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

Project No.: **13-AFLA1-Michailides**

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Objectives:

1. Complete the analysis of the toxigenic strains of *Aspergillus flavus* present in almond orchards.

2. Determine the survival and spread of the previously applied atoxigenic *A. flavus* strain AF36 and the displacement of aflatoxin-producing fungi in a research almond orchard.

3. Evaluate sorghum as an alternative to wheat for the formulation of the AF36 product for application in almond orchards.

4. Determine the risk almond mummies pose to aflatoxin contamination.

Interpretive Summary:

General**.** Cultural practices to limit aflatoxin accumulation by *Aspergillus flavus* and closely related fungi in susceptible crops are often insufficient to produce commodities below aflatoxin tolerance thresholds. The most effective strategy to limit aflatoxin accumulation in susceptible crops (i.e., maize, cottonseed, peanuts, and pistachio) is to use *A. flavus* strains that do not produce aflatoxins, known as atoxigenics, which both competitively displace aflatoxin producers when applied at a certain crop stage and influence aflatoxin biosynthesis when co-infecting a susceptible crop with toxigenic isolates [1]. The first *A. flavus* atoxigenic strain registered as a biocontrol agent, AF36, is used to limit aflatoxin accumulation of maize, cottonseed, and pistachio in regions of the US where this strain is endemic [2, 3]. AF36 has been repeatedly isolated from almond orchards across California by the Michailides Laboratory, and registration for use in commercial almond orchards is in progress. However, new biocontrol technologies are oriented to use 4 or more atoxigenics belonging to distinct vegetative compatibility groups (VCGs). Using a mixture of atoxigenics would aid to obtain

long-term and cumulative effects in treated fields [4]. Native, well adapted isolates to the target region/crop would ensure successful displacement of toxigenic fungi. Further, potential environmental risks usually accompanied by the introduction of exotic organisms when conducting biological control of plant diseases would be avoided. A mixture of atoxigenics should contain isolates with superior ability to reduce aflatoxin accumulation when co-infecting a substrate with isolates with high aflatoxin-producing potential. Mixtures of atoxigenics are successful in limiting maize aflatoxin contamination in both Kenya and Nigeria [5]. Research is being conducted to register mixtures of atoxigenics in other regions of the world, as well as in the US, including the Michailides Laboratory. In California, several susceptible crops (i.e., almond, pistachio, fig, walnut) are commonly planted in adjacent fields and it is important to identify how common are atoxigenic isolates across these crops to select isolates with the greatest chance to modify the fungal community structure in a regional scale.

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, or 2013, 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* S strain. After not applying the AF36 product in 2009 and 2010, these aflatoxin-producing fungi increased to 46% of the population. 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 aflatoxin-producing fungi 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 recovered *A. flavus* isolates were AF36. The results from applying the AF36 product in 2007/2008 and again in 2011/2012 demonstrated the ability of the AF36 product 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.
This project is doing research in support of gaining registration of the AF36 product for application in commercial almond orchards.

Comparison of biocontrol products**.** The atoxigenic strain *Aspergillus flavus* AF36 is currently dispersed using sterile wheat [*Triticum aestivum* L.], which serves as a food source to the fungus. AF36 reproduces on the wheat grain and disperses to the target crop either initially or after repeated reproduction cycles in other food sources [6]. Certain isolates interacting with the target crop may be more competitive than AF36 although the private food source provided by the biocontrol formulation gives reproductive and dispersal advantages to AF36 over aflatoxin-producers inhabiting in the soil matrix [7]. Sorghum [*Sorghum bicolor* (L.) Moench] is the main vehicle of dispersion of biocontrol agents used in Kenya and Nigeria [5, 8]. Because sorghum and the process to create the inoculum is cheaper and higher throughput than wheat, a sorghum-based product has been sought as an alternative to disperse AF36. In Arizona, experimental use of a sorghum-based AF36 formulation in cotton fields has yielded similar aflatoxin reduction levels to those obtained with the original wheat-based formulation. When comparing AF36's sporulating ability on wheat versus a sorghum-based formulation in a research pistachio orchard at the Kearney Agricultural Research and Extension Center, significantly more AF36 spores were produced on the wheat-based product. However, AF36's sporulating ability may differ among sorghum cultivars since both pericarp phenolic content and a pigmented testa (seed skin) concentrating tannins influence fungal reproduction [9]. Examination of AF36's sporulating ability on different sorghum cultivars with varying concentrations of phenolics and tannins would elucidate if other cultivar(s) provide(s) greater sporulation rates than those obtained using the previous formulations.

Objective 4 - Mummies were not collected in winter 2013/14.

Materials and Methods:

1. **Complete the analysis of the toxigenic strains of *Aspergillus flavus* present in almond orchards.**
   a) Selection of atoxigenic isolates**.** In 2013, 382 *A. flavus* isolates recovered from almond orchard soil during 2007 to 2011 were characterized as atoxigenic based in their inability to produce aflatoxins in a chemically defined liquid media [10] and were subjected to microsatellite analyses [11] to determine genetic variability among them (Picot, Cotty, & Michailides, *unpublished results*). DNA isolation, multiplex-PCR and microsatellite genotyping were conducted as described in previous publications [11, 12].

