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The Effects of Poultry Manures on *Aspergillus* Section *Flavi* Density, Aflatoxin Production, Plant Nutrients, and Characteristics of Pistachio Fruit

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A R T I C L E I N F O

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A B S T R A C T

Aflatoxins are secondary metabolites, which have documented in food and feed such as pistachio. Application of incorrect cultural practices such as animal manures resulted in increasing the density of *Aspergillus* section *Flavi* propagules and as consequences increase in aflatoxin content in pistachio nuts. Here, the density of *Aspergillus* section *Flavi* propagules, aflatoxin kernel content and characteristics of pistachio fruits in soil surface and fertilizer cannel applications of poultry manure in the orchards in randomized complete block design were compared with control treatment (without poultry manure application)**.** Application of poultry manure on soil surface increased *Aspergillus* section *Flavi* propagules by 4.5 and 11 times higher than compare to those treatments either which not received or in fertilizer channels, respectively. Aflatoxin content in pistachio nuts decreased by 85 and 51 % where poultry manures applied in fertilizer channels and no application compared to surface application, respectively. Overall, poultry manure in fertilizer channel reduced the frequency of blank fruits as well as increasing of splitted nuts and the nuts' sizes.

Introduction

Several species of *Aspergillus* are able to produce aflatoxins in food and feed throughout world (Mahbobinejhad et al., 2019). In tropical countries, climatic changes may intensify acute aflatoxicosis and deaths (Lewis *et al*., 2005). Host susceptibility may also directly influence by climate weather. This may increase peanut susceptibility to biotic and abiotic stresses (Wotton and Strange, 1987), maize kernel by increasing pests (Odvody *et al*., 1997), or early split in pistachio (Doster and Michailides, 1995; Moradi and Hokmabadi, 2011; Moradi *et al*., 2019; Hadavi, 2005). The presence of mycotoxins in food commodity is a major challenge all over the world (Bennet and Klich, 2003, Cotty and Jaime-Garcia, 2007; Yazdanpanah *et al*., 2006), in several plant species, such as cottonseed, peanuts, pistachios and maize (Molyneux *et al*., 2007; Tajabadipour *et al*., 2005; Mutegi *et al.*, 2009, Nesci and Etcheverry, 2002; Cheraghali *et al.*, 2007 and Singh *et al.*, 2001). Production of mycotoxins may remain adverse effects on health of human and animal as well as economic losses (CAST, 2003; Muller and Basedow, 2007; Kouba, 2003).

Pistachio (*Pistacia vera* L.) is among horticultural crops that is of high economic importance (Esalmi et al., 2019; Shamshiri *et al*., 2015) but its nuts are highly prone to be infected by *A. flavus* (Mirabolfathy *et al*., 2005; Moradi and Fani, 2018; Moradi, *et al.*, 2004) and as a consequences may cause serious health risks (Esmail pour *et al*., 2000; Mirabolfathy *et al*., 2005; Mohammadi Moghaddam *et al*., 2005). A survey in Spain (Burdaspal, *et al*., 2005) has been shown that the presence of aflatoxin B1 in 12 out of

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24 pistachio samples, with a range of 0.57 to 98.5 ng/g. It has been shown aflatoxin B1 (AFB1) is highly toxic and carcinogenic (Eaton and Gallagher, 1994; Muller and Basedow, 2007). As *A. flavus* present naturally in pistachio orchards, the establishment and contamination could occur in favorable environmental conditions (Moradi and Hokmabadi, 2011). Seasonal variation in mold density indicated that moisture content of substrates, relative humidity and temperature as well as water activity are key factors in contamination and predominant microorganisms to colonize the substrates (Blandino *et al*., 2008; Moradi *et al.,* 2010). Incorrect cultural practices may increase early splitting formation-exposing kernels to the airborne spores of molds. It has been shown that *A. flavus* contamination and aflatoxin production occurs before harvest especially in cracking, insect or bird damaged nuts (Sommer *et al*., 1986). Several factors affect the frequency of early splitting formation in pistachio nuts such as irrigation systems, nourishing, rootstock type, cultivar, age, quantity of crop, physicochemical properties of soil, and variation in temperature through the day and night (Tajabadipour, 1998). Doster and Michailides (1995) report that early split nuts with dried hull are contaminated by *Aspergillus* section *Flavi* three times higher than those early split nuts with soft and fresh hull.

Applications of organic fertilizers such as poultry manure can improve yield production and also the growth parameters of pistachio. Some scenarios concern the effects of poultry manures on increasing the population's density of *A. flavus* in pistachio orchards and consequently aflatoxin in pistachio nuts. Therefore, the present study is going to evaluate the risks of poultry manure applied in pistachio orchards on the density *A. flavus* group in soil, aflatoxin content in pistachio, and leaf elements, and also their correlations.

