Evaluation of the Atoxigenic *Aspergillus flavus* Strain AF36 in Pistachio Orchards

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Abstract

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The atoxigenic strain *Aspergillus flavus* AF36, which has been extensively used as a biocontrol agent in commercial corn and cotton fields to reduce aflatoxin contamination, was applied in research pistachio orchards from 2002 to 2005 and in commercial pistachio orchards from 2008 to 2011. AF36 was applied as hyphae-colonized steam-sterilized wheat seed (the same product and same application rate as used in cotton fields). In all orchards, applying the wheat-AF36 product substantially increased the proportion of vegetative compatibility group (VCG) YV36, the VCG to which AF36 belongs, within *A. flavus* soil communities. Application of the AF36 product in additional years further increased YV36 in the soil until it composed 93% of the *A. flavus* isolates in treated commercial orchards. Nonetheless, application of the AF36 product did not result in increased incidence of kernel decay of the nuts. For nuts harvested from commercial orchards, reductions in percentages of samples contaminated with aflatoxin from treated orchards (relative to that for untreated orchards) ranged from 20 to 45%, depending on the year. Because of the high value of pistachio nuts and the costs associated with rejection of shipments due to aflatoxin contamination, these reductions are significant and valuable to the pistachio industry.

Aflatoxins, which are potent toxins and carcinogens, are widely regulated by governments, with the tolerances set very low in food (43). Aflatoxins are mainly produced by two closely related fungi, *Aspergillus flavus* Link and *A. parasiticus* Speare, and they naturally grow in many crops, including corn, peanut, and cottonseed. These aflatoxin-producing fungi also grow at low levels in pistachio nuts (*Pistacia vera* L.) in commercial orchards in California, occasionally resulting in aflatoxin contamination (27). The incidence of aflatoxin-contaminated pistachio nuts in California is somewhat low. For example, it has been estimated at 1 aflatoxin-contaminated nut per 25,000 nuts (41). Nonetheless, due to very low tolerances of regulatory agencies and the problems associated with the rejection of shipments of pistachio nuts, these levels of aflatoxin contamination result in significant financial problems for the pistachio industry in California. The most common *Aspergillus* sp. found decaying pistachio nuts in California is *A. niger*, which is much more common than the aflatoxin-producing *A. flavus* and *A. parasiticus* (27). The aflatoxin-producing fungi typically grow in plant litter on the orchard floor of pistachio orchards (28) and, in late summer, can infect nuts on the tree, especially nuts with both hull and shell split known as early split nuts (27,29).

Naturally occurring isolates of *A. flavus* that are not able to produce aflatoxin ("atoxigenic" strains) have been used successfully to reduce aflatoxin contamination in cottonseed (3,5,14,19), peanut (24,26), and corn (7,8,12,25). Atoxigenic strains reduce aflatoxin contamination by displacing or excluding aflatoxin-producing fungi (17). The atoxigenic *A. flavus* AF36 has been developed as a biocontrol agent for preventing aflatoxin contamination of cottonseed (3,5). Currently, AF36 is used in commercial cotton production in Arizona, California, and Texas and in corn production in Texas and Arizona. Steam-sterilized wheat seed colonized by *A. flavus* isolate AF36 is applied in fields to the surface of the soil once each year in the late spring or early summer at the rate of 11.2 kg/ha. After exposure to moisture through irrigation, dew, rain, or high humidity, the wheat becomes rehydrated, allowing the fungal biocontrol agent to grow on the biomass of the dead wheat seed and produce spores for dispersal. The effectiveness of AF36 applications in cotton was determined by quantifying the effect on fungal populations and by measuring the reduction in aflatoxin contamination of the crop (3,5,14). The standard method used for identifying the AF36 fungus within *A. flavus* populations has been to determine whether *A. flavus* isolates belong to the same vegetative compatibility group (VCG) as the AF36 isolate, VCG YV36 (3,14,21). The major objectives of this research were to (i) quantify the occurrence of VCG YV36 (the VCG of the AF36 isolate) in commercial pistachio orchards in California prior to the application of the AF36 product in any commercial orchard, (ii) measure the effectiveness of applying the AF36 product in increasing the frequency of VCG YV36 in the fungal population in commercial pistachio orchards, (iii) evaluate whether application of the AF36 product results in increases in the incidence of decayed nuts, and (iv) determine the influences of application of the AF36 product on the aflatoxin contamination of the nuts.

Materials and Methods

Natural occurrence in commercial orchards. Incidences of VCG YV36, the VCG to which AF36 belongs, in commercial pistachio orchards was determined with a collection of 1,255 *A. flavus* isolates from pistachio orchards (38 different commercial orchards in eight counties) already present in our culture collection at the Kearney Agricultural Center. The *A. flavus* isolates had been obtained during our various studies of aflatoxin contamination in commercial pistachio orchards in California between 1990 and 2007, prior to application of the AF36 product in commercial orchards. Although most of the isolates were obtained from pistachio orchard soil, many isolates were also obtained from inflorescences,

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leaves, and nuts. The methods for obtaining these isolates have been described elsewhere (28,30). Incidences were determined by identifying those *A. flavus* isolates belonging to VCG YV36 (14,23). Briefly, nitrate-nonutilizing auxotrops were selected on a chlorate-containing medium and paired with complementary tester mutants (one cnx and one niaD) of AF36 (9,14). An isolate was identified as AF36 if it complemented one or both tester mutants, resulting in a zone of prototrophic growth where the mycelia met (14).

The AF36 product. *A. flavus* AF36 product was obtained from the Arizona Cotton Research and Protection Council, United States Department of Agriculture–Agricultural Research Service manufacturing facility in Phoenix, AZ, which produces the same AF36 product for application in commercial cotton fields. The AF36 product consists of steam-sterilized wheat seed that have been colonized by *A. flavus* isolate AF36. In 2002, the wheat colonized by the atoxigenic strain AF36 was produced in small amounts for this study (10). Starting in 2003, AF36 product was obtained from the production facility of the Arizona Cotton Research and Production Council in Phoenix AZ, where this product is produced for use in commercial corn and cotton. In all experiments, the AF36 product was applied once a year to the orchard floor at the same rate (11.2 kg/ha) as that applied to other crops. Rehydration of the AF36 product is necessary in order to produce fungal spores for dispersal of the biocontrol agent. In pistachio orchards in California, irrigation typically provides the moisture for rehydrating the AF36 product.

