Aspergillus Molds and Aflatoxins in Pistachio Nuts in California

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ABSTRACT

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A total of 14 *Aspergillus* species were isolated from the kernels of pistachio nuts, mainly early splits, from 11 commercial orchards in California in 1991 and 1992. Early splits are atypical nuts that have split hulls, exposing the kernel to invasion by molds and insects (normal nuts have intact hulls). *A. niger* was the only *Aspergillus* species that occurred frequently (in 30% of kernels from early splits). However, *A. flavus* or *A. parasiticus* (potential producers of the mycotoxins aflatoxins) were found in early splits from most orchards, and *A. ochraceus* or *A. melleus* (potential producers of the mycotoxins ochratoxins) were found in all orchards. Aflatoxins were detected in early splits from six of nine orchards in 1991 and five of eight orchards in 1992. Early splits with rough, shriveled hulls had more than twice the *A. niger* infection and more than three times as much *A. flavus* or *A. parasiticus* infection as early splits with smooth hulls. The rough early splits had over 99% of all the aflatoxins detected. Kernels infested by the insect navel orangeworm (*Amyelois transitella*) had substantially more infections by *A. niger, A. flavus* or *A. parasiticus*, and *A. ochraceus* or *A. melleus* and had 84% of all aflatoxin detected. The hulls of early splits frequently had low levels of aflatoxin. Hull rupture due to damage by birds or to cracking resulted in kernels infected with *Aspergillus* molds but at low levels. Fortunately, the pistachio nuts most likely to have mold and aflatoxin contamination, rough early splits infested with navel orangeworm, had several physical characteristics (weight, size, shell discoloration, hull appearance) distinct from normal nuts that could facilitate removal during processing.

Additional keywords: *Aspergillus tamarii, Pistacia vera.*

Molds in the genus *Aspergillus* frequently decay the kernels of such nuts as pistachios (*Pistacia vera* L.) (20), almonds (21,23), chestnuts (37), and pecans (15,38). Many *Aspergillus* species infect and decay nuts before harvest. For example, 13 species were isolated from pistachio kernels from orchards in Iran (20). Even when *Aspergillus* species occur infrequently, their presence can be a serious problem, because many produce toxins harmful to humans and animals (22). The most important of these toxins is aflatoxin, produced by *A. flavus* Link:Fr. and *A. parasiticus* Speare. There is widespread concern regarding this potent toxin and carcinogen, and many nations have regulations concerning the amount that can be present in foods for human and animal consumption (35). Aflatoxin has been found in pistachio nuts (31), almonds (12,29), and walnuts (12). Another mycotoxin of concern is ochratoxin, produced by *A. ochraceus* K. Wilh. and closely related *Aspergillus* species (22).

The shells of most pistachio nuts split naturally in the orchard prior to harvest. Fortunately, the hull usually remains intact, covering and protecting the kernel from invasion by molds and insects. Nevertheless, approximately 1–4% of the nuts in an orchard have the hull attached to the shell so that the hulls split with the shell (9), exposing the kernel to molds and insects. These nuts, called early splits (Fig. 1), have relatively high levels of aflatoxin (31). However, no research has determined the incidence of *Aspergillus* molds in early split pistachio nuts. The navel orangeworm (*Amyelois transitella* (Walker)) commonly infests nuts with ruptured hulls like early splits (25) and has been associated with very high levels of aflatoxin (31). In pistachio orchards, there are other instances of hull rupture besides early splits. When harvest is very late, the hull deteriorates and can become tattered, and these tattered pistachio nuts may contain aflatoxin (31). Bird damage (27) also causes hull rupture. After birds peck through the hull to eat some of the kernel, molds can infect the exposed kernel. Hulls also may rupture by cracking, a process distinct from early splits because the cracking occurs after the shell splits, and the location of the crack is not along the suture where the shell split (Fig. 1). The amounts of molds or aflatoxin in nuts that are bird-damaged or that have cracked hulls has not been investigated previously.

Because the removal of contaminated nuts during processing is probably the best way to reduce mold and mycotoxin contamination, it would be very beneficial to identify the distinctive features of contaminated nuts that would facilitate physical separation. For example, shell discoloration may be associated with mold and aflatoxin contamination of kernels. In addition, we have observed that many of the early splits have hulls that appear rough and shriveled, whereas others have smooth hulls similar to healthy pistachio fruits (Fig. 2). Preliminary results showed that nuts with rough, shriveled hulls had more *Aspergillus* mold and substantially more aflatoxin (7). The objectives of this research were to determine the *Aspergillus* species involved in kernel decay in commercial pistachio orchards, to evaluate characteristics of early splits that could be used in removing moldy and aflatoxin-contaminated nuts, and to determine the importance of other types of hull rupture, such as bird damage and cracking, for mold and mycotoxin contamination.

MATERIALS AND METHODS

Isolations of molds. Nut samples were collected from 11 commercial pistachio orchards (all cultivar Kerman) in California (nine orchards in Madera County, one in Tulare County, and one in Merced County). Samples were stored at 0–1 C. Various cultural practices had been used in these orchards. Five orchards had cover crops, whereas the rest were disced regularly for weed control. Orchards were irrigated by flood (four orchards), microjet (four orchards), and sprinkler (three orchards).

In 1991, many commercial orchards were harvested twice because of uneven maturing of pistachio nuts. Nuts were collected on 17 September for the early harvest and on 14 October for the last harvest. Because nuts matured earlier than normal in 1992, harvests also were earlier. Nuts were collected on 3 September 1992, except for one orchard on 22 September.

Pistachio fruits were separated into four categories: early splits, bird-damaged, fruits with cracked hulls, and normal. For bird-damaged and cracked fruits, only ones that had the hull ruptured exposing the shell were used. Hulls and shells were removed by hand from all fruits. All early splits were separated into groups according to whether the hull was rough and shriveled or smooth and whether navel orangeworm infested the kernel or not. For isolations from the early harvest in 1991, 200 early splits and 25 normal fruits were used from each of eight orchards. From the late harvest in 1991, between 68 and 100 early splits from each of six orchards and 50 bird-damaged fruits from each of five orchards were used. From the 1992 harvest, 200 early splits and 30 normal fruits from each of six orchards were used, between 37 and 200 bird-damaged fruits from each of five orchards were used, and between 100 and 767 pistachio fruits with cracked hulls from each of seven orchards were used.