Material and Methods

Experimental site and treatments

The experiments were conducted based on the randomized complete block design with three treatments in three replicates in a commercial pistachio orchard in Rafsanjan, Kerman province, Iran. The treatments are including, I. the application of poultry manure in soil surface (15 kg for each tree) II. The application of poultry manure in fertilizer channels (15 kg for each tree) and, III; No application of poultry manure as control. Each block was divided into three rows of pistachio trees by 500 m distances. The blocks were designed to be perpendicular to the wind direction in order to avoid the effect of experimental plots on each other such as distribution of spores, or to let further needed operations to be done.

Assessment of leaf nutrient

To analyze the contents of potassium, phosphorous, magnesium, calcium, ferrous, sodium, copper and manganese in leaves were collected according to the leaf samples of trees in each replicate during June. To analyze the effect of treatments on the frequency of cracked and early split pistachios, blank nuts, amount of aflatoxin in early split and cracked pistachio nuts, split and non-split pistachios, nuts ounce (number of nut in 28.3 gr) were investigated just after maturing (in September). This has been done by randomized sampling of pistachio nuts from the middle row of pistachio trees in each experimental unit.

The density of Aspergillus section Flavi in treatments

To find the density of *Aspergillus* section *Flavi*, some samples of the soil were also collected from a depth of 0-10 cm in each replicates. And meanwhile, to determine the populations density of *Aspergillus* section *Flavi*, a serial dilution method and AFPA and Czapeck media (*i.e.*, as described by Moradi *et al*. 2004) were applied.

Aflatoxin detection

Aflatoxin content was determined for the grounded kernels as described by Fani et al., 2014.

Analysis of soil and leaf samples

The air-dried soil samples were examined after passing through 2 mm sieve, and it's some physical and chemical properties were recorded. These include; the electrical conductivity of soil saturated extract (Rhoades, 1996), PH of saturated mud (Thomas, 1996), and texture, where the hydrometer method (Jay & Bauder, 1996) has been applied. Also, soluble calcium and magnesium using complexmetric via titration by EDTA, soluble sodium by atomic absorption, absorbable phosphorus by Olsen, absorbable potassium using ammonium acetate methods were measured (Aliahayaee, 1997; Jones, 2001, and Rhodes, 1996). In meantime, using DTPA and estimated through atomic absorption spectrometry (Wright and Stokzinski, 1996 and Aliahiaei, 1997), elements such as iron, zinc, manganese, and copper were also extracted. The drying process of leaves and fruits samples has been proceeded in an oven (65 ° C, 24h). And then the samples were milled, and 1 gram converted to ash at the level of 550 ° C for 5 hours. The ash was treated with 2 N hydrochloric acid and then extracted. During the extracting process, the concentrations of calcium and magnesium by applying complexometric titration, iron, zinc, manganese, and copper using atomic absorption spectrometry were determined. Phosphorus through colorimetric using a spectrophotometer at 470 nm, potassium by flame emission, Boron using azomethine H and total nitrogen in Kajaldal methods were also determined (Emami, 1996)

Statistical analyses

When the collection and registration processes of the observations, and crude data from laboratory analysis and nut characteristics of pistachio nut successfully finished, the information should be analyzed and performed by applying the variance and means comparison of data via so-called the Duncan's Multiple Range Test. Related F-value calculated via ANOVA (SPSS Inc., Chicago, IL, Version 12.0) was also found to be useful for descriptive and comparative statistics and therefore are going to be used for further discussions.

Results

In the research, the effects of poultry manure on leaf micro-and macro-elements, pistachio cracking and early splitting, population density of *Aspergillus* section *Flavi* in soil and aflatoxin kernel contents have been evaluated.

Analysis of variance (Table 1) reveals a significant difference among treatments in estimated parameters between soil surface or fertilizer channels applied poultry manure and the control treatment, where there are no significant differences for all estimated parameters between the soil surface and fertilizer channels with poultry manures, except for nut ounce.

On the other hand, treatments with no application of poultry manure had the higher percentages of early splitting (1.47%), cracking (35.0%) and blank (41.5%) nuts compared to other treatments with poultry manure. The same results can be observed in non-splitting nuts, the concentration of P, Ca, Mg, NA, and Zn in leaves in treatments either used poultry manures or not. Application of poultry manure in fertilizer channels has decreased nut ounce by 28.95% and consequently increased the nuts' weight.