In order to assess consistency of the data, over 20% of isolates were subjected to at least three repeats of independent PCR and genotyping assays for all the molecular markers.

b) Frequencies of the 12 atoxigenic groups across commercial orchards in California**.** In order to examine frequencies of each of these 12 atoxigenic genetic groups across the historical *A. flavus* fungal collection maintained at the Michailides Laboratory (which dates back to well over two decades), vegetative compatibility analyses (VCA) were utilized. VCA was conducted because of the large expense that would result if each isolate composing the collection was subjected to microsatellite analyses. Initially, tester pairs for each of the 12 groups of atoxigenics had to be generated. Briefly, *nit* mutants of the 42 atoxigenic *A. flavus* isolates belonging to the 12 groups were obtained from SEL agar (Czapek-Dox broth containing 25 g/l $KClO_3$, 50 mg/liter rose Bengal, 20 g/l bacto-agar, pH 7.0 [13]) by seeding a fungal suspension (25 µl) from each isolate into wells in the center of each plate. Plates were incubated for up to 30 days (31°C, dark), until auxotrophic sectors were observed. Spontaneous auxotrophic sectors were transferred into MIT agar (Czapek-Dox broth containing 15 g/liter $KClO_3$, 20 g/l bacto-agar, pH 6.5 [14]), and incubated for 3 days (31°C, dark) to allow for mutant stabilization. Mutants were then transferred to 5% V8™ agar and saved in vials containing sterile water until used in VCA. VCGs were found in VCA by pairing combinations of *niaD* mutants with either *cnx* or *nirA*$^-$ mutants in complementation agar (36 g/l dextrose, 20 g/l soluble starch, 20 g/l bacto-agar, pH 6.0 [15]). These three types of mutants spontaneously arose when isolates grow on SEL agar and each of them possesses different mutations in the nitrate reductase gene. In VCA, if mutants possess both the same alleles at each loci governing vegetative compatibility and complementary mutations, a heterokaryon will be formed and the mutation will be restored (complemented); the ability to utilize nitrate as a nutritive source by the anastomosing hyphae will be regained and a dense zone of prototrophic growth will occur indicating that mutants reacting belong to the same VCG. Fungal suspensions (25 µl) from each isolate were seeded into wells (3 per plate) 1 cm apart in the center of each plate, followed by incubation for 7 days (31°C, dark). Mutants complementing each other were assigned to the same VCG and those mutants producing the most vigorous prototrophic growth were designated as the official tester pair of the group and were used to check for vegetative compatibility with the rest of the isolates. More mutants were generated as described above to obtain tester pairs for those groups that remained to have a tester pair. This was performed until each of the 12 groups of atoxigenics had its tester pair. Mutants from each of the isolates belonging to the historical collection were generated as described above to check for vegetative compatibility with each of the tester pairs from the 12 atoxigenic groups.

c) Mechanisms of atoxigenicity**.** The 42 atoxigenic isolates belonging to the 12 atoxigenic VCGs were subjected to analyses to determine reasons for atoxigenicity. A rapid multiplex method using the polymerase chain reaction (PCR) was used to identify deletions in the aflatoxin biosynthesis gene cluster of each of the isolates, based on a protocol developed by Callicott & Cotty (In press, 2014). Briefly, PCR primers were developed for sixteen markers spaced approximately every 5 kb from 20 kb across the aflatoxin biosynthesis gene cluster. Multiplex PCR reactions were carried out using 0.08

µM of each primer, 1x AccuStart II PCR SuperMix (Quanta Biosciences), and 6 ng genomic DNA in a final reaction volume of 10 µl. Samples were subjected to an initial denaturation step of 1 min at 94°C; 30 cycles of 30 sec at 94°C, 90 sec at 62°C, and 90 sec at 72°C, with a final elongation step for 10 min at 72°C. PCR products were visualized on 1.4% agarose in 1x sodium boric acid buffer. Examining frequencies of the 12 almond-dominant atoxigenic VCGs in pistachio and fig fungal populations was performed in order to help determine which of these VCGs are better adapted to these three crops. It would be ideal if usage of certain VCGs in aflatoxin biocontrol strategies directed to almonds will positively influence fungal community compositions of other susceptible crops in nearby non-treated fields (i.e. pistachio and fig). Having VCGs able to thrive in several crops would potentially maintain a more stable fungal community structure with low aflatoxin-producing potential in comparison to using VCGs not well adapted to crops in nearby fields.

d) Efficacy of the 12 atoxigenic VCGs to limit aflatoxin accumulation when co-inoculated with a toxigenic isolate of either A. flavus or A. parasiticus. The ability to limit aflatoxin accumulation by members of each of the 12 atoxigenic VCGs was investigated. One isolate from each of VCG was co-inoculated on almond with a highly toxigenic isolate of either *A. flavus* (2A1L-11) or *A. parasiticus* (4C1P-11). Briefly, each isolate was grown for 7 days (31°C, dark) on 5% V8™ juice agar [16] and conidia were collected with a cotton swab and suspended in sterile deionized water. Conidial suspensions were quantified using a haemocytometer and diluted to a final inoculum concentration of 1.75 x 10^6 conidia/ml. Sterile glass vials (20 ml) containing approximately 5 g of mature-living almond kernels, previously sterilized using hot water (80°C, 45 sec), were either co-inoculated with a combination of a toxigenic and an atoxigenic isolate, or inoculated with a toxigenic isolate by itself. Each vial was inoculated with a fungal suspension containing approximately 350,000 spores per g of almonds, which was combined previously with the appropriate amount of distilled water to bring the almonds moisture content to 25%. This suspension was vortexed and inoculated evenly on the surface of almond kernels. The initial almond moisture content was determined to be 6%. When co-inoculation occurred, equal amounts of spores from the two isolates were used. Four vials containing almonds inoculated with water served as the negative controls. After inoculation flasks were covered with plastic caps and placed in a plastic container in a randomized complete block design (31°C, 7 days, dark). Four replicates per treatment were used. Each replicate consisted of a single glass vial. Atoxigenics across the globe are selected based on approaches similar to the one that we conducted. By using this approach, we are able to evaluate the competitive ability of atoxigenic isolates in reducing aflatoxin accumulation by influencing aflatoxin biosynthesis [6, 17, 18]. A second experiment for verifying the results of the first experiment will be conducted in August 2014.

