars of pistachio on the fifth and eighth day after inoculation (at %1). After growth of mold on different cultivars of nuts, the level of sporulation was evaluated according to the mentioned approach in the Materials and Methods section. As it was expected, in cultivars where the fungus had a higher rate of growth, its rate of spore creation was higher as well. As it is seen, the difference amongst of sporulation rate of various cultivars on the eighth day after inoculation is significant at a %1 level and Daneshmandi had the highest level of sporulation and in contrast, Garmeh addressed the lowest level of sporulation. (Table 2). In Fig 1, the rate of sporulation in pistachio cultivars of Daneshmandi, Sefid Badamy, Ghermez and Garmeh showed for 8 days after inoculation respectively.

Since the discovery of aflatoxins, *Aspergillus flavus* mold have always been mentioned as the most common source of mold contamination in food science which demonstrates the economical importance of this fungus. *A. flavus* illustrates a particular tendency to contaminate nuts and oil seeds. Peanuts, corn and pistachio are the major crops attacked by this fungus. (Mohammadi Moghadam and Hokmabadi, 2010). 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. Undoubtedly, as this fungus attacks a large spectrum of agricultural products, one of the most effective and useful approaches

Fig. 1. The percentage of sporulation levels of *Aspergillus flavus* mold on 4 Cultivars of Pistachio on the Eighth day after Inoculation

to solve this concern is to analyze the resistance of various cultivars of a product and select the most persistent cultivars to fungal growth and consequently, from the aflatoxin caused by its growth which eases lowering contamination levels by the help of choosing the best cultivar in a reforming program so as to reduce contamination to aflatoxin. As it was observed, the difference in the rate of fungal growth and sporulation on nuts was significant at %1 whose cause goes back to its genotype. 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. 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.

In most regions of the world, extensive research are being done for the purpose of identifying various crops' resistance level to aflatoxigenic *Aspergillus flavus* whose reports imply success. Mohammadi Moghadam *et al*. (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 *et al*. (1993) stated 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. Ghewande *et al*. (1993) performed studies regarding the resistance level of peanut cultivars relative to fungal 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.

REFERENCES

1. Aminshahidi, M., The study of aflatoxigenic *Aspergillus* in native contaminated Pistachio of Iran and study on capacity of producing aflatoxin in them. *M.Sc. thesis*, Microbiology group, College of Tehran University, 1997; pp: 180.
2. Bayman, P. and Cotty, P. J., Vegetative Compatibility and genetic diversity in the *flavus* population of a single field. *Can. Bot,* 1991; **69**: 1707-1711.
3. Doster, M. A. and Michailides, T. J., *Aspergillus* mold and aflatoxin in pistachio nuts in California. *Phytopathology*, 1994; **84**: 583-590.
4. FAO., Prevention of mycotoxins. FAO Food and Nutrition Paper No. 10. Rome 2008.
5. Geiser, D. M., Dorner, W., Bruce, W. H. and Taulor, J. W., The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. *Fung. Genet. Biol,* 2000; **31**: 169-179.
6. Ghewande, M. P., Nagaraj, G., Desai, S. and Narayan, P., Screening of groundnut bold seeded genotypes for resistance to *Aspergillus flavus* seed colonization and less aflatoxin production. *Seed Science & Technology*, 1993; **21**:45-51.
7. Gradziel, T. M. and Wang, D., Susceptibility of California almond cultivars to aflatoxigenic *A. flavus*. *Hort Science*, 1994; **29**: 33-35.
8. Mohammadi Moghaddam, M., Mohammadi Goltapeh, E., Hokmabadi, H. and Mortazavi, A. H., Evaluation of susceptibility of pistachio cultivars to aflatoxigenic *A. flavus* and aflatoxin B1 production. *Acta Hort*, 2006; **726**: 655-657.
9. Mohammadi Moghaddam, M. and Hokmabadi, H., 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.
10. Mojtahedi, H., Danesh, D., Haghighi, B and Fathi, S., Storagerelative humidity in Rafsanjan and impossibility of pistachio aflatoxicosis after nut processing. *Iran. J. Plant Path,* 1980; **16**: 1-4.
11. Pitt, J. I. and Hocking, A. D., *Aspergillus* and its telemorphs. In " *fungi and food spoilage* " (B.S.Schweigert, G.F. Stewart, eds .). *CSIRO*, Academic press Australia, 1985; 259 -313.
12. Trail, F., Mohanti, N. and Linz, J., Molecular biology of aflatoxin biosynthesis. *Microbiology*. 1995; **141**: 755-765.
13. Wogan, G. N., Chemical nature and biological effects of the aflatoxin. *Bacteriol, Rev.,* 1966; **30**(2): 460.