Aflatoxin Production among Almond Genotypes Is Not Related to Either Kernel Oil Composition or *Aspergillus flavus* Growth Rate

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Abstract. Genetic differences were observed in levels of aflatoxin production following controlled inoculations of California almonds [*Prunus dulcis* (Mill.) D.A. Webb, syn. *P. amygdalus*, Batsch.; *P. communis* (L.) Arcangeli, non Huds.]. Genetic variation was also observed in kernel oil composition, and in susceptibility to *Aspergillus flavus* Speare as indicated by rate of mold expansion on the surface of cut kernels. Several almond lines resulting from the introgression of peach [*P. persica* (L.) Batsch] germplasm had very low aflatoxin levels relative to commercial cultivars tested. Peach-derived almond breeding lines and cultivars also produced some of the highest oil quality as determined by the proportion of oleic acid, and by the oleic to linoleic acid balance. The proportion of linoleic acid to total oil ranged from 16% to almost 30%. No correlations were detected between aflatoxin production in inoculated almond kernels and either kernel oil composition or mold growth rate on injured kernel tissue.

Aflatoxin contamination in almond kernel is due to the filamentous fungus *Aspergillus flavus* and, to a lesser extent, *Aspergillus parasiticus* Speare. Aflatoxins are a very toxic, carcinogenic, and immunosuppressive class of mycotoxin (Abramson, 1998; Lillehoj, 1992). While aflatoxins have been detected in California almond samples at low frequencies (Fuller et al., 1977; Schatzki, 1996), a single contaminated kernel may contain very high levels (Schade et al., 1975). Almond production in California presently exceeds 370,000 t, with about half the crop being exported. Most countries importing California almonds limit aflatoxin levels in kernel meats to <5 µg·kg⁻¹ (Schatzki, 1996). Mold development by *A. flavus* has also led to crop losses both in the field and in storage, and we have observed that certain almond cultivars are more susceptible to mold development. Gradziel and Wang (1994) identified genetic resistances in al-

mond to *A. flavus* that are associated with seedcoat integrity and, to a lesser extent, kernel composition. Kernel dry-weight composition in these genotypes is 36% to 52% oil, with linoleic and oleic acids comprising ≈90% of total kernel oils (Abdallah et al., 1998). Kernel fatty-acid composition and metabolism have recently been reported to be associated with both resistance to *A. flavus* and subsequent potential for aflatoxin formation in groundnuts (*Arachis hypogaea* L.) (Reding and Harrison, 1994), maize (*Zeamays* L.) (Zeringue et al., 1996), and *Nigella sativa* L. (El-Sayed et al., 1997). The objectives of this paper were to identify almond genotypes producing low levels of aflatoxin and to evaluate their relationships with *A. flavus* mold growth rate and kernel fatty-acid composition.

Materials and Methods

Materials. Thirteen almond cultivars and four advanced almond breeding selections resulting from two to three backcrosses from almond × peach hybrids were selected for testing based on their economic importance and range of fruit and tree characteristics. Trees were grown under standard commercial practices at Univ. of California, Davis, evaluation plots at Manteca and Winters, Calif. Nuts were harvested at full maturity as indicated by complete hull split, dried at 60°C for

3 d to a moisture content of 3% to 4%, and stored at 4°C in airtight plastic containers until testing.

Fungal growth on different almond genotypes. Inoculations were made to injured kernels as described previously (Gradziel and Wang, 1994), using aflatoxin-producing *A. flavus* accession NRRL 25347. Injury was achieved by cutting kernels longitudinally across the cotyledons prior to inoculation (Fig. 1). Kernel halves containing the embryo were placed on trays and maintained at near-saturated humidities, with the remaining half discarded. Test samples were inoculated with 20 µL of an aqueous suspension of *A. flavus* spores (2 × 10⁶·mL⁻¹) applied to the center of the cut kernel surface, then incubated in the dark at 30 °C. Scanning electron microscope (SEM) images of inoculated kernels were made using standard methods as described by Hormaza and Polito (1996). Mold growth was recorded 3 d following inoculation using a Kodak DCS 420 digital camera (Eastman Kodak, Rochester, N.Y.). The maximum length of mold expansion along the longitudinal kernel axis was measured using Image-Pro Plus image analysis software (Media Cybernetics, Silver Spring, Md.). There were two replications of 10 kernels per treatment.

