Risk Factors, Spatial Patterns, and Biocontrol of Aflatoxin Contamination in California Almonds

Project No.: **12-AFLA1-Michailides**

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Objectives:

1. Complete the analysis of the toxigenic strains of *Aspergillus flavus* present in almond orchards. Determine at which stage of physiological development almond nuts are most susceptible to aflatoxin contamination.
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:

Toxigenicity of *Aspergillus flavus* isolates. The density of aflatoxin producing species and their toxigenicity was determined in almond orchards in three major almond producing regions for 2011 and compared to those in samples collected in 2010, 2007 and 2008. Previously, the method used to determine the toxigenicity of isolates included the HPLC quantification of aflatoxin production in Adye & Matales medium. A new method, both time and cost-effective, was used this year to more rapidly have access to the ratio of toxigenic species. This method consisted in inoculating spores of *Aspergillus* spp. on a coconut agar medium and observing blue fluorescence under UV light after 6 days of incubation at 30°C. Colonies surrounded by blue fluoresecence were defined as toxigenic while those without any fluorescence were

defined as atoxigenic. This new method is nevertheless less sensitive since a preliminary experiment showed that 3 isolates out of 52 were found to produce aflatoxins on A&M medium but did not show any blue fluorescence after 6 days at 30°C. The level of toxigenic species may be therefore slightly underestimated using this method.

The proportion of toxigenic species among the *A. flavus* L strain in 2011 was first determined using 262 with the A&M method. An additional 290 isolates were selected and their toxigenicity was determined using the CAM method. Ratios obtained with the 2 methods were similar, except for the isolates from the northern almond orchards for which the ratio of toxigenic species turned out to be slightly higher for the 290 isolates. In addition, the proportion of atoxigenic *A. flavus* L strains in soil of almond orchards from the central and south regions remained constant in 2011 as compared to that of 2010 population. In the northern region, a 10% decrease in the percentage of atoxigenic strains was observed.

Identify additional atoxigenic strains as potential biocontrol agents. To identify the best competitive atoxigenic strains of *A. flavus* for use as biocontrol agents, a funnel approach is being used. From our collection of atoxigenic fungi isolated from soils of commercial almond orchards, 400 were chosen for their absence of aflatoxin production under optimal conditions. They were first screened for their ability to inhibit aflatoxin production in mixed inoculation with a toxigenic isolate in liquid medium, followed by a second selection according to their fungal growth and ability to sporulate, under both a low and rich-nutrient environment. This method allowed us to select the 24 best growing and sporulating isolates. The aflatoxin and cyclopiazonic acid gene clusters will then be sequenced among those isolates to choose those least likely to revert to toxigenicity. Finally, the ability of the selected strains to displace toxigenic species of *Aspergillus* and suppress aflatoxin contamination of the nuts will be tested on soil and almond nuts, first under laboratory conditions and then under field experiments. The rationale of using a mixture of atoxigenic species will also be investigated.

Determine which developmental stage of almond nut is the most susceptible to infection by *A. flavus/A. parasiticus* and aflatoxin contamination. We were interested in examining when almonds become most susceptible to infection (*i.e.,* during or after the hull split). Furthermore, we wanted to determine whether differences in susceptibility are related to the nut physicochemical composition, such as the moisture content or the pH that are known to influence aflatoxin production. Is it related to the environmental factors that are known to contribute to the success of *Aspergillus* infection such as the climatic conditions, navel orangeworm (NOW) damage of the nuts, size of the hull opening, the density of *Aspergillus* toxigenic species in the soil, or the density of *Aspergillus* recovered from moths? Past experiments consisting in inoculating Nonpareil almonds with high aflatoxin-producing *Aspergillus* isolates every week during and after the hull split stage had already been conducted in 2011. Results showed that aflatoxin contamination in the nuts is very rare when using spore spray inoculation method. In contrast, a pre-inoculation of the nuts with NOW eggs leading to infestation a few days before the spore inoculation greatly enhanced the amount of aflatoxin contamination. Therefore, this year, a similar experiment is being repeated, with half of the branches pre- infested with NOW eggs placed between the hull and the shell seven to nine days before spore inoculation. A laboratory experiment, using almond kernels from different stages and inoculated with *Aspergillus* spp., is being conducted also to complete the results of the field study.

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 cottonseed and corn. 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 was applied in 2009 or 2010, although nut and soil samples continued to be collected to determine the survival and spread of AF36.

Before applying the wheat-AF36 product for the first time, 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 wheat-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 wheat-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 2007 and 2008 demonstrate that applying wheat with AF36 was very effective in increasing the amount of the atoxigenic strain AF36 under the conditions present in this almond orchard. The level of AF36 remained high in the soil with all of the *A. flavus* isolates evaluated from the soil collected in 2009 belonging to AF36 (even though no wheat-AF36 product was applied in 2009), indicating that the effect of application lasts more than a year and perhaps the wheat-AF36 product does not need to be applied every year. After further decrease in the level of AF36 in 2010, additional applications of wheat-AF36 were made in 2011 and 2012, resulting in high levels of AF36 again. The results from 2012 demonstrate again that the application of the wheat-AF36 product is effective in increasing the level of the atoxigenic strain AF36 in the soil.

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 always 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. The future plan

for this study is to do research in support of gaining registration of the atoxigenic *A. flavus* strain AF36 for application in commercial almond orchards. In addition, another area of research interest is to discover ways to improve the effectiveness of applying AF36 in almond orchards.

