Postharvest Pathology and Mycotoxin Contamination of Iranian Pistachio Nuts

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ABSTRACT

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Fresh, uncracked pistachio nuts were tested for *Aspergillus flavus* and aflatoxin before and after a commercial washing treatment. The wash water was a source of fungus contamination, but in spite of this, no aflatoxin was produced when the nuts were exposed to low humidity after immediate drying in the sun. Commercially hulled and dehulled nuts, inoculated with *A. flavus* spores, were incubated for as long as 4 mo at relative humidities of 25, 65, and 100%. No aflatoxin was detected on nuts stored at the lower humidities, but was detected at 100% relative humidity.

Previous experiments in our laboratory have shown that pistachio (*Pistachia vera* L.) nuts contain aflatoxin produced by *Aspergillus flavus* Link after ripening but before harvest (5). The greater the percent of open nuts the greater the percent of contamination (5).

Under field conditions in Rafsanjan, the Owhadi (kermani) cultivar of pistachio nut may be 75-90% open and remain on the tree for up to 3 mo following ripening. Yet in some years only 1-3 % nuts may be contaminated by only a trace to 5 μg/kg aflatoxin. In other years up to 75% of the nuts may be contaminated with up to 2,000 μg/kg (5). We also observed that unripened and unopened nuts damaged by insects contained significant amounts of aflatoxin at the same time that undamaged nuts were free of aflatoxin (10). The objectives of the present study were twofold: (i) to evaluate the importance of fungal contamination and aflatoxin production by analyzing wash water during the processing of commercial pistachio nuts (as designated second stage contamination by Suzangar et al) (9), and (ii) to investigate the role of relative humidity (RH) as a possible reason for the variation in percent and amount of aflatoxin contamination of pistachio nuts in the field.

MATERIALS AND METHODS

A typical processing plant in Rafsanjan, the major nut growing and export center of Iran, was chosen for the study. At this plant the commercial processing of pistachio nuts consists of: (i) dehulling of shells by machine; (ii) washing with water in a cement container (1×3×1m) to separate the aborted nuts; (iii) hand separation of uncracked nuts, and (iv) patio sun-drying or machine drying with hot air. After most of the pistachio nuts are ripe and have been harvested, uncracked nuts are soaked in water, hand cracked, and dried.

Wash water from a commercial washer was tested for fungal contamination by sterile serial dilutions of the water samples. The dilutions were plated on 2% water agar (WA). After incubation at 25 C for 4 days, the number of propagules of *A. flavus* and other fungi per milliliter of wash water was recorded.

For nut studies, 100 commercially-dehulled, washed and unwashed pistachio nuts were surface sterilized in 0.5% sodium hypochlorite for 10 min, placed in sterile petri plates containing salt malt agar (SMA) (1), and incubated at 25 C for 1 wk. After incubation, the percent of nuts contaminated with *A. flavus* was recorded. When whole nuts were used, they also were surface sterilized with sodium hypochlorite. In some instances following surface sterilization of nuts, they were manually cracked and the outer green shell, inner hard shell, and kernel aseptically removed and each placed separately on SMA plates. If the inner hard shell was unsplit before cracking, it was omitted from the test.

Spores of *A. flavus*, produced after 1 wk at 25 C on 2% potato-dextrose agar, were used to inoculate commercially processed, steam sterilized (120 C for 20 min) pistachio kernels. Cultures were incubated at 25 C for approximately 1 wk. Moldy nuts were shaken in sterile water containing trace amounts of "Tide" detergent and centrifuged at 7,000 rpm for 10 min. The supernatant liquid was decanted and the resulting pellets were resuspended in sterile distilled water. The number of viable propagules was estimated by the number of colonies on WA after 48 hr of incubation at 25 C. The *A. flavus* isolate that was used was cultured from Rafsanjan-grown pistachio nuts (number 7-1 in our collection). Identification of the fungus was confirmed by M. Mc Elroy of the United States Food and Drug Administra-

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tion. That the local strain of *A. flavus* produced aflatoxin B_1 was determined by 1-wk of incubation on rice substrate followed by chloroform extraction and aflatoxin determination by the official first action method (26.014-26.019) commonly designated as the CB method (7).

