Biocontrol of Aflatoxin Contamination and Selection of Atoxigenic Strains in California Almond Orchards

Project No.: **14-AFLA1-Michailides**

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Objectives:

Objectives 1 and 2 support the registration of the AF36 product for application in commercial almond orchards.

1. Conclude the study investigating the displacement of aflatoxin-producing fungi by the previously applied atoxigenic *A. flavus* strain AF36 in a research almond orchard.
2. Evaluate new formulations of the AF36 product involving sorghum and seed coating for application in almond orchards.
3. Evaluate the performance of predominant atoxigenic isolates collected from almond soil orchards in displacing toxigenic *Aspergillus* spp.

Interpretive Summary:

Biocontrol of *Aspergillus flavus* and reduction of aflatoxin contamination. The use of the atoxigenic *Aspergillus flavus* strain AF36 (a strain not able to produce aflatoxins) as a biopesticide has been successful in reducing aflatoxin contamination of pistachio nuts in commercial pistachio orchards in California in addition to reducing contamination of corn and cottonseed. After the AF36 product was registered by the US Environmental Protection Agency for use in pistachio orchards in 2012, about 75,000 acres of pistachio orchards were treated in California. Because application of this strain in pistachio and fig orchards in California had given promising results, we initiated a project with almonds in 2007 investigating the use of AF36 to reduce aflatoxin contamination in almond orchards. In the early summer of 2007, 2008, 2011, and 2012, an application of wheat infected with AF36 (the same commercial product used in pistachio orchards and in corn and cotton fields) was made to the ground in an almond orchard at the Nickels Soil Laboratory in Arbuckle, CA. No AF36 product was applied in 2009, 2010, 2013, or 2014, although nut and soil samples continued to be collected to determine the survival and spread of the AF36 fungus.

Applying the wheat-AF36 product in this almond orchard successfully reduced the percentage of aflatoxin-producing strains within the fungal population. Before applying the AF36 product, 92% of the isolates in the soil belonged to *A. parasiticus* or *A. flavus* S strain, both of which consistently produce high levels of aflatoxins. However, after applying the AF36 product, the frequency of *A. parasiticus* and *A. flavus* S strain in the fungal population decreased substantially until in 2009 only 2% of the isolates were *A. parasiticus* or *A. flavus* strain S. After not applying the AF36 product in 2009 and 2010, these aflatoxin-producing fungi increased to 46% of the isolates. However, after applying the AF36 product in 2011 and 2012, only 2% of the isolates were *A. parasiticus* or *A. flavus* S strain. These results demonstrate the effectiveness of applying the AF36 product in decreasing the frequency of these aflatoxin-producing fungi within the population of the *A. flavus* group in the almond orchard.

Although very little of the atoxigenic strain AF36 was present in the orchard soil before applying the wheat-AF36 product in June 2007 (only 2.5% *A. flavus* naturally belonged to AF36 in this orchard), after the application almost all of the *A. flavus* isolates evaluated were AF36. The results from applying the AF36 product in 2007/2008 and again in 2011/2012 demonstrated the AF36 product was very effective in increasing the amount of the atoxigenic strain AF36 under the conditions present in this almond orchard. The level of the AF36 fungus remained high in 2009 and 2013 even though no AF36 product was applied in these years, indicating that the effect of application lasts more than a year and perhaps the AF36 product does not need to be applied every year.

Applying AF36 did not significantly increase the incidence of hull decay of the nuts. The percentage of hulls decayed by *A. flavus* in treated areas was never significantly different from the decay in nuts from the untreated areas. And for comparison, the incidence of hull decay caused by *A. niger* was substantially higher than that by *A. flavus*.

The results so far demonstrate that applying the commercial AF36 product in an almond orchard in a manner similar to that done in commercial pistachio orchards is effective in increasing the frequency of this atoxigenic strain AF36 in the almond orchard. These results represent the completion of this study. In addition, these research results will be used to support the registration of the AF36 product for application in commercial almond orchards.

The study on the population of *Aspergillus* fungi in almond orchards have been completed and published in Plant Disease international journal of the American Phytopathological Society Please check publication by Donner et al., 2015.