Special care was taken with surface-sterilization of the kernels, because abundant *Aspergillus* spores infested the samples. Most *Aspergillus* species produced abundant conidia that were readily dispersed. Any kernel having obvious *Aspergillus* sporulation was not surface-sterilized but was examined, and isolations were made directly from the sporulation. To aid surface-disinfestation, kernels were first rinsed in ethanol (28). The exact procedure differed slightly with the different harvests. In 1991, the kernels were placed in 95% ethanol for 15 s, in 10% bleach (0.5% NaOCl) for 1 min, and in sterile distilled water to be rinsed for the early harvest but were not rinsed for the last harvest. In 1992, the kernels were placed in 70% ethanol for 15 s, in 10% bleach (0.5% NaOCl) for 5 min, and were not rinsed. For the late harvest in 1991, kernels were surface-sterilized individually, using sterile multiple-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ). In 1992, kernels were surface-sterilized in small groups of about 20. After surface-sterilization, kernels were transferred to sterile petri plates containing salt agar (6% NaCl, 0.5% agar). Surface-sterilizing and transferring were done in a laminar flow hood. The petri plates were incubated at 30 C for 6–14 days and then examined with a dissecting microscope for *Aspergillus* sporulation. All *Aspergillus* colonies (except some of the common black-spored *Aspergillus* colonies) were isolated into pure culture and identified by species by the method of Klich and Pitt (17). Certain isolates that produced abundant orange-yellow to rufous sclerotia were tentatively classified as *A. melleus* Yukawa by the descriptions of Raper and Fennell (24) and Christensen (1). In 1992, all nuts also were examined with a dissecting microscope (magnification 10–60×) for *Aspergillus* sporulation prior to surface-sterilization.

Aflatoxin analysis. For the early harvest in 1991, an equal number of fruits in the rough and smooth groupings were used for each orchard and then separated into two equal-sized samples (54–219 fruits per sample, depending on the orchard). An equal number of samples of normal fruits also were analyzed. For the late harvest from one orchard, 346 bird-damaged fruits and 692 early splits were divided into two and three samples, respectively. In 1992, between 350 and 2,750 early splits and between 50 and 550 normal fruits from each of eight orchards were used. Also in 1992, between 400 and 750 cracked fruits from each of three orchards and 250 bird-damaged fruits from one orchard were analyzed. In 1992, all fruits were divided into 50-nut samples. In 1991, extractions were performed on whole fruits, but in 1992, extractions were done separately on the kernels (with shell) and hulls. All samples were stored at −19 C and ground with a blender.

Aflatoxins were extracted from the nuts according to the Romer method, an AOAC official method (26,32). The extracts were derivatized with trifluoroacetic acid (TFA), and aflatoxins were quantified by high-pressure liquid chromatography (HPLC) with a C_{18} reversed-phase column and fluorescence detector (14,33). Samples were derivatized as follows: evaporated to dryness under a stream of nitrogen, 150 μl of TFA added, vial capped and vortexed, let stand 15 min, again evaporated to dryness with nitrogen, and 5 ml of injection solution, acetonitrile-water (10:90), added. Aflatoxins were quantified by HPLC with mobile phase, water-methanol-acetonitrile (62:20:18), and fluorescence detector (excitation 365 nm, emission 450 nm). Amounts of aflatoxins were calculated by summing the amounts of B_1, B_2, G_1, and G_2 and are presented as aflatoxin in nanograms per gram (fresh weight) of sample.

Characteristics of early splits. In 1991, the physical characteristics were measured for 100 early splits with rough hulls and 100 early splits with smooth shells gathered from a commercial orchard in Madera County, CA, on 17 September. In 1992, the physical characteristics were measured for rough early splits, smooth early splits, cracked fruits, and normal fruits from two commercial orchards (in Tulare and Madera counties, CA) harvested 27 and 31 August, respectively. The fresh weight for each fruit was taken. The hulls were removed by hand and in 1991 put aside to obtain hull weights. The shells were measured and visually examined for external shell discoloration, both the general and suture staining characteristic of early splits (Fig. 3). The shells were removed, and the kernels were examined for *Aspergillus* sporulation and navel orangeworm infestation with a dissecting microscope (magnification 10–60×). In 1991, the hulls and kernels were weighed in groups of 25, dried at 90 C for more than 16 h until weight did not decrease, and groups were weighed again for the dry weight. On 29 July 1992, all early splits present were marked with pieces of yarn (these were considered to have split very early).

Fig. 1. Types of hull rupture of pistachio fruits. From left to right, normal fruit with intact hull, cracked hull exposing shell, and early split exposing kernel.

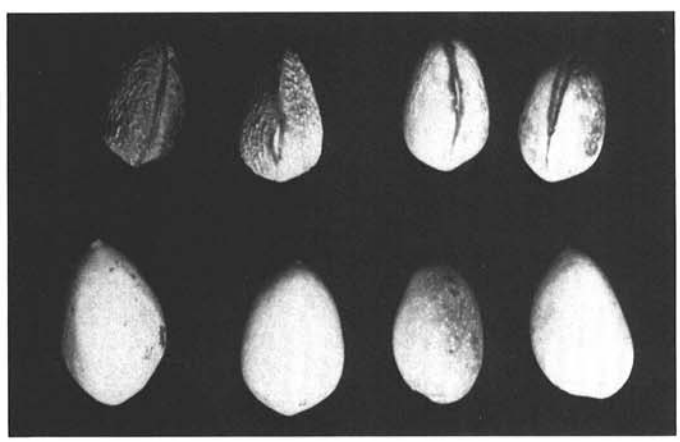

Fig. 2. Early split pistachio fruits with rough, shriveled hulls (top row, left two fruits) and smooth hulls (top row, right two fruits). The bottom row has normal fruits with intact hulls.