Application of AF36 product in research orchards. The atoxigenic *A. flavus* AF36 was applied to the ground in the late spring or early summer in two pistachio orchards ('Kerman') at the Kearney Agricultural Station in Parlier, CA. The two pistachio orchards differed in type of irrigation and irrigation frequency. Pistachio orchard A was irrigated by flooding every 10 to 14 days throughout the summer, while pistachio orchard B was irrigated with microsprinklers every 7 days. Both orchards had vegetation (which was mowed periodically) between the rows of trees but down the rows the soil surface was kept bare of vegetation for approximately 1 m on each side of the trees. For both experiments, the experimental design was a randomized complete block design with three replications. For orchard A, replicates were single trees with 6-by-6-m treatment area per replicate. For pistachio orchard B, each replicate consisted of 20 trees (four rows with 5 trees each) with a 24-by-27-m treatment area. In both orchards, the replicates were adjacent to each other. In orchard A, the AF36 product was only applied on 1 July 2002. In orchard B, the AF36 product was applied on 1 July 2003, 6 July 2004, and 29 June 2005. The experiment in orchard B had two treatments in addition to the untreated control. The first treatment was to apply the AF36 product in 2003 and 2004, while the second treatment was to apply the product in 2004 and 2005.

Application of AF36 product in commercial orchards. In late June or early July 2008, the AF36 product was applied to 1,200 ha of commercial pistachio orchards in Kern, Kings, Madera, Merced, and Tulare Counties in California. These applications complied with an Environmental Protection Agency (EPA)-approved Experimental Use Program which allowed treatment of the commercial crop without crop destruction. In June 2009, June 2010, and July 2011, the AF36 product was again applied to the same orchards and in the same manner as in 2008. All orchards had the Kerman, the predominant pistachio cultivar in California. In addition, all orchards were irrigated regularly by either drip or microsprinklers, the most common irrigation methods in California. Growers followed their normal irrigation practices.

Collection of samples from research orchards. Soil samples were collected to determine the effect of treatment with the AF36 product on fungal populations in the orchards. Each soil sample consisted of 25 cores (2 cm in diameter and 2 cm in depth). For all years and both research orchards, two separate samples were collected for each treated and control replicate. In order to assess the extent to which influences of applications are additive across years, soil was always sampled the day of but prior to application. Soil samples were also collected later in the summer. For pistachio orchard A, soil samples were collected on 1 July and 23 September 2002, 19 August 2003, 30 August 2004, 29 August 2005, and 21 August 2006. For pistachio orchard B, soil samples were collected 1 July and 23 September 2003, 6 July and 9 September 2004, 29 June and 12 September 2005, 25 September 2006, and 6 September 2007.

In order to compare kernel rot between treated and control plots, early split nuts, with the hull and shell split exposing the kernel, were collected because these are the main source of aflatoxin contamination in pistachio (27,41). There were too few early split nuts in orchard A to support meaningful comparisons due to the small block size. In orchard B, early split nuts were collected from trees on 23 September 2003 (416 early split nuts collected), 3 September 2004 (635 collected), 12 September 2005 (500 collected), 25 September 2006 (2,270 collected), and 6 September 2007 (4,242 collected). Normal nuts (nuts with the hull intact) were also collected on the same dates to quantify *Aspergillus* spp. on hull surfaces. Nut collection coincided with normal commercial harvest.

Collection of samples from commercial orchards. Soil samples were collected to determine treatment effects on fungal populations. For all sampling dates, three separate soil samples were collected from each orchard for seven pairs of orchards (a treated orchard and an adjacent untreated orchard). The mean size of these treated orchards was 204 acres, ranging from 112 to 400 acres. Orchards were in Kings, Madera, Merced, and Tulare Counties. The approximate distance between the two most distant orchards was 175 km. Each soil sample consisted of 10 cores (2 cm in diameter and 2 cm in depth) taken along a line of 30 trees (starting at least 240 m from the edge of the orchard). Soil samples were collected before applying the AF36 product in 2008 and 2009, as well as later in the summer during the harvest period (September) during all 4 years.

In the commercial orchards, the number of early split nuts collected and evaluated per orchard was 253 to 547 in 2008, 213 to 416 in 2009, 489 to 812 in 2010, and 1,788 to 3,167 in 2011. The total numbers of early split nuts were 1,151 and 1,279 in 2008, 928 and 947 in 2009, 2,004 and 2,199 orchards in 2010, and 4,951 and 4,674 orchards in 2011 for the untreated and treated orchards, respectively. As in the research orchards, normal nuts were also collected to quantify *Aspergillus* fungi on the hull surfaces. Three separate normal nut samples were collected from each orchard, each sample consisting of 10 nut clusters taken along a line of 30 trees (beginning at least 240 m from the edge of the orchard). Both types of nut samples were collected from three pairs of orchards on 29 August to 15 September 2008, 9 to 16 September 2009, and 9 to 21 September 2010, and from four pairs of orchards on 9 to 21 September 2011.

The amount of aflatoxin contamination in the nuts was quantified using "library samples" obtained from processors, who normally retain reference library samples for each pistachio load (approximately 23,000 kg of nuts) in order to determine how much growers will be paid. Library samples consist of 9 kg of fresh nuts taken at the processing plant. While being unloaded, nuts are sampled automatically and periodically to give a representative cross-section sample of the truck's load. The library samples for the orchards in this study were brought to the Kearney Agricultural Research and Extension Center, where three samples from the same orchard and with the same harvest date (±24 h) were combined to make a composite sample.

Evaluation of samples. Samples from research and commercial orchards were evaluated with the same methods. Soil samples were air dried, ground to pass a sieve (0.86-mm mesh opening), and thoroughly mixed. To quantify densities of *A. flavus* and *A. parasiticus* and to obtain isolates for VCG analyses, between 0.02 and 0.20 g of soil was sprinkled on the surface of a selective isolation (SI) medium containing chloramphenicol and dichloran (33) in each of 10 petri dishes and incubated at 30°C for 7 days. This method was selected because preliminary tests indicated that *A.*

flavus and *A. parasiticus* were at very low densities in orchard soil. The SI medium contained relatively high levels of dichloran to inhibit the growth of *A. niger*, which is typically at substantially higher densities than *A. flavus* and *A. parasiticus*. For many samples, soil plating was repeated until sufficient numbers of *A. flavus* isolates were obtained for VCG analyses. For comparison, the density of *Aspergillus* sect. *Nigri* was quantified because these fungi were not applied, are widespread naturally in pistachio orchards, and are related to *A. flavus*. To quantify *A. niger* and closely related fungi in *Aspergillus* sect. *Nigri*, 1.0 or 2.0 g of soil was added to 100 ml of sterile deionized water in 125-ml plastic bottles and shaken for 15 min on a mechanical shaker; then, 100 µl of the soil suspension was spread evenly on 10 plates of dichloran chloramphenicol peptone agar (DCPA; 2), and incubated at 30°C for 5 to 7 days.

