Aflatoxin Variability in Pistachios

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Pistachio fruit components, including hulls (mesocarps and epicarps), seed coats (testas), and kernels (seeds), all contribute to variable aflatoxin content in pistachios. Fresh pistachio kernels were individually inoculated with *Aspergillus flavus* and incubated 7 or 10 days. Hulled, shelled kernels were either left intact or wounded prior to inoculation. Wounded kernels, with or without the seed coat, were readily colonized by *A. flavus* and after 10 days of incubation contained 37 times more aflatoxin than similarly treated unwounded kernels. The aflatoxin levels in the individual wounded pistachios were highly variable. Neither fungal colonization nor aflatoxin was detected in intact kernels without seed coats. Intact kernels with seed coats had limited fungal colonization and low aflatoxin concentrations compared with their wounded counterparts. Despite substantial fungal colonization of wounded hulls, aflatoxin was not detected in hulls. Aflatoxin levels were significantly lower in wounded kernels with hulls than in kernels of hulled pistachios. Both the seed coat and a water-soluble extract of hulls suppressed aflatoxin production by *A. flavus*.

The aflatoxins are a group of toxic and carcinogenic polyketide secondary metabolites that are produced by strains of *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*, and these compounds can be produced when these molds contaminate crops such as tree nuts, peanuts, corn, and cotton. The incidence of aflatoxin contamination in tree nuts is low, but aflatoxin levels can be quite variable and high levels can develop in a small percentage of nuts (27-29). For instance, it has been estimated that only 1 in 28,250 walnuts (10), 1 in 26,500 almonds (28), and 1 in 25,000 pistachios (31) develop high levels of aflatoxin contamination. However, a single pistachio with an aflatoxin concentration of 60,000 ppb can contaminate an otherwise aflatoxin-free 10-lb (ca. 4.5-kg) test lot (approximately 3,000 nuts) at the U.S. Food and Drug Administration action level of 20 ppb. Thus, it is of interest to determine why certain nuts are susceptible to developing high levels of aflatoxin.

The drupaceous pistachio fruit consists of an edible kernel (seed) and a seed coat (testa) encased in a hardened shell (endocarp), all of which are surrounded by a fleshy hull (mesocarp and epicarp) which is removed during processing (4). The pistachio shell splits as the nut matures, a desirable feature for marketing and consumption; however, the hull remains intact in the majority of mature pistachios at harvest (30). *A. flavus* is a weak plant pathogen (a wound pathogen), and aflatoxin is rarely detected in pistachio kernels with intact hulls (26, 31). In a small percentage (1 to 5%) of pistachios, the shell and the still adhering hull split together (31). Studies have correlated these early-split pistachios, in which the kernel can be exposed to fungal spores, with high levels of aflatoxin contamination (7, 31). The split hull also allows entry of the navel orangeworm, one of the major insect pests of pistachios (22), and high levels of aflatoxin contamination are found more frequently in insect-damaged kernels than in undamaged kernels (7, 31). Similarly, kernel injury appears to enhance *A. flavus* colonization in almonds, while the seed coat acts as a barrier to colonization (11).

In this study, mature pistachios with intact hulls were col-

lected from orchards. In-hull pistachios were subjected to hull injury, and kernels were either injured or left intact, simulating conditions which could occur under preharvest conditions. The seed coats of hulled and shelled pistachios were removed or left intact, and kernels were injured or left intact. Kernels were inoculated with spores of a toxigenic *A. flavus* strain in order to determine the effect of hull, seed coat, and kernel injury on *A. flavus* colonization and aflatoxin accumulation in pistachios.

MATERIALS AND METHODS

Pistachio samples and treatments. Mature pistachios with undamaged and nonadhering hulls were collected from a University of California Experimental Station orchard. The hulls were aseptically sliced along the shell suture, and pistachios with unopened shells were discarded. A total of 500 pistachios with opened shells were selected for this study. Hulls that were cut to simulate early-split pistachios were left on 100 nuts. The hulls and shells were aseptically removed from the other 400 nuts, and the entire seed coat was aseptically removed from 200 of these nuts. One-half of all kernels were injured by using a scribe to make a puncture wound approximately 1 mm wide by 2 mm deep in each kernel. The following six samples of pistachios were prepared: (i) intact kernels with artificially split hulls (50 nuts), (ii) wounded kernels with artificially split hulls (50 nuts), (iii) intact kernels without hulls or shells (100 nuts), (iv) wounded kernels without hulls or shells (100 nuts), (v) intact kernels without hulls, shells, or seed coats (100 nuts), and (vi) wounded kernels without hulls, shells, or seed coats (100 nuts).

