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Prevention and Control of Fungi Contaminated Stored Pistachio Nuts Imported to Saudi Arabia

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Abstract

To evaluate the contamination risk of the improper storage of pistachio nuts was studied in the major location of Saudi Arabia by studying the fungi associated with non and salted pistachio nuts. The infection with *Aspergillus flavus* and *A. niger* and treatment of this infection with some abiotic factors , salting and fumigation with acetic acid on the invasion and colonization were also studed. High percentage infection (100%) were found in salted pistachio of Maddinah , while low infection (68.75%) was found in non salted pistachio of Jeddah.Referring to the total fungal counts (9845.5 and 5681.8 CFU/g nuts) were detected on malt extract yeast agar and rose bengal agar media respectively. *Aspergillus niger* and *A. flavus* were found common in all pistachio samples collected from the three locations on the two media used. The both fungi were grew at temperatures between 20 and 35°C , also as the relative humidity increased the fungal growth increased reached its maximum at 100% RH. Sodium chloride at 20 and 25 % completely stopped the linear of the both fungi on malt yeast extract agar medium. Application of nuts with sodium chloride was found to increased the resistance of pistachio nut to invasion and colonization by the fungi during storage. Also, the resistance to invasion was increased by increasing the doses of fumigation with acetic acid applied to the pistachio nuts reached 0% infection at the higher dose of acetic acid (60%).

Key words: Pistachio, Mould, Invasion, Cholonization, abiotic agents, Salting, Fumigation.

Introduction

The pistachio (*Pistacia vera L.*) is a perishable nut susceptible to fungus infection in the orchard and during improper storage conditions. Damaged nuts caused by either pistachio weevil (*Amyetois transitalla*) or mechanically during harvest and transportation are particularly subjected to fungal invasion and decay.

Several fungi are capable of infecting growing pistachio nuts and causing damage to hulls and kernels (Michailides, 2006 and Denizel *et al.,* 2006). Infection is often facilitated by early splitting of hulls which leads to infestation by a number of hemipteran insects which feed on the nuts and serve as non-specific vectors for diseases (Sallam,2007).

Common saprophytic fungi including species of *Alternaria, Aspergillus, Chladosporium, Eurotium Fusarium, Penicilium, Trichoderma, Ulocladium, Epicoccum and Rhizopus* decay kernels of pistachio (Michailides, 2006). Abdel-Gawad and Zoharii (1993) identified a wide range of moulds from five kinds of nut seeds for human consumption in Saudi Arabia. Denizel *et al*. (2006) reported that the dominant external mycoflora of the immature pistachio nuts from three areas at Turkey consisted of *A. niger*, *A. flavus* and *Penicillium spp*. They also add that the stored dehlled nuts and dust sample taken from ware-houses were extensively contaminated with *A. flavus*, *A. niger* and *A. ochraceus*.

Late season rains will promote activity of *Botrydiplodia dathidea* on pistachio hulls and kernels (Michailides *et al*., 1995). Because mould counts on nuts going into storage can be high (Heperkan *et al*., 1994) , it is important that proper storage conditions (especially low RH ,absence of standing water)should be maintained to avoid serious problems.

The greatest postharvest disease threats are from

Aspergillus flavus and *A. paraziticus*. The danger is particularly serious because these fungi can produce aflatoxins. In their study of *Aspergillus* molds in California pistachios, Doster and Michalides (1994) reported that early split nuts had over 99% of the aflatoxin detected and now-infected nuts had substantially more infection by several *Aspergillus spp*. as well as over 84% the aflatoxin detected.

Several chemicals have been used as preservatives in stored grain to prevent mould growth during ambient drying. Acetic acid (AA) vapour applied at 0.78ml/kg to high moisture content of grains inoculated with conidia of *A. flavus* effectively prevent the fungal growth for 120 days at 20°C (Sholberg and Guance,1996) and also on stone fruit to control post harvest decay.

The objective of this work to evaluate the contaminatioin risk of the improper storage of pistachio nuts in the major locations of Saudi Arabia by studying 1-the fungi associated with pistachio nuts. 2-focusing on the influence of abiotic factors (temperature and relative humidity) on the growth of *Aspergillus flavus* and *A. niger* and 3- the effect of salting with NaCl or fumigation with acetic acid on the invasion and colonization of pistachio nuts with *A. flavus* and *A. niger*.

Material and methods

Pistachio samples

Twelve kg of commercially pistachio nut samples were collected randomly from supermarkets of three locations in Saudi Arabia,i.e., Elmaddinah, Makkah and Jeddah. The samples were stored in paper bags at 10°C until examination.