To quantify fungi on hull surfaces, 25 nuts with intact hulls were placed in a 250-ml sterile plastic bottle, 15 ml of sterile 0.005% (vol/vol) Tween solution was added, and bottles with the nuts were then shaken (15 min, 180 rpm) on an oscillating shaker. Then, 200 µl of the washings was spread evenly on each of 10 DCPA plates and 400 µl on each of 10 SI plates. After 5 to 7 days at 30°C, *Aspergillus* colonies were enumerated.

Kernels of early split nuts were examined for fungal decay with a dissecting microscope (magnification ×10 to 60) after removing the hulls and shells by hand. All *Aspergillus* fungi observed, except for those belonging to *Aspergillus* sect. *Nigri*, were isolated into pure culture.

Identification of fungi. In general, *Aspergillus* spp. were identified using the descriptions and methods of Klich and Pitt (38). Each colony of *A. flavus* and *A. parasiticus* (up to 30 colonies per soil sample) was isolated into pure culture on Czapek yeast extract agar (38) in order to verify identification and to obtain isolates for strain determination. All isolates of *A. flavus* were classified as belonging to one of two morphotypes, the L strain or S strain, according to whether a few large sclerotia or abundant small ones, respectively, were produced (13). The identification of isolates to these morphotypes was done because isolates of *A. flavus* S strain (along with isolates of *A. parasiticus*) from pistachio orchards in California consistently produce aflatoxins (28).

Vegetative compatibility analyses. The incidence of the applied atoxigenic isolate AF36 among *A. flavus* isolates recovered from various substrates was determined by identifying those isolates belonging to VCG YV36 (the VCG of the isolate AF36) using vegetative compatibility analyses, as described above, for analyses of *A. flavus* populations endemic to California pistachio (14,23). The number of *A. flavus* isolates from the soil that were evaluated was 10 isolates per sample for the research orchards (two samples per treatment per block) and 15 isolates per sample for the commercial orchards (three samples per orchard). Occasionally, fewer isolates per sample were evaluated because an insufficient number of isolates were obtained from the sample. For the commercial orchards, the total number of *A. flavus* isolates actually evaluated for all orchards was 524 to 585 isolates, depending on sampling date.

Table 1. Natural occurrence of *Aspergillus flavus* vegetative compatibility group YV36, to which the atoxigenic isolate AF36 belongs, among isolates of *A. flavus* obtained from commercial pistachio orchards in important pistachio-growing counties in California[y]

County	Acreage (%)[z]	Isolates evaluated (*n*)	AF36 (%)
Kern	44.3	276	12.7
Madera	19.6	581	7.2
Tulare	9.2	277	2.9
Fresno	8.9	65	3.1
Merced	4.6	20	15.0
Counties combined	86.6	1,219	7.4

[y] Most *A. flavus* isolates were obtained from orchard soil but many were also obtained from inflorescences, leaves, and nuts. These isolates are further described in the Materials and Methods section.

[z] Percentage of total acreage of pistachio trees grown in California that are grown in specified county (42).

Aflatoxin analysis of nuts. Library nut samples from the 2008 harvests were analyzed for aflatoxins by the DFA of California (formerly known as the Dried Fruit Association of California) using the AOAC Official Method Number 991.31, which uses immunoaffinity columns (Vicam, Inc.) for cleanup and high-performance liquid chromatography (HPLC) with post-column derivation and fluorescence detector for quantification. The limit of detection for total aflatoxins was 0.3 ng g^{-1}. These library samples analyzed were from six pairs of orchards (treated orchard and an adjacent untreated orchard). The approximate distance between the two most distant orchards for aflatoxin sampling in 2008 was 85 km.

Library nut samples from the 2009, 2010, and 2011 harvests were also analyzed for aflatoxins at the Kearney Agricultural Research and Extension Center, using a method similar to that used in 2008. After the in-shell pistachio nuts were ground finely and mixed thoroughly in a Blixer 6 vertical cutter-mixer (Robot Coupe), 50 g of the ground nuts were blended with 5 g of NaCl and 200 ml of methanol-water (60:40; vol/vol) using a Waring Blender. After filtration, 25 ml of the filtrate was passed through an AflaTest immunoaffinity column (Vicam, Inc.). The column was washed with 20 ml of deionized water and dried. Aflatoxins were eluted by passing 1 ml of methanol through the column. Aflatoxins were quantified by HPLC with a C18 column and a fluorescence detector (360 and 440 nm as wavelengths for excitation and emission, respectively). A mixture of methanol–water (45:55, vol/vol) at 0.8 ml min^{-1} flow-rate was used as the mobile phase. A photochemical reactor enhancement detection system (Aura Industries) was used for post-column photolytic derivatization to increase the sensitivity and selectivity of the fluorescence detector. The limit of detection for aflatoxins B1, B2, G1, and G2 were all below 1.0 ng g^{-1}. Total aflatoxins were calculated by summing the amounts of B1, B2, G1, and G2. More extensive aflatoxin analyses were done with the library samples from the 2009 to 2011 harvests compared with 2008. Library samples were from 13 pairs of orchards (treated orchard and adjacent untreated orchard). These orchards were in Kern, Kings, Madera, Merced, and Tulare Counties. The approximate distance between the two most distant orchards for aflatoxin sampling was 220 km.

Data analysis. Analysis of variance was used with least significant difference (LSD) for separation of means. Density data (propagules per gram) were log transformed prior to being subjected to Fisher's protected LSD test (39). For aflatoxin values, one-sided Fisher's exact tests were used on two-by-two contingency tables to test whether the AF36 treatment was effective. All analyses were done using SAS (release 9.2; SAS Institute Inc.).