Statistical analysis. All analyses were done with SAS (SAS Institute Inc., Cary, NC, release 6.04). In general, analysis of variance was used with the least significant difference (LSD) for mean separation. For counts, chi-square or Fisher's exact test was used. Data frequently needed to be transformed. All measurements of aflatoxin were transformed by the log transformation, log [amount of aflatoxins (ng/g) + 1]. In general, data in the form of percents were transformed with the standard arcsine transformation (arcsine of the square root of the proportion) (19). In some cases however, percents were not transformed if the range of values was small (18), and the variances did not fail the *F*-max test for homogeneity of variances (10).

RESULTS

Occurrence of *Aspergillus* in orchards. Many different *Aspergillus* species were isolated from early split pistachio nuts, but only *A. niger* Tiegh. was found in more than 1.0% of the early splits (Table 1). The same species were found in kernels of bird-damaged fruits and fruits with cracked hulls, except *A. carbonarius* (Bain.) Thom, which was isolated once from the kernel of a cracked fruit. *A. niger* was much more common in all orchards than any other *Aspergillus* species. The percentage of early splits with *A. niger* in an orchard ranged from 10 to 40% in 1992. Species that have a potential for ochratoxin production (*A. ochraceus* or *A. melleus*) were isolated from kernels from every orchard. Species that have a potential for aflatoxin production (*A. flavus* or *A. parasiticus*) were isolated from kernels in six of the 10 orchards used in 1991 or 1992. No clear effect of cultural practices (presence or absence of cover crops or type of irrigation) on occurrence of *Aspergillus* species or aflatoxin in kernels was observed.

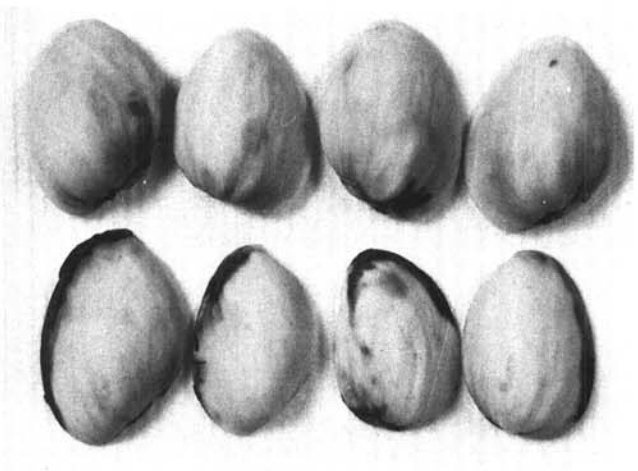

Fig. 3. Staining of shell along the suture or split characteristic of early split pistachio nuts (bottom row). Normal nuts with intact hulls do not have such staining (top row).

TABLE 1. Occurrence of *Aspergillus* species in kernels of early split pistachio nuts[x]

Aspergillus spp.[y]	Percentage of nuts	Percentage of orchards[z]
A. niger	30.2	100
A. ochraceus	0.9	80
A. flavus	0.7	60
A. melleus	0.5	40
A. sydowii	0.2	40
A. tamarii	0.2	20
A. wentii	0.2	20
A. parasiticus	0.1	10

[x]Isolated from 3,524 surface-sterilized kernels from commercial orchards in California in 1991 and 1992.
[y]Other species found but in less than 0.10% of the nuts: *A. terreus*, *Eurotium amstelodami*, *A. japonicus*, *A. carneus*, *A. alliaceus*, and two unidentified *Aspergillus* isolates. *A. carbonarius* was isolated from the kernel of a fruit with a cracked hull.
[z]Ten commercial orchards with between 200 and 500 nuts per orchard.

Comparison of early splits with rough and smooth hulls. Although many early splits had hulls similar in appearance to the hulls of normal fruits, all orchards had some early splits that had rough, shriveled hulls. The percentage of early splits with rough hulls in an orchard ranged from 16 to 69% in 1991 (nine orchards) and 25 to 72% in 1992 (eight orchards). The mean percentage of rough early splits was 39% in 1991 and 52% in 1992.

Early splits with rough, shriveled hulls had substantially more mold in the kernel compared to early splits with smooth hulls. The rough early splits had more than twice the *A. niger* infections in both 1991 and 1992 (Table 2) and more than six times the *A. flavus* or *A. parasiticus* infections (Table 3) compared to

TABLE 2. Differences in kernel infection by *Aspergillus niger* for early split pistachio fruits with hulls of different appearance and with infestation of kernel by the insect navel orangeworm (NOW)

	Kernels with *A. niger* (%)[x]		
	Before incubation[y]	After incubation[z]	
Characteristics	1992	1991	1992
Rough hull; NOW	31.0	61.6	55.0
Rough hull; no NOW	26.6	48.3	30.5
Smooth hull; NOW	16.5	21.1	27.4
Smooth hull; no NOW	9.3	14.0	15.1
$LSD_{0.05}$	12.0*	23.8*	13.1**
Rough hull	27.5	52.6	39.9
Smooth hull	9.9	17.3	18.3
$LSD_{0.05}$	6.7**	23.8*	11.5*
NOW infested	26.4	50.6	45.5
Not infested	15.5	31.2	20.9
$LSD_{0.05}$	14.4ns	7.1**	15.9*

[x]Approximately 200 pistachio fruits were harvested from each of seven commercial orchards on 3 September 1992 (one orchard was harvested 22 September). Approximately 100 pistachio fruits were harvested from each of six commercial orchards on 14 October 1991. ns = not significantly different ($P > 0.05$); * = significantly different ($0.05 > P > 0.001$); ** = significantly different ($P < 0.001$).
[y]Sporulation of *A. niger* was observed when shells were removed but before surface-sterilization or any incubation.
[z]Kernels were surface-sterilized and incubated at 30 C for 6–10 days.

