Occurrence and Interaction of *Aspergillus flavus* with Other Fungi on Almonds

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This work was supported in part by the Almond Board of California (Cooperative Agreement 12-14-5001-51).

We thank Steven L. Purcell and Donald T. Wicklow for identification of aspergilli; W. C. Snyder for identification of fusaria; David F. Farr for the identification of several other of the fungi used in this study; Mary Nelson and Mary Haskin, biological research aides, for their technical assistance; and George Stanley, Dried Fruit Laboratory, Fresno, for commercial almond samples.

Accepted for publication 29 January 1979.

ABSTRACT

PHILLIPS, D. J., B. MACKEY, W. R. ELLIS, and T. N. HANSEN. 1979. Occurrence and interaction of *Aspergillus flavus* with other fungi on almonds. Phytopathology 69: 829-831.

The occurrence of the aflatoxin-producing fungi of the *Aspergillus flavus* group (AF) on samples from 89 locations in California was influenced by other fungi on the kernels. The influence of these other fungi on AF was evaluated in 1975 and 1976 in field plots. Fruit of almond cultivars Milo, Nonpareil, and Ne Plus were inoculated before hull dehiscence with dry conidia of aflatoxin-producing aspergilli alone or in combination with groups of fungi (1975) or individual isolates (1976). Fungi used included yeasts, *Monilinia fructicola, Ulocladium atrum, U. chartarum, Drechslera spicifera, Fusarium roseum, Rhizopus stolonifer, Cladosporium cladosporioides, Penicillium funiculosum,* and *Aspergillus ficuum.* The presence of other fungi with *A. flavus* or *A. parasiticus* generally reduced the number of isolates of AF that were recovered from hulls and kernels. Fungi requiring high moisture appeared antagonistic to AF in colonizing almond kernels. *U. chartarum* reduced the isolation of *A. flavus* most significantly.

Additional key words: Biological control, mycotoxin, *Prunus dulcis.*

The drupe of the edible sweet almond has a distinct pericarp enclosing the kernel. This pericarp consists of an outer fleshy hull and inner hard shell. Fruits usually mature on the tree, and a longitudinal suture on one side of the hull splits, exposes the shell, and allows rapid drying of the fruit.

Many species of fungi may colonize drying fruit of the almond (*Prunus dulcis* (Mill.) D. A. Webb) (10), including *Aspergillus flavus* Link and *A. parasiticus* Speare (11). These two *Aspergillus* spp. (AF) are widespread on seed and other crops (9,15) and may produce aflatoxins before or after harvest if environmental conditions are favorable. The AF group has been noted frequently on almond hulls and kernels, but under natural conditions the fungi rarely penetrate the undamaged kernel (5,11,13). In contrast, when almond fruit was inoculated experimentally in the orchard, the frequency of AF colonization of undamaged kernels was high (2–63%) and depended somewhat on the almond cultivar (11). Because of this difference between natural and experimental colonization, we evaluated the frequency of occurrence of various fungi with AF on almond kernels and initiated experiments on the interaction among fungi on almond hulls.

MATERIALS AND METHODS

Examination of fungi on almonds. Surface disinfested and nondisinfested almond kernels or hulls were tested for the presence of fungi. For surface disinfestation, samples were dipped in 70% (v/v) ethanol/H_2O for 10 sec, then immediately soaked in 0.5% sodium hypochlorite solution for 5 min. The disinfested samples were aseptically plated on malt-salt medium (MSM) containing 7.5% NaCl, 2% malt extract (Bacto, Difco Laboratories, Detroit, MI), and 2% agar. Nondisinfested samples were plated directly on MSM plus 13 mg/ml of 2,6-dichloro-4-nitroaniline to inhibit growth of some *Rhizopus* spp. Five almond kernels or hull sections were placed on each plate. After incubation for 1 wk at 30 C, fungi visibly growing on each kernel or hull piece were counted. Most fungi were tentatively identified by genus. *Aspergillus* spp. were classified to a "group" by color and morphology (12). The species of representative isolates then were identified (12).

