Risk Factors and Spatial Patterns Associated with Aflatoxin Development in California Almonds

Project No.: **09-AFLA1-Michailides**

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Objectives:

1. Determine the spatial pattern of *Aspergillus* strains in northern, central, and southern almond- growing regions in California.
2. Identify risk factors and spatial patterns associated with aflatoxin development in California almonds.
3. Apply the atoxigenic *A. flavus* strain AF36 in a research almond orchard to study the establishment and survival of AF36 and the displacement of aflatoxin-producing fungi.
4. Determine the incidence of the atoxigenic strain AF36 among *A. flavus* isolates naturally occurring in commercial almond orchards throughout the almond-growing regions of California.

Interpretive Summary:

In 1971, the U. S. Food and Drug Administration (FDA) alerted the almond industry of California that the results of a recent FDA study indicated that almonds, as well as other tree nuts, are sometimes contaminated with aflatoxins (AFs). In recent years, an increasing number of notifications through the European Commission Rapid Alert System for Food and Feed (RASFF) indicated that those maximum levels have been

exceeded in almonds exported from the United States of America. In 2008, RASFF reported a total of 43 alerts or informational notifications concerning high levels of aflatoxins in almonds. Most of these notifications (30, 69.8%) were for almonds originating from the United States, followed by Australia (9, 20.9%). These notifications essentially amounted to a rejection or requirement for reprocessing of the shipment. These rejection costs which are further compounded because the affected shipper is subjected to more intensive testing of subsequent exported lots. The frequency of Rapid Alerts results in serious socio-economic consequences for consumers and producers. Consumers reduce their nut purchases based on bad publicity by the media while producers and exporters make sure that utilize all the available technology to avoid aflatoxin contamination and preventing any aflatoxin formation during storage and shipment so that the crop does not exceed regulatory limits. Positive samples result in potential rejection of loads and subsequent destruction, if costs are prohibitive of further sorting (Molyneux et al., 2007).

We have collected and stored about 1,500 isolates of *Aspergillus flavus* and *A. parasiticus* collected from almond orchards representing three major geographical regions. The results of this study showed that the aflatoxin producing isolates of *A. flavus* L-strains were predominant in both years. Overall, the incidence of toxigenic L-strains was 63% in 2007 and 80% in 2008. Incidences of toxigenic isolates varied widely among the orchards and years. In conclusion, the high incidences of aflatoxin producing *A. flavus* communities in orchards of California increase the risk of aflatoxin contamination of almonds.

In 2009 we collected almond samples from the same commercial orchards that were sampled in 2008 from northern, central, and southern California as soon as the nuts were shaken from the trees and again when the nuts were swept into windrows and picked up. Now, all the samples have been analyzed for aflatoxins. According to the data, orchard location has a major influence on aflatoxin contamination. While none of the almond samples from the three different orchards in northern California in both 2008 and 2009 crops contained any aflatoxins, 4.2% and 2.1% of the samples originating from central California in 2008 and 2009 crops, respectively, were contaminated with aflatoxins (mean of positive samples of 82.8 ng/g [ng/g = parts per billion, ppb). Aflatoxins were even more commonly found in almonds originating from southern California. For instance, 20.8 and 14.6% for the 2008 and 2009 samples, respectively, were contaminated with aflatoxins (mean of positive 31.5 ng/g total aflatoxins) **(Table 1).** Aflatoxins B1 and B2 were more common than G1 and G2 suggesting more infection by *A. flavus* than by *A. parasiticus.* However, a few samples had G1 aflatoxin which suggests that infections by *A. parasiticus* also occurred.

Interestingly, none of the samples from the orchards in northern California had any detectable aflatoxin levels from the five almond samples which were analyzed to quantify aflatoxin levels in parts per billion (ng/g kernels) per orchard. In general, more samples from the southern orchards had aflatoxins than in those from the central region. Furthermore, in 2008 all three orchards in the central region had G1 aflatoxin, which implies that almonds were infected by *A. parasiticus.*

The distribution of AFs between samples collected immediately after shaking the trees and those collected just before removing the nuts from the field, suggesting that aflatoxin can develop both on the tree as a result of infection by aflatoxigenic fungi before and at harvest and also during the time the nuts are drying on the orchard ground.