Inoculation and incubation. Pistachios were placed in petri dishes (five nuts per plate). Each nut was inoculated on the kernel (or the wound in the kernel) with approximately 200 spores of *A. flavus* NRRL 25347 (identified as strain 42-12 at the Western Regional Research Center), an aflatoxigenic strain isolated from pistachios, in 5 μ l of 0.05% Tween 80. The petri plates were incubated for 7 or 10 days at 25°C.

Seed coat agar. Shelled pistachios (179 g) were immersed in boiling deionized water for 1.5 min. After the pistachios were drained, the seed coats were peeled off the kernels with tweezers. The seed coats were dried to a constant weight at 55°C. A total of 14 g of seed coats was obtained. The water used to boil the pistachios was blended with the seed coats. The seed coat solution was added to modified Czapek yeast autolysate agar (24) at concentrations of 1, 2, 3, 4, 5, and 8.3% (wt/vol) equivalent dried seed coats per ml. Deionized water was used to dilute the preparations to the final volume. The pH of the resulting seed coat agar ranged from 6.61 (8.3% seed coats) to 6.84 (1% seed coats). Petri plates (diameter, 60 mm) containing each concentration were prepared in triplicate. Single-point inoculations were made with approximately 200 spores of *A. flavus* NRRL 25347 in 5 μ l of 0.05% Tween 80. The plates were incubated at 25°C for 5 days.

Pistachio agar. Shells were removed from raw pistachios (1 kg), and the kernels were ground with a blender (Waring). Pistachio agar was prepared by blending with a Waring blender purified water (Barnstead) and 4% (wt/vol) ground pistachios and then adding 2% (wt/vol) agar (Scott). This medium was

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FIG. 1. Hulled and shelled pistachio kernels with seed coats removed and cuticular layers intact that were inoculated with *A. flavus* NRRL 25347 and incubated for 10 days. No visible fungal colonization was observed, and no aflatoxin was detected in any of these kernels.

autoclaved, and petri plates (diameter, 100 mm) were prepared in replicates of five. Single-point inoculations were made with approximately 200 spores of *A. flavus* NRRL 25347 in 5 μ l of 0.05% Tween 80. The plates were incubated for 10 days at 25°C.

Pistachio hull extract. Freeze-dried, mature hulls (675 g) were ground with a Waring blender and sequentially extracted with 4 liters each of hexane, ethyl acetate, acetone, methanol, and water. The water extract was stirred overnight with 2 kg of Amberlite XAD-2 (Mallinckrodt). The Amberlite XAD-2 was filtered and washed with 4 liters of methanol. The methanol was evaporated under reduced pressure, and the residue was freeze-dried, which resulted in 18 g of XAD-2-adsorbed material.

Freeze-dried, unextracted hulls were added at a concentration of 4% (wt/vol) to pistachio agar prior to autoclaving. XAD-2-adsorbed material from the water extract was added at a concentration of 4% (wt/vol) equivalents (equal to 0.1% [wt/vol]) to pistachio agar prior to autoclaving. Petri plates (diameter, 100 mm) were prepared in replicates of five. Single-point inoculations were made with approximately 200 spores of *A. flavus* NRRL 25347 in 5 μ l of 0.05% Tween 80. The plates were incubated for 10 days at 25°C.

Aflatoxin analysis. *A. flavus* NRRL 25347 produces aflatoxin B₁ (AFB₁) and trace levels of AFB₂. In this study, individual kernels were assayed only for AFB₁. Hulls and shells were removed before in-hull kernels were extracted; individual hulls were extracted and assayed separately. Each kernel or hull was extracted in 20 ml of methanol by crushing it with a flattened stirring rod, and a 1-ml aliquot was brought to dryness under N₂ at 40°C and derivatized with hexane and trifluoroacetic acid (24). Extracts were quantitatively analyzed for AFB₁ by

reversed-phase high-performance liquid chromatography (HPLC) with a Hewlett-Packard model 1050 apparatus with peak detection by fluorescence as described previously (24). The limit of detection was 0.01 μ g of AFB₁ per kernel or hull.