Comparison of sporulation of AF36 on wheat and sorghum used as carriers of the biopesticide product. The manufacturer who produces the wheat-AF36 inoculum has now started using sorghum as carrier of the AF36 inoculum in addition to the wheat-AF36 product, roasting of the seed instead of sterilizing it, and coating it with the propagules of AF36 instead of inoculating and incubating the seeds. The sporulation of AF36 on wheat and sorghum support these changes in the production of the AF36 inoculum. Although the sporulation on sorghum is delayed during the first 3 weeks after application in the field, it catches up with the sporulation on wheat by 4 or 5 weeks. Approximately 80% of the seeds of either wheat or sorghum found with sporulation of AF36 4 weeks after the application in the field. Furthermore, both the wheat-AF36 product and the sorghum-AF36 product sporulated well in a pistachio orchard at similar levels as those obtained in the almond orchards, although the irrigation schedules of these orchards differed.

Almond mummies as a risk factor for aflatoxin contamination in addition of serving as overwintering sites of NOW. To determine the risk almond mummies pose to aflatoxin contamination, a large number of mummies need to be examined and analyzed. The research progress on this objective depends on the availability of the almond mummies. More mummies will be collected in winter of 2014 to determine NOW levels in them and contamination of NOW larvae emerged from mummies with *A. flavus* / *A. parasiticus.* Interestingly, NOW moths collected from two almond fields in 2012 showed three peaks of high levels of *A. flavus* / *A. parasiticus.* Since early in the season there are not very many propagules of *Aspergillus* fungi in the orchard environment, this high level early in the season (peak 1 in April) probably is due to the fact that there is contamination of NOW moths emerging from mummies infected by *A. flavus /A. parasiticus.* The *Aspergillus* density increases in the orchard later in the season which may explain peak 2 and peak 3, since NOW moths can pick up spores of these fungi from the tree surface. These results suggest that NOW moths can bring propagules of the aflatoxigenic fungi to the right site (hull split) for infection by the pathogen. It is also expected that once AF36 is applied in an orchard to have more AF36 propagules reaching the tree canopy than toxigenic *Aspergillus flavus / A. parasiticus* and most likely the proportion of NOW moths carrying AF36 propagules to be higher than NOW moths carrying toxigenic *Aspergillus* species. This phenomenon was shown in two pistachio orchards treated with AF36.

Materials and Methods:

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

a) Toxigenicity of Aspergillus populations from almond orchards. This portion of research was reported in previous annual reports since this was a continuing objective. However, we have done additional work on determining the ratio of *A. flavus* producing aflatoxins and the quantities of aflatoxins produced by the isolates of the 2011 collection (**Figure 1**).

The ability to produce aflatoxin production in the A&M medium was defined for 262 *A. flavus* L isolates. The quantification of aflatoxin production by *Aspergillus* isolates in the A&M medium was described in previous reports. In addition, the ratio of toxigenic *A. flavus* L strains was determined for an additional 290 *A. flavus* L strains, using a new method based on the property of aflatoxins to fluoresce under UV light in a coconut agar medium (CAM) which seems not to interfere with the fluorescence of aflatoxins (Degola et al., 2007). This method consists in inoculating 20 µL of a spore suspension on plates of CAM. After 6 days growth of the isolates at 30°C, the bottom of the plates were exposed to UV light at wavelength of 365 nm. After a few minutes under UV light, a blue fluorescence surrounding the colonies appears, which is an indication of the ability of an isolate to produce aflatoxins.

b) Identify additional atoxigenic strains as potential biocontrol agents. Although this was not part of our initial objective, we started selecting atoxigenic strains among the isolates of *A. flavus* collected from almond orchards. There is a trend now for research to use mixtures of atoxigenic strains instead of one and we anticipate that this will be the case eventually in all crops that have an atoxigenic strain of *A. flavus* registered.

To identify the best competitive atoxigenic strains of *A. flavus* for use as biocontrol agents, a funnel approach was used. Our collection of fungi isolated from commercial soil orchards now includes more than 1500 *A. flavus* L-strains among which 400 were chosen due to their absence of aflatoxin production under optimal conditions. They were then screened for their ability to inhibit aflatoxin production in mixed inoculation with a toxigenic isolate in liquid medium, using a high throughput method described by Degola et al. 2012. Following this 96-well microplate experiment, the 50 best isolates were selected and their inability to produce aflatoxins was double checked, using an almond meal agar medium. Two isolates were able to produce trace amount of aflatoxin on this medium and were therefore ruled out of our study. The 48 remaining isolates were then single- inoculated on almond kernels and on an almond soil extract meal agar (SOM), used to model growth conditions in soil (Hestbjerg et al., 2002, in our study, maize soil was replaced by almond soil).The radial fungal growth on SOM along with the sporulation ability on SOM (measured after 7 days at 30°C) and growth on almond kernels (measured after 3 days at 30°C) were performed twice in two separate experiments. The aflatoxin and cyclopiazonic acid gene clusters will then be sequenced. Finally, the ability of the selected strains to displace toxigenic species and suppress aflatoxin contamination of the nuts will be tested on soil and almond nuts, first under laboratory conditions and then under field experiments. The rationale of using a mixture of atoxigenic species will also be investigated.

c) Determine in which stage of physiological development almond nuts are most susceptible to fungal infection and aflatoxin contamination. In the summer 2013, every week during and after the hull split stage and until harvest, branches of Nonpareil almond trees were sprayed with a spore suspension of a highly aflatoxin-producing *A. flavus* S strain, *A. parasiticus,* or water control. In addition, half of the branches were pre- infested with NOW eggs placed between the hull and the shell seven to nine days before spore inoculation. At each spray timing, almonds were collected and the size of the hull opening, the moisture content, and the pH of the nuts were determined. On August 29th, all branches were cut off to simulate the harvest. Five days later, the bags were collected from the field. All nuts will be then examined for visible infections by *Aspergillus* and analyzed for aflatoxin levels.