TABLE 3. The association of hull appearance and navel orangeworm (NOW) infestation with kernel decay of early split pistachio fruits by potential aflatoxin producers (*Aspergillus flavus* [Af] or *A. parasiticus* [Ap]) and potential ochratoxin producers (*A. ochraceus* [Ao] or *A. melleus* [Am]) in commercial orchards[x]

	Kernels with *Aspergillus* spp. (%)	
Characteristic	Af or Ap	Ao or Am
Rough hull	2.7 (9.5)[y]	1.4 (6.7)
Smooth hull	0.4 (3.8)	0.6 (4.5)
$LSD_{0.05}$	... (4.3)*[z]	... (5.2)ns
NOW infested	4.5 (12.2)	3.4 (10.6)
Not infested	0.7 (4.7)	0.7 (4.8)
$LSD_{0.05}$	... (6.1)*	... (5.7)*

[x]Only orchards from which specified fungi were isolated were included in the analysis. Some orchards had too few NOW-infested nuts to be included. The number of orchards ranged from three to eight. The data for 1991 and 1992 were combined.
[y]Values in parentheses are the means of the transformed data, using the standard arcsine transformation. The statistical analysis was performed on the transformed data.
[z]ns = not significantly different ($P > 0.05$); * = significantly different ($0.05 > P > 0.001$).

smooth early splits. *Alternaria* spp. were found sporulating in more kernels of rough early splits (13.3%) than in smooth early splits (6.5%) ($LSD_{0.05} = 3.6$). However, with *Penicillium* spp. there was no significant difference ($P = 0.37$) between rough (7.5%) and smooth early splits (12.5%).

Early splits with rough hulls had substantially more aflatoxin than did early splits with smooth hulls. Almost all of the aflatoxin detected was in rough early splits in both 1991 (Table 4) and 1992 (Table 5). Only one sample of smooth early splits had aflatoxin in 1991 (5 ng/g) and 1992 (31 ng/g). In contrast, five and 21 samples of rough early splits had aflatoxin in 1991 and 1992, respectively. In 1992, most of the samples of rough early splits had high levels of aflatoxin (greater than 100 ng/g). Aflatoxin was detected in six of nine and five of eight orchards in 1991 and 1992, respectively. Normal nuts with intact hulls had no aflatoxin in 1991 and 1992. Aflatoxin B_1 was detected in all samples that had aflatoxin, but aflatoxin G_1 was in only 17 and 30% of the samples with aflatoxin in 1991 and 1992, respectively. Aflatoxins B_2 and G_2 were detected, but less frequently and in smaller amounts, than aflatoxins B_1 and G_1.

Navel orangeworm. Navel orangeworm (NOW) infested early split kernels in all orchards, but the severity of infestation varied greatly from orchard to orchard (from 10 to 52% with a mean of 31% in 1991 and from 4 to 33% with a mean of 15% in 1992). NOW-infested kernels had substantially more mold than noninfested kernels. In both 1991 and 1992, NOW-infested kernels had more *A. niger* infection than did noninfested kernels (Table 2). Likewise, more than six times the potential aflatoxin producers and more than four times the ochratoxin producers were found in NOW-infested early splits than in noninfested early splits (Table 3). Although in 1992 NOW-infested early splits had 40% more *Alternaria* sporulation and 54% more *Penicillium* sporulation, these were not significant differences ($P = 0.215$ for *Alternaria*, $P = 0.054$ for *Penicillium*). NOW infestation was associated with more kernel infections, but was not necessary for infection, because many of the noninfested nuts also were infected (Tables 2 and 3).

NOW-infested early splits had substantially more aflatoxin than did noninfested early splits (Table 5). NOW-infested samples averaged more than twenty times the aflatoxin of noninfested samples, and 84% of all aflatoxin detected was found in NOW-infested early splits (Table 5). However, in terms of the number of samples, there were only slightly more samples with aflatoxin for NOW-infested (12 samples) than for noninfested (10 samples). NOW infested more early splits with rough hulls (mean 46 and 20% in 1991 and 1992, respectively) than early splits with smooth hulls (mean 20 and 7%). The combination of rough hull and NOW infestation resulted in the most kernel infection by *A. niger* (Table 2) and the most aflatoxin (Table 5).

Characteristics of early splits. Besides hull appearance being distinct, early splits with rough hulls were different from early splits with smooth hulls in many physical characteristics. The early splits with smooth hulls were >80% heavier than early splits with rough hulls in both years. Also, rough early splits had more staining of the shell and had smaller shells compared to smooth early splits (Table 6). In 1991, rough early splits were substantially lighter in fresh weight for hulls (0.25 versus 0.89 g per hull) and for kernels (0.62 versus 0.97 g per kernel) than were smooth early splits ($P < 0.001$). However, there was little difference between rough and smooth early splits in the dry weight of the hulls and kernels. Also, in 1991, the hull and kernel moisture contents of rough early splits (17 and 9%, respectively) were substantially less than those of smooth early splits (77 and 44%, respectively) ($P < 0.001$). The hulls of rough early splits tended to rupture earlier in the season than the hulls of smooth early splits (Table 6).

Shell discoloration or staining differed greatly for the different types of pistachio nuts (Table 6). Early splits had more shell staining than did normal or cracked fruits. The external surface of the shells of most early splits had a distinctive pattern of discoloration along the suture or split (Fig. 3). None of the shells of normal fruits with intact hulls had this characteristic suture staining, although 4–5% of the shells of fruits with cracked hulls did (Table 6). Many of the early splits had other shell discoloration as well as suture staining (Fig. 4). There was a large variability in the severity of shell discoloration for early splits, although, in general, shells of rough early splits were more discolored than shells of smooth early splits (Fig. 4; Table 6). Although a few early splits had extensive shell discoloration, most early splits had relatively little. For example, 54 and 73% of the early splits had less than 11% of the shell external surface discolored for two orchards in 1992.

Comparison of hull and kernel. In addition to pistachio kernels being contaminated with aflatoxin, the hulls of early splits also had aflatoxin (Table 7). Slightly fewer hull samples (13 samples)

TABLE 4. The association of hull appearance with aflatoxins in early split pistachio fruits from the 1991 harvest[w]

Characteristic	Samples with aflatoxins[x] (%)	Aflatoxins per sample (ng/g)	Total aflatoxin[y] (%)
Rough hull	63	7.79 (0.94)[z]	99.1
Smooth hull	13	0.25 (0.10)	0.9

[w] Only data for the four commercial orchards (of the eight studied) that had aflatoxins are presented. The amounts of aflatoxins B_1, B_2, G_1, and G_2 were added together.

[x] The same number of fruits was analyzed for each type of fruit in the two replications for each orchard. The number of fruits per sample ranged from 72 to 122 depending on the orchard. No aflatoxins were detected in fruits with intact hulls.