Table 1. The effects of poultry manure application on estimated parameter

Parameters	Unit	Treatment					
		No application		Surface application		Fertilizer Channel	
Early splitting nuts		1.47	A	0.95	B	0.74	B
Cracking nuts		35.0	A	33.63	AB	31.69	B
Blank percentage		41.55	B	22.0	A	33.33	A
Splitting percentage		46.75	B	67.2	A	54.54	AB
Non- splitting percentage		11.69	A	10.75	A	12.12	A
Leaf Phosphor		0.24	A	0.25	A	0.25	A
Leaf calcium		1.73	A	1.7	A	1.53	A
Leaf magnesium	%	0.68	A	1.28	A	0.68	A
Leaf potassium		0.43	B	1.5	AB	1.63	A
Leaf sodium		0.08	A	0.1	A	0.06	A
Leaf iron		58.33	B	79.0	A	81.33	A
Leaf copper		5.2	B	6.53	AB	6.63	A
Leaf Manganese	PPM	21.3	B	25.0	A	23.23	AB
Leaf Zinc		0.09	A	9.66	A	9.66	A
Ounce number of nuts in 28.3	Gram	31.56	B	30.32	B	28.95	A
Aflatoxin B1	ng/g	93.11	B	295.4	A	45.24	B
Aflatoxin B2	ng/g	11.13	B	24.93	A	3.7	B

Means with the same letters at each row are not significantly different at p< 0.05

Application of poultry manure on soil surface has increased *Aspergillus* section *Flavi* propagules by 4.5 and 11 times higher than those treatments which not received or in fertilizer channels, respectively. There are no significant differences for *Aspergillus* section *Flavi* propagules for fertilizer channels in the presence of poultry manures. On the other hands, application of poultry manure in soil surface has increased the propagules of *Aspergillus* section *Flavi* by 2.5 * 10^4/g higher than prior of the application (8.2 *10^3) which is significant.

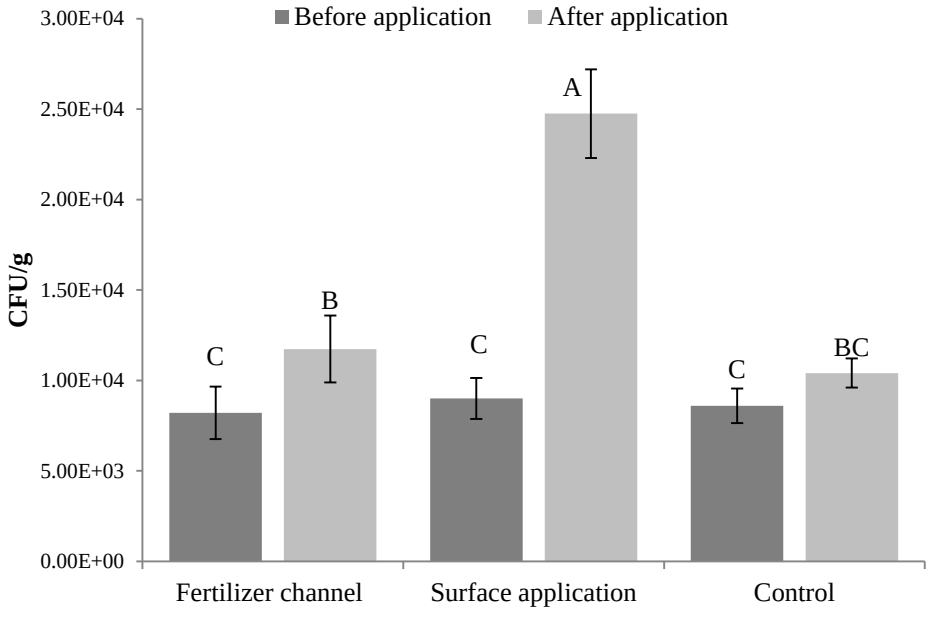

Fig. 1. The effects of poultry manure application on the density of *Aspergillus flavus* in soil

It can be seen that, there are significant differences between plant nutrient content and pistachio nuts cracking. For example, the frequency of early splitting pistachio was negatively correlated with the concentration of calcium and potassium in leaves and was positively correlated with copper content (0.65), which is significant (P <0.05).

The percentage of irregular cracking with leaf zinc has a positive and significant correlation (0.67). The percentages of potassium found in leaves were negatively correlated with iron, manganese, and copper by -0.61, -0.71, and -0.69, respectively. This mostly indicates that potassium adsorption from the soil competes with iron, manganese and copper, and therefore rising concentrations of iron, manganese, and copper in soil leads to less potassium absorption and a higher frequency of early splitting pistachios.

Aflatoxin contents in pistachio nuts have declined by 85 % and 51 % in presence and absence of poultry manures in fertilizer channels, respectively, in the applied surfaces.