The contents of agar plates were extracted in 50 ml (60-mm plates) or 100 ml (100-mm plates) of methanol by crushing the contents with a flattened stirring rod; 1-ml aliquots were derivatized and assayed for AFB₁ as described above.

Statistical analyses. The statistical significance of treatment effects (wounded versus unwounded; with seed coat versus without seed coat) was determined by performing an analysis of variance by using MINITAB (Minitab, Inc.). The AFB₁ content of pistachio kernels within treatments was highly variable and did not follow normal distributions. Data were $\log_{10}(x + 1)$ transformed in order to reduce the variance and achieve normality. Both log-transformed and nontransformed data sets were analyzed by analysis of variance. Statistically significant effects were accepted at the $P < 0.05$ level.

RESULTS

Effect of kernel wounding and seed coat on colonization and aflatoxin content of hulled pistachios. There was no visible evidence of *A. flavus* colonization on any of the 100 hulled intact kernels without seed coats (Fig. 1), and AFB₁ was not detected in any of these nuts (Table 1). Fungal colonization was observed on 20% of the intact hulled kernels with seed coats after 7 days and on 62% of the intact hulled kernels with seed coats after 10 days. The majority of the colonized kernels had sparse mycelial growth with little sporulation, and the AFB₁ concentrations were less than 5 μ g per kernel. However, heavy colonization was observed on six intact kernels with seed coats after 10 days, and the AFB₁ concentrations ranged from 20 to 460 μ g per kernel.

Wounding had highly significant effects on the AFB₁ content of hulled pistachio kernels ($P < 0.001$, as determined by analysis of variance) and on *A. flavus* colonization. Extensive fungal colonization was observed on 100% of the wounded kernels with or without seed coats. After 10 days, wounded kernels without seed coats had fungal growth that covered the entire kernel surface (Fig. 2), and wounded kernels with seed coats had fungal growth that covered approximately one-half the kernel surface. After 7 days, wounded kernels with seed coats had one-half the average AFB₁ content of wounded kernels without seed coats, but after 10 days the average AFB₁ contents of wounded kernels with and without seed coats were the same (Table 1). The average AFB₁ content of all hulled pistachio kernels was significantly greater on day 10 than on day 7 ($P < 0.001$, as determined by analysis of variance).

The AFB₁ concentrations were more variable for the

TABLE 1. Aflatoxin contents and *A. flavus* colonization of in-hull and hulled pistachios

Prepn	No. of kernels or hulls colonized/no. tested		No. of kernels or hulls with AFB ₁ /no. tested		AFB ₁ concn (μ g/kernel) ^a	
	7 days	10 days	7 days	10 days	7 days	10 days
In-hull intact kernels						
Hulls	NDI ^b	47/50	NDI	0/50	NDI	0
Kernels	NDI	NDK ^c	NDI	17/50	NDI	0.73 $\pm$ 0.6
In-hull wounded kernels						
Hulls	NDI	50/50	NDI	0/50	NDI	0
Kernels	NDI	NDK	NDI	38/50	NDI	1.8 $\pm$ 0.8
Hulled intact kernels						
With seed coats	10/50	31/50	3/50	22/50	0.28 $\pm$ 0.6	15 $\pm$ 19
Without seed coats	0/50	0/50	0/50	0/50	0	0
Hulled wounded kernels						
With seed coats	49/50	50/50	48/50	50/50	86 $\pm$ 37	290 $\pm$ 62
Without seed coats	50/50	50/50	50/50	50/50	180 $\pm$ 31	290 $\pm$ 40

^a Means $\pm$ 95% confidence intervals for 50 kernels.

^b NDI, not determined (in-hull kernels were incubated for 10 days).

^c NDK, not determined (kernel colonization could not be distinguished from hull colonization).