Sampling of commercial almonds. During 1974, a sample of 200 shelled almonds was obtained from each of 89 orchards in the Central Valley of California; 100 surface disinfested and 100 nondisinfested kernels were analyzed for fungi. The relative frequency of occurrence of the various fungi on the kernels was recorded, and simple correlation coefficients on arcsin square root proportions were computed.

Moisture content was determined on a fresh weight basis for each sample from the weights of a 50-kernel subsample before and after oven drying for 48 hr at 86 C.

Preparation of inoculum. Fungi isolated from almond hulls were established in pure culture. Inocula of some fungi other than *Aspergillus* spp. were prepared from the fungi grown separately on steam-sterilized carnation straw (10 g of air-dried straw and 15 ml of water) in wide-mouth jars closed with muslin. After about 2 wk at room temperature (21 C ± 2 C) the colonized straw, which had been air-dried, was crumbled with a glass rod. This crumbled straw was used as inoculum in field-plot testing for antagonism to aflatoxin-producing *Aspergillus* spp.

Conidia and conidial heads of *A. flavus* or *A. parasiticus* were collected, mixed with dry talc, held several weeks, and used to inoculate almond fruits. The conidia were from 1-wk-old toxigenic cultures grown on potato dextrose agar at room temperature.

Inoculation of almond fruits with *A. flavus* and other fungi. In 1975 and 1976, 45 Milo, Nonpareil, and Ne Plus soft-shelled almond trees near Fresno, CA, were selected for testing. The trees were in five blocks, each with three trees per cultivar. About 2 wk before hull-split, representative fruits were sprayed with 0.01% sodium hypochlorite solution, rinsed with water, and allowed to dry.

Crumbled bits of dry straw colonized by the test fungi were held in place with masking tape over the suture line of each of two adjacent disinfested fruits. Approximately 2 cm^2 of fruit was covered with the test inoculum. The two fruits were enclosed together in two muslin bags, one bag inside the other but separated

by paper toweling, and then were exposed to AF by means of a powder blower inserted through the end of the bags. The bags and paper toweling acted as a filter to reduce contamination. The treated fruit remained on the tree until normal harvest; at that time the bags were not opened but were cut from the tree.

The almonds in the bags were allowed to air dry and were held in dry storage for about 2 mo, after which sections of the hulls or whole kernels were surface disinfested and plated on MSM. At harvest, the moisture content of the whole fruit was about 20% (fresh weight basis) or equivalent to a water activity of about 0.8 (11).

In 1975, the test inoculum was a mixture of fungi, grouped according to their probable moisture requirements (6). The treatment groups were the mixture of *A. flavus* and *A. parasiticus* isolates alone (treatment F) and F combined with each of the following four groups: (A) an unidentified yeast and *Monilinia fructicola* (Wint.) Honey; (B) *Ulocladium atrum* Preuss, *U. chartarum* (Pr.) Simmons, *Drechslera spicifera* (Bainier) Matsushima; (C) *Fusarium roseum* (Lk.) Snyd. & Hans., 'Gibbosum,' *F. roseum* (Lk.) Snyd. & Hans., *Rhizopus stolonifer* (Ehr. ex Fr.) Vuill., *Cladosporium cladosporioides* (Fresen) de Vries; (D) *Penicillium funiculosum* Thom, *Aspergillus ficuum* (Reich.) Hennings. In one treatment, no inoculum was added. This procedure resulted in two fruits per treatment, in six treatments on each of the 45 trees that comprised the three different cultivars.

In 1976, individual isolates from the 1975 groups with reduced AF colonization and other isolates were selected for further testing. Treatments on each tree were a mixture of aflatoxin-producing isolates, *A. parasiticus* 72-71, *A parasiticus* 72-70, *A. flavus* 72-68, and *A. flavus* 72-46 (H); H plus tape with no test inoculum (I); and H with (T) *Saccharomyces* sp.; (U) an unidentified yeast; (V) *Ulocladium atrum;* (W) *U. chartarum;* (X) *Fusarium roseum;* (Y) *Cladosporium cladosporioides;* and (Z) a mixture of T, U, V, W, X, and Y. In one treatment, no inoculum or tape was used. This procedure resulted in two fruits per treatment, 10 treatments on each of the 45 trees that comprised three different cultivars.