2. Determine the survival and spread of the previously applied atoxigenic *A. flavus* strain AF36 and the displacement of aflatoxin-producing fungi 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, or 2013. 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, and 23 August 2013.

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) 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.

3. Evaluate sorghum as an alternative to wheat for the formulation of the AF36 product for application in almond orchards.

Four sorghum cultivars provided by Chromatin, Inc. (New Deal, TX) were utilized for this study (see **Table 4**): a white-pericarp sorghum hybrid, 12GS9013; two red-pericarp hybrids, 12GS9016 and 12GS9022; and a red-pericarp with pigmented testa hybrid, 12GS9023. From each cultivar, five batches of 1,000 kernels were counted and weighted independently. Only non-damaged, free of debris kernels composed each batch. Each cultivar was sent for phenolics quantification at the Cereal Quality Laboratory of the Department of Soil and Crop Sciences, Texas A&M University, College Station, TX. The total polyphenol contents of the samples were determined by the Folin-Ciocalteu method [19] and expressed as mg gallic acid equivalent per g of sample (dry basis). Biocontrol formulations of ACRPC-w and ACRPC-s were prepared previously by the ACRPC. For the other sorghum cultivars, biocontrol formulations were prepared by adjusting the industrial scale methodology of biocontrol preparation [6] to a laboratory scale. Sterile 96-well polystyrene plates (Thermo Fischer Scientific Inc., Waltham, MA) were used to evaluate AF36's ability to sporulate on the different

nutrient sources provided by the sorghum formulations. Plates were divided in four equal sections (24 wells each), and each section served as one of the five repetitions of each of the 8 biological control formulations (two per cultivar). Biocontrol formulations were randomly placed across plates. For each repetition, a single kernel of biocontrol product was placed in each of the 24 wells using sterile forceps. A total of 15 plates were used per experiment. Sterile water was added to the spaces between and outside wells to keep humidity high. Plates were randomly placed inside crispers and incubated at 31°C for 7 days. After incubation, plates were visually inspected and the number of kernels with visible AF36 growth was recorded. For each formulated biocontrol product's repetition, four kernel pairs with visible growth of AF36 were used to quantify spore production. Three hundred µl of sterile distilled water were used to wash the spores from each kernel pair and were combined with 1,200 µl of distilled water in sterile 2 ml Eppendorf tubes. A mathematical model was constructed to convert % transmittance using a spectrophotometer [20] to the number of spores based on a standard curve developed by using known amounts of serially-diluted spore suspensions of AF36 previously quantified with a haemocytometer. For the % transmittance readings, 500 µl of the spore suspensions contained in each 2 ml Eppendorf tube were combined independently with 1.5 ml of sterile purified water on quartz cuvettes and then converted to values of spores per ml using the mathematical model. Then the average of spore values of each repetition (eight kernels) were extrapolated to the number of kernels per gram of each cultivar with a final adjustment by multiplying the number of spores per gram by the percent of kernels that allowed AF36 growth. Weight, gallic acid content and AF36 reproduction data was subjected to Analysis of Variance (ANOVA) and means were separated with Tukey's Honest Significant Difference (HSD) test using SAS 9.4 4 (SAS Institute, Cary, NC).

Results and Discussion:

Selection of atoxigenic isolates**.** Microsatellite analyses using POPULATION software package Version 1.2.32 and Bayesian clustering program Structure 2.2.3 [21] revealed that the most common atoxigenic isolates across almond orchards in California, 42 total, belong to 12 genetically distinct groups. Each of these groups is considered to independently descend from a single clonal lineage. Minimal variation among members of the same group may exist, although such variation was not enough to consider isolates to belong to different groups. Such variation may arise from mutations in the microsatellite markers that arose over the years due to biotic and/or abiotic factors.