For inoculation of pistachio, nuts were immersed for 40 min in a suspension of *A. flavus* containing 10^6 spores/ml before incubation at 20-25 C and RH of 25 ± 5, 65 ± 5, and 100%. Twenty-five percent RH was chosen as that of the mean prevailing in Rafsanjan during nut ripening (6) and was achieved in this experiment by incubating the tested nuts under room conditions in the laboratory. Sixty-five percent ± 5% RH, slightly higher than the highest mean RH recorded over the past 5 yr in the pistachio growing area (6), was maintained by placing the nuts in an air-circulating incubator in which dishes with water and wet paper towels were placed. Care was taken to replenish the water supply daily to maintain the humidity. The RH and temperature were recorded by a hygrothermograph. Placing nuts and wet paper towels in a large closed desiccator produced 100% RH as indicated by water droplet formation on the glass cover. Contamination of nuts with *A. flavus* was determined by periodically culturing them on SMA. After dipping nuts in the spore suspension, but before culturing them, the surfaces of the nuts at 25% RH were sterilized with sodium hypochlorite, and those at 65% and 100% RH were dried at 60 C for 48 hr before being tested for fungal growth and aflatoxin. Preliminary experiments showed that treatments of the nuts by either method resulted in the same degree of *A. flavus* contamination. For routine aflatoxin analysis 100 g of nuts (hard shells plus kernels) were used and the toxin determination was made by employing official method (26.020-26.024, commonly known as BF method (7). When whole nuts were used, the outer green shells of nuts previously tested were removed, dried at 60 C for 48 hr, then tested for aflatoxin content by the Pons et al method (8).

Spore density on nuts contaminated with *A. flavus* in the laboratory were compared with those naturally contaminated in the field. Petri plates of SMA were exposed for various time periods in a commercial

TABLE 1. Number of fungal propagules in wash water and mean percent of pistachio nuts contaminated with *Aspergillus flavus* before and after washing[a]

Expt. year[b]	Sequence of sampling	No. of fungal propagules/ml[c]		Mean percent of nuts contaminated with *A. flavus*[d]	
		A. flavus	Total fungi	Hardshell	Kernel
1975	Before washing	0	15 ± 2	3 ± 1	3 ± 1
	After washing	1,500 ± 110	8,450 ± 1,650	50 ± 12	66 ± 7
	Wash water + 0.5% NaOCl[e]	0	0	6 ± 4	10 ± 4
1976	Before washing	...	...	7 ± 5	2 ± 1
	After washing	...	...	35 ± 8	46 ± 5

[a]All nut samples were analyzed for aflatoxin and none was detected.
[b]The field plots for 1975 were separated by 20 km from those used in 1976.
[c]Tabulated numbers are the mean of two replicates with standard error.
[d]Tabulated numbers are the means of five replicate groups of 20 nuts each.
[e]The nuts became discolored during drying.

TABLE 2. Infection and aflatoxin production in dehulled pistachio nuts inoculated with a spore suspension of *Aspergillus flavus* and incubated at different relative humidities

Incubation[a] time (days)	Nuts contaminated at			Aflatoxin[b] produced at		
	25% RH (%)	65% RH (%)	100% RH (%)	25% RH (μg/kg)	65% RH (μg/kg)	100% RH (μg/kg)
0 (S)	5	0	...	0	0	...
2 (S)	0	0	...	0	0	...
4 (S)	0	0	...	0	0	...
6 (S)	...	...	...	0	...	...
8 (S)	...	0	...	0	0	...
10 (S)	...	0	...	0	0	...
0 (A)	100	0	0	0	0	0
2 (A)	100	100	100	0	0	0
4 (A)	100	100	100	0	0	0
6 (A)	...	...	100	0	...	178
8 (A)	...	100	100	0	0	48
10 (A)	...	100	100	0	0	60
120 (A)	95	93	...	0	0	...