RESULTS

Sampling of commercial almonds. Fungi were isolated from more than 90% of the kernels sampled from 89 commercial orchards. The *A. niger* (group) and *Alternaria* spp. were most common on the disinfested kernels, but *Aspergillus* spp. and *Penicillium* spp. predominated on nondisinfested kernels (Table 1). The AF was common on nondisinfested kernels (59.7%) but infrequent on disinfested kernels (0.4%). The presence of various other fungi reduced the frequency of AF in the samples. The *A. glaucus* (group), *Rhizopus* spp., and unknown isolates were negatively correlated with AF. *Rhizopus* spp. and unknown isolates were positively correlated with increasing moisture in the kernels. Occurrence of *Penicillium* spp. was positively correlated with occurrence of AF.

Field plots. The inoculation of almond hulls with the test fungi in 1975 generally lowered the frequency of recovering AF from the disinfested hulls or kernels (Table 2). The no-inoculum treatments had no AF and were not analyzed statistically. Groups B and D reduced AF on the hulls. Groups A, B, and C significantly reduced the frequency of AF isolation from the kernels. Generally those fungi considered hydrophilic or mesophilic (4) appeared to antagonize AF colonization of the kernels.

Individual isolates tested in 1976 usually reduced the frequency of AF isolated from the kernel, but the recoveries of AF from hulls inoculated with the yeasts tended to be high. *U. chartarum* and the mixture of all test fungi significantly reduced AF isolation from both hull and kernels (Table 3). The AF was not recovered from the no-inoculum treatment.

In 1975, more AF was isolated from the kernels and hulls of Nonpareil and Milo cultivars than from Ne Plus, but in 1976 more was found on the hulls of Ne Plus than on the others. Differences in colonization could have been related to the environment before the different harvest dates or during storage, rather than to inherent differences in the susceptibility of the fruit.

DISCUSSION

A. flavus growth and/or toxin production was reduced by several other *Aspergillus* spp. and other fungi (1–4,7,8,14,16). In situ, *A. niger* prevented penetration of peanut kernels by *A. flavus* (6). Similarly, *A. flavus* colonization of gnotobiotically grown peanuts was reduced by *Trichoderma viride* but was stimulated by *P. funiculosum* (17). In general, the presence of a mixture of organisms is considered to reduce the production or accumulation of mycotoxins (9,12).

In our tests, *U. chartarum* was an especially effective antagonist of AF on almonds, and most of the fungi inoculated onto almond hulls before harvest reduced the colonization of the kernels by toxigenic isolates of *A. flavus* or *A. parasiticus*. The more effective antagonists were not fungi commonly associated with the dry-shelled kernels, but rather fungi generally thought to require high to moderate moisture conditions (6). Thus, the moisture requirements of the fungi seem to be involved in their antagonistic ability on almonds. The nutrients available at hull-split support fungal colonization; hence, the antagonism may have resulted from competition for hull nutrients. AF colonization of kernels appears to depend on the presence and composition of the microflora on the hull during a critical period, probably beginning with hull-split and

TABLE 1. Fungi isolated from almond kernels in 1974

Fungus Isolated	Disinfested kernels[a]			Nondisinfested kernels		
		Correlation coefficient			Correlation coefficient	
	% colonization[b]	With *A. flavus*	With kernel moisture	% Colonization[b]	With *A. flavus*	With kernel moisture
Alternaria spp.	18.8	−0.08	0.00	0.3	−0.07	−0.12
Rhizopus spp.	8.5	−0.08	0.09	19.4	−0.24[c]	0.30[d]
Other fungi	14.2	−0.03	0.00	24.4	−0.23[c]	0.23[c]
A. niger group	35.2	0.10	0.03	98.8	−0.06	0.08
A. flavus group	0.4	...	0.07	59.7	...	−0.18
A. glaucus group	2.9	0.07	−0.01	30.0	−0.37[d]	0.09
Other Aspergilli	0.7	−0.03	−0.16	37.0	−0.14	0.00
Penicillium spp.	0.4	0.03	0.10	27.2	0.34[d]	−0.11
No fungus	9.9	−0.11	0.01	0.3	0.00	0.00

[a] Kernels dipped in 70% ethanol for 10 sec and then 0.5% sodium hypochlorite solution for 5 min.
[b] Means of 89 samples of 100 kernels.
[c] Statistically significant, $P = 0.05$.
[d] Statistically significant, $P = 0.01$.

ending when the moisture content of the kernel falls below a water activity of 0.70 (11).