Frequencies of the 12 atoxigenic groups across fig and pistachio orchards in California**.** Tester pairs for each of the 12 groups of atoxigenics were successfully generated. These groups were designated as VCGs G01, G02, G03, G04, G05, G07, G08, G09, G10, G11, G12, and G13 (**Table 1**). None of the tester pairs from one group complemented with any of the other tester pairs from the other groups, nor with the tester pair of the VCG to which AF36 belongs to, leading us to conclude that each of the 12 groups of atoxigenic VCGs descend independently from single clonal lineages. Results so far indicate that the most common atoxigenic group in almond, VCG G03, is the most common and the second most common VCG in the fig and pistachio populations, respectively. VCG G10 is the most common VCG in the pistachio population but was not detected in the fig population; G10 ranks in the 4th most common VCG in the almond population. G13 is an important member of the populations of the three crops studied. G05 is an important member of the populations of both almond and fig, but not in

pistachio. Neither G02 nor G07 were detected in either pistachio or fig populations. Current results indicate that VCGs G03, G05, G10, and G13 may complement the biological control agent AF36 in limiting aflatoxin accumulation of almonds, as well as in pistachios and figs, if a mixture of these isolates is used in a biological control program. Pending to evaluate are the results from VCGs G11 and G12. Modifying fungal community structures to benefit a target crop (i.e., almond) may benefit other susceptible crops, which in turn will help to reduce the frequencies of toxigenic isolates across the target area. The decision of selecting atoxigenic isolates well adapted to several crops may promote the persistence of such isolates in the environment for longer terms, which will result in decreased aflatoxin-producing potentials of the *Aspergillus* population residing in a target area, in this case the almond-growing regions of California, and potentially will reduce the period in which biocontrol agents need to be reapplied because of the ability of the atoxigenics conforming the mixture to thrive in other crops. In order to obtain the greatest positive effect in reducing the frequencies or aflatoxin-producing fungi, atoxigenic fungal populations interacting with other crops may need to be considered when selecting biocontrol agents for the almond regions of California.

Mechanisms of atoxigenicity of the 12 atoxigenic Aspergillus flavus VCGs**.** Analyses of genetic markers in the aflatoxin biosynthesis gene cluster of each isolate belonging to the 12 atoxigenic *A. flavus* VCGS revealed that VCGs G02, G03, G08, and G11 possess deletions in the segments that were examined, which are sufficient to explain the atoxigenicity of those VCGs (**Figure 1**). However, this approach examines only a fraction of the whole aflatoxin biosynthesis gene cluster and the reasons for atoxigenicity for VCGs G01, G04, G05, G07, G09, G10, and G12 was not resolved when using this approach. Deletions in other portions of the cluster and/or genetic defects that introduce a signal that prevent aflatoxin biosynthesis may be the reason for atoxigenicity of those VCGs. Indeed, the biocontrol agent AF36 possess an intact aflatoxin biosynthesis gene cluster and the reason for its atoxigenicity is due to a genetic defect in the polyketide synthesis gene, *pksA*, a gene that codes for the production of a protein fundamental for aflatoxin formation [22]. This genetic defect was not found in the VCGs in which deletions were not observed for the examined markers (data not shown). More research needs to be conducted to determine the exact cause for atoxigenicity of these VCGs.

Efficacy of the 12 atoxigenic VCGs and AF36 to limit aflatoxin accumulation when co-inoculated with a toxigenic isolate of either A. flavus or A. parasiticus***.*** All of the tested atoxigenic *A. flavus* VCGs were linked to reduce aflatoxin accumulation of almonds when co-inoculated with a toxigenic isolate of either *A. flavus* or *A. parasiticus* in comparison to almonds inoculated with a toxigenic isolate alone (**Table 2**). Indeed, aflatoxin reductions for both B_1 and G_1 aflatoxins were never below 98.5% and in many cases reached 99% in comparison to those levels obtained when inoculating toxigenic isolates alone. Both of the toxigenic isolates accumulated significantly ($P<0.0001$) higher aflatoxin concentrations than those concentrations in any of the co-inoculations. However, significant differences were detected in the ability to reduce aflatoxins accumulation among VCGs although the observed aflatoxin reductions, in all cases, were well over the desirable reduction that an atoxigenic isolate may possess in order to be considered successful in limiting aflatoxin accumulation (>80% aflatoxin reduction) [1, 17]. Results from this experiment suggest that the 12 atoxigenic VCGs are suitable candidates to integrate a biocontrol formulation with multiple isolates because of their superior competitive ability to inhibit aflatoxin biosynthesis.

Biocontrol of *A. flavus* and reduction of aflatoxin contamination**.** Applying the wheat-AF36 product was very effective in increasing the population of the atoxigenic strain AF36 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 2**). 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 2**), 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 2**).

In the untreated areas (control), the level of AF36 was frequently high even though the AF36 product was never applied in those areas (**Figure 2)**. 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 2**). Furthermore, sometimes the percentage of AF36 in the untreated areas was high, such as 74% in September 2008 and 61% in September 2013 (**Figure 2**). 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 the total population of *A. flavus*/*A. parasiticus* (toxigenic and atoxigenic) in the soil varied through the years (**Figure 3**). 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 3**). In 2011 and 2012 the density remained low even though the AF36 product had been applied both years (**Figure 3**). 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 176 and 112 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 4**), 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 4**). 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 4**), 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 4**). After applying the AF36 product again in 2012, only 2%

of the isolates were *A. parasiticus* or *A. flavus* S strain (**Figure 4**). However, in 2013 when no AF36 product was applied, the level of these aflatoxin-producing fungi started to increase again reaching 16% (**Figure 4**). 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 3**), 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 3**). For example, 1.53% of the nuts from treated areas had hulls decayed by the *A. niger* group in 2013 compared to none decayed by the *A. flavus* group (**Table 3**). 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. The results from this study should help in obtaining registration of the AF36 product for use in commercial almond orchards in California.

