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Effect of Irrigation Systems on the Contamination of *Aspergillus flavus* and Aflatoxin Production in Shahpasand Pistachio Cultivar

Mahdi Mohammadi-Moghadam[*1], Ali Reza Tavakoli[2], Amir Hossein Mohammadi[3], Mohammad Moradi[3], Hossein Hokmabadi[4]

[1] *Plant Protection Research Department, Agricultural and Natural Resources Research and Education Center of Semnan Province (Shahrood), AREEO, Shahrood, Iran*

[2] *Associate Prof, Agricultural Engineering Research Institute. Agricultural Research, Education and Extension Organization, (AREEO), Karaj, Iran*

[3] *Pistachio Research Center, Horticultural Science Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Rafsanjan, Iran*

[4] *Education and Extension Institute, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran*

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ABSTRACT

Aflatoxins are secondary metabolites produced by *Aspergillus flavus*, *A. parasiticus*, *A. tamari*, *A. bombyci*s and *A. nomius* species. Relative humidity (RH) is one of the most important parameters influencing the development of aflatoxins in pistachio orchards by *Aspergillus* species. The type of irrigation system affects density and frequency of the *Aspergillus* species in the soil. In the current study, to evaluate fungus population density three irrigation treatments (flood, drip-surface, and subsurface irrigation) were applied. In order to investigate the population density of *Aspergillus flavus* in different irrigation systems, sampling was done from soil and kernels of Shahpasand cultivar at Damghan's Pistachio Research Station under various types of irrigation systems implementation. The samples were inoculated on AFPA medium using serial dilution method. The results indicated that the population density of *A. flavus* ranged various in pistachio kernels and soils between different types of irrigation system. Through subsurface irrigation, the population density of fungal colonies was significantly at the lowest level. The results showed that the use of modified adopted subsurface irrigation system had been effective in reducing soil surface moisture and led to decrease of population density in comparison with the conventional surface drip irrigation.

Introduction

Iran is the most important pistachio-producing counties (Sharifkhah et al., 2020; Norozi et al., 2019) considered to be the world's leading in producing 479000 hectares of pistachio orchards and the largest producer around the world and in the country is Kerman province with more than about 52% of Iran total

[*]**Corresponding author**: Email address: mm.moghadam52@gmail.com
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productions. In addition, Razavi Khorasan, Yazd, Fars, Semnan, South Khorasan, Qom, Markazi and Sistan and Baluchestan provinces follow Kerman in rank (Ahmadi *et al*., 2017). Currently, Iran is the world's largest exporter of pistachio of the (Alipour, 2018), USA, Turkey, Greece, Syria and Afghanistan, respectively (Ahmadi *et al*., 2017). In addition, Economical value of Iran pistachio export is more than 1.5 milliards dollars per year that account as a second source of foreign exchange earnings in comparison with oil exports. Therefore, it is quite clear that pistachio is an important agricultural commodity, which needs product preparation considerations for international trade and market.

Overall, contamination of pistachio to *Aspergillus flavus* and aflatoxin is one of the great challenges currently facing export industry, so aflatoxin contamination of pistachio could threat the source of foreign exchange earnings, which negatively affects Iran's international trade (Mahbobinejhad et al., 2019). Due to the importance and significance of the subject, different aspects of pistachio contamination by *Aspergillus flavus* and aflatoxin should be monitored seriously (Mohammadi Moghadam *et al*., 2020). So comparing the population density of *Aspergillus flavus* and aflatoxin producing in different irrigation systems can be used as a basic research not only to create a more comprehensive and credible study on aflatoxin(AF) in pistachio around the world and particularly in Iran, but also as a guide to manage fungal contamination in orchards.