Table 2. Correlation coefficients (r^2) between estimated parameters

Treatment	K^+ %	Cu^{+2} PPM	Mn^{+2} PPM	Zn PPM	Fe PPM	Na^+ %	Mg^{+2} %	Ca^{+2} %	P %	Cracking %	Early splitting %
Early splitting %	-0.37*	0.65*	0.23	0.27	0.38	-0.27	0.015	-0.38*	0.34	-0.18	1
Cracking %	0.43	-0.005	-0.16	0.67*	-0.44	-0.38	-0.06	0.09	0.25	1	
P of leaf %	-0.28	0.29	0.7*	0.03	0.07	0.025	0.06	0.35	1		
Ca+2 of leaf (%)	-0.31	-0.11	0.45	-0.56	-0.14	-0.04	-0.22	1			
Mg+2 of leaf (%)	-0.29	0.27	0.15	0.09	-0.14	0.5	1				
Na+ of leaf (%)	-0.08	-0.43	0.39	-0.4	0.16	1					
Fe of leaf PPM	-0.61*	0.37	0.55	-0.17	1						
Zn of leaf PPM	0.41	0.23	-0.39	1							
Mn+2 of leaf PPM	-0.71*	0.29	1								
Cu+2 of leaf PPM	-0.69*	1									
K+ of leaf %	1										

Discussion

Aflatoxin contamination can occur during the ripening of pistachio nuts, which may show itself direct or indirect (Moradi and Fani, 2018; Moradi et al., 2004, and Doster and Michailides, 1999). Direct contamination may occur by *Aspergillus* spp. in early splitting cultivars result in aflatoxin production, while indirect one may arise by the factors affect the *Aspergillus* spp. density in the orchards, and cracking the nuts. The contamination may also be intensified by the factors which favor *Aspergillus* section *Flavi* growth and aflatoxin production during processing, transporting, and storing the nuts after harvest (Moradi and Hokmabadi, 2011). The results showed that the application of poultry manure on the soil's surface has increased the density of *A. flavus* group 4.5 and 9 times higher respect to the control treatment and fertilizer channel. This may indicate the application of poultry manure on soil's surface could increase organic matter, and result in the density of *Aspergillus* section *Flavi* propagules in the soil and subsequently in the air-borne spores. It shall increase the risk of contamination for pistachio nuts, especially for early splitting and cracking nuts. Therefore, it is recommended that, the poultry manure should be applied in the fertilized channels to avoid such contamination.

It has been demonstrated that *Aspergillus* species are the most common air-borne mold in pistachio orchards which can distribute widely in soil. The density of air-borne spore of *Aspergillus* spp. are

influenced by the cultural practices, the time and frequency of irrigation, plant's debris and animal manures application in soil (Moradi *et al*., 2004). Doster and Michailides (1994) reported that the spores of *Aspergillus* species in the soil could disperse in dust to aerial parts of trees by cultural practices such as disking.

It has been shown that poultry manure increased the density of fungal population in soils of cotton in China (Huang *et al*., 2006), sugarcane in Australia (Pankhurst *et al*., 2005), rice in India (Dinesh *et al*., 1998), and forest in Spain (Acea and Carballas, 1996).

The results has been shown cultural practices, plant litter, frequency and time of irrigation, frequency of toxigenic strains of *A. flavus*, early splitting and cracking of nuts, animal manures, environmental factors, distribution of aflatoxin in pistachio bulks and harvesting date are critical in the frequency of aflatoxin content in pistachios (Doster and and Michailides, 1994; Doster and and Michailides, 1995; Moradi et al., 2004; Ghadarijani and Javanshah, 2005; Moradi and Hokmabadi, 2011; Sommer et al., 1986).

The results also show that the application of poultry manure negatively affect the frequency of early splitting nuts formation by 0.74 % and 0.95 % in the fertilizer channel and soil's surface application, respectively. The same results have been observed with the formation of cracking nuts. On the other hands, poultry manure could improve the frequency of blank, splitting, and non-splitting nuts, which may therefore increase market-friendly. No significant differences were observed between the application of poultry manure on the soil's surface or in fertilizer channel assessing market-friendly characteristics.

Poor plant health and stress factors may have direct or indirect effects on the formation of aflatoxins (Sander *et al*., 1985 and Payne *et al*., 1989), and therefore, the poultry manure shall be used in channels to reduce the risk of aflatoxin in pistachio. More importantly, it is necessary to understand cultural techniques, such as planting time, crop density and cultivars, soil and leaf analysis, and also salinity and drought. Application poultry manure in fertilizer channel shall impose a suitable nourishing condition for pistachio trees and therefore decrease the percentage of cracking nuts. The positive effects of nutrients on fruit yield and quality were also reported (Keshavarz et al., 2011; Norozi et al., 2019). Plants with no poultry manure application may have a significantly higher frequency of early split nuts, which may subject to higher values of fungal and aflatoxin contamination compared to those applied in the depth of soil. Therefore, proper nutrition of pistachio trees with potassium and calcium fertilizers can reduce the pistachio nut cracking and consequently aflatoxin content in pistachios.

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