Evaluate sorghum as an alternative to wheat for the formulation of the AF36 product for application in almond orchards**.** Significant differences were detected in the weight of 1,000 sorghum kernels among the sorghum cultivars ($P < 0.0001$, **Table 4**). The sorghum cultivars 12GS9016 and 12GS9013 had the lowest (27.3) and highest (44.4) number of kernels per gram, respectively (**Table 4**). Gallic acid content was significantly different among the four sorghum cultivars ($P < 0.0001$, **Table 5**). Sorghum cultivar 12GS9023 contained the highest quantity of gallic acid (8.80 mg gallic acid/g of seed). Such concentration is within the range of tannin-containing sorghum cultivars. A pigmented testa observed after dissection of kernels of this sorghum cultivar confirmed the accumulation of tannins in that region. The gallic acid content in the other two red-pericarp sorghum cultivars is associated with phenolics found in the pericarp, but not in the testa. The white-pericarp sorghum cultivar, 12GS9013, contained the lowest quantity of gallic acid (1.18 mg gallic acid per g of seed), which is characteristic of white sorghums although there are other white-pericarp sorghum cultivars that possess lower phenolic concentrations. In general, all sorghum cultivars used in the current study only promoted fungal growth of AF36. Presence of microorganisms other than *A. flavus* was not detected. Growth of AF36 ranged from 98.6% to 100% of kernels across the examined cultivars. Spore suspension readings using a spectrophotometer (% transmittance) were negatively correlated with readings using a haemocytometer ($R = -0.9076$, $P < 0.0001$, $y = -0.6489x + 83.494$). Highly significant differences in AF36's spore production were detected among cultivars ($P < 0.0001$, **Table 5**). 12GS9013, the white-pericarp sorghum hybrid, had significantly higher spore production (**Table 5**) and 12GS9023 had significantly less ($P < 0.0001$) spore production than any other sorghum cultivar (**Table 5**). Results from this study indicate that certain sorghum cultivars should be selected and others avoided promoting greater sporulation rates and increasing displacement of aflatoxin-producing fungi by AF36. The smaller size of sorghum kernels in comparison to wheat kernels increases the area in which the biocontrol agent is able to sporulate and subsequently disperse. Both phenolics and

tannins inhibit growth of several fungal genera, including *Aspergillus* [9, 23]. In addition, both families of compounds deter feeding by insects, rodents, and birds [24, 25]. Utilization of biocontrol products by higher organisms is a concern because product loss may reduce significantly the success in limiting aflatoxin contamination of the treated crop [26].

Gallic acid content influence substrate utilization by micro and macro organisms; substrates with high gallic acid concentrations usually are not utilized as a food source by a large number of micro and macro organisms unless they possess the ability to lyse gallic acid in the form of phenols and tannins [9, 27]. In the present study, we observed that a white seeded sorghum promotes greater AF36's reproduction than the other cultivars tested. That cultivar promoted around 40% more spore reproduction than any of the other cultivars tested. On the other hand, red-pericarp pigmented testa sorghum, 12GS9023, allowed low AF36's reproduction rates. High-tannin containing cultivars should be avoided when selecting sorghum as a vehicle of dispersion unless the biological control isolate possess the ability to lyse tannins. It appears that AF36 has nonfunctional tannase genes due to its poor reproduction on the red seeded cultivars, especially in 12GS9023. Using sorghum cultivars with both low phenol and no tannins in biocontrol formulations would promote superior AF36 sporulation and will allow for establishment of long-term fungal communities with low aflatoxin-producing potential to ensure almond and pistachio production with little or no aflatoxin accumulation. Testing of sorghum biocontrol formulations with both low polyphenol and no tannins needs to be evaluated in research almond orchards to determine AF36's ability to limit aflatoxin accumulation in both crops when using improved formulations. This approach is currently being tested in a research almond orchard of the Kearney Agricultural Research Center by using four genetically distinct atoxigenic isolates in a variety or sorghum substrates with varying gallic acid content. Results of both displacement of aflatoxin-producing fungi and reduction of aflatoxin accumulation in comparison to untreated controls will be analyzed after almond harvest. In addition, current research includes examination of DNA regions associated with tannin lysis in several atoxigenic isolates that commonly inhabit almond and other crops orchards (i.e., pistachio, fig, and walnut). Identifying isolates able to utilize sorghum cultivars containing high tannin concentrations as a private food source would ensure utilization solely by the superior isolate and would deter utilization by other microorganisms and/or higher organisms. This may aid in increasing rates of both dispersion of biological control agents and subsequent displacement of aflatoxin-producing fungi.

Research Effort Recent Publications:

Doster, M.A., Cotty, P.J., and Michailides, T.J. 2014. Evaluation of the atoxigenic *Aspergillus flavus* strain AF36 in pistachio orchards. Plant Disease 98:948-956.

Palumbo, J.D., Mahoney, N.E., Light, D.M., Siegel, J., Puckett, R., and Michailides, T.J. 2014. Spread of *Aspergillus flavus* by navel orangeworm (*Amyelois transitella*) on almond. Plant Disease 98: (in press).

Table 1. Frequencies of atoxigenic *Aspergillus* flavus VCGs detected in almond, pistachio, and fig commercial orchards in California from 2007 to 2011.