[a]Dehulled nuts placed in sterile distilled water (S) or 10^6/ml *A. flavus* spores in suspension (A).
[b]Sum of aflatoxins B_1 plus B_2, the only ones that were detected. Analyses based on 100-g nut samples.

pistachio garden in Rafsanjan.

Recording hygrothermographs were placed in three locations in a commercial pistachio warehouse and continuous readings were made between 20 September, when the warehouse was empty, till 15 December, when the harvest had been completed for 1 mo but before shipping had started. Mean weekly RH was calculated by multiplying the number of hours by the highest RH in each ten-degree range beginning at 10 to 80 RH (the lowest and highest RHs recorded). The values for each range then were added and divided by 168 (total hours in a week).

RESULTS

The wash water was free of *A. flavus* and nearly so of other fungal contaminants before nut washing (Table 1). Following washing of commercial quantities of nuts, the wash water became heavily contaminated with fungi including *A. flavus*. Also, after washing the mean percent nut contamination increased. Nevertheless, aflatoxin analysis of washed and unwashed nuts indicated that both groups of nuts were free of aflatoxin at the level of analytical detection that was employed (0.5 μg/kg). To rule out wash water as a possible source of fungal inoculum, sodium hypochlorite was added to the water in a final concentration of 0.5% which eliminated the fungi of the wash water. The mean percent contamination of the nuts with *A. flavus* after washing remained essentially the same as for those not washed with sodium hypochlorite (Table 1). Since the sodium hypochlorite treatment did not reduce the number of *A. flavus* propagules, it would appear that the infection of the nuts took place prior to harvest and was deepseated. Larger batches of nuts also were treated with sodium hypochlorite but this resulted in shell and kernel discoloration, which lowered their commercial value according to the growers. Two groups of dehulled nuts (without the outer green shell) were tested in the laboratory. One group was placed in sterile water, the other was inoculated with the local strain of *A. flavus* and both were incubated for various times at three different RHs to determine the extent of aflatoxin production after drying and storage of the nuts. Essentially none of the dehulled nuts dipped in sterile water was contaminated with *A. flavus* nor was there any aflatoxin detected (Table 2). Dehulled nuts inoculated with *A. flavus* were 100% contaminated; despite this, no aflatoxin was detected at the end of 4 mo of storage at 25 and 65% RH. In contrast, at 100% RH substantial amounts of aflatoxin were detected in nuts after only 6 days (Table 2).

At 25% RH in sterile water no contamination of whole nuts (green-shell on) by *A. flavus* resulted nor was aflatoxin produced (Table 3). At 65% RH, the whole nuts in sterile water were contaminated within 2 days and the amount of aflatoxin remained essentially constant during incubation. As with dehulled nuts, 100% of the whole nuts inoculated with *A. flavus* were contaminated at all RHs. No aflatoxin was detected at 25% RH. However with whole nuts at 65% RH the aflatoxin present remained essentially constant and only at 100% RH was there a progressive increase in aflatoxin with time of incubation. Aflatoxin first appeared in the outer green shell, then in the hard shell and kernel in whole nuts incubated at 100% RH (Table 4). The percent of the total whole nut aflatoxin increased with time and most of the aflatoxin was in the treated hard shell and kernel. Meanwhile after 4 days the percent of the total whole nut with aflatoxin in the green shell remained essentially constant for most of incubation period.

The time required to attain moisture equilibrium of whole and dehulled nuts at 65% RH is shown in Fig. 1. The nuts with the green outer shell present required more time (10 days) to reach moisture equilibrium as compared with dehulled nuts (8 days).

In the field, *A. flavus* spore density was 2.7/hr on 10-cm-diameter plates or 0.0344 spores/ml/hr. Since the time between ripening and harvest may be as long as 3 mo, the nuts theoretically could be exposed to 2,184 hr or a total of 75 spores/ml in the field. The laboratory spore suspension was essentially 10^5 times greater than that of the Rafsanjan field air, while the ambient RH in Rafsanjan at the time of harvest during the past 5 yr varied betwen 10 and 25% except during actual rainfall

TABLE 3. Infection and aflatoxin production in whole pistachio nuts inoculated with a spore suspension of *Aspergillus flavus* and incubated at different relative humidities