Because approval for the new formulation of AF36 was delayed, the experiments to evaluate the new formulations of the AF36 product involving sorghum and seed coating for application in almond orchards has been postponed and is being conducted this summer. In the meantime, for the registration of AF36 on almonds, EPA representatives who are involved in reviewing the submitted file requested additional studies, which were performed in the spring and summer 2015. Results have been reported to the EPA during a conference call and it is expected that no additional questions will be raised and the registration of AF36 for almonds will be straightforward.

Materials and Methods:

1. Conclude the study investigating the displacement of aflatoxin-producing fungi by the previously applied atoxigenic *A. flavus* strain AF36 in a research almond orchard.

The atoxigenic *A. flavus* strain AF36 was applied as wheat seed that had been colonized by the fungus. This is the same commercial product registered for application in pistachio orchards and in corn and cotton fields. The wheat-AF36 product was applied to the soil surface at a rate of 10 pounds per acre (same application rate as used for other crops) in a research almond (cv. Nonpareil) orchard at the Nickels Soil Laboratory on 28 June 2007, 2 July 2008, 3 June 2011, and 26 June 2012 (the AF36 product was applied to the same areas each year). No AF36 product was applied in 2009, 2010, 2013, or 2014. The experimental design was a randomized complete block design with 3 replications. Soil samples were collected just before the application of the wheat-AF36 product. Nuts and additional soil samples were collected during the period of commercial harvest on 9 August 2007, 2 September 2008, 1 September 2009, 2 September 2010, 2 September 2011, 21 August 2012, 23 August 2013, and 13 August 2014. To quantify the density of *A. flavus* and *A. parasiticus* in the soil and to obtain isolates for strain determination, between 0.02 g and 0.20 g of soil was sprinkled on the surface of a selective isolation medium (containing chloramphenicol and dichloran) of each of 10 petri dishes and incubated at 30 °C for 7 days. To quantify *A. niger* (including closely related fungi in the *A. niger* group), 1.0 or 2.0 g soil was added to 100 ml of sterile deionized water in sterile plastic bottles. After the bottles with the soil solution were shaken for 15 min on a mechanical shaker, 100 µl of the soil solution was spread evenly on 10 plates of dichloran chloramphenicol peptone agar, and the plates incubated at 30 °C for 5 to 7 days. The hulls and the external surface of shells of nuts collected at harvest time (9,000 nuts per treatment for each year 2007-2012 and 4,500 nuts per treatment in 2013 and 2014) were evaluated for visible decay by *A. flavus* and *A. parasiticus*. Any fungal colonies observed decaying the hulls and shells that possibly could belong to *Aspergillus* (except *A. niger*) were isolated into pure culture in order to identify the species. Isolates of *A. flavus* obtained from soils and nuts were tested to see if they belonged to the atoxigenic strain AF36 using a vegetative compatibility group method.

In order to evaluate new formulations of the AF36 product involving sorghum and seed coating for application in almond orchards we had to wait until the EPA approval. The approval was granted in late June, new formulations were obtained from the Arizona Cotton Council of Research, and the studies were initiated. Because during conference calls questions were raised about the involvement of bees in spreading *Aspergillus* fungi among amond flowers, and the presence of *Aspergillus* on immature developing fruit, a study was initiated to answer these questions and satisfy data to EPA to support the AF36 registration in almond.

2. Evaluate new formulations of the AF36 product involving sorghum and seed coating for application in almond orchards.

Because of an unreasonable delay in the approval of new formulations of AF36 by EPA, this objective has just been started. In the meantime, experiments were done to answer questions raised by EPA representatives who are involved in the review of the AF36 registration for almonds.

Blossoms. Four trees per cultivar were randomly selected and 30 blossoms per tree were collected. Collection was conducted twice (18 February and 2 March 2015). Immediately after collection, blossoms were placed in sterile plastic screens inside sterile plastic containers. Sterile water was added to containers to maintain high humidity. Plastic containers were incubated at 30°C for 7 days in the dark. Macroscopic and microscopic examination of the developing fungi was conducted until each fungal isolate was assigned to its corresponding species.

Honey bees. Over a hundred honey bees pollinating almond blossoms of the almond research orchard at UC Kearney Agric. Research & Extension Center were collected individually using plastic bags on 6 and 7 March 2015 and plated in modified rose Bengal culture media (Cotty, 1994). This medium is selective for growth of *Aspergillus* section *Flavi*. Plated honey bees were incubated for 5 days at 30°C. Macroscopic and microscopic examination of the developing fungi was conducted until each fungal isolate was assigned to its corresponding species. Recovered *A. flavus* isolates were subjected to vegetative compatibility analyses to determine if they belong to the same genetic group as AF36.