VCG[a]	Frequencies (%)[b]		
	Almond	Pistachio	Fig
G01	0.8	0.9	0
G02	1.3	0	0
G03	1.8	5.0	6.6
G04	0.8	IP[c]	IP
G05	1.1	0	4.4
G07	0.8	0	0
G08	0.8	1.9	2.2
G09	1.1	3.4	0
G10	0.8	5.6	0
G11	0.8	IP	IP
G12	0.8	IP	IP
G13	0.3	4.1	2.2

[a] Tester pairs of VCGs were developed after an initial study examining genetic diversity of *A. flavus* isolates using microsatellite markers. That study grouped (12 groups) isolates based on genetic markers similarities. Tester pairs were developed for each of those 12 groups to check for vegetative compatibility in other fungal populations.

[b] Frequencies were calculated as the percentage of isolates belonging to each VCG, in each of the examined crops. The number of isolated tested for vegetative compatibility was 382, 320, and 45 for the almond, pistachio, and fig populations, respectively. Relatively few of the VCGs remained to be examined in both the pistachio and fig populations, and these tests will be conducted during August 2014. Future plans include examining frequencies of these VCGs in other fungal populations associated with susceptible crops across California (i.e. walnut).

[c] IP = In progress.

Table 2. Ability to reduce aflatoxin content of almonds by 12 atoxigenic *Aspergillus flavus* VCGs endemic to almond orchards and AF36 when co-inoculated with a toxigenic isolate of either *A. flavus* or *A. parasiticus.*

	A. flavus 2A1L-11[a]		*A. parasiticus* 4C1P-11[a]			
Co-inoculated with	*Aflatoxin* B_1 (µg/g)[b]	% B_1 Reduction[c]	*Aflatoxin* B_1 (µg/g)[b]	% B_1 Reduction[c]	*Aflatoxin* G_1 (µg/g)[b]	% G_1 Reduction[c]
None	1,131 a	-	1,368 a	-	234 a	-
G01	3.3 cde	99.7	5.5 cde	99.6	1.0 d	99.6
G02	25.6 b	97.7	20.3 b	98.5	4.8 b	97.9
G03	4.7 cde	99.6	7.4 bcde	99.5	2.0 bcd	99.1
G04	3.0 cde	99.7	3.5 e	99.7	0.7 d	99.7
G05	9.8 bc	99.1	4.8 de	99.6	1.2 d	99.5
G07	6.3 bcd	99.4	18.9 b	98.6	3.9 bc	98.3
G08	1.1 e	99.9	4.6 cde	99.7	1.8 bcd	99.2
G09	7.8 bcd	99.3	13.1 bcd	99.0	4.1 bc	98.2
G10	4.1 cde	99.6	7.3 cde	99.5	1.6 cd	99.3
G11	24.5 b	97.8	14.7 bc	98.9	4.3 bc	98.1
G12	25.4 b	97.8	6.8 bcde	99.5	1.7 bcd	99.3
G13	3.9 cde	99.7	5.9 cde	99.6	1.9 bcd	99.2
AF36	2.2 de	99.8	5.2 cde	99.6	2.3 bcd	99.0

[a] These toxigenic isolates were selected based on their ability to produce large quantities of aflatoxins in several substrates when conducting distinct experiments in the Michailides Laboratory. Aflatoxins were calculated using a HPLC.
[b] Aflatoxin values followed by same letters within a column do not differ significantly according to Tukey's honestly significant difference test (P = 0.05).
[c] Percent reduction of was calculated as [1 – (aflatoxin content in almonds co-inoculated with both the toxigenic and atoxigenic isolate/ aflatoxin content in almonds inoculated only with the toxigenic isolate) x 100. These was calculated only for the aflatoxin B_1 concentration for flasks inoculated with *A. flavus* 2A1L-11, and independently for both aflatoxins B_1 and G_1 for flasks inoculated with *A. parasiticus* 4C1P-11.

Table 3. Incidence of hulls of Nonpareil almonds decayed by various *Aspergillus* fungi for almonds 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

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

Table 4. Various characteristics and weight of the four sorghum cultivars used in the current study.

Cultivar	Color	Moisture content (%)	n[a]	Avg. weight (g)[b]	Avg. number of kernels per g
12GS9013	White	12.1	5	22.5 d	44.4
12GS9016	Red	12.4	5	27.8 a	36.0
12GS9022	Red	11.9	5	25.9 b	38.7
12GS9023	Red	11.9	5	24.4 c	41.0

[a] Each batch of nuts was composed of 1,000 intact kernels.
[b] Average weight of the five 1,000 kernel batches of each cultivar. Means separation of weight values was conducted using Tukey's HSD test (α = 0.05). Values followed by different small case letters differ significantly.

Table 5. Gallic acid content and *Aspergillus flavus* AF36 sporulation ability on the four sorghum cultivars that contain different amount of gallic acid.

Cultivar	mg gallic acid per g[a]	Kernels with AF36 growth (%)[b]	Spores per g[c]		*t*-test[d]	
			Exp. 1	Exp. 2	Exp.1	Exp.2
12GS9013	1.18 d	100	290.8 a	224.9a	0.0679	0.1913
12GS9016	1.44 c	98.6	171.5 b	159.1b	0.0483	0.0521
12GS9022	1.97 b	100	199.8 b	161.1b	0.0243	0.4891
12GS9023	8.80 a	99.6	115.9 c	119.8c	0.1899	0.2697

[a] Gallic acid content was determined at the Cereal Quality Laboratory of the Department of Soil and Crop Sciences, Texas A&M University, College Station, TX using a HPLC. Means separation of gallic acid values was conducted using Tukey's HSD test (α = 0.05). Values followed by different small case letters differ significantly.