[y] Total aflatoxins were calculated by summing for all samples the products of the amount of aflatoxins found in a sample and the sample weight.

[z] Values in parentheses are the means of the log transformed data. Statistical analysis was performed on the transformed data. Fruits with rough hulls had significantly ($P = 0.043$) more aflatoxins than fruits with smooth hulls. $LSD_{0.05} = 0.81$ for transformed data.

TABLE 5. The association of hull appearance and navel orangeworm (NOW) infestation with aflatoxins in kernels of early split pistachio fruits from the 1992 harvest[w]

Characteristics	No. of 50-nut samples	Samples positive for aflatoxins[x] (%)	Aflatoxins per nut[y] (ng)	Total aflatoxins[z] (%)	No. of samples with specified amount of aflatoxin (ng/g)			
					1–10	11–100	101–1,000	>1,000
Rough hull; NOW	18	61 a	2,998 a	83.7	5	0	1	5
Rough hull; no NOW	51	20 b	141 b	16.2	3	1	5	1
Smooth hull; NOW	5	20 a–c	2 c	0.1	0	1	0	0
Smooth hull; no NOW	47	0 c	0 c	0.0	0	0	0	0

[w] Data from the three commercial orchards of eight that had the highest levels of aflatoxins.

[x] Numbers followed by the same letter are not significantly different ($P = 0.05$) by pairwise comparisons, using Fisher's exact test. No aflatoxins were detected in samples with intact hulls.

[y] Statistical analysis was performed on log transformed data. Numbers followed by the same letter are not significantly different ($P = 0.05$) by pairwise comparisons, using Fisher's LSD test. $LSD_{0.05} = 0.712$ for log transformed means. Values presented were back transformed from the means for the log transformed data.

[z] Total aflatoxins were calculated by summing for all samples the products of the amount of aflatoxins measured for a sample and the sample weight.

had aflatoxin than kernel samples (19 samples). However, hull samples had substantially less aflatoxin than kernel samples (Table 7). The maximum aflatoxin detected was 803 and 10,841 ng/g for hull and kernel samples, respectively. There was only a slight correlation of aflatoxin in kernels and hulls. For example, only 21% of the kernel samples with aflatoxin also had aflatoxin in the corresponding hulls. Conversely, only 31% of the hull samples with aflatoxin had aflatoxin in the corresponding kernels. As with kernels, hulls from rough early splits had aflatoxin more frequently and in larger amounts than smooth hulls (Table 7). In addition, hulls that had NOW infestation of the kernel more frequently had aflatoxin (20% of samples) than hulls from noninfested nuts (13% of samples).

Other types of hull rupture. Bird-damaged pistachio fruits frequently had molds decaying the kernels (Table 8). In both 1991 and 1992, the *Aspergillus* species that had the potential to produce aflatoxin or ochratoxins were found in bird-damaged fruits (Table 8). In the 1991 late harvest, there was little difference in the frequency of *Aspergillus* molds between bird-damaged nuts and early splits. In 1992, some fungi were more frequent in early splits than were bird-damaged nuts, but for other fungi, there was little difference (Table 8). Few bird-damaged nuts were analyzed for aflatoxin, but in 1991, one orchard had a sample with aflatoxin (3 ng/g) (one early split sample from this orchard had aflatoxin [14 ng/g]). In 1992, only one orchard was tested for aflatoxin in bird-damaged fruits, and no aflatoxin was detected in any of five 50-nut samples (9% of the early split samples from this orchard had aflatoxin).

Fruits with cracked hulls had much less kernel decay than early splits but still had some fungal infections (Table 9). Although very rare (both less than 0.1%), potential aflatoxin producers and potential ochratoxin producers sporulated on kernels of cracked fruits. The NOW infestation was similar in cracked fruits and smooth early splits (Table 6). Hull rupture for cracked fruits did not occur as early as rupture in some of the early splits (Table 6). Shell size of cracked fruits was significantly smaller than that for normal fruits with intact hulls but larger than shells of smooth early splits (Table 6). No aflatoxin was detected in kernels from cracked fruits (21 samples of 50 nuts) from the three orchards that had the most aflatoxin in early splits in 1992 (18% of the early split samples had aflatoxin).

DISCUSSION

Early split pistachio nuts are commonly infected with mold, because their hulls split along the shell suture (unlike normal pistachio fruits that have intact hulls), exposing the kernel to invasion by molds and insects. We isolated *A. niger* very frequently from early split kernels and 12 other *Aspergillus* species infrequently (Table 1). In Iran, *A. niger* was the most common *Aspergillus* species isolated from pistachio nuts (not necessarily early split), and 12 other *Aspergillus* species also were isolated (20). However, there were several differences between our findings for pistachios in California and those of Mojtahedi et al (20) in Iran. *Aspergillus* molds were found at much higher frequencies in Iran than in California. For example, in Iran for three of four harvests,

TABLE 6. Comparison of the characteristics of pistachio fruits that have different types of hulls for the 1992 harvest[r]

Hull[s]	No. examined	NOW[t] (%)	Split very early[u] (%)	Fruit fresh weight (g)	Shell staining		Shell dimension (mm)	
					Suture (%)[v]	General[w]	Length	Width
Rough ES	396	17.2 a[x]	18 a[x]	1.64 a[y]	99 a[x]	2.2 a[y]	19.1 a[y]	13.3 a[y]
Smooth ES	597	5.6 b	2 b	3.18 b	81 b	1.2 b	20.7 b	14.4 b
Cracked	115	7.0 b	0 bc	NM[z]	4 c	1.3 b	21.8 c	15.0 c
Normal	279	0.0 c	0 c	4.10 c	0 d	0.0 c	22.5 d	15.6 d

[r] Fruits were harvested from a commercial orchard on 27 August 1992. Similar results were observed in 1991 and in an additional orchard in 1992.
[s] Early splits (ES) and cracked fruits both have ruptured hulls, but for early splits, the hull split corresponds to the split of the shell. Normal fruits have intact hulls. Rough and smooth early splits have hulls that appear rough (shriveled) and smooth, respectively.
[t] Kernels infested with navel orangeworm (NOW).
[u] Fruits that had hull rupture prior to 29 days before harvest.
[v] Nuts that had discoloration on shell along the suture where the shell splits.
[w] A pretransformed scale was used to rate the amount of discoloration on shell surfaces: 0 = 0% of shell surface discolored; 1 = 1–10%, 2 = 11–35%, 3 = 36–64%, 4 = 65–89%, 5 = 90–99%, and 6 = 100% discolored.
[x] Numbers followed by the same letter are not significantly different ($P = 0.05$) by pairwise comparisons, using Fisher's exact test on the counts.
[y] Numbers followed by the same letter are not significantly different ($P = 0.05$) by pairwise comparisons, using Fisher's LSD test.
[z] NM = not measured.