Aflatoxin production under controlled conditions. Undamaged kernels with seedcoats were ground to a fine powder. A mixture of 5% almond kernel powder and 1.5% agar in 40 mL water was autoclaved and 10 mL sterile solution poured into 60-mm petri dishes. Each petri dish was inoculated with 200 spores of *A. flavus* and incubated at 30 °C for 7 d. Samples were then derivatized and analyzed for aflatoxin by high-performance liquid chromatography with fluorescence detection as described by Goodrich-Tanrikulu et al. (1995), with four petri dish samples being evaluated for each genotype.

Oil content and composition. Total fat content and fatty-acid methyl esters (FAMES) were determined according to the procedure of Garces and Mancha (1993), as reported previously (Abdallah et al., 1998). A 100-mg sample of kernel tissue and 0.5 mL (10 g·L⁻¹ in methanol) of margarine acid (17:0) were boiled (at 80 °C for 2 h under N₂ gas) with a reagent mixture containing methanol : heptane : benzene : 2,2-dimethoxypropane : H₂SO₄ (37:36:20:5:2, by volume). After cooling at room temperature, two phases formed. The upper phase, containing FAMES, was collected. FAMES were separated using an FID-GC gas chromatograph equipped with an SP-2330 column (30 m, 0.25 mm i.d., 0.2-µm film thickness) (Supelco, Bellefonte, Pa.). The FAMES were identified based on retention times of known standards (Sigma, St. Louis). The presence of 17:0 as an internal standard allowed the calculation of the total lipids based on the area of the standard. The extraction solutions were prepared as follows: Solution (A) contained 185 mL methanol, 100 mL benzene, 25 mL 2,2-dimethoxypropane, and 10 mL H₂SO₄; solution (B) contained heptane. For each sample, 0.5 mL of 17:0, 3 mL from A, and 2 mL from B were added.

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Data were recorded on a dry-weight (DW) basis and analyzed using the SAS analysis of variance procedure for balanced data and the SAS REG procedure for regression analysis (SAS Institute, 1988). Fisher's least significant difference (LSD) was used for comparison of genotype effect within treatments.

Results

Fungal growth on different genotypes. All genotypes showed rapid mold growth under test conditions (Table 1). Statistical differences existed among genotypes, though no appreciable field resistance was observed in this germplasm. No significant correlations were observed between *A. flavus* mold growth and any of the other variables tested. Microscopic examination of 'Nonpareil' kernel tissue, including the seedcoat and associated wax layers (Fig. 1), showed the bulk of kernel meat tissue to be composed of storage cells packed with oil-filled spherosomes. Similar structures were observed in SEMs of other genotypes, although the thickness of both the seedcoat and waxy layers varied both within and among genotypes.

Aflatoxin production under controlled conditions. Sizable and significant differences in aflatoxin production were observed within the germplasm tested. Aflatoxin production under the conducive environments of these controlled experiments ranged from 0.04 to 0.33 $\mu\text{g}\cdot\text{g}^{-1}$ DW (Table 1). Three of the peach-derived genotypes (having PA prefix) showed the lowest levels of toxin production, while the fourth peach-derived line, PA-F7,1-1, had the highest toxin level. Remaining almond cultivars showed the full range of intermediate values with moderate variation about the means. No correlation was detected between aflatoxin production and either fungal growth rate or kernel oil content and composition (Table 1).