Aflatoxin control requires effective management strategies to prevent fungal infections that occur on crops in the orchards and storage room. Hence, current detoxification methods to eradicate AFs from yield seems to be difficult and cost a lot, so it’s believed that fungal contamination control of the crop in the orchards is more effective (Nigam *et al*., 2009, Samapundo *et al*., 2007). Developing an effective program to control aflatoxins requires identification and management the critical elements, which cause significant reduction of toxin-production in orchards or storage conditions. The management techniques aiming to inhibit AFs production in crops at pre-harvest procedure has always been included pest control, biological control, use of resistant varieties and proper irrigation management (Fani *et al*., 2014). In addition, carrying out orchards practices is a suitable method to decrease humidity in order to reduce pistachio contamination risk to *A. flavus* and declines contamination with aflatoxins. However, humidity is the most important factor for the growth of *A. flavus* and favorable for growth of aflatoxigenic strains in many orchards and it has been indicated that, the conditions of high humidity increased fungal contamination level, enormously. In recent years, due to water scarcity and economic value and impacts of pistachio product, orchards are subjected to change irrigation system. Therefore, irrigation performance and efficiency is becoming increasingly important and the irrigation systems have a key role in reducing or increasing fungal population because of various soil moisture as the crop protection perspective. Totally, favorable conditions in orchards are needed to occur the growth of aflatoxigenic strains and AFs production that leads to fungal contamination and toxin-producing. As it’s noted, humidity is one of the most important factors that cause fungal growth, which type of irrigation, irrigation systems performance, changing and modification the systems are the most effective solutions to control fungal contamination. According to this hypothesis, the population density of fungus was assessed in three treatments including flood, drip-surface, and subsurface irrigation at orchards with Shahpasand cultivar.

However, aflatoxins contamination is considered to be one of the most serious food safety problems around the world (Williams *et al*., 2004). Hence, contamination of food crops such as peanut, pistachio, maize and

cotton by aflatoxins is an important issue that affect food health, food industry, and food processing which follows significant regulations and standards (Williams *et al*., 2004, Van Egmond *et al*., 2007). Several studies have shown that pistachio fruit in contact with orchards surface could be contaminated with *A. flavus*, so it's necessery to prune the barks close to the soil surface (Moradi *et al*., 2002; Moradi *et al*., 2000). In another study, assaying colonization of waste material from processed procedure and leave showed that Aspergillus species could colonize on the remained material in initial stage and adequate supply of moisture by irrigation during the fall could play an important role (Moradi *et al*., 2002). Survey results showed that the level of contamination for pistachios to the ground associated with plant residues and other organic fertilizers in orchards, so performing the orchard sanitation practices could reduce fungal contamination, significantly (Moradi *et al*., 2000).

Materials and Methods

Sampling method

Determination the population density of Aspergillus flavus between various irrigation methods

Soil sampling of pistachio orchards

In order to study the population density of *Aspergillus flavus* in soil of pistachio orchard with various irrigation systems, soil sampling carried out from Shahpasand orchards, which included three systems (flood, drip-surface, and subsurface irrigation). Initially, Soil samples were collected up to 10cm depth included 5 primary samples, each 1 kg sample obtained from the shade tree canopy, then samples were mixed thoroughly and 500g of mixed used as a main source. Soil samples were inoculated in the AFPA plates by serial dilution method (completely randomized design with three repetitions). This fungal group produces conidia from yellow to olive-green color on AFPA medium, typically. The plates were kept in incubator at 26°C, then colonies of *A. flavus* were counted and contamination level in different samples estimated (Mohammadi Moghadam *et al*., 2020).

Kernel sampling

In order to study the population density of *Aspergillus flavus* in pistachio kernel, sampling was done during harvest time in orchards under various irrigation systems. Hence, to evaluate contamination level of *A. flavus*, grounded samples were cultivated on AFPA medium by serial dilution method (completely randomized design with three repetitions). Based on this method, 10g pistachio kernel of each sample was added to 90ml 0.1% pepton. Then, 0.1ml of each dilution was spread onto the AFPA plates, and kept at 26°C for 2-3 days. In the following, colonies of *A. flavus* were counted and by this method, contamination level in different samples compared together. Finally, Duncan's multiple tests was used to count mean difference of fungal colonies (Mohammadi Moghadam *et al*., 2020).