Developing almond fruits. Twenty developing almonds of four trees of each of the four cultivars (Nonpareil, Carmel, Butte, and Padre) were randomly selected every 15 days starting on 1 April 2015 and weekly collections started after 15 July. Immediately after collection, five developing almonds from each of the sampled trees were washed in 100 ml of sterile distilled water in 250 ml Erlenmeyer flasks by agitation for 10 minutes. From each wash, 100 µl were combined with 9.9 ml of sterile water, vortexed and 100 µl aliquots were plated in acidified potato dextrose agar plates. Inoculated plates were incubated at room temperature for 7 days. Macroscopic and microscopic examination of the developing fungi was conducted until each fungal isolate was assigned to its corresponding species. Recovered *A. flavus* isolates were subjected to vegetative compatibility analyses to determine if they belong to the same genetic group as AF36.

3. Evaluate the performance of predominant atoxigenic isolates collected from almond soil orchards in displacing toxigenic *Aspergillus* spp.

These experiments are in progress and we have no information to report at this time. An additional experiment under this objective will be to check almonds of various cultivars for infection by *Aspergillus flavus* and/or *A. parasiticus* and the production or inhibition of production of aflatoxins in the inoculated nuts of the various cultivars.

Results and Discussion:

Applying the wheat-AF36 product was very effective in increasing the frequency of the atoxigenic strain AF36 within the fungal population under the conditions present in this almond orchard. Although the frequency of the atoxigenic strain AF36 was very low in the soil before applying the AF36 product in 2007, after the applications in 2007 and 2008 almost all of the *A. flavus* isolates were AF36 (**Figure 1**). The frequency of AF36 remained high in the soil in treated areas from August 2007 to July 2008, which is evidence that the AF36 fungus survived the winter and spring well. In September 2009 (approximately 14 months since the last application) the level of AF36 in soil remained high in treated areas, indicating that the effect of application lasts more than a year and perhaps the AF36 product does not need to be applied

every year. However, by September 2010 the level of AF36 decreased substantially (**Figure 1**), suggesting that an additional application of the AF36 product would be needed. After additional applications of AF36 were made in this orchard in 2011 and 2012, the frequency of AF36 increased substantially to 73% in 2012 and 76% in 2013 (**Figure 1**).

In the untreated areas (control), the level of AF36 was frequently high even though the AF36 product was never applied in those areas (**Figure 1**). For all of the sampling dates after the start of the study, the percentage of AF36 in the untreated areas was higher than the 5% at the beginning of the study (**Figure 1**). Furthermore, sometimes the percentage of AF36 in the untreated areas was high, such as 74% in September 2008 and 61% in September 2013 (**Figure 1**). These results suggest that the atoxigenic fungus AF36 readily moves from treated areas to the untreated areas, thereby providing benefits to the untreated areas.

The effect of application of the wheat-AF36 product on the density of *A. flavus*/*A. parasiticus* in the soil varied through the years (**Figure 2**). Applications in 2007 and 2008 resulted in a high density of *A. flavus*/*A. parasiticus*, which was followed by moderate decreases in 2009 and again in 2010 (when no additional AF36 was applied) (**Figure 2**). In 2011 and 2012 the density remained low even though the AF36 product had been applied both years (**Figure 2**). Similarly, the density remained low in 2013, as would be expected because no AF36 product was applied in 2013. For comparison, the density of *A. niger* in the soil during the period of the study was typically higher than that of *A. flavus*/*A. parasiticus*. The density of *A. niger* ranged from 27 to 499 propagules / g soil (depending on the sample date) with mean values of 173 and 110 propagules / g soil for the AF36-treated and untreated areas, respectively.