FIG. 2. Hulled and shelled pistachio kernels, with seed coats removed and cuticular layers punctured, that were inoculated with *A. flavus* NRRL 25347 and incubated for 10 days. All kernels had heavy fungal colonization, but aflatoxin concentrations ranged from 30 to 710 μg per kernel.

wounded kernels with seed coats than for the wounded kernels without seed coats. After 7 days, 48 of the 50 wounded kernels with seed coats had lower AFB₁ concentrations than the wounded kernels without seed coats (Fig. 3A); however, the AFB₁ concentrations ranged from 0 to 678 μg per kernel for kernels with seed coats and from 1.7 to 418 μg per kernel for kernels without seed coats. After 10 days, only 23 of the 50 wounded kernels with seed coats had lower AFB₁ concentrations than the wounded kernels without seed coats (Fig. 3B); the AFB₁ concentrations ranged from 1.3 to 890 μg per kernel for kernels with seed coats and from 30 to 710 μg per kernel for kernels without seed coats. The differences in the standard deviations of the AFB₁ concentrations in wounded kernels with and without seed coats after 10 days were not significant ($P > 0.05$, as determined by an F test; one-tailed test).

In vitro effect of seed coats on AFB₁ production by *A. flavus*. Seed coats at a concentration of 1.7% in modified Czapek yeast autolysate medium inhibited AFB₁ production by 50% (Fig. 4). Growth, as measured by colony diameter and appearance (data not shown), was not affected even at a seed coat concentration of 8.3%, even though AFB₁ production was 98% inhibited.

Effect of hulls on *A. flavus* colonization and AFB₁ content of pistachios. Nearly all (97%) of the in-hull pistachios were colonized by *A. flavus* within 10 days. Although the kernels were the site of inoculation, the hulls were heavily colonized by *A. flavus* (Fig. 5); however, AFB₁ was not detected in any of the 100 hulls examined (Table 1). More than twice as many wounded kernels as intact in-hull kernels contained AFB₁; however, all of these kernels had comparatively low AFB₁ levels (Fig. 6), and the average AFB₁ contents of the two sets did not differ significantly (Table 1). After 10 days of incubation, the AFB₁ content of in-hull intact kernels was only 5% of the AFB₁ content of hulled intact kernels with seed coats, and the AFB₁ content of in-hull wounded kernels was only 0.6% of the AFB₁ content of hulled wounded kernels.

In vitro effect of hulls and hull extract on AFB₁ production by *A. flavus*. After 10 days, radial growth of *A. flavus* on both pistachio agar and pistachio agar with hulls had covered the petri plates (internal diameter, 82 mm); however, the *A. flavus*

growth on the hull-containing medium was much more luxuriant, with considerably more dense mycelial growth and sporulation, than the growth on pistachio agar alone. Nevertheless, even with the enhanced growth of *A. flavus*, aflatoxin production was inhibited 71% on the hull-containing medium (Table 2). Radial growth and sporulation of *A. flavus* were restricted on pistachio agar containing the pistachio hull extract, and aflatoxin production was inhibited 99.9%.

DISCUSSION

Great variability in aflatoxin content has been observed among individual seeds (3). Furthermore, studies such as those of Cucullu et al. (5) and those conducted in our laboratory (25) have demonstrated an unexplained mosaic of aflatoxin distribution even within individual seeds. These widely ranging aflatoxin concentrations have not been satisfactorily explained. In this study, in which we used one strain, one level of inoculation, one temperature, and one length of incubation, we eliminated major sources of variability. In addition, we used an inoculum of 200 spores per nut as a realistic representation of field conditions. It has been estimated that 75 spores land on a pistachio in the 3-month period after ripening and before harvest (19). By contrast, many laboratory studies have used 2 to 4 orders of magnitude more spores. There is evidence that the number of spores in the inoculum and the resulting aflatoxin concentration may be inversely related (2, 20); variability of aflatoxin content may be affected as well.

Trace metals, especially zinc (17, 18), and water activity (8) have been reported to affect aflatoxin biosynthesis. It is doubtful that either the zinc content or the water activity of the mature, intact pistachio nuts used in this study varied enough to affect the 700-fold variability in aflatoxin concentration observed.

In an attempt to explain the aflatoxin variability observed in nuts, we examined the major components of the pistachio fruit (the hull, the seed coat, and the kernel) as a function of colonization by *A. flavus* and the aflatoxin content of individual kernels.