Fatty acid composition. Total kernel oil content varied by genotype from 36% to 51% of the kernel DW (Table 1), which is similar to the range of 35% to 61% DW reported by Vezvaei and Jackson (1996) for cultivars grown in Australia. The oil content of 'Monterey' was low, while that for 'Padre' was high, with the remaining genotypes varying between these limits in a fairly uniform distribution. The oil contents of selections possessing peach germplasm were intermediate. The predominant fatty acids in kernel oil were oleic, linoleic, and palmitic. Palmitoleic, stearic, and linolenic acid were also detected at low levels as previously reported (Abdullah et al., 1998). The major differences in fatty-acid composition among these genotypes were the proportions of oleic, a monounsaturated fatty acid, and linoleic, a polyunsaturated fatty acid. The proportion of oleic acid was highest, ranged from $\approx 62\%$ to 76%, and was highly and negatively correlated with linoleic acid levels ($r = 0.99$). High oleic acid levels were observed in the cultivar Le Grand, which has peach germplasm in its lineage (Kester et al., 1991) and in three of the four breeding selections derived from peach. The exception was the selection PA-25-75, which contained lower oleic and

Fig. 1. Scanning electron micrograph (SEM) of section of 'Nonpareil' almond kernel showing the (partially separated) seedcoat (C) with external (W) and internal (w) wax layers, and oil-filled spherosomes in kernel storage cells (arrow). (Bar = 0.1 mm). Inset shows the location of *A. flavus* inoculation (X) and area of the SEM (box). (SEM courtesy of V.I. Polito, Pomology, Univ. of California, Davis).

Table 1. Mold growth and aflatoxin production in relation to almond kernel oil composition and their Pearson's correlation coefficients (r). (Sample standard deviations given in parenthesis).

Almond genotype	Mold growth (mm)	Aflatoxin production ($\mu\text{g}\cdot\text{g}^{-1}$ dry wt)	Total oil (% dry wt)	Oil composition (%)		
				Palmitic acid	Oleic acid	Linoleic acid
PA-F7,2-9	6.4 (0.2)	0.04 (0.003)	43.4 (1.2)	5.9 (0.1)	73.0 (1.3)	19.1 (0.9)
PA-F7,5-11	8.4 (0.6)	0.05 (0.005)	44.8 (1.0)	6.3 (0.2)	71.3 (0.3)	20.6 (0.4)
PA-25-75	5.3 (0.8)	0.07 (0.003)	45.9 (0.6)	6.7 (0.4)	61.8 (2.1)	29.2 (1.8)
Wood Colony	5.7 (0.5)	0.11 (0.008)	46.4 (2.0)	5.6 (0.3)	70.5 (2.7)	22.4 (3.1)
Monterey	7.2 (2.2)	0.12 (0.02)	36.0 (2.5)	6.3 (0.4)	67.5 (2.1)	24.2 (2.4)
Padre	4.9 (0.6)	0.17 (0.03)	50.6 (1.0)	5.3 (0.2)	70.9 (2.1)	21.8 (1.9)
Butte	4.2 (0.5)	0.17 (0.04)	47.5 (3.1)	6.2 (0.3)	64.7 (1.4)	26.9 (1.0)
Nonpareil	9.1 (1.6)	0.17 (0.02)	38.8 (0.3)	6.4 (0.3)	66.8 (0.8)	25.0 (1.2)
Carmel	6.6 (1.2)	0.18 (0.03)	40.6 (0.8)	6.0 (0.3)	66.6 (2.4)	25.5 (2.0)
LeGrand	4.7 (0.4)	0.20 (0.05)	48.4 (0.5)	5.8 (0.2)	76.0 (0.4)	16.3 (0.2)
Fritz	4.9 (0.8)	0.20 (0.03)	38.4 (2.4)	5.8 (0.5)	70.6 (1.1)	21.8 (0.4)
Mission	7.4 (1.0)	0.20 (0.04)	47.3 (1.2)	5.3 (0.4)	71.9 (2.3)	20.6 (1.6)
Price	7.1 (1.5)	0.22 (0.03)	38.1 (2.0)	5.0 (0.1)	72.8 (2.4)	20.3 (2.4)
Sonora	9.0 (2.9)	0.25 (0.05)	43.8 (2.3)	5.8 (0.3)	69.3 (2.3)	22.9 (1.7)
Ne Plus Ultra	9.4 (2.9)	0.25 (0.06)	45.5 (3.1)	6.1 (0.1)	70.7 (0.1)	21.4 (0.1)
Aldrich	6.6 (0.7)	0.31 (0.01)	47.9 (1.9)	5.5 (0.4)	71.0 (1.2)	21.8 (0.9)
PA-F7,1-1	8.1 (1.2)	0.33 (0.04)	44.4 (1.5)	5.8 (0.1)	72.8 (1.4)	19.5 (1.3)
LSD	1.3	0.05	3.0	0.6	3.2	2.8
Variable	r					
Mold growth	---	0.24	0.32	0.14	0.10	0.10
Aflatoxin	0.24	---	0.10	-0.44	0.30	0.24
production						
Linoleic acid	0.10	0.24	0.17	0.57*	-0.99***	---