Before the first application of the wheat-AF36 product in 2007, 92% of the isolates in the soil belonged to *A. parasiticus* or *A. flavus* S strain (**Figure 3**), both of which consistently produce high levels of aflatoxins. However, after applying the AF36 product, the percentage of *A. flavus*/*A. parasiticus* isolates belonging to the aflatoxin-producing *A. parasiticus* or *A. flavus* S strain decreased substantially until almost none of the isolates in the treated areas were *A. parasiticus* or *A. flavus* S strain (**Figure 3**). These results provide evidence of the potential for the atoxigenic strain AF36 to displace aflatoxin-producing fungi. Even in September 2010 (which was 26 months after the last application of the AF36) the percentage of isolates belonging to *A. parasiticus* or *A. flavus* S strain remained very low (**Figure 3**), suggesting that the AF36 product might not need to be applied every year. However, after not applying the AF36 product in 2009 and 2010, eventually these aflatoxin-producing fungi increased again to 46% of the isolates in 2011 (**Figure 3**). After applying the AF36 product again in 2012, only 2% of the isolates were *A. parasiticus* or *A. flavus* S strain (**Figure 3**). However, in 2013 when no AF36 product was applied, the level of these aflatoxin-producing fungi started to increase again reaching 16% (**Figure 3**). These results demonstrate the effectiveness of applying the AF36 product in decreasing the frequency of these aflatoxin-producing fungi within the population of the *A. flavus* group in the almond orchard.

Applying the AF36 product did not significantly increase (P=0.05) the incidence of hull decay by *A. flavus* for nuts from the treated areas (**Table 1**), indicating that applying the fungus AF36 to the orchard floor did not result in increased fungal decay of the crop. Furthermore, for all years, substantially more nuts were decayed by fungi of the *A. niger* group than by those of the *A. flavus* group even in the areas treated with AF36 (**Table 1**). For example, 2.75% of the nuts

from treated areas had hulls decayed by the *A. niger* group in 2014 compared to only 0.07% decayed by the *A. flavus* group (**Table 1**). These results should reassure growers that applying the AF36 product will not impact negatively the almond crop.

In general, the favorable results from this study support the use of the AF36 product in almond orchards. Application of the AF36 product was effective in increasing the biocontrol agent AF36 in the almond orchard without increasing any fungal decay on the nuts. This study investigating the application of AF36 in a research almond orchard is now completed. The results from this study should help in obtaining the registration of the AF36 product for use in commercial almond orchards in California.

The results analysis of fungal communities associated with almond blossoms, honey bees, and developing almonds throughout the season are given below separately for each of item:

Blossoms. Results of the two tests were similar and were combined for analyses. The recovered fungi were classified based on macroscopical and microscopical morphology with *Alternaria* spp. being the most commonly identified group (82% of blossoms), followed by *Fusarium* spp. (2%), *A. niger* (1.5%), and *Penicillium* spp. (0.2%); 16% of blossoms did not yield any fungus. Padre was the least susceptible ($P < 0.05$) cultivar to *Alternaria* spp. while Butte was the most susceptible ($P < 0.05$) to *A. niger*. Neither *Fusarium* spp. nor *Penicillium* spp. incidences differed ($P > 0.05$) among cultivars. Noteworthy, none of the examined blossoms harbored *A. flavus*, regardless of (i) high *A. flavus* densities existent on the examined plot stemming from deliberate dispersal of toxigenic and atoxigenic isolates during the summer of 2014 and previous years and (ii) natural frequencies of *A. flavus* throughout the environment. *Aspergillus flavus* is a pathogen of plants, animals, and insects. Relevant to insects, there were reports on diseases of bees by *Aspergillus* spp. including *A. flavus.* Since no *Aspergillus flavus/A. parasiticus* were detected in almond blossoms, there is no concern that bees will acquire any *Aspergillus flavus* and/or *A. parasiticus* from the almond blossoms as they visit flower to flower to collect nectar and pollinate the blossoms.

Honey bees. Examining the fungal microflora found on bees collected from almonds at full bloom in 2015, we determined the followings fungal taxa: the recovered fungi were classified based on macroscopical and microscopical morphology. *Alternaria* spp. was the most commonly identified group (22.6%), followed by *Rhizopus* spp. (22.2%), *A. niger* (20.2%), *Fusarium* spp. (19.1%), *A. flavus* (6.4%), *Penicillium* spp. (5.3%); *A. fumigatus* (2.1%), *Trichoderma* spp. (1.1%) and *Botrytis* spp. (1.0%). All of the *A. flavus* isolates were subjected to vegetative compatibility analyses and none of them belong to the same VCG as AF36. The presence of *A. flavus* on bees, even in very low levels, is an indication that the bees acquire these fungi from other sources where contamination by *A. flavus* is common.