The hull, as the outermost layer of the pistachio fruit, is covered with a cuticle which provides a protective barrier against pathogen attack (1). Workers have isolated cutinases from pathogenic fungi which degrade plant cuticle and provide a point of entry into the host plant (13). Wound pathogens, such as *A. flavus*, lack the ability to penetrate the cuticular layer (6). They gain entry into the host plant through breaks in the cuticle caused by abrasions or insects. Pistachio hulls with intact cuticles are resistant to *A. flavus* colonization (26, 31); however, *A. flavus* rapidly colonizes hulls once the cuticular layer has been damaged, as demonstrated in this study with the pistachios with cut hulls. Despite extensive fungal colonization of cut hulls, aflatoxin was not produced in the hulls. The lower aflatoxin content of kernels with hulls compared with hulled kernels is also probably the result of the aflatoxin-inhibitory effect of the hulls. In this study, injured, hulled kernels contained an average of 160 times more aflatoxin than injured kernels with hulls contained. Early-split pistachios with shriveled hulls contain substantially more aflatoxin than pistachios with early-split, fleshy hulls (7). Shriveled, desiccated hulls may lack the moisture required for *A. flavus* colonization, thus limiting growth to the kernel. High aflatoxin levels could accumulate in these kernels since *A. flavus* would not be exposed to the aflatoxin-inhibitory effect of the hulls. The aflatoxin-inhibitory effect of the hulls was also observed in in vitro assays; the addition of hulls or a partially purified water extract

FIG. 3. Ranked distribution of aflatoxin levels in hulled and shelled wounded kernels with and without seed coats that were inoculated with *A. flavus* NRRL 25347 and incubated for 7 days (A) and 10 days (B).

of hulls to pistachio kernel agar resulted in a significant reduction in aflatoxin production.

Another cuticle layer which presents a barrier to *A. flavus* infection of kernels is found below the seed coats of tree nuts (9). This cuticular layer has been examined previously as a barrier to *A. flavus* colonization in peanuts (14), cotton bolls (15), and corn (12). In corn, *A. flavus* appears to enter the seed through breaks in the seed coat cuticle (21). In our study, none of the 100 intact pistachio kernels without seed coats was colonized by *A. flavus* after 10 days, and aflatoxin was not detected in any of these kernels. *A. flavus* does not appear to be able to germinate on or penetrate this cuticular layer. Evaporation of the 5- μ l inocula used in this study on the hydrophobic surfaces of kernels stripped of their seed coats may have resulted in a water activity too low for germination.

The seed coat matrix appears to be more conducive to adhesion and/or germination of *A. flavus* spores than the under-

FIG. 4. Inhibition of aflatoxin production by pistachio seed coats. Seed coats were incorporated into Czapek yeast autolysate (CYA) agar, and plates were inoculated with *A. flavus* NRRL 25347 and incubated for 5 days.

FIG. 5. In-hull pistachios with artificially split hulls and intact kernels. The kernels were inoculated with *A. flavus* NRRL 25347 and incubated in their hulls for 10 days. The hulls were heavily colonized, but aflatoxin was not detected in the hulls; 66% of the kernels had no detectable aflatoxin.

lying cuticular layer is. *A. flavus* was able to germinate on and colonize 62% of intact pistachios with seed coats after 10 days. However, most of the colonization was weak, and the levels of aflatoxin in the kernels were low (less than 5 μg per kernel). Only 12% of intact kernels with seed coats developed significant colonization and had aflatoxin levels of more than 20 μg per kernel after 10 days. Whereas the frequency of kernel colonization tripled between days 7 and 10, from 20 to 62%, the frequency of aflatoxin-containing kernels increased sevenfold, from 6 to 44%. The latter increase implies that there was an initial delay in aflatoxin production. In *in vitro* plate assays, seed coats inhibited aflatoxin production but not the growth of *A. flavus*. Aflatoxin production in kernels with seed coats may require penetration into the kernels, and the delay in aflatoxin production observed in kernels with seed coats may represent the period of time between initial colonization of the seed coat

TABLE 2. Effect of pistachio hulls and hull extract on aflatoxin production and growth of *A. flavus*

Medium	Colony diam (mm) ^a	AFB ₁ concn ($\mu\text{g}/\text{plate}$) ^b
Pistachio agar	82	35 $\pm$ 8
Pistachio agar with hulls	82	10 $\pm$ 4
Pistachio agar with hull extract	31	0.05 $\pm$ 0.08

^a Means of five plates.