Quantification of aflatoxins produced in pistachio kernels

For quantification of the aflatoxin, kernels of pistachio cultivars were dried inside an oven and aflatoxin content of samples was measured by using Waters e2695 (USA) HPLC, consisting of a chromolith C18, 100 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. The limit of detection (LOD) for AFs was 0.3 $mg.ml^{-1}$. For this purpose, pistachio samples were slurried up with water in a ratio of 1/3 for 15 minutes, then slurried samples were extracted (30 g) with 90 ml of pure methanol on a

Waring blender (Waring, USA) for 3 minutes and filtered through Whatman paper No. 4. Filtrates (8 ml) were mixed with phosphate buffer (42 ml). Immunoaffinity columns were used for purification of samples. First, 20 ml of phosphate buffer was passed (transmitted) through the column to ready it, then 25 ml of the extract mixed with the phosphate buffer was passed (transmitted) through the column; and the column was again washed with 20 ml of phosphate buffer. After drying the column, 1500 µl of methanol (with the purity special for liquid chromatography) was passed through the column. By one minute, 750 µl of methanol was again passed through the column. After collecting the total methanol phase, 1750 µl of water was added to it, and finally 200 µl of the preparation was injected into the HPLC apparatus. Aflatoxins B1, B2, G1 and G2 were measured by comparing the peak areas with a 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 0.4–2.7 ng/ml aflatoxins B1. In the case of mobile phase HPLC, the methanol/water (40/60) used for the derivation of potassium bromide, nitric acid and Kobra cell. The chromolite column (10cm) with an internal diameter of 4.6mm (Partisil 5 ODS3, USA) was used. The column temperature was set to 35 ºC with a moving phase of 2.5 $ml.min^{-1}$. Fluorescence detector was set at wavelengths ex=365 nm and em=355 nm.(Mohammadi Moghadam *et al*., 2020).

Results

Investigating and comparing the population density of Aspergillus flavus under various irrigation systems

The results of *A. flavus* colonies counting in various soil samples showed that contamination level was differed among various irrigation systems. Also, statistical analysis and comparison of colony number in various soil samples (Duncan's multiple tests) identified that between various irrigation systems, the highest contamination level to *A. flavus* in pistachio soil obtained from flood system and the lowest contamination level to *A. flavus* belonged to subsurface drip irrigation system (Table 1).

Table 1. Comparison of mean difference about colony number of *A. flavus* in pistachio soil under various irrigation systems.

Irrigation type	Mean of colony number of *A. flavus*	Grouping variables (α =5%)
Flood	443	a
surface drip	390	b
Subsurface drip	320	c

Results of pistachio kernel testing

Generally, table 2 shows the population density of *A. flavus* in kernel samples at orchards under various irrigation systems. Colony number in different pistachio samples was determined by statistical and variance analysis (Duncan's multiple tests) with the least significant difference of contamination level to *A. flavus* under various irrigation systems.

Table 2. Comparison of mean difference about colony number of *A. flavus* in pistachio kernels under various irrigation systems.

Irrigation type	Mean of colony number of *A. flavus*	Grouping variables(α =5%)
Flood	250	a
surface drip	230	a
Subsurface drip	220	a

Results of aflatoxin producing in various samples

Assaying aflatoxin production level in pistachio kernels is shown in table 3. According to statistical analysis, no significant difference in aflatoxin production at kernel samples was observed at 5% level under various irrigation systems. Therefore, pistachio nut samples with these three irrigation systems placed in the same group of contamination level to aflatoxin and in all the cases, contamination samples to AFs was lower than thresholds.

Table 3. Frequency (percentage) of contamination samples to aflatoxin in pistachio kernel under various irrigation systems.

Irrigation type	Contamination samples frequency (percentage)	Grouping variables (α=5%)
Flood	33%	a
surface drip	33%	a
Subsurface drip	33%	a

Average followed by the same letter are not significantly different at level of 5%, by Duncan's Multiple Range Test.