Insect feeding damage, especially damage caused by NOW (*Amyelois transitella*) on almonds and other nuts before and/or after harvest might contribute to the invasion and development of aflatoxigenic fungi. The results of NOW trapping from an almond orchard and plating showed that contamination of NOW with *A. flavus* during March through July increased two to threefold in August through October. The increased contamination of NOW moths later in season can be explained by the fact that kernels are infected after hull split and more propagules of *Aspergillus* fungi are found on the crop late in season. This contention is also supported by the results of the transmission experiment. Larvae that get contaminated with *Aspergillus* spores from a source of *Aspergillus* (sporulation on infected fruit) can crawl to healthy nuts, transfer the spores there, create wounds, and result in infection of healthy nuts by *A. flavus* and aflatoxin contamination. Therefore, NOW larvae serve a dual role in infection by *Aspergillus* and aflatoxin contamination of nut kernels: a) bringing the propagules of the aflatoxigenic fungus to the almond kernel; and b) creating wounds that are needed for infection. Because the almond seed coat imposes a barrier to infection (Gradziel and Wang, 1994), breaking the seed coat which is a barrier to fungal infection is essential for infection of nuts by *A. flavus/parasiticus* and aflatoxin contamination, which explains to close association between NOW damage and severity of aflatoxin contamination.

Mummies collected in two seasons and plated on specific media showed very high levels of infection by *A. flavus/parasiticus* and other *Aspergillus* spp. Almond mummies collected in winter 2009 from an orchard in Madera County were highly “contaminated” with *Aspergillus* section Flavi fungi. Surprisingly, 96% of the unsterilized kernels plated produced *Aspergillus* section Flavi along with 100% of the section *Nigri*. Furthermore, even the kernels that did not show any NOW damage were highly “contaminated” (84%) with *Aspergillus* section Flavi along with 100% of the section *Nigri.* Mummies from winter 2006 kept at cold storage (near 32ºF) were also highly “contaminated” with both *Aspergillus* section Flavi (98%) and section *Nigri* (98%). The high incidence of section Flavi in mummies explains why NOW moths emerged from mummies were highly contaminated with isolates of the Flavi section reported in last year’s final report (Almond Board of California, Final Reports 2008-09). All the isolates of *Aspergillus* section Flavi from these mummies were stored for future studies to determine the fungal species and the incidence of aflatoxigenic strains.

Application of the wheat product infested by the atoxigenic strain-AF36 to the floor of an almond orchard resulted in a moderate increase in *A. flavus*/*A. parasiticus* in the soil in 2007, the first year of the study, and this AF 36 strain survived well through the winter and spring. Although the level of the atoxigenic strain AF36 was very low in the soil before applying the wheat-AF36 product in 2007, after application almost all of the *A.*

flavus isolates were AF36. However, the level 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 after the final application) the level of AF36 in soil remained high in treated areas, indicating that the effect of application lasts more than a year. The increase in the percentage of AF36 isolates in the untreated soil over time from 1.7% before applying AF36 in June 2007 to 74% in September 2008 demonstrates the movement of AF36 from the treated areas to the untreated areas and its ability to displace other strains.

Before applying the wheat-AF36 product, the most common aflatoxin-producing fungus present in this orchard was *A. parasiticus*, which consistently produces aflatoxins at a high level in almonds. In addition, the S strain of *A. flavus* (which also tends to produce high levels of aflatoxins) was at approximately the same level as the L strain of *A. flavus* (which includes AF36 and many other atoxigenic strains). However, after applying the wheat-AF36 product, the level of the L strain of *A. flavus* increased until almost none of the *A. flavus*/*A. parasiticus* isolates in the treated areas belonged to the aflatoxin-producing *A. parasiticus* or *A. flavus* S strain. Thus isolates capable of producing high levels of aflatoxin were almost completely displaced by isolates producing low levels or no aflatoxins in the treated areas. In the untreated areas, the percentage of *A. flavus*/*A. parasiticus* isolates belonging to the L strain of *A. flavus* increased from 20% in June 2007 to 92% in September 2009, which was probably due to movement of fungi from the treated areas. Furthermore, applying AF36 did not significantly increase the level of hull decay by *A. flavus* for nuts from the treated areas. Substantially more nuts were decayed by fungi of the *A. niger* group than by those of the *A. flavus* group. Because the treatment is unlikely to have caused more decay by *A. niger* group or *A. ochraceus* group, the higher levels of decay were probably due to factors other than applying AF36.

Materials and Methods:

1. Determine the spatial pattern of *Aspergillus* strains in northern, central, and southern almond- growing regions in California.

This portion of research was reported in last year's annual report 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 (see objective 2).

2. Identify risk factors and spatial patterns associated with aflatoxin development in California almonds.

Toxigenicity of Aspergillus populations.