Mould incidence

Twenty cotyledons from the 200 nuts examined were randomly picked and surface sterilized with 2% sodium hypochlorite for 2 min, followed by rinsing with three washes of sterile distilled water. Four cotyledons were plated together equispaced from each on the two media used {malt extract yeast agar (MEYA) and rose bengal agar media (RBA)}. Two of the cotyledons had their inner surface turned up and the remaining two had their outer surfaces turned up. Before pouring plates streptomycin and penicillin were added each at a concentration of 50 mg /L of medium. Plates were incubated upright at 28°C for 5- 8 days during which the number of cotyledons that yielded colonies was recorded. Also, the colonies were noted enumerated and subculured for identification. Fungal incidence was expressed as a percentage of the 100 cotyledons plated according to Adebajo and Dyaolo (2003).

Estimation of total fungal counts

A serial decimal dilution was performed by adding randomly 10g of seed sample with 90 ml of sterilized water and shacked for 15 min at 200 rpm followed by ten fold serial dilutions up to 10^{-5}. Duplicate 1 ml of each dilution were added to Petri dishes containing 15 ml of the two above mentioned media (MEYA and RBA). The plates were incubated at 27°C for 5 days and the fungal colonies exerted were encountered daily and the result were expressed as CFU per gram of sample. The fungal colonies recovered were identified using the most documented keys in fungal identifications (Raper and fennel, 1973, Gilman, 1975, Barnett and Hunter,1984 and Domsch *et al*.,1993).

Effect of temperature and relative humidity on fungal radial growth

Two isolates of the most frequently isolated fungi ,*A. flavus* and *A. niger* were inoculated on plates of malt extract yeast agar medium using 5 mm discs and incubated at 15, 20, 25, 30, and 35°C in incubator and the radial growth of the fungi were determined.

Five levels of RH were maintained according to Ayyasamy and Baskaran (2005). The required RH was maintained by mixtures of appropriate combinations of concentrated sulphuric acid and distilled water (Table, 1). Mixtures were taken in the desiccators for each level of RH. Petri plates with malt extract yeast agar medium with the fungus, kept in the desiccators and covered with lids and sealed off with cellophane tape. The radial growth (mm)

Table 1. preparation of solutions for maintenance of different RH levels. (according to Ayyasam and Baskaran,2005)

Serial No.	Distilled water (ml)	H_2SO_4 (ml)	RH %
1	100.0	0	100
2	88.5	11.5	95
3	80.0	20.0	90
4	75.0	27.0	80
5	70.0	30.0	70

were determined at the end of the experiment.

Salt stress test

1-In-vitro effect

The effect of sodium chloride on the radial growth of *A. flavus* and *A. niger* was studied using MEY agar plates. A disc (5mm dm) from a pure culture of the two fungi was placed in the centre of the medium plate supplemented with Na Cl at 0, 2.5, 5, 10, 15 and 20%. Petri plates were incubated at 27°C for 7 days and the two diameters of every dish were measured . Four replicates were used for each particular treatment.

2-In-vivo effect

The effect of Na Cl on pistachio nut resistance to invasion and colonization by *A. flavus* and *A. niger* was also studied according to method of Hasan (1998). Spore suspensions were prepared from 8 days old cultures. Aseptic pistachio nuts as mentioned before soaked separately in o (distilled water as control), 10,15, and 20% solution of Na Cl for one hour. Treated nuts were aseptically placed in a sterile 15-cm diameter Petri-dish and left to dry and then 2 ml of a spore suspension of the two strain of fungi (approximately 1x 10^7 conidia /ml was applied for each dish and four replicates were used for each treatment. The nuts were gently rolled around the dish to spread the inoculums evently over their surface. The dished were put in a closed desiccators containing water and incubated at 27°C for 2 weeks. The nuts were visually examined for invasion and the colonization percentage were assayed.

Fumigation with acetic acid (AA)

The effect of fumigation with acetic acid to control the invasion and colonization by *A. flavus* and *A. niger* was evaluated according to method described by Sholberg and Guance (1996). As mentioned before sterilized pistachio nuts were fumigated for 1hr with 20, 40, and 60% acetic acid. Four replicates were used for each treatment. Treated nuts were asceptically placed in a sterile Petri dishes and then inoculated with fungal conidia immediately as mentioned before.

Statistical analysis

Standard deviation and least significant differences (LSD) at 5% were calculated to compare the variance between treatments (Gomez and Gomez,1984).