Developing almond fruits. Among the mycoflora of developing almond fruit, over 3,500 isolates have been collected and identified and only five isolates belonged to *A. flavus.* Other genera found include *Fusarium, Alternaria, Penicillium, Botrytis, Cladosporium, Aureobasidium, Rhizopus, Acremonium, Coniothyrium, A. niger* and *A. fumigatus.* Examination of fungal communities associated with the developing almond fruits is still being conducted. Vegetative compatibility analyses with the recovered isolates will be conducted once almond washings are finished. The goal of this experiment is to determine whether AF36 propagules reach the right

sites where aflatoxin contamination may occur if a toxigenic *A. flavus/A. parasiticus* invaded the nuts.

Although a diverse fungal community was associated with almond blossoms, honey bees, and developing almond fruits, particular importance will be given to *Aspergillus flavus*. The absence of *A. flavus* in blossoms and honey bees suggests that this fungus remains inactive during the almond blossom and pollination period of the examined almond cultivars. Either the propagules of the fungus are very low during bloom of almonds or blossoms are not good substrates for colonization by *A. flavus*. Therefore, honey bees that move from flower to flower and thus pollinate the almonds are not exposed to *A. flavus* during this process. Use of AF36 on almond orchards during crop development (summer) would not contribute to either *A. flavus* blossom infection or contamination or to subsequent honey bee diseases related to collecting pollen and nectar from blossoms (winter). Our results suggest that frequencies of *A. flavus* that contaminates honey bees during almond pollination, if occurring at all, will be very low. Subsequently, at least in California, infection of bee adult and larvae by *A. flavus* seem to originate from sources unrelated to the almond pollination process. For example, it has been reported that other organisms contribute to bee's disorders in a greater extent (Cornman *et al.* 2012, Schäfer *et al.* 2014, Eiri *et al.* 2015, Tozkar *et al.* 2015) even though *Aspergillus* species have the potential to severely diminish both larvae and adult honey bee populations (Foley *et al.* 2014).

Examination of mycoflora associated with developing almond fruits revealed that *A. flavus* is not an important member of the communities during April to mid-July. Other fungal species dominate these communities during this period. It is expected that in the following weeks an exponential growth of aflatoxin producing fungi will occur, especially after heavy infestation by NOW. These studies are currently being conducted and final results will be ready after the summer 2015. Once the timeframe in which aflatoxin-producing fungi increase is determined, optimal application dates of biopesticides will be determined.

Research Effort Recent Publications:

Donner, M, Lichtemberg, PSF, Doster, M, Picto A, Cotty, PJ, Puckett RD, Michailides, TJ, 2015. Community structure of *Aspergillus flavus* and *A. parasiticus* in major almond-producing areas of California, United States. Plant Disease 99:1161-1169.

Doster MA, Cotty PJ, Michailides TJ, 2014. Evaluation of the atoxigenic *Aspergillus flavus* strain AF36 in pistachio orchards. *Plant Disease* 98: 948-956.

(Attention: Although this is on the use of AF36 atoxigenic strain of *A. flavus* on pistachio, it is relevant to almonds since a major goal of our research is to get the registration of AF36 for almonds.)

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Donner, M, Lichtemberg, PSF, Doster, M, Picto A, Cotty, PJ, Puckett RD, Michailides, TJ, 2015. Community structure of *Aspergillus flavus* and *A. parasiticus* in major almond-producing areas of California, United States. Plant Disease 99:1161-1169.

Doster MA, Cotty PJ, Michailides TJ, 2014. Evaluation of the atoxigenic *Aspergillus flavus* strain AF36 in pistachio orchards. *Plant Disease* 98: 948-956.

Eiri DM, Suwannapong G, Endler M, Nieh JC, 2015. Nosema ceranae Can Infect Honey Bee Larvae and Reduces Subsequent Adult Longevity.

Foley K, Fazio G, Jensen AB, Hughes WO, 2014. The distribution of Aspergillus spp. opportunistic parasites in hives and their pathogenicity to honey bees. *Veterinary microbiology* 169(3): 203-210.

Schäfer MO, Genersch E, Fünfhaus A, Poppinga L, Formella N, Bettin B, Karger A, 2014. Rapid identification of differentially virulent genotypes of Paenibacillus larvae, the causative organism of American foulbrood of honey bees, by whole cell MALDI-TOF mass spectrometry. *Veterinary microbiology* 170(3): 291-297.

Tozkar CÖ, Kence M, Kence A, Huang Q, Evans JD, 2015. Metatranscriptomic analyses of honey bee colonies. *Frontiers in genetics* 6.