In the laboratory/growth chamber, almonds were collected from six Nonpareil almond trees at the same time the field experiment was conducted. Under laboratory conditions, the almonds were shelled carefully so that the surface of the nut was not harmed, and the kernels (wounded and non-wounded) inoculated. After several weeks of incubating at 30°C, the nuts will be examined for a visible infection of the fungus and tested for aflatoxin as previously described.

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 wheat-AF36 product was applied to the same areas each year). No wheat-AF36 product was applied in 2009 or 2010. 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, and 21 August 2012. 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 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.

Sorghum as a carrier for the AF36 fungus was compared with the currently used wheat-AF36 product in a research almond orchard at the Kearney Agricultural and Extension Center.
In a first experiment, the atoxigenic *A. flavus* strain AF36 wheat- or sorghum-product was applied to the soil at a rate of 10 pounds of product per acre (the same product and same application rate as used for commercial cotton fields and commercial pistachio orchards) on 24 June 2013. The experimental design was a randomized complete block design with 3 replications. For each replication, each product was applied around the surface of one tree. Note that various cultivars

were included in this experimental design. A control treatment with no treatment was also included.

After applying the wheat and sorghum-AF36 product to the orchard floor, the orchard was drip-irrigated on that day and at its normal schedule throughout the summer (twice per week). Soil samples were collected just before the application of AF36 products and during harvest period, i.e. 19 August 2013. For the soil sampling, 12 cores (2cm x 2cm) were collected in the north and south part of each tree, separately. To quantify the density of *A. flavus* and *A. parasiticus* in the soil and to obtain isolates for strain determination, 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. Isolates of *A. flavus* obtained from soils were tested to see if they belonged to the atoxigenic strain AF36. Strain identification was done using the Vegetative Compatibility Group procedure.

In a second experiment, the production of spores on the newly formulated seed-coated sorghum-AF36 product and the wheat-AF36 product were compared in the same research orchard. The experimental design included 4 replications, each of which consisting of one tree area. For each replication, both products were applied separately on both sides of the tree. Two cultivars were included in this experimental design: Carmel and Padre. The products were applied to the soil on the same day and at the same rate as the previous experiment, *i.e.* 10 pounds of product per acre, but in a smaller area in order to facilitate the sampling of seeds over time. Sporulation was quantified in several ways. The percentage of seeds showing sporulation was recorded by examining under a microscope the presence of sporulation on 25 randomly collected seeds. This measurement was performed during 38 days, every 2 days the first 3 weeks and then once a week. In addition, the amount of spores produced per gram of each product was quantified. Twenty-five other seeds were placed in sterile plastic bottles containing 10 mL Tween 0.01% and shaken for 15 minutes on a mechanical shaker before quantification of *A. flavus* spores using a hemacytometer. The spore solutions were then further diluted and 100 µL of the diluted spore suspension were spread evenly on DCPA plates. After incubating the plates at 30°C overnight, the number of *A. flavus* colonies was counted as a measurement of the number of viable spores produced. The amount of spores produced was quantified every week for 6 weeks.

Results and Discussion:

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

a) Toxigenicity of Aspergillus populations. The proportion of atoxigenic *A. flavus* L strains in soil almond orchards remained constant in 2011 as compared to that of 2010, except for the northern region for which the percentage of atoxigenic strains decreased by 10% in 2011, although this decrease may not be significant (**Figure 1;** statistics will be completed at a later date)**.** The amounts of aflatoxin produced by the toxigenic L strain isolates varied significantly and it does not seem to differ from region to region (**Table 1**). These isolates had the ability to produce high levels of aflatoxins in the proper substrate which is an indication that their presence in the almond orchards constitutes a major risk factor. Therefore, having an atoxigenic strain registered for use in almonds definitely will be needed in order to displace the

toxigenic strains and reduce aflatoxin contamination in almonds. It is also mandatory to select for more atoxigenic strains among the isolates in order to have them ready for use when mixtures of atoxigenic strains are pursued for registration in the near future.

b) Identify additional atoxigenic strains as potential biocontrol agents. The selection process for atoxigenic *A. flavus* L strains as potential biocontrol agents is still underway. We were able to screen more 400 atoxigenic strains of *A. flavus* L strain, based on their ability to reduced aflatoxin production in mixed inoculation with a toxigenic species of *A. flavus* in a liquid medium. The innate ability to grow fast and/or sporulate abundantly was then screened for 48 isolates on almond kernels and on an almond soil organic matter agar (Hestbjerg et al., 2002). **Figure 2** below summarizes the results of fungal growth and sporulation ability on SOM and almond kernels. For each variable (*i.e.,* fungal growth on SOM, production of spores on SOM and on almond kernels), a number was attributed to each isolate according to its ranking (48 for the best and 1 for the worst isolate) in order to select the 24 « best isolates » to be included in the next screening step (*i.e.,* sequencing of the aflatoxin and cyclopiazonic acid gene clusters) to make sure that these isolates will not revert back in producing aflatoxins.

In a nutshell, to identify new biological control strains from our collection, several criteria are being considered among which are a) ability to compete with toxigenic *A. flavus* strain; b) ability to control aflatoxin production; c) adaptability to the environment; and d) the absence of aflatoxin and cyclopiazonic acid production due to deletions and/or mutations in the toxin biosynthetic gene clusters.

c) Determine in which stage of physiological development almond nuts are most susceptible to fungal infection and aflatoxin contamination. The almonds have not harvested yet. Results should be available for the Annual ABC Conference.