Results and Discussions

Table (2) show that 68.75-100.0% (average 90.62%) of pistachio nuts were naturally infected with fungi on malt extract and rose bengal agar media respectively of samples collected from different locations. High percentage infection (100%) were found in salted pistachio of Maddinah , while low percentage infection (68.8%) was found in sample of non salted pistachio of Jeddah.

Referring to the total fungal count associated to nuts, the high count (387.3 colonies/100 nuts) was detected from salted pistachio of Makkah on malt extract agar medium, while the low colony count (212.5 colonies/100 nuts was detected on rose bengal agar medium from salted pistachio of Maddinah and non salted sample of Jeddah.

The results on fungal counts (9848.5 and 5681.8 CFU/ g nuts) were detected on MEYA and RBA media respectively. The high frequency of moldy and large counts probably indicate poor storage conditions and the high temperatures as well as higher relative humidity (Heperkan *et al*.,1994).

Occurrence of fungi in different locations

The data of all samples from different regions per principle fungal species or genera given in Table (3) indicating that the surface contamination occurred within intact nuts of each samples. Great variation in types and in numbers of propagations among sample was noted and many fungal isolates which were found in water surface washing of salt pistachio nuts were absent or present in the non salted nuts.

Aspergillus niger and *A. flavus* showed similarities where in both species were found common in all pistachio samples collected from the three locations on the two media used. As the results on CFU /g showed that *A. niger* and *A.flavus* prevailed in the non pistachio nuts collected from Makkah with maximum values of 250 and 112.5 respectively on rose bengal agar medium.

Also, *A. sydowi* was detected in the same pistachio of different locations,except samples of Maddinah placed on malt extract agar medium. Other fungi included *A. ochraceus, Fusarium spp. Penicillium and Phycomycetes* were found to occur sporadically and to constitute small numbers of the fungal isolates on rose bengal agar medium. The predominance of *Aspergillus* and *Penicillium spp*. in stored pistachio nuts was also shown by Doster and Michalides (1994) in a work carried out with samples from different countries. For example, it was reported that up

Table 2. Percentage of fungal infection and number of fungi associated with salted and non salted Pistachio nuts obtained from different locations in Saudi Arabia.

Location	Pistachio treatment	% of infection		No. of fungi (No./100 nuts)		CFU colonies / g nuts	
		Malt extract	Rose bengal	Malt extract	Rose bengal	Malt extract	Rose bengal
Maddinah	Salted	100	100	27.5	212.5	56.8	511.4
	Non	100	81.3	275.0	218.8	113.7	113.7
Makkah	Salted	75.0	81.3	387.3	243.8	757.6	558.18
	Non	100	100	262.5	2438	757.6	4924.2
Jeddah	Salted	100	87.5	375.0	318.8	5681.8	9848.5
	Non	68.8	68.8	213.8	212.5	132.6	113.7

Table 3. occurrence of fungi in salted and nonsalted pistachio nuts collected from three locations in Saudi Arabia

(CFU/100 nuts)

location	Pistachio treatment	Fungal genera and species							
		A. flavus	*A. niger*	*A.. ochrac-eus*	*A. sydowi*	*Fusarium spp.*	*Penicillium Spp.*	*Phyco-mycetes spp.*	*Rhizop-us Spp.*
On malt extract agar medium									
Maddinah	Salted	25.0	137.5	62.5	-	-		-	50.0
	Non salted	50.0	150.0	62.5	-	-	25.0	-	50.0
Makkah	Salted	50.0	150.0	-	12.5	50.0	-	-	50.-0
	Non salted	112.5	250.0	-	62.5	-	62.5	-	-
Jeddah	Salted	25.0	87.5	-	25.0	225.5	-	-	-
	Non salted	62.5	75.0	-	25.0	-	87.5		-
On rose bengal agar medium									
Maddinah	Salted	12.5	112.5	-	25.0	-	-	-	37.5
	Non salted	12.5	62.5	-	50.0	-	-	-	25.0
Makkah	Salted	50.0	75.5	-	25.0	-	-	25.0	12.5
	Non salted	25.0	87.5	-	50.0	25.0	-	-	12.5
Jeddah	Salted	25.0	100.0	-	25.0	12.50	-	112.5	-
	Non salted	50.0	87.5	-	137.5	-	25.0	-	-

to 13 species were isolated from pistachio kernels from orchards in Iran and 14 species from orchard in the United States (Boutrif,1997). Similar results were also obtained in Turkey by Denizel *et al.* (2006).

Effect of temperature on the linear growth of *A. flavus* and *A. niger* on malt extract yeast agar medium.