Results

Natural occurrence. VCG YV36, the VCG to which the atoxigenic isolate AF36 belongs, was relatively common and widespread in California prior to the application of biocontrol products containing the AF36 fungus as the active ingredient (Table 1). Out of 1,219 isolates of *A. flavus* collected from commercial pistachio orchards, 7.4% belonged to the VCG YV36. Furthermore, *A. flavus* isolates belonging to YV36 were detected in commercial pistachio orchards in all the major pistachio-growing counties in California, although the frequency varied substantially between the various counties (Table 1).

Soil: research orchards. Applying the AF36 product in orchard A resulted in a substantial decrease in *A. parasiticus* within the *Aspergillus* sect. *Flavi* population. This orchard originally had high levels of *A. parasiticus* (62.2% of the *A. flavus* or *A. parasiticus* isolates belonged to *A. parasiticus*) but, after application of the AF36 product, no *A. parasiticus* isolates were recovered from the soil from the treated areas in 2002. Even though, over time, the percentage of *A. parasiticus* increased, even in 2006 (slightly more than 4 years after application of the AF36 product), the percentage of *A. parasiticus* (18.3% of the isolates) was statistically significantly less than the percentage before application (62.2%). In con-

trast, orchard B had very low proportions of *A. parasiticus* prior to applying the AF36 product (0.9% of the *A. flavus* or *A. parasiticus* isolates) and throughout the duration of the study (mean percentage 1.6%). Although isolates of the S strain of *A. flavus* were occasionally recovered, the percentage of S strain among *A. flavus* or *A. parasiticus* isolates was always very low for both orchards. For orchard A, the mean percentage of the S strain among *A. flavus* or *A. parasiticus* isolates over the duration of the study (2002 to 2006) was 0.3 and 1.4% for the treated and untreated areas, respectively. For orchard B, the mean percentage of S strain over the period of the study (2003 to 2007) was 0.1% for both the untreated and the treated areas.

After applying the AF36 product in the research orchards, the frequency of VCG YV36 within the *A. flavus* soil population increased substantially in both orchard A and orchard B (Fig. 1). Prior to applications, YV36 was detected in both orchards at very low frequencies (0.9 and 2.5% of the *A. flavus* isolates for orchards A and B, respectively). In orchard A, a single application in July 2002 resulted in percentages of YV36 over 90% in the soil for 2002 through 2004 and levels over 60% in 2005; even in 2006, 4 years after the only application, the frequency of YV36 remained above the frequency prior to application at 8% (Fig. 1A). Compared with orchard A, in orchard B, applications did not have as large an effect on frequencies of YV36 in soil (Fig. 1). Only after two applications (either 2003 and 2004 or 2004 and 2005) were the percentages of YV36 above 70% (Fig. 1B). Furthermore, the percentage of YV36 among *A. flavus* isolates decreased the second year after the last application (for example, in 2006, the frequency of YV36 among *A. flavus* dropped to 48% for the areas treated in 2003 and 2004). Also, frequencies of VCG YV36 increased consistently over the winter and spring in orchard B (Fig. 1B). For example, in areas first treated on 6 July 2004, the percentage of *A. flavus* composed of YV36 increased from 27.0 to 55.3% between early September 2004 and late June 2005.

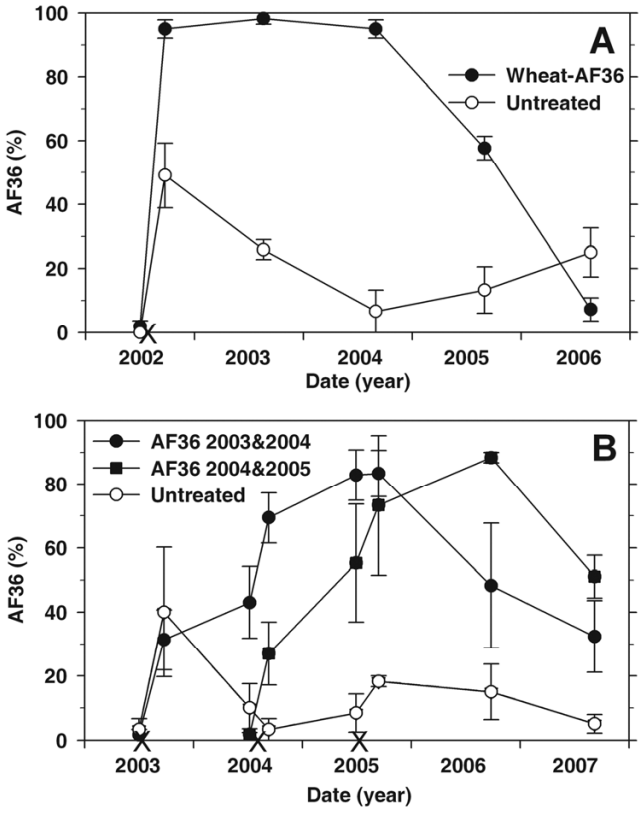

Fig. 1. Percentage of *Aspergillus flavus* isolates belonging to the atoxigenic strain *Aspergillus flavus* AF36 for isolates from soil collected from the areas treated with the AF36 product or from untreated areas in research orchards. Values are means of three replications ± standard errors. Dates when the AF36 product was applied are marked with an X. **A,** Orchard A. The AF36 product was applied only once on 1 July 2002. **B,** Orchard B. For one treatment, the AF36 product was applied on 1 July 2003 and 6 July 2004 whereas, for the other treatment, the AF36 product was applied on 6 July 2004 and 29 June 2005.

Soil: commercial orchards. In all commercial orchards, the *A. flavus* L strain was substantially more common than either the *A. flavus* S strain or *A. parasiticus* (Table 2). After 4 years of applying the AF36 product, significantly more of the *A. flavus* or *A. parasiticus* isolates were *A. flavus* L strain in treated orchards (99.3%) than in untreated orchards (83.4%). After application, the S strain typically occurred at lower frequencies in treated orchards than in untreated orchards, though differences were not statistically significant for any of the sampling dates (Table 2). A statistically significant smaller percentage of the *A. flavus* or *A. parasiticus* isolates were *A. parasiticus* in treated orchards than in untreated only in 2010 (0.5 and 3.3% for the treated and untreated orchards, respectively) and 2011 (0.3 and 11.0%) (Table 2).