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.

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 wheat-AF36 product in 2007, after the applications in 2007 and 2008 almost all of the *A. flavus* isolates were AF36 (**Figure 3**). The frequency of AF36 remained high in the soil in treated areas from August 2007 to July 2008, which is evidence that AF36 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 wheat-AF36 product does not need to be applied every year. However, by September 2010 the level of AF36 decreased substantially (**Figure 3**), suggesting that an additional application of the wheat-AF36 product would be needed. After additional applications of wheat-AF36 were made in this orchard in 2011 and 2012, the frequency of AF36 increased substantially to 73% (**Figure 3**). In the untreated areas (control), the level of AF36 was frequently high for the various sampling dates with a high of 74% in September 2008 compared to 5% prior to application of the AF36 product (**Figure 3**), which suggests the movement of AF36 from the treated areas 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 4**). 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 4**). In 2011 and 2012 the density remained low even though the wheat-AF36 product had been applied both years (**Figure 4**). For comparison, the density of *A. niger* in the soil during the period of the study ranged from 48 to 499 propagules / g soil (depending on the sample date) with mean values of 182 and 122 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 5**), both of which consistently produce high levels of aflatoxins. However, after applying the wheat-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 5**). 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 wheat-AF36) the percentage of isolates belonging to *A. parasiticus* or *A. flavus* S strain remained very low (**Figure 5**), suggesting that the wheat-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 5**). After applying the AF36 product again in 2012, only 2% of the isolates were *A. parasiticus* or *A. flavus* S strain (**Figure 5**). These results demonstrate the effectiveness of applying the wheat-AF36 product in decreasing the frequency of these aflatoxin-producing fungi within the population of the *A. flavus* group in the almond orchard.

Applying AF36 did not significantly increase (P=0.05) the incidence of hull decay by *A. flavus* for nuts from the treated areas (**Table 2**), 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 2**). For example, 0.43% of the nuts from treated areas had hulls decayed by the *A. niger* group in 2012 compared to none decayed by *A. flavus* group (**Table 2**).

In general, the favorable results from this study support the use of the wheat-AF36 product in almond orchards. Application of the wheat-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 wheat-AF36 product for use in commercial almond orchards in California.

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

In order to improve efficiency and increase the amounts of inoculum produced, the manufacturer who produces the wheat-AF36 inoculum has now started using sorghum as carrier of the AF36 inoculum in addition to the wheat-AF36 product, roasting of the seed instead of sterilizing it, and

coating it with the propagules of AF36 instead of inoculating and incubating the seeds. The sporulation of AF36 on wheat and sorghum support these changes in the production of the AF36 inoculum. Although the sporulation on sorghum is delayed during the first 3 weeks after application in the field, it catches up with the sporulation on wheat by 4 or 5 weeks (**Figure 6**). Approximately 80% of the seeds of either wheat or sorghum were found with sporulation of AF36 4 weeks after the application in the field. Furthermore, both the wheat-AF36 product and the sorghum-AF36 product sporulated well in a pistachio orchard at similar levels as those obtained in the almond orchards, although the irrigation schedules of these orchards may have been a little different from each other (**Figure 7**). Although we determined the initial propagules levels of *A. flavus /A. parasiticus* in the almond orchard where the biological control products were applied (**Figure 8**), the results in the changes of the propagule levels and the densities of AF36 in the treated vs. the untreated areas will be available by the end of the season. It takes a long time to complete all the vegetative compatibility group tests in order to determine the levels of AF36 strain.

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

This objective is in progress and depends on the availability of the almond mummies. More mummies will be collected in winter of 2014 to determine NOW levels in them and contamination of NOW larvae emerged from mummies with *A. flavus* / *A. parasiticus.* Interestingly, NOW moths collected from two almond fields in 2012 showed three peaks of high levels of *A. flavus* / *A. parasiticus.* Since early in the season there are not very many propagules of *Aspergillus* fungi in the orchard environment (Michailides & Morgan, 1990), this high level (peak 1 in **Figure 9**) probably is due to the fact that there is contamination of NOW moths emerging from mummies infected by *A. flavus /A. parasiticus.* The *Aspergillus* density increases in the orchard later in the season which may explain peak 2 and peak 3 (**Figure 9**), since NOW moths can pick up spores of these fungi from the tree surface. These results suggest that NOW moths can bring propagules of the aflatoxigenic fungi to the right site (hull split) for infection by the pathogen. It is also expected that once AF36 is applied in an orchard to have more AF36 propagules reaching the tree canopy than toxigenic *Aspergillus flavus/A. parasiticus* and most likely the proportion of NOW moths carrying AF36 propagules to be higher than that of NOW moths carrying toxigenic *Aspergillus* species. This phenomenon was shown in two pistachio orchards treated with AF36. NOW moths trapped from the AF36-treated vs. the nearby untreated orchard and tested in Si10 agar media showed significantly higher incidence of AF36 than the incidence of other *A. flavus/A. parasiticus*.

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Table 1. Distribution of aflatoxin-producing and atoxigenic *Aspergillus flavus* L-strain isolates among three regions of California in 2011 in 2 media: A&M and CAM. The mean of aflatoxin concentration of the L-strains was only determined using HPLC quantification of aflatoxin in A&M medium.