The two fungi were incubated at five different degrees of temperatures:15, 20, 25, 30 and 35°C. Results in Table (4) reveal that the radial growth of *A. flavus* and *A. niger* was maximal at 25 °C (57.25mm) and 30°C (78.00 mm) respectively. Both fungi grow at temperatures between 20 and 35°C . The two fungi were unable also to grow at temperature 15°C.

Effect of relative humidity on the linear growth of *A. flavus* and *A. niger* on malt extract yeast agar medium.

It is clear from the results in Table (5) that *A. flavus* and *A.niger* grow at a wide range of relative humidity, however, the growth was much enhanced by high RH. Also, as the RH increased the growth increased to reach its maximum at 100% RH.

Effect of sodium chloride salt on the linear growth (mm) of *A. flavus* and *A. niger* on malt extract yeast agar medium

Table (6) show that all concentrations of sodium chloride used had affected the linear growth of the two tested fungi. Concentrations at 20 and 25% proved in this concern to

Table 4. Effect of different levels of temperatures on the radial growth (mm) of *A. flavus* and *A. niger* on malt extract yeast agar medium.

Temperature °C	*A. flavus*	*A. niger*
15	0.0*	0.0
20	27.0	37.25
25	57.25	54.75
30	46.25	78.00
35	14.00	47.25
LSD at 5%	2.038	6.151

* Each value is an average of data of 4 replicates .

Table 5. Effect of different levels of relative humidity on the radial growth (mm) of *A. flavus* and *A. niger* on malt extract yeast agar medium.

RH%	*A.flavus*	*A. niger*
100	62.50*	82.25
90	42.25	62.25
80	31.75	45.25
70	27.25	26.75
60	14.50	19.5
LSD at 5%	6.349	6.500

* Each figure is an average of data of 4 replicates and incubated at 25°C for A. flavus and at 30°C for A.niger.

Table 6. Effect of sodium chloride salt on the linear growth (mm) of *A. flavus* and *A. niger* on malt extract yeast agar medium.

Fungi	Sodium chloride conc. (%)							LSD at 5%
	0	2.5	5	10	15	20	25	
A.flavus	90.0*	81.0	76.75	34.75	12.00	0.00	0.00	2.26
A. niger	90.0	88.5	84.75	46.50	18.00	0.00	0.00	3.78

* Each value is an average of data of 4 replicates and incubated at 25°C for A. flavus and 30°C for A.niger

be the most effective as they completely stopped their growth.. Also, it is evident that as the concentration of salt increased the antifungal activity against fungal growth was significantly increased. Conforming results were available by other workers including Thamaboripat *et al.* (1992), they reported that high concentrations of NaCl may adversely affect the water activity required for fungal growth or it may be that sodium ions interfere with ion transport in the organism. Similar observations were also obtained by Hasan (1998).

Effect of sodium chloride on seed colonization percentage

Pistachio nut treated with different concentrations of sodium chloride were evaluated for colonization by *A. flavus and A. niger*. In general, data in Table (7) show that the mean percentage of colonized seeds ranged from 95.0 to 97.0% for *A. flavus* and from 90.0 to 97.5% for *A. niger* during the 1st and 2nd week storage respectively.

Application of seeds with sodium chloride increased the resistance of pistachio nut to invasion and colonization by the two fungi. As the percentage of seed colonization were decreased by increasing the dose of Na Cl applied to the inoculated seeds and reached its minimum at the higher concentrations during different period of storage than the control (without Na Cl). Such results were greatly agreed with those obtained by Chatterjee (1989) and Thamaboripat *et al*. (1992).

Effect of fumigation with acetic acid (AA) on seed colonization percentage

Results in Table (8) show that the mean percentage of seeds colonized by *A. flavus* was 97.50% during the 1st and 2nd and were 85.75 and 87.00% in seeds colonized by *A. niger* during the same two periods respectively.

Fumigation with AA was found to increase the resistance of pistachio nuts to invasion and colonization during storage. In general, the resistance was increased by increasing the doses of AA applied to the nuts. As the percentage of infection reached o% at the higher concentration of AA (60%) in nuts inoculated with *A. flavus* at the 1st and 2nd week and during the 1st week in *A. niger*. This is in accordance with the limits given by Sholberg and Guance (1996) who reported that acetic acid vapour applied to canola, rice, and wheat inoculated with *A. flavus* effectively prevented *A. flavus* growth. Similar observations were showed by Sholberg (1998) and Sholberg and Guance (1996).