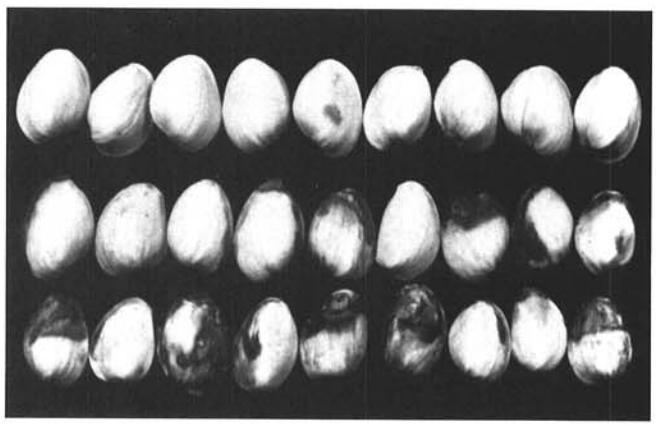

Fig. 4. Discoloration of pistachio shells of normal nuts (top row), early splits with smooth hulls (middle row), and early splits with rough, shriveled hulls (bottom row).

TABLE 7. Aflatoxins in different components of early split pistachio fruits from the 1992 harvest of two commercial orchards

Part of fruit	Hull	Samples positive for aflatoxins[x] (%)	Aflatoxins per fruit[y] (ng)	Total aflatoxins[z] (%)
Kernel + shell	Rough	35 a	958.4 a	98.4
	Smooth	3 c	0.8 b	0.1
Hull	Rough	20 ab	5.9 b	1.5
	Smooth	8 bc	0.1 b	0.0

[x] Numbers followed by the same letter are not significantly different ($P = 0.05$) by pairwse comparisons, using Fisher's exact test.
[y] Statistical analysis was performed on log transformed data. Numbers followed by the same letter are not significantly different ($P = 0.05$) by pairwise comparisons, using Fisher's LSD test. $LSD_{0.05} = 1.44$ for the means for the log transformed data. Values presented were back transformed from the means of the log transformed data.
[z] Total aflatoxins were calculated by summing for all the samples the products of the amount of aflatoxins measured for sample and the sample weight.

A. niger was isolated from 65 to 77% of all pistachio nuts, whereas in California *A. niger* was found in only 30% of early splits, the nuts most likely to have mold (typically only 1–4% of all nuts are early splits [9]). Although some of the rarer *Aspergillus* spp. were found only in Iran or in California, most of the more common species were found in both. One exception was *A. fischeri*, which was very common in Iran but not in California. Another important exception was that in Iran *A. ochraceus* was isolated only after the nuts were processed, and the closely related *A. melleus* was not isolated at all, whereas in California both species were relatively common (Table 1). The species with potential for aflatoxin production, *A. flavus* and *A. parasiticus*, were found both in Iran and California, although *A. flavus* was much more common in Iran than in California. For pecans in Georgia, *A. niger* was the most common *Aspergillus* species, and more than 15 other *Aspergillus* species were found (15). In almond kernels from California, the *A. niger* group was the most common, but the *A. flavus, A. glaucus, A. ochraceus,* and *A. wentii* groups also were found (individual species were not identified) (23). In all these studies in nut orchards, *A. niger* was the most common *Aspergillus* species, although many others also were found.

Even though all the *Aspergillus* species found, except *A. niger*, occurred at very low levels in pistachio nuts from California (Table 1), many of these species are known to produce mycotoxins, and their presence could be a problem. Aflatoxins, produced by *A. flavus* and *A. parasiticus*, are potent toxins and carcinogens and are the most widely regulated mycotoxin (35). In 1976, *A. flavus* was found in 12% of all pistachio kernels (representing all types of pistachio nuts and not just early splits) before harvest in California (34). In our study, *A. flavus* was found only at very low levels in pistachio nuts: never in normal nuts (with intact hulls), 0.7% of the early splits (Table 1), 1.2% or less of the bird-damaged nuts (Table 8), and only 0.02% in nuts with cracked hulls (Table 9). The incidence of aflatoxin in pistachio nuts from California is estimated to be very low (one aflatoxin-contaminated nut per 25,000 nuts) (31). Therefore, it is not surprising that *A. flavus* was found only infrequently in pistachio nuts. No previous study had determined the incidence of *A. flavus* in early splits, although the levels of aflatoxin were quantified in one study (31). Most likely, in some cases, *A. flavus* can decay pistachio kernels without producing aflatoxins, because only 43% of *A. flavus* isolates from California pistachio orchards were able to produce aflatoxins when tested (8). The presence of *A. parasiticus* in pistachio nuts in California orchards (Table 1) has not been previously reported as far as we know, although *A. parasiticus* would be expected because G_1 aflatoxin has been detected in pistachio nuts (31) and is typically produced only by *A. parasiticus* and not by *A. flavus* (6). Even though *A. parasiticus* is much rarer than *A. flavus* in California pistachio orchards (Table 1), its presence is important because isolates of *A. parasiticus* are more likely to produce aflatoxins than are *A. flavus* isolates (8). Similarly, in Iran, *A. parasiticus* was present in pistachio nuts but was much less common than *A. flavus* (20). Most of the commercial orchards we studied had kernel infections by *A. flavus* or *A. parasiticus* and aflatoxin contamination. Surprisingly, these aflatoxin levels were much higher than expected considering the low levels detected in processed pistachio nuts from California. The reasons for this discrepancy could be that our method was more sensitive to the presence of low levels of aflatoxin because we used early splits (the nuts most likely to have aflatoxin) and the nuts we used were gathered from orchards before processing (which would remove many contaminated nuts).