		North			Central			South		
A&M medium	Number of isolates	110	ratio	Amount of AF	72	ratio	Amount of AF	80	ratio	Amount of AF
	Afla+	48	44%	37470 ± 76396	39	54%	81651 ± 366159	53	66%	62121 ± 81070
	Afla-	62	56%		33	46%		27	34%	
CAM	Number of isolates	130	ratio		53	ratio		107	ratio	
	Afla+	77	59%		29	55%		66	62%	
	Afla-	53	41%		24	45%		41	38%	

Table 2. 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

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

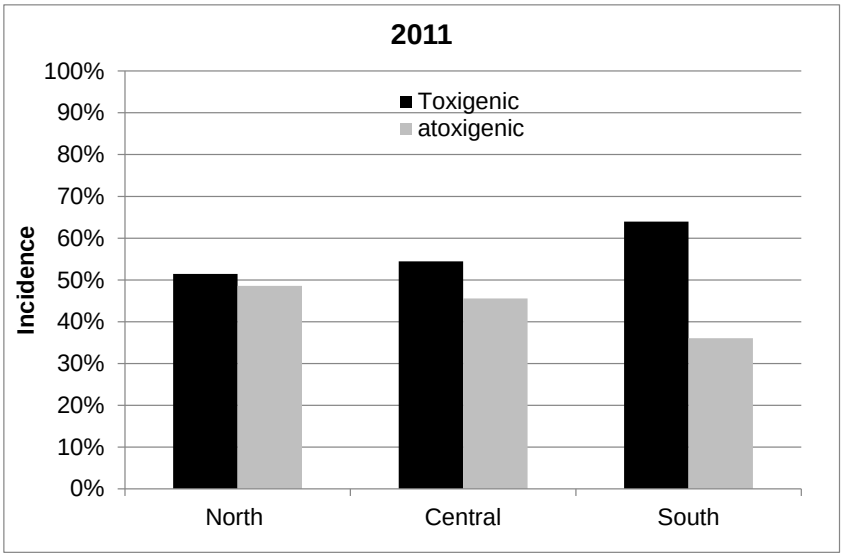

Figure 1. Distribution of aflatoxin-producing and –nonproducing (atoxigenic) *Aspergillus flavus* L-strain isolates among three regions of California in 2011, average of the two methods (A&M and CAM).

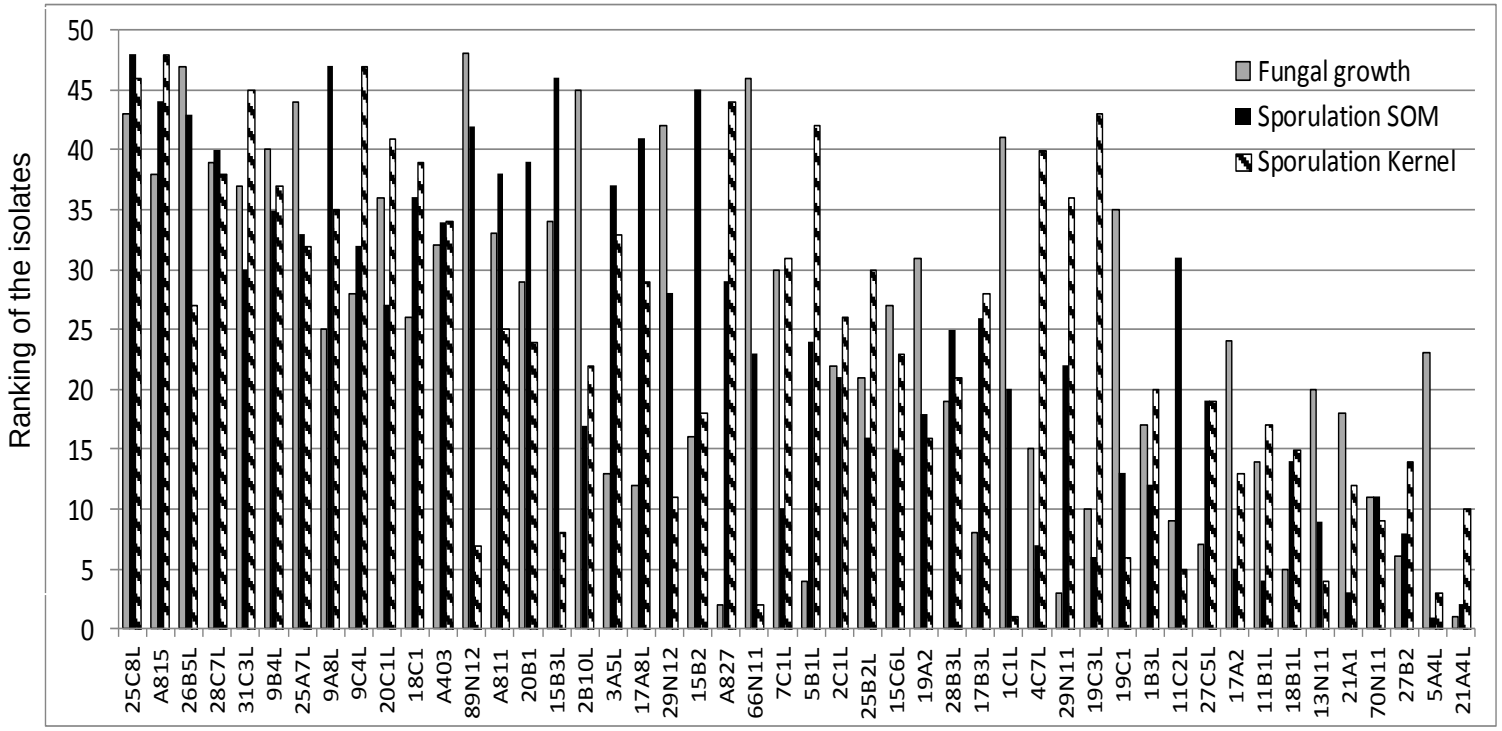

Figure 2. Screening of 48 atoxigenic isolates of *Aspergillus flavus* on SOM for their growth and sporulation ability on almond soil extract meal agar (SOM) and on almond kernels (averages from two separate experiments).