Table 7. Effect of salting with sodium chloride on the percentage of infection of pistachio nuts infected with *A. flavus* and *A.niger* during storage for one and two weeks

Treatment	*Aspergillus flavus*		*Aspergillus niger*	
	Storage periods / week			
	One week	Two weeks	One week	Two weeks
Control (non-inoculated)	0.0	0.0	0.0	0.0
Inoculated nuts and treated with NaCl at.				
0%	95.0	97.0	90.0	97.5
10%	70.0	75.5	81.0	85.5
15%	51.0	82.0	59.5	62.5
20%	49.5	49.5	36.0	41.0
LSD at 5% for				
• Treatment	• 20.23		• 22.30	
• Period	• 12.79		• 44.61	
• interaction	• 28.62		• 31.54	

Table 8. Effect of fumigation with acetic acid on the percentage of infection of pistachio nuts infected with *A. flavus* and *A.niger* during storage for one and two weeks.

Treatment	*Aspergillus flavus*		*Aspergillus niger*	
	Storage periods / week			
	One week	Two weeks	One week	Two weeks
Control (non-inoculated)	0.0	0.0	0.0	0.0
Inoculated nuts and treated with NaCl at.				
0%	97.5	95.5	85.75	87.0
10%	28.75	77.5	66.0	70.5
15%	13.75	22.5	11.25	42.5
20%	0.0	0.0	0.0	20.0
LSD at 5% for		20.23		22.30
Treatment	12.79		44.61	
Period	28.62		31.54	
interaction				

Further research is needed to explore other means of preventing mould infection. These could include prevention of contamination in the field through the use of biological control agents ,that are pathogenic to the mycotoxin producing fungi such as yeast or harmless bacteria or fungi.

Conclusion

This study recommended that it must be stored the pistachio nut in a controlled proper storage (temperature and humidity) as soon as possible to control the infection caused by *Aspergillus spp*. Also, it can be used the NaCl and fumigation with acetic acid to prevent any sporulation of mycotoxin producing fungi.

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دراسات على منع التلوث الفطرى والتخلص منها فى الفستق المستورد والمخزن فى المملكة العربية السعودية

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الملخص

تم دراسة وتقييم خطورة التخزين الردىء للفستق المملح والغير مملح والمجموع من ثلأث مناطق كبرى فى المملكه العربية السعودية شملت مكة والمدينة وجدة وتم دراسة مدى احتوائها على الفطريات ودراسة مدى إصابتها بفطريات الاسبرجلس فلافس وأسبرجلس نيجر. كما أجريت دراسة عن مدى تأثير بعض العوامل البيئية مثل الحرارة والرطوبة والتمليح بكلوريد الصوديوم والتبخير بحمض الخليك على اصابة الفستق ومدى مقاومته للاصابة بالفطريات السابقة . أوضحت النتائج أن أعلى نسبة إصابة للفستق كانت فى العينات المملحة من فستق المدينة وأقلها اصابة فى العينات الغير مملحة من فستق جدة. وقد بلغ العدد الكلى للفطريات ٥٦٨١,٨ - ٩٨٤٥,٥ وحدة فطرية /جرام فستق على بيئة مستخلص المولت والخميرة وعلى بيئة الروزبنجال على الترتيب. كما وجد أن فطريات الاسبرجلس فلافس ونيجر من أكثر الفطريات الموجودة والتى تم عزلها من فستق المناطق الثلاثة. بأختبار تأثير بعض العوامل البيئية والكيماوية على نمو الفطريات الاسبرجلس فلافس ونيجر والتى أمكنها النمو على درجات حرارة بين ٢٥ ، ٥٣٥ م ويزداد نموها بزيادة الرطوبة النسبية حتى ١٠٠٪. كما وجد أن كلوريد الصوديوم بتركيز ٢٠ -٢٥ ٪ يمكنة تثبيط نمو الفطريات كليتا عند اختبارها على البيئة الصناعية من مستخلص المولت والخميرة . كما بينت الدراسة أن معاملة الفستق المعقم والمحقون بفطريات الاسبرجلس فلافس ونيجر بواسطة كلوريد الصوديوم أو التبخير بحمض الخليك يزيد من مقاومة الفستق للاصابة بالفطريات وذلك خلال التخزين لدرجة أنه أمكن منع الاصابة بالفطريات باستعمال التبخير بحمض الخليك بتركيز ٦٠٪. نوقشت النتائج فى ضوء الابحاث المقارنة كما نوقش امكانية استخدامها ضمن سياسة متكاملة للحد من انتشار الفطريات بالفستق.