The frequency of VCG YV36 in the soil of the commercial orchards increased substantially after application (Fig. 2). Prior to

Table 2. Percentage of isolates that belong to *Aspergillus flavus* (strain L or strain S) or *A. parasiticus* for isolates obtained from soil from commercial orchards treated with the AF36 product and from adjacent untreated orchards in 2008 to 2011

	Isolates that belong to specified fungus (%)[y]		
Sample date, treatment	***A. flavus* L**	***A. flavus* S**	***A. parasiticus***
June 2008[z]			
AF36	92.5 ns	2.3 ns	4.2 ns
Untreated	96.1	1.7	1.1
September 2008			
AF36	94.5 ns	0.6 ns	4.0 ns
Untreated	96.6	0.5	2.4
June 2009[z]			
AF36	93.8 ns	0.6 ns	5.0 ns
Untreated	85.7	1.2	11.8
September 2009			
AF36	99.6 ns	0.0 ns	0.4 ns
Untreated	97.7	0.7	1.3
September 2010			
AF36	98.9 ns	0.1 ns	0.5 a
Untreated	95.4	0.5	3.3 b
September 2011			
AF36	99.3 a	0.2 ns	0.3 a
Untreated	83.4 b	4.1	11.0 b

[y] Statistical analyses were performed on arcsine-transformed data. Values presented were transformed back from the means of the arsine-transformed data. Numbers followed by the same letter are not significantly different ($P = 0.05$) by pairwise comparison using the least significant difference test; ns = values for treated and untreated are not significantly different ($P > 0.05$).

[z] These samples were collected prior to applying the AF36 product for that year.

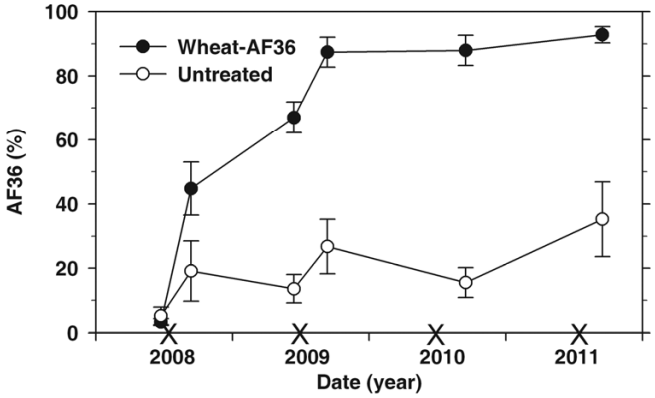

Fig. 2. Percentage of *Aspergillus flavus* isolates from soil belonging to the atoxigenic strain AF36 for isolates collected from commercial orchards treated with the AF36 product or from untreated orchards. Dates when the AF36 product was applied are marked with an X. Values are means of seven orchards ± standard errors.

application, only 3.3 and 5.1% of the *A. flavus* isolates from the soil belonged to the VCG YV36 for orchards to be treated and left untreated, respectively. Three months after application, 45% of the *A. flavus* isolates from soil in treated orchards belonged to VCG YV36. The YV36 increased through winter and spring and continued to increase following subsequent applications of the AF36 product, until over 90% of the isolates belonged to VCG YV36 (Fig. 2). For the seven treated orchards, the mean percentage of the *A. flavus* isolates in YV36 strain for individual orchards was 61.0 to 82.7% (averaged over the sampling dates of September 2008 to September 2011). Even though the soil samples in untreated orchards were collected at least 240 m from the adjacent treated orchards, the percentage of VCG YV36 in the untreated orchards also increased from 5% prior to any application to 35% in September 2011 (Fig. 2).

Nuts: research orchards. The density of *A. flavus* or *A. parasiticus* on the hull surface of mature nuts in orchard B did not differ significantly between treatments for any of the years 2004 to 2007 (results not presented in a table because there were no statistically significant differences). For all treatments and years, the density of *A. flavus* or *A. parasiticus* on the hull surface was very low. For the years 2004 to 2007, densities of *A. flavus* or *A. parasiticus* were 0.16 to 0.57, 0.07 to 1.94, and 0.03 to 0.29 CFU/nut for the first AF36 treatment (applied in 2003 and 2004), second AF36 treatment (applied in 2004 and 2005), and untreated control, respectively. Comparisons were made with *Aspergillus* sect. *Nigri*, because these fungi were not applied, are widespread naturally in pistachio orchards, and are related to *A. flavus*. For every year, densities of *Aspergillus* sect. *Nigri* on hulls were always substantially greater than that of *A. flavus* or *A. parasiticus*. For example, in 2005, the density of *Aspergillus* sect. *Nigri* was12.5 to 28.5 CFU/nut (depending on treatment), while *A. flavus* or *A. parasiticus* was 0.1 to 0.8 CFU/nut. Mean densities of *Aspergillus* sect. *Nigri* on hulls over the 4 years was 78.1, 61.0, and 56.0 for the first AF36 product treatment (2003 and 2004), the second AF36 treatment (2004 and 2005), and the untreated control, respectively.

Early split nuts from the areas treated with the AF36 product in orchard B never had significantly more kernel decay by *A. flavus* or *A. parasiticus* than those from untreated areas (results not presented in a table because there were no significant differences). No kernels were observed with *A. flavus* or *A. parasiticus* for any of the samples except for kernels from the areas receiving treatments in 2003 and 2004, with 1 of 210 kernels colonized by *A. flavus* in 2003 and 1 of 1,289 kernels colonized by *A. flavus* in 2007. Across all years, substantially more early split nuts were decayed by fungi in *Aspergillus* sect. *Nigri* than by *A. flavus* or *A. parasiticus*. For example, in 2007, incidences of decay by *A. flavus* or *A. parasiticus* were 0.00 to 0.02% whereas incidences of decay by *Aspergillus* sect. *Nigri* were 2.2 to 4.4%.

Nuts: commercial orchards. Densities of *A. flavus* or *A. parasiticus* on hull surfaces of mature nuts did not differ between treated and untreated commercial orchards for any year (Table 3). Densities of *Aspergillus* sect. *Nigri* on nut hulls were always substantially higher than the densities of *A. flavus* or *A. parasiticus* for both treated and untreated orchards. For example, densities of *A. flavus* or *A. parasiticus* on hull surfaces in treated orchards were 0.5 to 6.1 CFU/nut for the different years, whereas densities of *Aspergillus* sect. *Nigri* were 17.7 to 35.0 CFU/nut (Table 3).