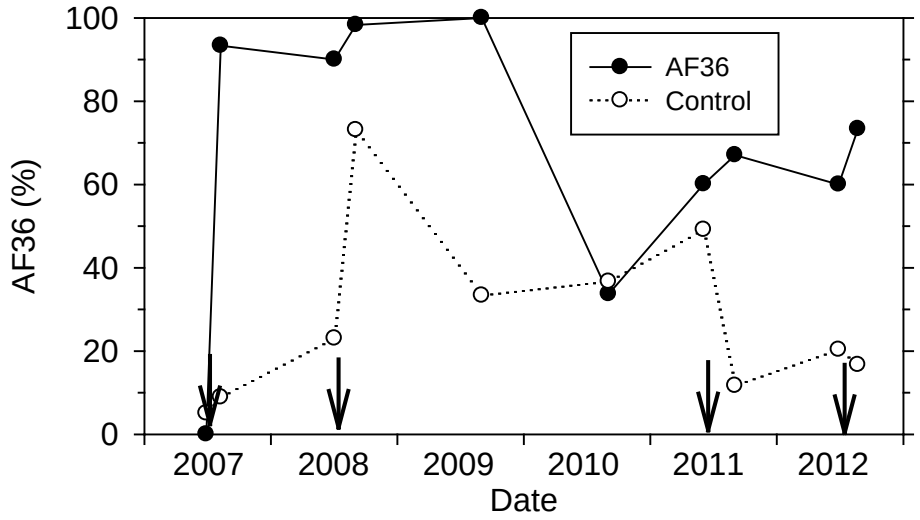

Figure 3. Percentage of *Aspergillus flavus* isolates belonging to the atoxigenic strain AF36 for isolates from soil collected from the areas treated with the wheat-AF36 product or from untreated areas in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product was applied on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

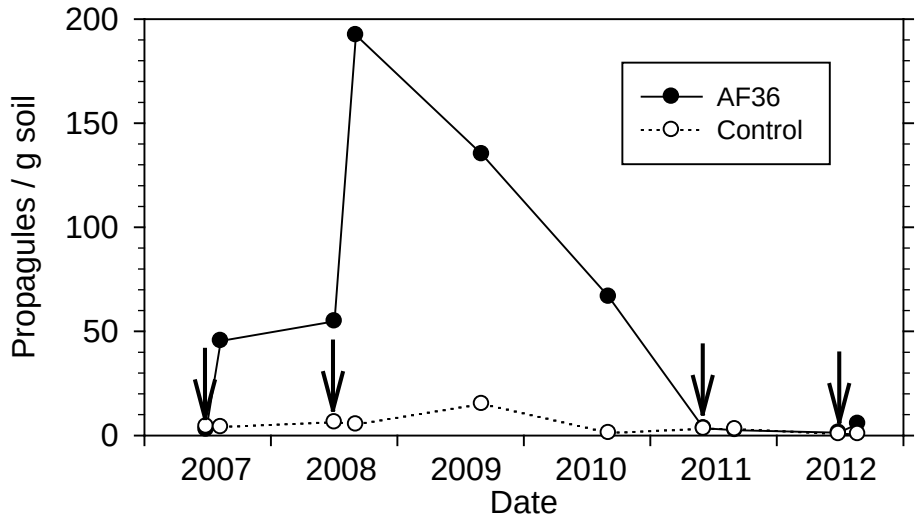

Figure 4. Density of *Aspergillus flavus*/*A. parasiticus* in soil collected from areas treated with the wheat-AF36 product or from untreated areas in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product was applied on 28 June 2007, 2 July 2008, 3 June 2011, and 26 June 2012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

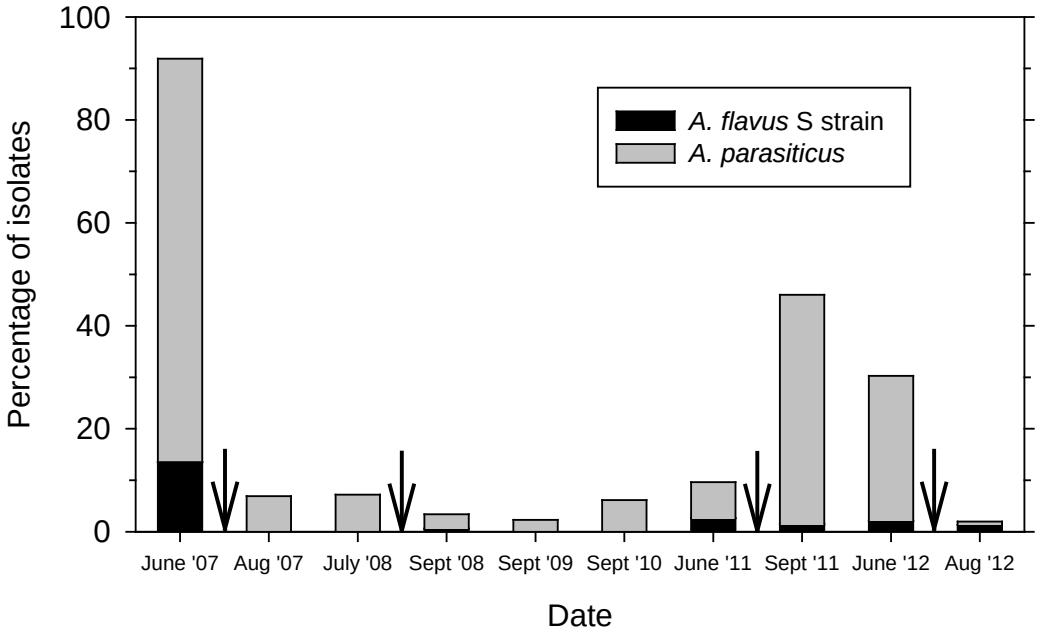

Figure 5. Percentage of *Aspergillus flavus*/*A. parasiticus* isolates that are the aflatoxin-producers *A. parasiticus* and *A. flavus* S strain for isolates from soil collected from areas treated with the wheat-AF36 product in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product was applied on 28 June 2007, 2 July 2008, 3 June 2011, and 26 June 2012 (arrows).