The natural AF colonization of the disinfested kernels appeared to be low (0.4%), even though it was greater than 50% on the nondisinfested kernels.

Certain fungi interfere with AF colonization of almond kernels, but it is not known whether the interference is due primarily to the presence of antagonistis or to the physical or chemical environment of the hull. The chemical composition of the fruitlike hull, which is quite unlike that of the kernel, or high temperatures during drying in the orchard may especially influence the interaction among fungi on the hull and subsequent kernel colonization by AF.

Further study is in progress to evaluate these interacting environmental factors and the possible suppression of aflatoxin contamination by the specific antagonists used in our investigation.

TABLE 2. The colonization of almond fruit by *Aspergillus flavus* or *A. parasiticus* after inoculation with other fungi

Cultivar	Proportion of sample (%) colonized by *Aspergillus* spp. when inoculated with indicated fungal group[a]					Cultivar[b] means
	A + F	B + F	C + F	D + F	F	
Kernel						
Nonpareil	20	23	23	37	38	28 a
Milo	0	3	10	14	38	13 b
Ne Plus	7	0	3	10	7	6 c
Means	9[c]	9[c]	11[c]	20	28	
Hull						
Nonpareil	57	77	83	62	97	75 a
Milo	83	80	90	63	86	80 a
Ne Plus	76	33	72	72	63	63 b
Means	72	63[c]	82	66[c]	82	

[a]A = Unidentified yeast, *Monilinia fructicola*; B = *Ulocladium atrum, U. chartarum, Drechslera spicifera;* C = *Fusarium roseum, F. roseum 'gibbosum,' Rhizopus stolonifer, Cladosporium cladosporioides;* D = *Penicillium funiculosum, Aspergillus ficuum;* F = *A. parasiticus* 72-71, *A. flavus* 72-68, *A. parasiticus* 72-70, *A. flavus* 72-30.

[b]Overall cultivar means within a hull or kernel column without a letter in common differ at the 5% significance level. Each datum represents about 30 kernels or hulls from each cultivar.

[c]Significantly different from group F at the 5% level.

TABLE 3. Colonization of almond fruit by *Aspergillus flavus* or *A. parasiticus* after inoculation with a specific fungus

Cultivar	Proportion of sample (%) colonized by *Aspergillus* spp. when inoculated with indicated fungus[a]									Cultivar[b] means
	T	U	V	W	X	Y	Z	H	I	
Kernel										
Nonpareil	14	10	20	3	20	20	10	27	21	16 a
Milo	13	10	10	0	3	20	10	18	17	11 a
Ne Plus	3	7	10	10	21	14	13	23	33	15 a
Means	10[c]	9[c]	13	5[c]	15	18	11[c]	23	24	
Hull										
Nonpareil	83	63	60	37	57	53	47	60	60	58 a
Milo	87	77	57	28	33	47	33	57	72	54 a
Ne Plus	83	93	93	97	93	93	93	93	90	92 b
Means	84	77	70	54[c]	61	64	58[c]	70	74	

[a]T = *Saccharomyces* sp.; U = Yeast; V = *Ulocladium atrum;* W = *U. chartarum;* X = *Fusarium roseum;* Y = *Cladosporium cladosporioides;* Z = Mixture of T, U, V, W, X, and Y; H = *A. parasiticus* 72-71, *A. parasiticus* 72-70, *A. flavus* 72-68, *A. flavus* 72-46; I = same as H with no tape.

[b]Overall cultivar means within a hull or kernel column without a letter in common differ at the 5% significance level. Each datum represents about 30 kernels or hulls from each cultivar.

[c]Significantly different from group H at the 5% level.

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