Applying the AF36 product in the commercial orchards did not result in an increase in kernel decay. The incidence of kernel decay of early split nuts by *A. flavus* or *A. parasiticus* did not differ significantly between treated and untreated orchards for any year (Table 4). Furthermore, in treated orchards, the incidences of kernel decay by *Aspergillus* sect. *Nigri* were always substantially higher than for decay by *A. flavus* or *A. parasiticus* (Table 4). For 2008 to 2011 combined, significantly more *A. flavus* isolates from nut hulls from treated orchards (49.5%) belonged to VCG YV36 than for isolates from the untreated orchards (22.6%) (P value = 0.000; one-sided Fisher's exact test).

Aflatoxin analyses. Nuts from orchards treated with the AF36 product were less likely to contain detectable aflatoxin concentrations than those from untreated orchards for each harvest from 2008 through 2011 (Table 5). Percent reductions in numbers of nut samples with detectable aflatoxin contamination was 20.4% to 44.9%, depending on the year (Table 5). For 2008 to 2011 combined, significantly fewer nut samples from treated orchards (6.9%) were contaminated with aflatoxins than for nuts from the untreated orchards (11.5%) (P value = 0.003; one-sided Fisher's exact test). Sometimes a second harvest ("reshakes") is done a few weeks after the first harvest. For these second harvests, the percent reduction in numbers of samples with detectable aflatoxin contamination in treated orchards relative to untreated orchards was 23.6, 85.4, and 58.1% for 2009, 2010, and 2011, respectively (there were too few second-harvest samples in 2008 for a useful comparison). For 2009 to 2011 combined, second-harvest nuts from treated orchards were less frequently contaminated with aflatoxins (9.1% were contaminated) than second-harvest nuts from untreated orchards (20.0% were contaminated; 54.6% reduction; P value = 0.011; one-sided Fisher's exact test). For the five individual ranches (with treated and adjacent untreated orchards) with more than 100 samples, four had a lower percentage of contaminated samples in the treated orchard than in the adjacent untreated orchard (percent reduction of 21.6 to 78.7%, with mean reduction of 57.0%), while the fifth ranch had approximately the same percentage of contamination in the treated (3.3% of samples contaminated) as in the untreated (3.1%). Nuts from orchards treated with

Table 3. Density of *Aspergillus flavus* or *A. parasiticus* and *Aspergillus* sect. *Nigri* on hull surfaces of freshly harvested pistachio nuts from commercial orchards treated with the AF36 product for consecutive years and from adjacent untreated orchards[y]

		Density (CFU/nut) of each fungus[z]	
Sample date	**Treatment**	***A. flavus*/*A. parasiticus***	***A.* sect. *Nigri***
September 2008	AF36	6.1 ns	27.3 ns
	Untreated	9.1	32.5
September 2009	AF36	3.1 ns	30.9 ns
	Untreated	2.0	119.0
September 2010	AF36	0.5 ns	35.0 a
	Untreated	0.2	102.0 b
September 2011	AF36	0.9 ns	17.7 ns
	Untreated	0.4	20.3

[y] Samples were collected from three pairs of orchards in 2008 to 2010 but four pairs of orchards in 2011.

[z] Statistical analyses were performed on log-transformed data. Values presented were transformed back from the means of the log-transformed data. Numbers followed by the same letter are not significantly different (P = 0.05) by pairwise comparison using the least significant difference test; ns = values for treated and untreated are not significantly different (P > 0.05).

Table 4. Incidences of kernels decayed by *Aspergillus flavus* or *A. parasiticus* and *Aspergillus* sect. *Nigri* for early split nuts collected from commercial orchards treated with the AF36 product for consecutive years and from adjacent untreated orchards

		Kernels colonized by specified fungus (%)[z]	
Year	**Treatment**	***A. flavus*/*A. parasiticus***	***A.* sect. *Nigri***
2008	AF36	0.09 ns	6.39 ns
	Untreated	0.16	9.85
2009	AF36	0.10 ns	5.80 ns
	Untreated	0.03	5.95
2010	AF36	0.06 ns	3.01 ns
	Untreated	0.01	3.17
2011	AF36	0.09 ns	2.00 ns
	Untreated	0.05	2.21

[z] Statistical analyses were performed on arcsine-transformed data. Values presented were transformed back from the means of the arsine-transformed data. Numbers followed by the same letter are not significantly different (P = 0.05) by pairwise comparison using the least significant difference test; ns = values for treated and untreated are not significantly different (P > 0.05).

the AF36 product were less likely to contain total aflatoxin concentrations greater than 15 parts per billion (ppb) than those from untreated orchards for each harvest from 2009 through 2011 but not in the first year of the application in 2008 (Table 6).

Discussion

The atoxigenic *A. flavus* VCG YV36, the VCG to which isolate AF36 belongs, was relatively common and widespread in California prior to the application of the biocontrol product containing AF36 as the active ingredient. VCG YV36 occurred naturally in commercial pistachio orchards in all the major pistachio-growing counties of California (Table 1). This widespread natural occurrence of the AF36 strain suggests that this biocontrol agent is well adapted for developing and surviving in California pistachio orchards. The relatively high frequency at which VCG YV36 naturally occurs in cotton fields in Arizona was one of the reasons that isolate AF36 was originally selected for use as a biocontrol agent (17). VCG YV36 also naturally occurred at a relatively high frequency in California pistachio orchards, with 7.4% of the naturally occurring *A. flavus* isolates belonging to VCG YV36 (Table 1). Compared with 15 other atoxigenic *A. flavus* VCGs, the YV36 strain was the most common *A. flavus* VCG resident in pistachio and fig orchards in California (31,32). Frequencies of strain YV36 in Arizona cotton fields prior to biocontrol applications have been reported as between 1 and 9% of the *A. flavus* isolates (6,15,22), which is roughly similar to the frequencies observed in California pistachio orchards (Table 1). Furthermore, incidences of VCG YV36 within *A. flavus* populations in soils were 0.2 to 7.1% in Texas prior to treatment with the AF36 product (20) and averaged approximately 1% in northern Mexico (40). Members of the same *A. flavus* VCG share a common gene pool distinct from that of other VCGs and with linkage equilibrium across vast distances (35). Therefore, the natural occurrence of VCG YV36 in California indicates that applications of the AF36 product do not carry risks associated with introducing a new microorganism into the California environment.