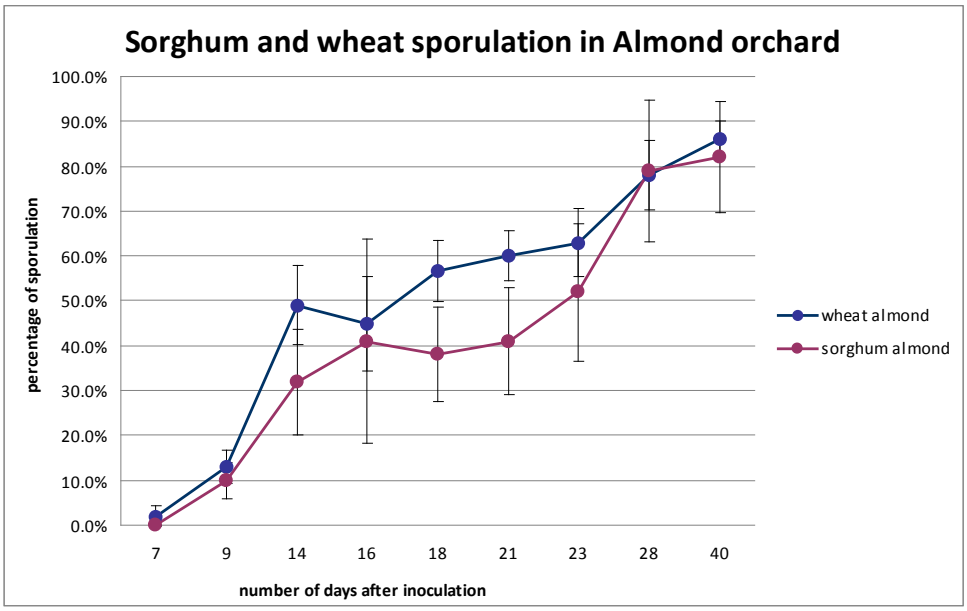

Figure 6. Sporulation incidence of *Aspergillus flavus* strain AF36 on wheat and sorghum products applied in an experimental **almond orchard** in 2013 at Kearney Agricultural Center. (Points are the average of four replications of 25 seeds examined in each sampling date.)

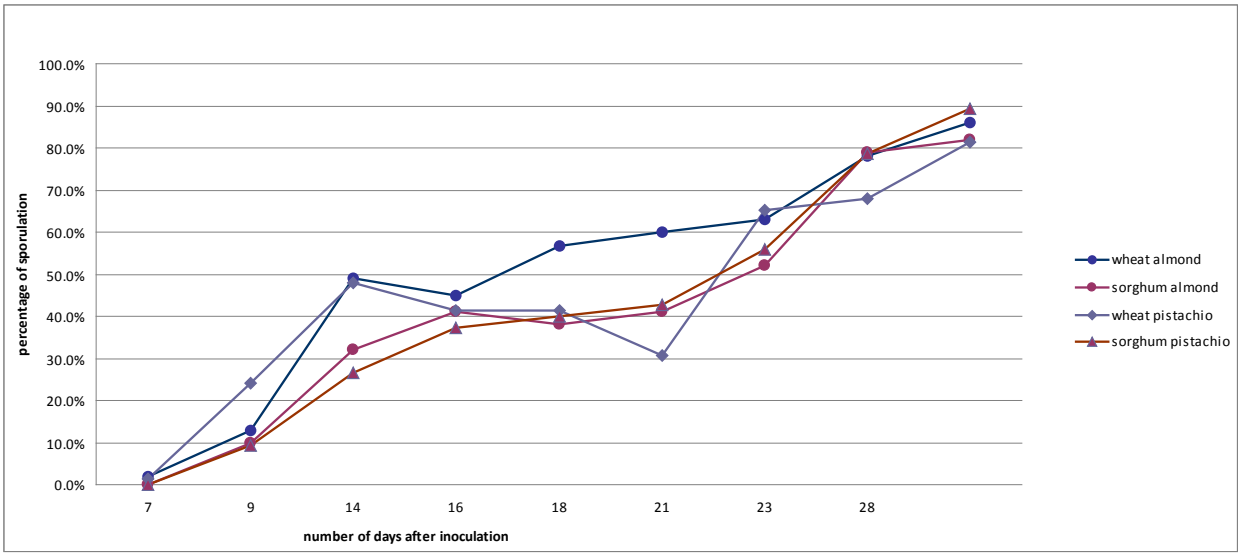

Figure 7. Sporulation incidence of *Aspergillus flavus* strain AF36 on wheat and sorghum products applied in experimental **almond and pistachio** orchards in 2013 at Kearney Agricultural Center. (Points are the average of four replications of 25 seeds examined in each sampling date.)