A major risk factor for aflatoxin contamination in almonds orchards is the aflatoxin-producing ability of the *Aspergillus* section Flavi communities within the field. In order to determine which fungi produce the greatest quantities of aflatoxin and the frequency of occurrence of atoxigenic *A. flavus* strains, the aflatoxin-producing ability was quantified from *A. flavus* isolates taken from almond orchard soil across California. In 2007 and

2008, over 900 isolates belonging to the *A.* section Flavi were isolated, from 28 almond producing orchards located in the northern, central, and southern valley of California. From each orchard, 5 to 15 isolates were randomly picked and over 500 were analyzed for aflatoxin content in liquid fermentation. The aflatoxin was extracted with acetone and methylene chloride and quantified with a High Performance Liquid Chromatography (HPLC). This research is still in progress.

Aflatoxin contamination of almond nuts in the field.
Samples of almond nuts were collected throughout at harvest (August to September) in both 2008 and 2009. Only samples of the cultivar Nonpareil were collected in the study because this cultivar represents the largest portion of almond cultivated acreage in California (37.7%) and it seems to be most susceptible to aflatoxin contamination mainly due to its extra soft and thin shell and high navel orangeworm (NOW) damage. Sixteen samples per orchard from three orchards in each northern (Glenn County), central (Stanislaus County), and southern (Kern County) regions were collected on the day the nuts were shaken to the ground and similar samples when the nuts were swept in the middles on the day before removing the nuts to take them to the processing plant or stockpile. **Figure 2** represents a map of the sites where samples were collected. Samples included in shell nuts with the hulls attached and weighed approximately 10 kg. These nuts left to dry for 3 to 5 additional days under the sun on a cemented flat surface and sent to a processor for shelling under commercial shelling conditions. All the kernels samples were stored at -18^{o}C to inactivate any further mold development and aflatoxin production. A total of 288 samples were used for aflatoxin analyses.

After shelling, approximately 2 kg of almond kernels were ground in a Blixer® 6 vertical cutter-mixer (Robot Coupe, Ridgeland, MS, USA) at high speed for 30 sec. to fulfil the requirement of “grind finely” and “mixed thoroughly”, which is a critical point in the EC directive (European Commission, 1998). Standards of aflatoxins B_1, B_2, G_1, and G_2 were purchased from Sigma-Aldrich (St. Louis, MO, USA) as a crystalline powder form. Aflatoxin analyses were done with a HPLC at Kearney Agricultural Center. Vicam AflaTest® immunoaffinity columns (IAC) were used. The limits of detection (LOD) of aflatoxins were 0.29 ppb for B_1 and G_1, 0.12 ppb for B_2, and 0.15 ppb for G_2. For recovery studies, AFs-free samples (50 g almonds) were spiked at three levels of each aflatoxin (2, 5, and 10 ng g^{-1}). Three replicates for levels of 5 and 10 ng g^{-1} and ten replicates for level of 2 ng g^{-1} were performed. AFs concentrations were determined using the HPLC as previously described.

Statistical analyses.
Analyses were performed with the Statistical Analysis System (SAS Institute, Inc., v.9.2). Fisher’s least significant difference (LSD) test at 5% probability level was used to test comparisons between the AFs contents in two times of sampling: immediately after shaking the trees and at the time when the nuts were removed from the orchard.

Navel orangeworm (NOW) studies.
In 2009 and 2010, we continue sampling periodically NOW moths from an almond orchard in Madera County to follow on the population levels of NOW moths carrying

viable propagules of *Aspergillus* section Flavi fungi. A total of 50 moths were plated per trap when there were >50 moths per trap and all the moths were plated when there were <50 moth per trap. Moths were plated onto Si10 salt medium; the plates were incubated at 30ºC (86ºF), and recorded after one week incubation.

Transmission of Aspergillus by NOW.
To determine how NOW larvae contribute to aflatoxin contamination, we designed an experiment by placing larvae 7-days after hatching in 9-cm Petri dishes together with 7 healthy almond kernels, using the following 4 treatments: Trt 1: larvae only, without *A. parasiticus* as a noninfested control; Trt 2: five larvae were infested with spores by crawling on top of a sporulating culture of *A. parasiticus* and enclosed with the healthy almonds in the Petri dish; Trt 3: an *A. parasiticus* infected fruit was enclosed in the center of the Petri plate with the healthy almond kernels and 5 NOW larvae of the same age as above in Trt 2; and Trt 4: as in Trt 3 by placing an infected fruit in the center of the plate but without any larvae. The *A. parasiticus* isolate used was selected because of its ability to produce high aflatoxin levels. Five replicated Petri dishes were used per treatment and all the plates were incubated at room temperature 23±1ºC (77ºF) for 10 days. All almond kernels in each Petri dish were collected as a replicate sample and analyzed for aflatoxins using a HPLC. The experiment was repeated twice.