Discussion

Aflatoxins are polyketide products of some Aspergillus species like *Aspergillus flavus, A. parasiticus, A. nomius, A. bombycis, A. pseudotamari, A. ochraceoroseus* and two anamorph of Aspergillus, namely, *Emericella venezuelensis* and *E. astellata*. Since the discovery of aflatoxin, *A. flavus* is the main source of AF contamination in scientific reports, which raise economic concerns (Abbas *et al.*, 2005, Brown *et al*., 2013). Moradi *et al*., (2004) reported Aspergillus genus as a soil-borne in pistachio growing areas and the fungal population in soil are affected by the time and frequency of irrigation. Recent studies show that contamination to toxin causative agents starts from orchards and develops during pistachio processing and storage period. The amount of Early-split pistachios from one orchard to another and one year to another was variable depending on soil type, nutrition, variety, climate and irrigation system type (Tajabadipour, 2003). The results demonstrated the correlation between population density level on the soil surface and frequency of irrigation, indicating that, the type of irrigation system led to decrease or increase of population density of fungi during the year (Moradi *et al*., 2002). According to the studies, it's critical to avoid irrigation and soil manipulation in orchards during the sensitive period (late August and September) of pistachio nut contamination. So that, release of the conidia and rise in the population density of *A. flavus* in orchards do not occur. Field study on fallen pistachio nuts in orchards revealed a relatively positive correlation between contamination level and frequency of irrigation and the presence of plant residue, especially different type of organic matter. It is clear that, orchards sanitation and hygiene decreases contamination significantly and will be of primary concern (Moradi *et al*., 2002). Several studies have been conducted to compare the effect of irrigation on early splitting of pistachios and associated contaminations. Further studies on the relationship of irrigation deprivations, the draught stress and early splitting during the growing season showed that inadequate water supply for irrigation will increase early splitting of pistachio nuts during early spring (Doster *et al*., 2001). Similarly, Sommer *et al*., (1986) reported that, the prevalence of aflatoxin contamination in early-split nuts was about 50 times more than naturally splitting pistachios. However,

one of the major constraints and key factors for early splitting has been water shortage in the late spring and consequently, early-split nuts were the critical source of fungal, pests and aflatoxin contamination, which showed regular irrigation as one of main effective ways to reduce percentage of early-split nuts. Also, Tajabadipour, (2003) identified that the percentage of early-split pistachios changed in different orchards and during various years, depending on soil type, nutrition, variety, climate and irrigation system type.

Furthermore, fungal disease, Alternaria late blight is dependent upon high humidity and occurs in pistachio grown areas of California, which increases cracking of shells, splitting and shell staining and results in marketability reduction. This problem in orchards with flood irrigation system, poor drainage and without chemical control for improving permeability is severe. However, it seems that by altering environmental conditions and reducing wet surface in orchards can decrease Alternaria disease damage. Therefore, the effect of subsurface drip and flood irrigation estimated in pistachio orchard with Kerman variety. In subsurface drip irrigation plot, dew formation period and relative humidity reduced while temperature was higher that led to reduction of leave contamination to Alternaria. At harvest time, 10 and 55% of pistachio leaves were extensively contaminated with fungi in subsurface drip irrigation and flood irrigation systems, respectively. Hence, subsurface drip irrigation declined pistachio nuts contamination in around half. Apparently, other study identified that control of Alternaria disease in orchards under flood system could be achieved with switching to subsurface irrigation (Goldhamer *et al.*, 1996). The results obtained in the present study showed that by switching the irrigation system from flood to surface or subsurface drip irrigation, the soil population of *Aspergillus flavus* decreased because of a significant reduction of soil's moisture content. In consequence, fungal population on pistachio nuts and aflatoxins were effectively controlled by reducing soil fungal population that resulted in producing healthier crops with high quality. Therefore, it's recommended to replace the existing surface irrigation system to modified adopted subsurface drip in order to reduce humidity and then decrease the emergence of fungi.

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