Other mycotoxins are also of concern and may be present in pistachio nuts, although we analyzed nut samples only for aflatoxins. *A. flavus* and *A. tamarii* Kita, both present in pistachio nuts in California (Table 1), can produce the toxin cyclopiazonic acid (5,6). Some isolates of *A. flavus* that do not produce aflatoxins produced cyclopiazonic acid (13). *A. ochraceus* and *A. melleus*, both relatively common in early splits in California (Table 1), have been shown to produce two types of mycotoxins, ochratoxins and penicillic acid (2). Ochratoxin was produced by isolates of *A. ochraceus* from pistachio nuts in Turkey (3). Although an isolate of *A. ochraceus* from pistachio nuts in Iran did not produce detectable levels of ochratoxin, moldy feed decayed by this isolate

TABLE 8. Comparison of bird-damaged and early split fruits for amount of molds in pistachio kernels from commercial orchards[v]

Year	Incubation[w]	Fruit	Kernels with specified *Aspergillus* mold (%)[x]		
			A. niger	Af or Ap[y]	Ao or Am[z]
1991	After	Early split	34.4	0.8	5.1
		Bird damaged	40.8	1.2	5.2
		$LSD_{0.05}$	11.2[ns]	1.1[ns]	4.6[ns]
1992	Before	Early split	16.3	0.0	0.7
		Bird damaged	6.3	0.0	0.5
		$LSD_{0.05}$	4.5[*]	...	0.9[ns]
	After	Early split	24.7	1.5	1.3
		Bird damaged	14.6	0.4	0.4
		$LSD_{0.05}$	5.0[*]	2.1[ns]	1.6[ns]

[v] Between 50 and 100 fruits were harvested on 14 October 1991 per type of fruit for each of five orchards. Between 37 and 200 fruits were harvested on 3 September 1992 per type of fruit for each of five orchards.

[w] Sporulation observed when kernel was examined prior to surface-sterilization or incubation. Normal fruits with intact hulls had no fungal sporulation on kernel. Kernels were surface-sterilized and incubated at 30 C for 6–10 days and then examined for fungal growth. Normal fruits with intact hulls had none of these fungi in the kernel except *Aspergillus niger*, which developed in 0.1 and 3.0% of the kernels in 1991 and 1992, respectively.

[x] ns = not significantly different ($P > 0.05$); * = significantly different ($0.05 > P > 0.001$).

[y] Af = *A. flavus*; Ap = *A. parasiticus*. These species are potential aflatoxin producers.

[z] Ao = *A. ochraceous*; Am = *A. melleus*. These species are potential ochratoxin producers.

TABLE 9. Comparison of pistachio fruits with cracked hulls and early split fruits for mold levels in the kernel for seven commercial orchards[u]

Fruit	Kernels with mold sporulation (%)[v]				
	Aspergillus spp.			*Alternaria* spp.	*Penicillium* spp.
	A. niger	Af or Ap[w]	Ao or Am[x]		
Early split	16.7 (24.2)[y]	0.20 (2.6)	0.63 (4.6)	9.1 (17.6)	7.1 (15.4)
Cracked hull	5.7 (13.8)	0.02 (0.9)	0.00 (0.3)	0.5 (3.9)	0.7 (4.9)
$LSD_{0.05}$	... (6.1)[*z]	... (3.0)[ns]	... (2.0)[*]	... (5.0)[**]	... (11.5)[ns]

[u] For the early splits, 200 nuts were examined for each orchard. For the nuts with cracked hulls, between 100 and 767 nuts were examined for each orchard.

[v] Sporulation observed when kernel was examined prior to surface-sterilization or incubation. Normal fruits with intact hulls had no fungal sporulation.

[w] Af = *A. flavus*; Ap = *A. parasiticus*. These species are potential aflatoxin producers.

[x] Ao = *A. ochraceous*; Am = *A. melleus*. These species are potential ochratoxin producers.

[y] Data were transformed by the standard arcsine transformation. Numbers in parentheses are means for the arcsine transformed values. Percent values for the means were calculated from the means of the transformed data.

[z] ns = not significantly different ($P > 0.05$); * = significantly different ($0.05 > P > 0.001$); ** = significantly different ($P < 0.001$).

and fed to ducklings killed the ducklings (20). Although *A. niger* is very common in nuts such as pistachio, it has not been established whether potent mycotoxins are produced by these isolates. *A. niger* can produce the toxins naphtho-γ-pyrones (11), and at least some isolates of *A. niger* caused the deaths of chickens (36) and ducklings (20) given feed decayed by the isolates.

Many of the early splits had rough, shriveled hulls (Fig. 2). The most probable reason that rough early splits had more *Aspergillus* mold (Tables 2 and 3) and more aflatoxin (Tables 4 and 5) than smooth early splits is that rough early splits split earlier in the season, so the kernel was exposed earlier and longer to mold infection (Table 6). We think that the hull becomes rough or shriveled on these early splits because the process of hull rupture allows moisture to be lost (hulls of rough early splits had a moisture content of only 17%, whereas hulls of smooth early splits had 77%). The characteristic of rough hulls gives another option for processors to use for separating mold- and mycotoxin-contaminated pistachio nuts from healthy nuts. Also, our results suggest that the early splits that split several weeks before harvest are the nuts that contribute most to the mold and mycotoxin problem.

The navel orangeworm insect is a serious pest of nut crops, including pistachios, in California. NOW eggs are usually found only on pistachio fruits with ruptured hulls, and the developing larvae feed on the pistachio kernels (25). *A. flavus* was found more often in insect-damaged, mainly by NOW, pistachio nuts than nuts with no insect damage (34). We found that not only were there substantially more of the potential aflatoxin producers, *A. flavus* or *A. parasiticus*, in NOW-infested early splits, but also substantially more *A. niger* and potential ochratoxin producers, *A. ochraceus* or *A. melleus* (Tables 2 and 3). Aflatoxin was more frequently found in NOW-infested pistachio nuts than in noninfested nuts (31). In 1992, we found that NOW-infested early splits had aflatoxin more frequently, had higher levels of aflatoxin, and even though relatively few early splits were NOW-infested (15%), more than 80% of all the aflatoxin detected was in infested early splits. NOW are not necessary to allow molds to penetrate the hull or shell because early splits already have the hull ruptured and the shell split. NOW is not even necessary for infection because many noninfected early splits have kernel decay. However, NOW-infested nuts tend to have very high levels of aflatoxin (Table 5) suggesting an effect on *A. flavus* beyond favoring infection.