Table 1. Incidence of hulls of Nonpareil almonds decayed by various *Aspergillus* fungi for nuts harvested from areas treated with the wheat-AF36 product or from untreated areas in a research almond orchard at the Nickels Soil Laboratory.

		Percentage of hulls with decay by specified fungi			
Year	Treatment	*A. flavus* group	*A. niger* group	*A. ochraceus* group	Other *Aspergillus*
2007	AF36	0.197 ns[y]	nd[z]	0.078 ns	nd
	Untreated control	0.000	nd	0.024	nd
2008	AF36	0.028 ns	1.028 ns	0.004 ns	0.033 ns
	Untreated control	0.007	0.262	0.000	0.011
2009	AF36	0.028 ns	1.008 ns	0.015 ns	0.000 ns
	Untreated control	0.004	0.641	0.000	0.028
2010	AF36	0.000 ns	0.059 ns	0.072 ns	0.006 ns
	Untreated control	0.000	0.059	0.043	0.000
2011	AF36	0.000 ns	0.138 ns	0.028 ns	0.000 ns
	Untreated control	0.000	0.132	0.015	0.000
2012	AF36	0.000 ns	0.431 ns	0.011 ns	0.000 ns
	Untreated control	0.000	0.280	0.004	0.000
2013	AF36	0.000 ns	1.529 ns	0.043 ns	0.007 ns
	Untreated control	0.000	0.811	0.043	0.007
2014	AF36	0.067 ns	2.750 ns	0.586 ns	0.000 ns
	Untreated control	0.007	1.205	0.294	0.000

[y] Not significantly different (P=0.05).
[z] Not determined.

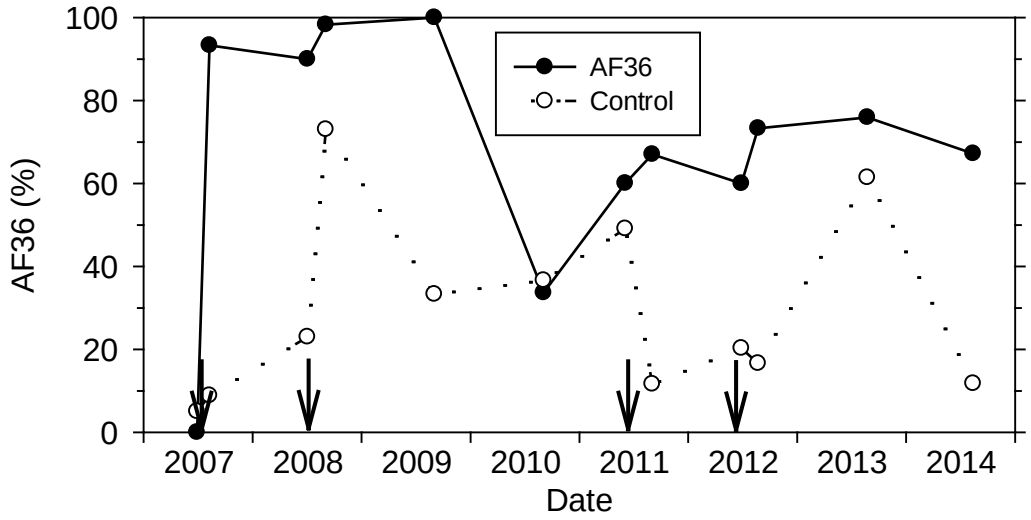

Figure 1. Percentage of *Aspergillus flavus* isolates belonging to the atoxigenic strain AF36 for isolates from soil collected from the areas treated with the wheat-AF36 product or from untreated areas in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product was applied on 28 June 2007, 2 July 2008, 3 June 2011, and 26 June 2012 (arrows).