Applications of the AF36 product in pistachio orchards successfully increased the proportion of VCG YV36 within the *A. flavus* population in the soil (Figs. 1 and 2). For comparison, the AF36 product applied in a cotton field in Arizona resulted in increasing incidences of YV36 in the *A. flavus* soil population, from 0% before application to 69% at the harvest (14). In most cases, it is difficult to precisely compare our results with those for the AF36 product or other atoxigenic-strain products in other crops, because different types of data were gathered and reported. In many cases, the fungal levels on the crop after application of the AF36 product were reported instead of in the soil. For example, 63% of the *A. flavus* population on the crop was the AF36 strain for 71 cotton fields treated with the AF36 product in 2001 (5). The results from applying the AF36 product in Arizona cotton fields over the years have been summarized as increasing the AF36 strain in the soil and on the crop to around 80% (19). These results suggest that applying the AF36 product in cotton fields was more effective in increasing the AF36 strain than applying the product in commercial pistachio orchards, where 45% of the *A. flavus* population belonged to the AF36 strain after a single application (Fig. 2). However, the proportion of the *A. flavus* population belonging to the AF36 strain continued to increase in treated pistachio orchards over the following winter and spring (Fig. 2). This suggests that competitive exclusion by the applied AF36 continued through periods not typically considered when designing aflatoxin management programs and may provide insights on the use of competitive microbes throughout crop cycles, not only for aflatoxin reductions but also for potential management of other mycotoxins and even non-mycotoxin plant diseases. Furthermore, pistachio is a perennial tree crop and differences in the speed of displacement between pistachio and annual crops may result from the need to modify *A. flavus* populations associated with established, overwintering crops.

In some studies of biocontrol of aflatoxins with atoxigenic strains, instead of the applied atoxigenic strain, the toxin production of *A. flavus* isolates was reported in treated areas. For example, peanut fields treated with the commercial product afla-guard (consisting of barley coated with conidia of the atoxigenic *A. flavus* strain NRRL 21882) reduced the aflatoxin-producing *A. flavus* in the soil from 71% of the *A. flavus* population to only 4% (26). Also, application in corn fields of another atoxigenic *A. flavus*

Table 5. Percentage of pistachio nut library samples contaminated with aflatoxin from commercial orchards treated with the AF36 product and from adjacent untreated orchards in 2008 to 2011

Year	Treatment	Samples (*n*)	Contaminated with aflatoxin (%)	Reduction of contaminated samples (%)	*P* value[z]
2008	AF36 product	38	29.0	20.4	0.3399
	Untreated	33	36.3	…	…
2009	AF36 product	163	11.0	38.6	0.0390
	Untreated	228	18.0	…	…
2010	AF36 product	245	2.9	44.9	0.1500
	Untreated	212	5.2	…	…
2011	AF36 product	162	3.7	36.7	0.2563
	Untreated	171	5.9	…	…
2008–2011	AF36 product	608	6.9	39.9	0.0033
	Untreated	644	11.5	…	…

[z] One-sided Fisher's exact test for two-by-two contingency table.

Table 6. Percentage of pistachio nut library samples that were contaminated with greater than 15 parts per billion (ppb) total aflatoxins for samples from commercial orchards treated with the AF36 product and from adjacent untreated orchards in 2008 to 2011[y]

Year	Treatment	Samples (*n*)	Contaminated with >15 ppb aflatoxin (%)	Reduction of samples with >15 ppb (%)	*P* value[z]
2008	AF36 product	38	15.8	–4.2	0.6539
	Untreated	33	15.2	…	…
2009	AF36 product	163	4.9	20.1	0.3868
	Untreated	228	6.1	…	…
2010	AF36 product	245	0.8	71.2	0.1002
	Untreated	212	2.8	…	…
2011	AF36 product	162	2.5	29.6	0.4092
	Untreated	171	3.5	…	…
2008–2011	AF36 product	608	3.3	31.7	0.1108
	Untreated	644	4.8	…	…

[y] The pistachio industry standard as defined by the Federal Marketing Order for Pistachios is a tolerance of 15 ppb total aflatoxins.
[z] One-sided Fisher's exact test for two-by-two contingency table.

strain, K49, as a wheat product substantially reduced the proportion of *A. flavus* isolates from soil that produced aflatoxins (1). For example, in areas where the atoxigenic strain K49 was applied, between 6 and 16% (depending on sampling date) of the *A. flavus* isolates produced aflatoxins compared with between 49 and 75% for the untreated areas (1). This type of comparison is less precise and complicates comparisons among studies, as does the use of different rates of the atoxigenic products. For example, afla-guard was typically applied at 22.5 kg/ha (26) and the atoxigenic K49-wheat product at 20 kg/ha (1) whereas, in our study and other studies with the AF36 product, the rate was 11 kg/ha (the label rate for the commercial AF36 product). In general, however, studies on atoxigenic strains have shown this strategy to have useful benefits for aflatoxin management.

Application of the AF36 product in commercial pistachio orchards successfully reduced aflatoxin contamination of pistachio nuts (Tables 5 and 6). Application of atoxigenic strains in other crops such as cotton, peanut, and corn also effectively reduced aflatoxin contamination. In commercial cotton fields, application of the AF36 product has consistently reduced aflatoxin contamination in cottonseed (3,5). As an example, 82% of the cotton fields treated with the AF36 product in 2001 had cottonseed with aflatoxins at less than 20 ppb, whereas all the control seed lots were over 100 ppb (3). In commercial peanut fields, application of the atoxigenic *A. flavus* NRRL 21882 (afla-guard) reduced aflatoxin levels by 85% (26). In addition, an example with corn is that the application of the atoxigenic *A. flavus* strains CT3 and K49 in corn fields resulted in a reduction in aflatoxin levels of 60 to 86%, depending on year and strain (1). Also, with corn, applying a mixture of four atoxigenic *A. flavus* strains to corn fields in Nigeria resulted in aflatoxin reductions of 67 to 95% (7). For comparison, in commercial pistachio orchards treated with the AF36 product, the percent reduction in aflatoxin contamination was 20 to 45%, depending on year, with a reduction of 40% for all years combined (Table 5). In addition, the percent reduction in samples with aflatoxins at greater than 15 ppb (the pistachio industry standard as defined by the Federal Marketing Order for Pistachios) was 32% for all years combined (Table 6). In conclusion, the reduction in aflatoxin contamination associated with applying the AF36 product in pistachio orchards appeared to be significantly less than the reduction for the AF36 product and other atoxigenic *A. flavus* strains in other crops. Nonetheless, this level of reduction in aflatoxin contamination for pistachio observed in our study is valuable to the California pistachio industry. The AF36 treatment itself is inexpensive (17). In addition, the California pistachio industry benefits by producing a consistently high-quality product with low aflatoxin contamination (44). A thorough economic analysis of aflatoxin contamination of pistachio nuts has been done (34). Because the confidence of consumers and of buyers (those intending to further process the pistachio nuts) is so important for the marketing of pistachio nuts (34), the use of the AF36 product in pistachio orchards should be economically beneficial to the California pistachio industry.