The hull covers the pistachio nut until it is removed after harvest during the processing of the nut. Previously, it has been shown that aflatoxin can be produced in the hulls after artificial inoculation with *A. flavus*, though at much lower levels than in the kernel (4,20,30). We measured the aflatoxin in naturally infected hulls from commercial orchards. Aflatoxin was slightly less frequent but at substantially lower levels in hulls than in kernels with shells (Table 7). Similar to the situation with kernels, substantially more aflatoxin was found in rough hulls than in smooth hulls (Table 7). Surprisingly, there was only a slight correlation between aflatoxin in hulls and in kernels. This suggests that infection by *A. flavus* or *A. parasiticus* usually occurs separately in the kernel or hull, and there is little cross-contamination. Because of the presence of aflatoxin in hulls from commercial orchards, some care should be taken if hulls are to be used for animal feed. Similarly, although pistachio culls or unmarketable nuts are nutritionally acceptable as livestock feed (16), care should be taken that the culls used for feed are not contaminated with aflatoxin.

Bird-damaged pistachio fruits frequently had moldy kernels (Table 8). In both 1991 and 1992, potential aflatoxin and ochratoxin producers were found in bird-damaged nuts. Although aflatoxin was detected in one sample (at the low level of 3 ng/g) of bird-damaged nuts, not enough samples were analyzed to fully reveal the severity of aflatoxin contamination of bird-damaged nuts. Even though bird damage to pistachio nuts is widespread in California, most of the damaged nuts are removed from the trees by the birds (27). Because relatively few bird-damaged nuts are actually harvested (except in certain orchards with severe bird problems), the importance of bird damage to the total mold and mycotoxin problem is probably less than that of early splits. However, if the goal is to completely eliminate aflatoxin from pistachio nuts, then consideration should be given to bird damage.

Cracking of hulls is another type of hull rupture (Fig. 1). In early splits, the hull splits in the same location that the shell splits, but in cracked fruits, the location of the rupture of the hull does not correspond to the split in the shell. Many, but not all, early splits had the hull split before any pistachio fruits had cracked hulls (Table 6), which may explain why cracked fruits had less kernel mold than early splits (Table 9). *A. flavus* sporulated on some kernels of cracked fruits, although in fewer than 0.1% of the kernels. However, no aflatoxin was detected in kernels from cracked fruits even though 21 samples of 50 nuts were analyzed (from three orchards that had high levels of aflatoxin in early splits). Although aflatoxin may be present because *A. flavus* decayed cracked fruits, the level should be very low. However, if the harvest is delayed for a long period, it would probably allow the fungi to develop further and perhaps more aflatoxin would be detected. When pistachios were harvested late, nuts with tattered hulls had aflatoxin-contaminated kernels (31). Although the causes for the cracking of the hull are not well understood, at least some of the cracked fruits had a small part of the hull still attached to the shell (M. A. Doster and T. J. Michailides, *personal observations*), and nuts with cracked hulls had significantly smaller shells than normal nuts that had intact hulls (Table 6).

A very important method for reducing the number of mold- and aflatoxin-contaminated nuts sold to consumers is the physical removal of nuts that appear undesirable during processing. However, because the shell is not removed from pistachio nuts, the kernel cannot be examined directly for decay, and other features must be used. Before the hull is removed, early splits may be identified by the rupture in the hull. However, using the characteristic of rough, shriveled hull would facilitate removal of contaminated nuts because the rough hull is more distinctive visually than the hull split, and rough early splits are the nuts most likely to have *Aspergillus* mold, NOW, and aflatoxin. Another promising option before hull removal is to use the fruit fresh weight, which shows substantial differences between types of pistachio nuts. Rough early splits had less than half the fruit fresh weight of normal nuts (Table 6). Although all of these characteristics are promising, processors currently evaluate pistachio nuts after hull removal and are not set up to evaluate pistachio fruits prior to hull removal. Currently, evaluation of the staining or discoloration of the shell by machines or humans is widely used by processors. This method should remove much of the mold and aflatoxin contamination, because many of the early splits are heavily stained (Fig. 4). However, many of the early splits are only slightly stained (Figs. 3 and 4; Table 6). At present, many of the pistachio nuts showing the suture shell-staining characteristic of early splits (Fig. 3) are not being removed during processing. For example, between 0.6 and 3.4% of the nuts in samples bought at retail outlets, representing five processors, showed suture staining (M. A. Doster and T. J. Michailides, *personal observations*). If the processor does not remove these lightly stained nuts, then moldy nuts, perhaps with aflatoxin, would be sold to consumers. Other nut characteristics that could be used are shell size (Table 6) and nut weight (kernel fresh weight for rough early splits was only 64% of that for smooth early splits in 1991). Fortunately, the pistachio nuts most likely to have mold or aflatoxin contamination are very different from normal, healthy nuts, and there are several promising characteristics that could be used to aid in separating contaminated nuts. It should be easy to reduce substantially molds and aflatoxin in pistachio nuts, but eliminating aflatoxin completely may not be possible (some countries do have a zero tolerance [35]).

Possible recommendations for reducing molds and mycotoxins in pistachio nuts in California are somewhat limited. Growers can attempt to decrease NOW levels in orchards and maybe limit bird damage, although birds are not as important as navel orangeworm. At this time, our results do not support recommending that growers change cultural practices, such as use of cover crops or type of irrigation. However, we are currently investigating the

effect of irrigation on the formation of early splits. It is very important that processors remove moldy nuts that are aflatoxin contaminated. The nuts most likely to be contaminated are early splits with rough hulls and infested with NOW, and care should be taken that these nuts are separated out during processing.

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