*,***Significant at $P \leq 0.05$ or 0.001, respectively.

higher linoleic acid levels. PA-25-75 resulted from a cross to *P. mira* Koehne, a wild peach species, followed by backcrosses to almond, and similar ratios have been reported by Rikhter (1990) in the generative organs from such hybrids. While a weak correlation of 0.57 was calculated between palmitic acid and linoleic acid, no relationship was observed between oil content or composition and the other variables tested (Table 1).

Discussion

Previous studies have shown the presence of natural barriers to *A. flavus* infection in the seedcoat and associated wax layers (Fig. 1) (Gradziel and Wang, 1994). Although the fungus is widespread in nature, occurring both in air and soil, virtually all field infections of almond have been associated with insect damage to these kernel barriers exposing the nutritionally rich kernel contents (Phillips et al., 1976; Schatzki, 1996). While genetic variation was found for the almond kernel characteristics studied, including kernel oil content and composition, rate of mold expansion, and aflatoxin production, no clear association was observed between these traits as has been in certain annual crops (El-Sayed et al., 1997; Reding and Harrison, 1994; Zeringue et al., 1996). An explanation for this discrepancy may lie in the finding of a high negative correlation of $r = 0.99$ between oleic and linoleic acid. Similar high correlations in pistachio (*Pistacia vera* L.) and hazelnut (*Corylus avellana* L.) led Garcia et al. (1992, 1994) to propose the presence in these tree crops of a unique oleic desaturase that regulates the conversion of oleic to linoleic acid. Differences in fatty-acid metabolism for these species could preclude the type of association between fatty-acid composition and level of *A. flavus* damage observed in the other annual species, since biochemical studies have indicated that the specific by-products of linoleic fatty acid metabolism may actually promote or suppress disease (Brown et al., 1996; de Luca et al., 1995; Reding and Harrison, 1994; Zeringue et al., 1996). A different fatty-acid pathway in almond would produce different reaction by-products with different disease relationships.

While genetic differences in oil composition (Abdallah et al., 1998) and *A. flavus* susceptibility (Gradziel and Wang, 1994) have

been reported previously in almond, this study is the first to demonstrate a low aflatoxin production potential in California almond germplasm. High levels of oleic relative to linoleic acid should also reduce susceptibility to kernel rancidity, and thus to postharvest losses. Improved health benefits to the consumer of such high oleic to linoleic acid ratios have been reported (Sabate and Hook, 1996; Sabate et al., 1996). The demonstration of genetic differences in aflatoxin metabolism offers the possibility for breeding improved almond varieties with low potential for aflatoxin production following natural *A. flavus* infection.

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