Applying the AF36 product to the ground of pistachio orchards did not increase the density of *A. flavus* or *A. parasiticus* on the hull surface of mature nuts (Table 3) or the incidence of kernel decay during harvest (Table 4). In cotton fields, applying the atoxigenic strain AF36 did not increase the quantity of *A. flavus* on the harvested crop or the incidence of infected locules (14). Similarly, applying the atoxigenic *A. flavus* NRRL 21882 (afla-guard) in peanut fields did not increase the quantity of *A. flavus* or *A. parasiticus* in the peanut (24). Thus, in general, applying atoxigenic strains does not result in significantly increasing levels of crop decay. Decay data from the current study and the overall results from other crops should reassure pistachio growers that applying atoxigenic fungi to orchards will not increase the decay in the crop at harvest.

The proportion of VCG YV36 within *A. flavus* populations also increased in the untreated pistachio orchards that were adjacent to orchards treated with the AF36 product (Figs. 1 and 2), suggesting that the fungus had spread from the treated areas. Similarly, substantial increases in the proportion of AF36 among the *A. flavus* community have been observed in Arizona in untreated fields adjacent to cotton fields treated with the AF36 product (21). Furthermore, conidia of the applied AF36 strain were readily dispersed in the air into untreated fields near treated cotton fields (11). In addition, the conidia of atoxigenic *A. flavus* strains applied to the soil surface in peanut fields tended to remain close to the soil surface rather than move deep into the soil, which would facilitate aerial dispersal of the conidia (36). In addition to aerial dispersal, water moving in the furrows in peanut fields also transported conidia (36). However, the aflatoxin-producing strains would also be spread in similar ways as the atoxigenic strains, moving from untreated areas into treated areas. Therefore, atoxigenic products are preferably applied over large areas for maximum benefit (18). Furthermore, it is likely that our study underestimated the full effectiveness of applying the AF36 product in pistachio orchards because of the movement of *A. flavus* AF36 from treated into nearby untreated areas, thereby decreasing aflatoxin contamination in untreated control areas. Movement of AF36 between orchards also suggests that improvement in the effectiveness of AF36 in pistachio orchards might be achieved through applications to other tree crops either adjacent to or in the vicinity of pistachio orchards. This likely will provide at least some protection from aflatoxins to those crops as well.

The AF36 fungus persisted in the soil of pistachio orchards for years after the final application of the AF36 product (Fig. 1), suggesting that an application has multiple-year effects. A similar effect has been observed in Arizona cotton fields treated with AF36 product (16,17). For example, a cotton field that had 2% atoxigenic strain prior to treatment with AF36 product (without subsequent treatment) went to 96, 52, and 47% atoxigenic strain for 1, 2, and 3 years after treatment, respectively (16). However, greater reductions in aflatoxin resulted from repeated applications (18). Nonetheless, the AF36 fungus might persist well enough that, once sufficiently established, application does not need to be made every year.

Pistachio is different from crops such as cotton, peanut, and corn in that the part of the plant to be protected from aflatoxin contamination (that is, the nut on the tree) is more distant from the soil surface where the atoxigenic product is applied. One consequence of this might be that displacement of the aflatoxin producers in the soil might occur considerably before competition on the crop. In commercial pistachio orchards *A. flavus* and *A. parasiticus* commonly grow on pistachio litter on the orchard floor (28); thus, displacement of aflatoxin producers might initially be in this organic matter. In fact, because the incidence of kernel decay by aflatoxin-producing fungi is so low in pistachio orchards (41), probably little or no displacement of aflatoxin-producing fungi is occurring directly on or in the pistachio nuts. A second consequence of preharvest nut distance from the soil is that the fungi infecting the nuts likely do not come primarily from directly below the tree but, rather, disperse across a distance while rising to the required height. This phenomenon is likely of less importance on row crops with fruit relatively close to the soil surface such as cotton or peanut and a reason pistachio has a need for larger areas to be treated in order to minimize movement of aflatoxin producers onto crops from untreated areas.

Individual pistachio orchards differed greatly, with the AF36 product being more effective in some orchards compared with others. For example, the AF36 product was more effective in changing the soil fungal population in orchard A than in orchard B (Fig. 1). These differences among orchards might be due to several factors. In order for the AF36 product to produce conidia, the wheat-based product must rehydrate to sufficient moisture to allow fungal growth and sporulation (10). Therefore, anything affecting moisture and humidity could influence the effectiveness of AF36 product for producing spores. In Arizona cotton fields, the recommendation is to apply the AF36 product near canopy closure followed by an irrigation, resulting in favorable moisture and humidity for growth and sporulation of the fungus on the wheat seed

(18,37). In contrast, pistachio orchards do not have a canopy that traps humidity and moisture the way the cotton canopy closure maintains a relatively high humidity. Furthermore, the moisture and humidity in different pistachio orchards probably varies depending on cultural practices such as irrigation and cover crops. Soil type might also influence AF36 effectiveness. For example, the AF36 product may be less effective in light, sandy soils, probably due to the reduced moisture levels (4).

Although the atoxigenic AF36 product has been effective in reducing aflatoxin contamination, future research in several areas might improve the effectiveness in pistachio orchards in California. For example, the optimal time for applications in pistachio orchards has not been determined. In cotton fields, earlier application of the AF36 product resulted in better displacement of aflatoxin-producing fungi (37). Earlier applications may also improve the effectiveness in pistachio orchards. Optimal irrigation practices to maximize the sporulation on the AF36 product also need to be determined. Results from the current study supported the biopesticide registration of the AF36 product with the U.S. EPA for pistachio in California, Arizona, Texas, and New Mexico (Arizona Cotton Research and Protection Council; *Aspergillus flavus* AF36, biopesticide label granted 29 February 2012; EPA Reg. No. 71693-1). The AF36 product likely will also be useful for reducing aflatoxins in almond and fig orchards in California (31). Once biopesticide registrations for these crops are achieved, true area-wide management of aflatoxins in California tree crops can be realized, with the potential for greatly reducing the aflatoxin-producing potential of *A. flavus* populations throughout the vast regions within which almond and pistachio are commercially cultivated in California.

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