Incubation[a] time (days)	Nuts contaminated at			Aflatoxin[b] produced at		
	25% RH (%)	65% RH (%)	100% RH (%)	25% RH (μg/kg)	65% RH (μg/kg)	100% RH (μg/kg)
0 (S)	0	0	0	0	0	...
2 (S)	0	100	...	0	13	...
4 (S)	0	100	...	0	13	...
6 (S)	0	...	...	0	...	...
8 (S)	0	100	...	0	11	...
10 (S)	0	100	...	0	13	...
0 (A)	0	0	0	0	0	0
2 (A)	100	100	100	0	30	7
4 (A)	100	100	100	0	33	82
6 (A)	100	...	100	0	...	63
8 (A)	100	100	100	0	27	95
10 (A)	100	100	100	0	37	159

[a]Whole nuts placed in sterile distilled water (S) or 10^6/ml *A. flavus* spore suspension (A).
[b]Sum of aflatoxin B_1 plus B_2, the only ones that were detected. Analyses based on 100-g nut samples.

(6). Thus though nuts were subjected to greater challenge in the laboratory than in the field, at 25% RH no aflaxtoxin was produced in 4 mo.

Because the warehouse of one of the principal nut buyers in Rafsanjan was filled with nuts, the RH increased from 22.1%, in September, and reached a mean maximum of 63.4% during the week of 7-14 November, when harvesting was completed in 1976. Thereafter the warehouse RH decreased to essentially a constant level of 48.6 to 49.5%. Changes in warehouse RH occurred despite a nearly constant outside RH of 25-30%. The maximum RH recorded was 71-80% for 1 hr when the house was almost completely filled from 30 October- 6 November.

DISCUSSION

These results confirm those previously reported, that *A. flavus* inoculum is disseminated via the wash water (10) This investigation has shown that the inoculum carryover is on the surface of nuts and that time is required to initiate infection. Previously, we reported delaying surface sterilization for 48 hr after nut dipping in wash water infested with *A. flavus* significantly increased the percent of nuts contaminated (9). Also, surface sterilization with sodium hypochlorite on a commercial scale had no effect on nuts already contaminated in the field. Similar difficulty of sterilization of rice grains contaminated with *A. flavus* has been pointed out by Christensen and Mirocha (2).

The second stage of possible nut contamination by *A. flavus* appears to be of minimal importance in aflatoxin production under the prevailing conditions of a typical commercial processing plant in the Rafsanjan area. Immediately after washing, the nuts are exposed to patio sun-drying in which the RH ranges 14-23% (6). Placing fresh pistachio nuts in a suspension of 10^6 *A. flavus* spores/ml for 40 min of patio drying as per commercial nuts, and incubating them at 25% RH for up to 4 mo resulted in no detectable aflatoxin. This may be contrasted with field-contaminated nuts treated in the same way which were estimated to be exposed to approximately 75 spores over the same time period. These results confirm those of Emami and coworkers (5) that the percent of aflatoxin-contaminated nuts at harvest did not change after 3 and 6 mo of storage.

TABLE 4. Amount and percent of total aflatoxin in different parts of whole pistachio nuts inoculated with a spore suspension of *Aspergillus flavus* and incubated at 100% relative humidity

Incubation time (days)	Aflatoxin detected[a]			
	Green shell		Hardshell + Kernel	
	Amount (μg/kg)	Total (%)	Amount (μg/kg)	Total (%)
0	0	0	0	0
2	7	100	0	0
4	12	15	70	85
6	13	21	50	79
8	15	16	80	84
10	7	4	152	96

[a]Sum of aflatoxin B_1 plus B_2. Analyses based on 100-g samples.