[b] Percentage of kernels of each cultivar in which AF36 surface growth was visible.

[c] Two experiments were conducted, and two batches of each cultivar were used and combined on each experiment. Each batch was composed of 5 repetitions, and each repetition consisted of the average of the spores produced on four kernel pairs. Values represent thousandths of the average spores produced by *Aspergillus flavus* AF36 on the cultivars used in the current study and reflect the adjustment performed by multiplying the predicted spores per g of kernels by the percent kernels with visible growth of each cultivar. Means separation of spores per g values was conducted using a Repeated-Measures approach with Tukey's HSD test (α = 0.05). Values followed by different small case letters differ significantly.

[d] The two batches of each cultivar in each experiment were subjected to Student's *t*-test to determine significant differences between them.

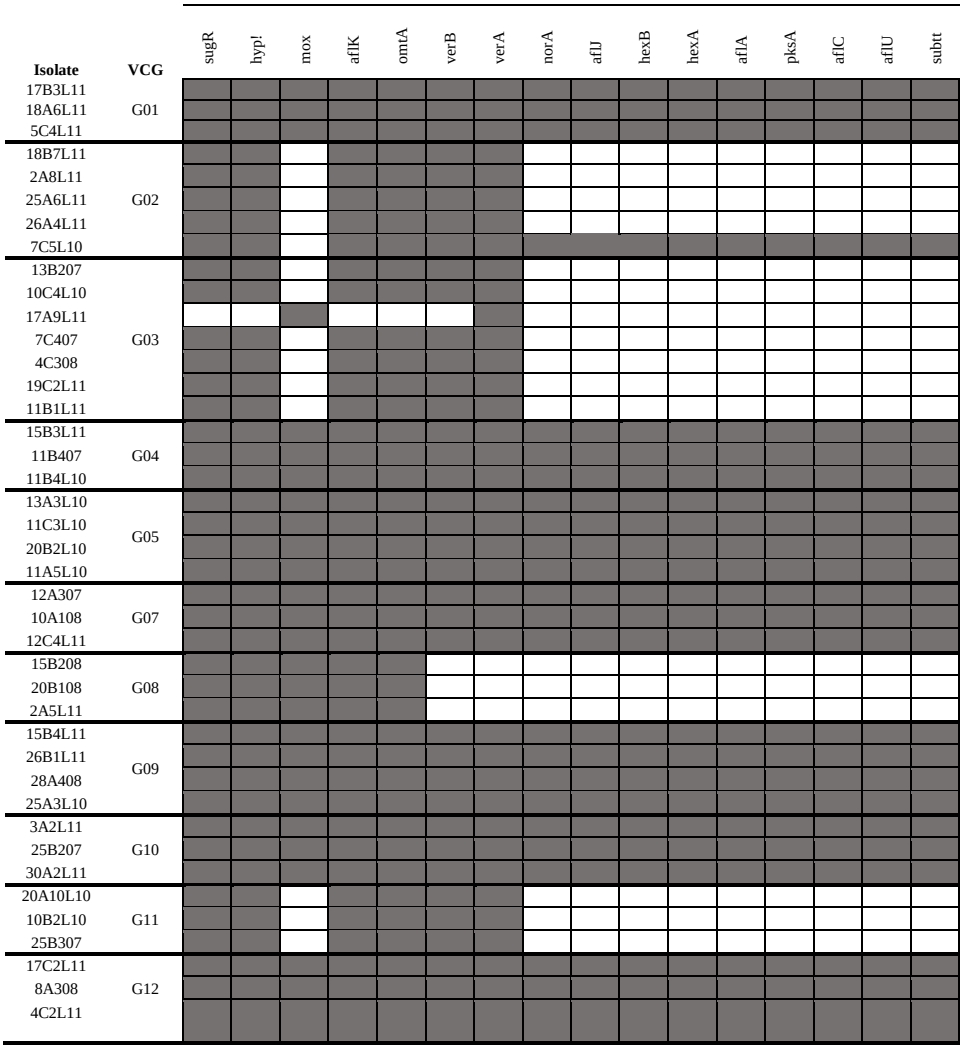

Figure 1. Different PCR amplification of the regions belonging to the aflatoxin biosynthesis gene cluster examined for the 42 isolates belonging to the 12 groups of dominant atoxigenic isolates across almond orchards. Gray boxes indicate presence of the genetic marker; white boxes indicate absence of genetic marker. Note: G13 is pending to be examined.