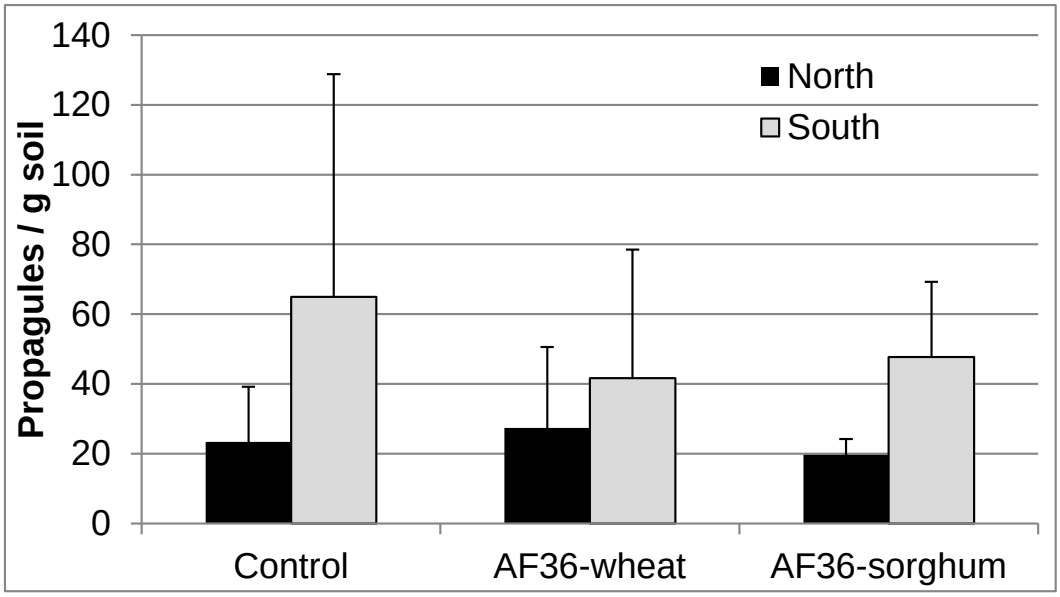

Figure 8. Density of *Aspergillus flavus*/*A. parasiticus* in soil collected from the northern and southern areas of trees before treatment with the wheat-AF36 product and the sorghum-AF36 product in a research almond orchard at Kearney Agricultural Research Center. Bars show standard deviation of three replications.

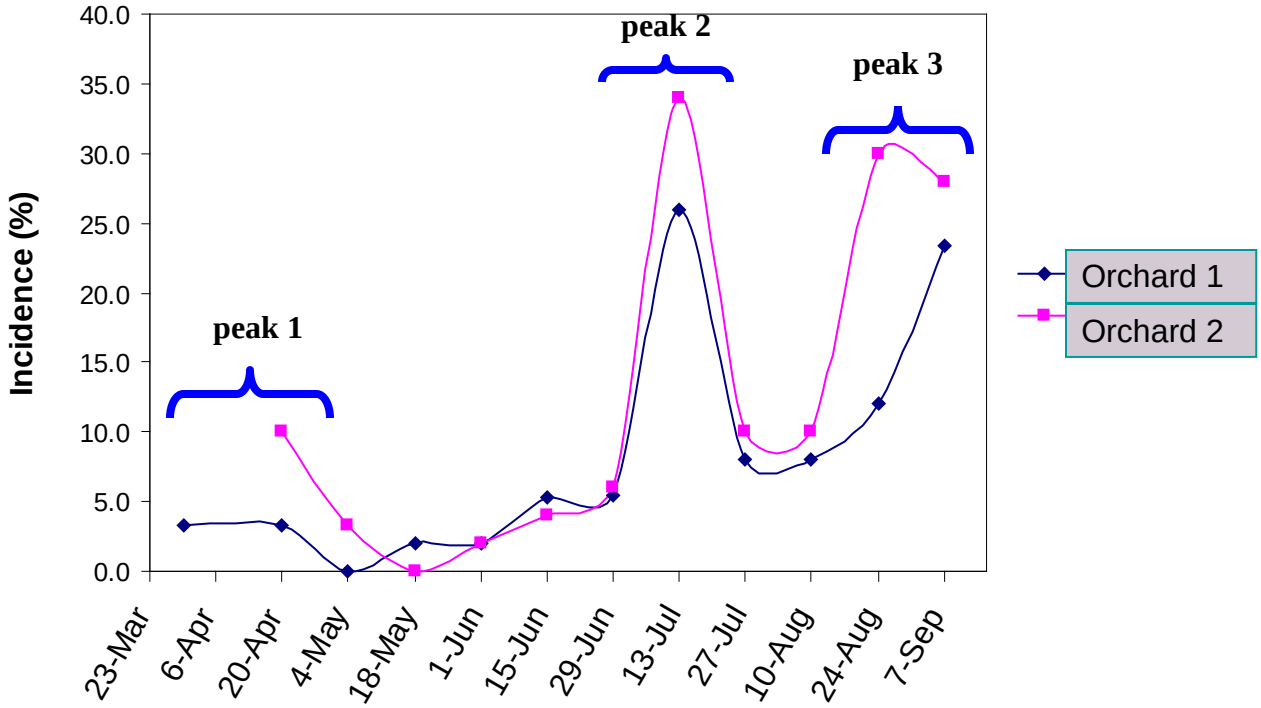

Figure 9. *Aspergillus* sect. *Flavi* (*A. flavus, A. parasiticus,* and *A. tamarii*) isolated on Si10 agar media after plating navel orangeworm (NOW) moths trapped with sticky traps in **two almond orchards**, one each in Fresno and Madera Counties in 2012.