^b Means $\pm$ standard deviations of five plates.

and the time when hyphae come into contact with a cuticular lesion.

Kernel injury was a significant factor in colonization of kernels by *A. flavus* and in the accumulation of high aflatoxin concentrations. With the protective cuticle layer breached, the frequency and degree of colonization of wounded kernels were greater than the frequency and degree of colonization of unwounded kernels. High aflatoxin levels in early-split pistachios have been correlated with insect-damaged kernels (7, 31). Colonization was less extensive on wounded kernels with seed coats, and the level of visible colonization was only one-half that observed on wounded kernels without seed coats after 10 days. Gradziel and Wang (11) observed that seed coats could delay *A. flavus* colonization of almond kernels for up to 3 days. Wounded kernels with seed coats also contained an average of 50% less aflatoxin than wounded kernels without seed coats after 7 days, but after 10 days wounded kernels with and without seed coats had similar levels of aflatoxin. Thus, even for kernels with cuticular lesions, which are susceptible to rapid and extensive *A. flavus* colonization, the presence of a seed coat can delay aflatoxin production for several days.

Wounded kernels with similar levels of *A. flavus* colonization had extremely variable aflatoxin contents. By day 10, the AFB₁ contents of wounded kernels with seed coats ranged from 1.3 to 890 μg per kernel. Even without seed coats, which appear to contribute to variable aflatoxin contents, the kernel AFB₁ concentrations ranged from 30 to 710 μg per kernel.

We suggest that aflatoxin variability can be classified into the

FIG. 6. Ranked distribution of aflatoxin levels of in-hull intact and wounded kernels inoculated with *A. flavus* NRRL 25347 and incubated for 10 days.

following two types: type I variability, in which a nut either does or does not contain aflatoxin; and type II variability, in which there is a range of aflatoxin concentrations among nuts. Type I variability appears to be a function of the integrity of kernel barriers (e.g., cuticular layers and seed coats). A lesion in kernel layers would result in all infected nuts containing aflatoxin. In this study, 100% of the nuts in which we sliced through kernel layers contained aflatoxin on day 10, while only 22% of the intact kernels contained aflatoxin. In a previous study, 100% of pistachio kernels subjected to three physical treatments which damaged cell structure (freezing and thawing; autoclaving; drying and rehydrating) contained aflatoxin (23).

Type II variability (why one wounded nut contains 1 µg of AFB₁ and another contains 900 µg of AFB₁) is more difficult to explain. Much research on aflatoxin production has been and is being conducted at the molecular level and on crops; however, relatively little information at the cellular level is available. We propose that the hypha-plant interaction is a possible source of type II variability. Fungitoxic compounds in cells play a role in plant defense only if the fungus enters the cell. Aflatoxin production by *A. flavus* that infects a kernel could be reduced if a hypha penetrates a cell or subcellular structure containing an aflatoxin-inhibitory compound. In corn seeds, *A. flavus* hyphae have been found intracellularly as well as intercellularly (21). A component of the pistachio seed coat inhibited aflatoxin production in *in vitro* assays, yet on the nut, the seed coat does not inhibit aflatoxin production. The inhibitory component in the seed coat may be compartmented, explaining the seed coat's contribution to variable aflatoxin concentration. The degree of variability in the AFB₁ contents of injured kernels with seed coats versus injured kernels without seed coats, while not statistically greater at the 5% level, was significantly greater at the 10% level. Compartmentation of an aflatoxin-inhibitory compound would also explain the lack of uniform distribution of aflatoxin within a highly contaminated peanut kernel (5). It could also explain the highly heterogeneous distribution of aflatoxin among kernels of corn from one ear and between ears of identically treated corn (16), and among the pistachios that were inoculated and incubated identically in this study.

Further chemical analysis of the layers surrounding the kernel should assist in the identification of compounds which contribute to variable aflatoxin levels in pistachios.

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