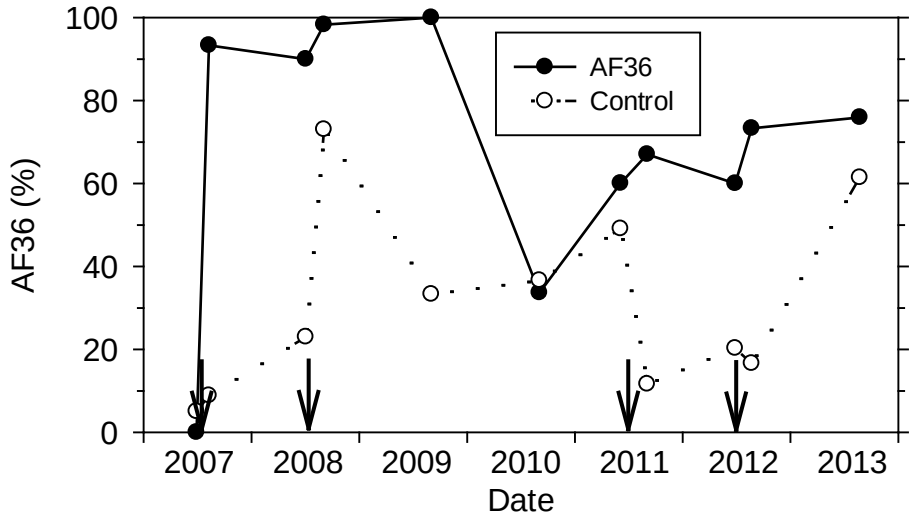

Figure 2. Percentage of *Aspergillus flavus* isolates belonging to the atoxigenic strain AF36 for isolates from soil collected from the areas treated with the wheat-AF36 product or from untreated areas in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product was applied on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

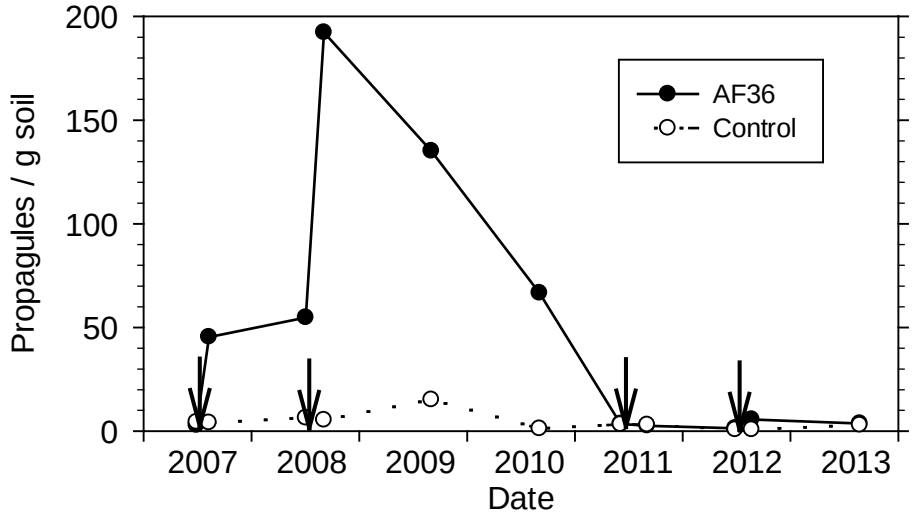

Figure 3. Density of the total population of A. flavus/A. parasiticus (toxigenic and atoxigenic) *Aspergillus flavus*/*A. parasiticus* in soil collected from areas treated with the wheat-AF36 product or from untreated areas in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product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

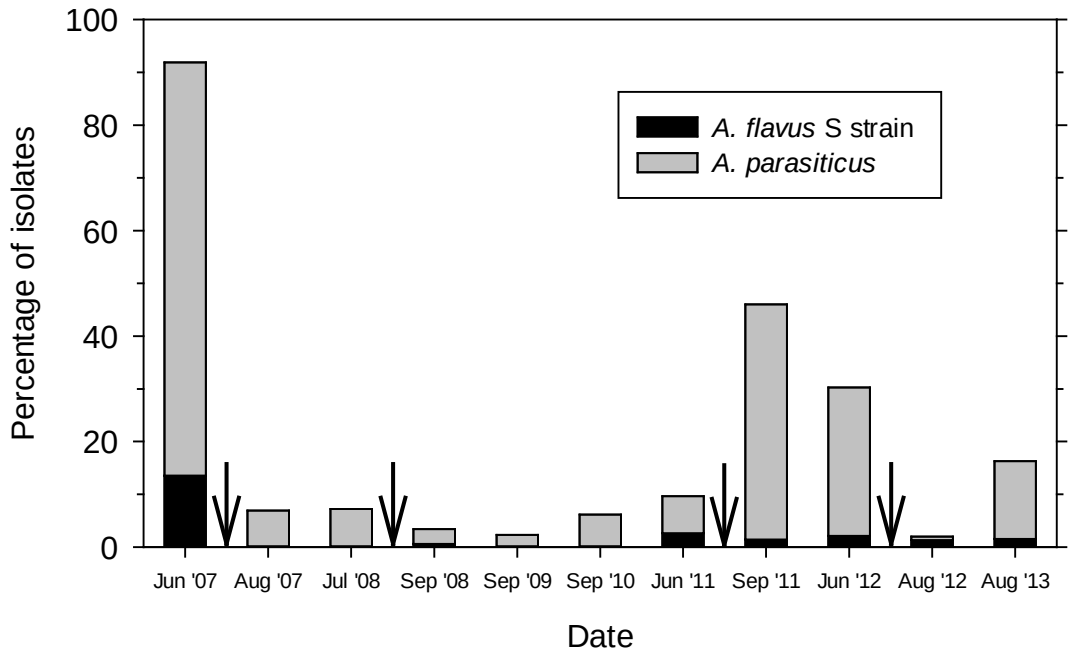

Figure 4. Percentage of *Aspergillus flavus*/*A. parasiticus* isolates that are the aflatoxin-producers *A. parasiticus* and *A. flavus* S strain for isolates from soil collected from areas treated with the wheat-AF36 product in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product was applied on 28 June 2007, 2 July 2008, 3 June 2011, and 26 June 2012 (arrows).

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