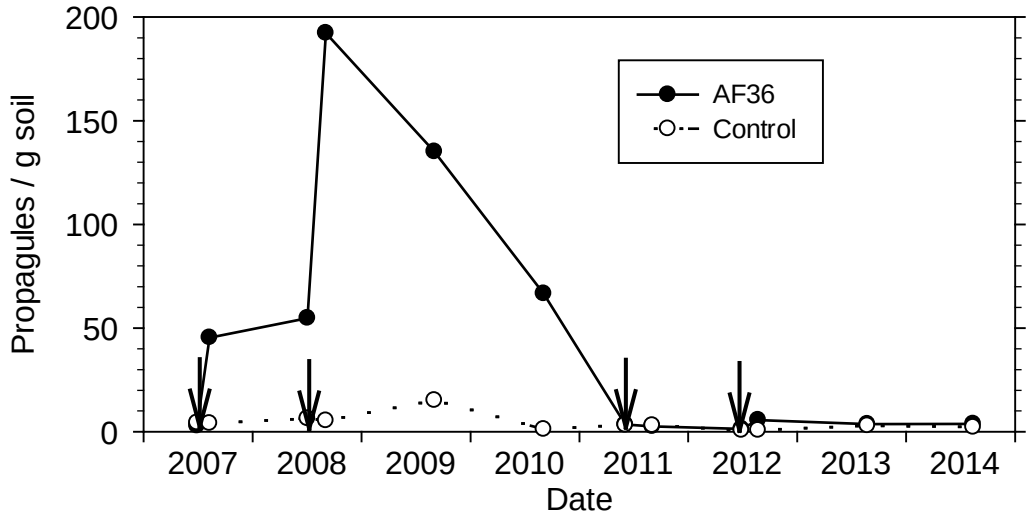

Figure 2. Density of *Aspergillus flavus*/*A. parasiticus* in soil collected from areas treated with the wheat-AF36 product or from untreated areas in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product was applied on 28 June 2007, 2 July 2008, 3 June 2011, and 26 June 2012 (arrows).

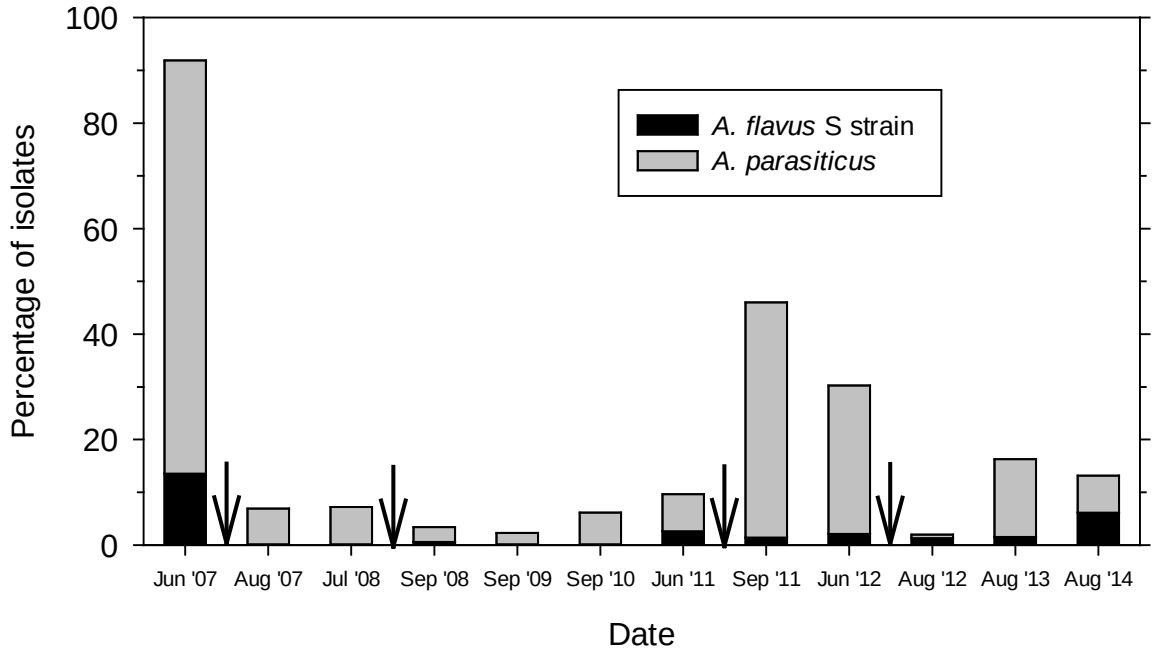

Figure 3. Percentage of *Aspergillus flavus*/*A. parasiticus* isolates that are the aflatoxin-producers *A. parasiticus* and *A. flavus* S strain for isolates from soil collected from areas treated with the wheat-AF36 product in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product was applied on 28 June 2007, 2 July 2008, 3 June 2011, and 26 June 2012 (arrows).