The ecology of aflatoxin production by *A. flavus* appears to have been thoroughly explored for stored peanuts (4), rice (2), and Turkish pistachio nuts (3), in which a minimum of 85% RH is required for aflatoxin production. Only dehulled nuts inoculated with *A. flavus* spores and incubated at 100% RH in the laboratory exhibited increasing amounts of aflatoxin over a 10 day period at 25 C. Nuts incubated at the same temperature at 25 and 65% RH exhibited no aflatoxin even after 4 mo. Further, when whole nuts were dipped in sterile distilled water and incubated at similar RHs only the outside green shell cover contained detectable amounts of aflatoxin after storage at 65% and 100% RH. Also, the amount of aflatoxin in the green shell was essentially constant at 65% RH regardless of whether the nuts were placed in sterile water or inoculated with *A. flavus* spores. Dehulled nuts inoculated with *A. flavus* spore suspension and incubated at 65% RH were not observed to produce aflatoxin. RH of 83–85% has been observed to be the minimum for aflatoxin production in Turkish pistachio nuts (3). The essentially constant amount of aflatoxin observed in the present experiments on nuts in the green shell may derive from the 2-day requirement for the RH to change in whole nuts when placed at 65% RH. During this 2-day period, therefore, the humidity is high enough to allow aflatoxin production, but thereafter it is too low and the amount of aflatoxin produced over the 10-day period would be that produced the first 2 days.

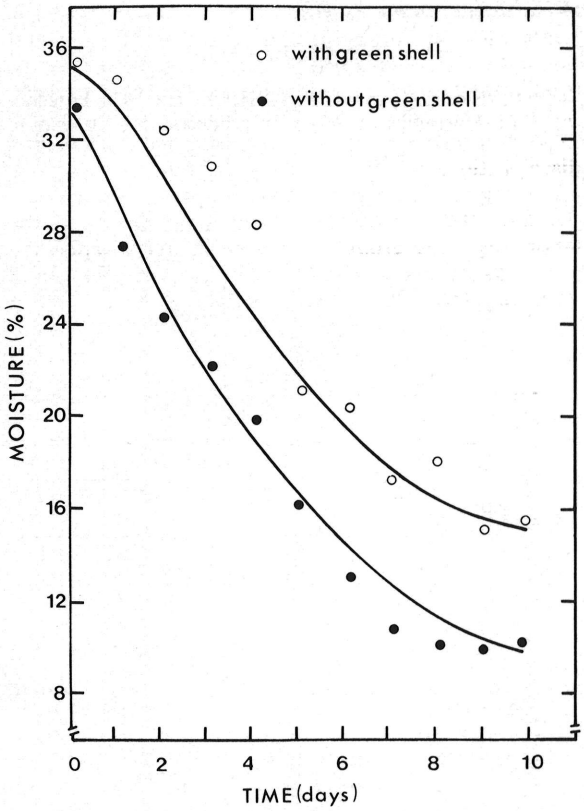

Fig. 1. Daily mean percent water content in hardshell plus kernel of whole or outer green shell dehulled pistachio nuts incubated at 65 ± 5% relative humidity and 25 C for 10 days.

Emami and his coworkers (5) report that the water content of the pistachio nuts before ripening on the trees is as high as 76%. The water content of the nuts decreases rapidly prior to ripening on the trees and over the subsequent 145-day period. Thus, it would appear that the liquid flow from the tree to the nut apparently stops, or at least decreases, at the time of nut ripening. The foregoing explanation of the yearly rate of aflatoxin contamination of Iranian pistachio nuts also would explain why insect-damaged nuts were observed to be aflatoxin contaminated before ripening. The unripened nuts have a high water content and there appears to be no lessening of water flow from tree to nut as occurs at ripening, when up to 90% of the outer green shell of Owhadi pistachio nuts are split. Thus, in the unripened state, conditions that are maximally maintained to conserve water are most conducive to aflatoxin production, if the nut integument is damaged to allow entry of *A. flavus*. Thus, when whole pistachio nuts are inoculated with spores of *A. flavus* at concentrations far greater than nuts exposed in the field, they do not become aflatoxin contaminated if incubated at 25% RH. From the present results it may be concluded that a substantial amount of aflatoxin contamination of pistachio nuts would be predicted only when it rains (essentially 100% RH) and the nuts are on the trees. This conclusion is in accord with previous observation of the yearly variation and correlation between United States rejection rates due to aflatoxin nut contamination and rainfall during only the 3-mo harvest time in the Iranian growing and export area of Rafsanjan (*unpublished*).

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