Aspergillus in almond mummies.
To determine the incidence of *Aspergillus* section Flavi on mummies, more than 500 mummies were collected from a Nonpareil almond orchard in Madera County. The mummies were shelled and the kernels were classified as healthy or mouldy. The mouldy kernels were classified according to their color as “black,” green,” and “white.” Pieces of mouldy kernels (without surface sterilization) of the “green” category were plated on CYA media; the plates were incubated at 86ºF, and the *Aspergillus* species were recorded 1 week later. *Aspergillus* isolates were classified in the sections Flavi (*A. flavus, A. parasiticus,* and *A. tamarii*) and section Nigri (*A. niger, A. japonicus,* and *A. carbonarius*).

3. Determine the spread and survival 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 hyphae-colonized steam sterilized wheat seed to the soil at a rate of 10 pounds of wheat per acre (the same product and same application rate as used for commercial cotton fields and commercial pistachio orchards) in a research almond (cv. Nonpareil) orchard at the Nickels Soil Laboratory on 28 June 2007 and 2 July 2008 (the wheat-AF36 product was applied to the same areas for both years). No AF36 was applied after the application in July 2008. The experimental design was a randomized complete block design with 3 replications. After applying the wheat-AF36 product to the orchard floor, the orchard was irrigated with micro-sprinklers on that day and at its normal schedule throughout the summer. 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, and 1 September 2009. 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 (86ºF) 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 (86ºF) 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. Strain identification was done using the Vegetative Compatibility Group procedure (to fulfill objective 4).

Results and Discussion

1. Determine the spatial pattern of *Aspergillus* strains in northern, central, and southern almond - growing regions in California.

Results for this objective were presented in last year's annual report (refer to Final Research Projects 2008-2009 of Almond Board of California).

2. Identify risk factors and spatial patterns associated with aflatoxin development in California almonds.

Toxigenicity of Aspergillus populations.

The results of this study showed that the aflatoxin producing isolates of *A. flavus* L-strains were predominant in both years. Overall, the incidence of toxigenic L-strains increased from 63% in 2007 to 80% in 2008. Incidence of atoxigenic *A. flavus* communities decreased in all regions of the valley from 2007 to 2008. In year 2007, atoxigenic isolates were more common in the southern region (52%) and less in the north (42%). In 2008 there was no difference among the regions observed. Incidences of toxigenic isolates varied widely among the orchards and years. In conclusion, the high incidences of aflatoxin producing *A. flavus* communities in orchards of California increase the risk of aflatoxin contamination of almonds.

Aflatoxin contamination of almond nuts in the field.

The occurrence and levels of AFs in California almonds from nine different orchards in three geographic areas in California for both 2008 and 2009 crops are summarized in **Table 1.** The contamination levels in 20 out of 288 almond samples (6.9%) ranged from 2.17 to 102.32 µg kg^{-1} (ppb) for AFB_1 and from 2.17 to 103.33 µg kg^{-1} for total AFs. **Figure 1** shows the distribution of total AFs in almonds for 2008 and 2009 samples. The mean contamination levels in positive almond kernels were 38.1 and 40.5 ng g^{-1} for AFB_1 and total AFs, respectively. Sixteen out of 20 positive samples exceeded the maximum limit of 4 µg kg^{-1} set by EU regulations for total AFs. The four remaining contaminated samples contained total AFs in the range of 2.17-3.76 µg kg^{-1}. However,

all the contaminated samples showed levels higher than the suggested EU limit (2 µg kg^{-1}) for AFB_1. The results confirm previous observations by Schade et al. (1975), who reported that 10 of 74 (13.5%) samples of unsorted, inshell California almonds had up to 107 ng g^{-1} total AFs and 13 of 27 samples of diced almonds had 119 ng g^{-1} total AFs. In another study, on average, only 1 kernel from the field in 26,500 (approximately 30 kg) of shelled almonds contained AFs, with the high correlation of contamination with damaged nuts which are removed by standard sorting procedures (Fuller et al., 1977). Gurses (2006) detected AFs in 3 of 13 hard shell Turkish almond samples at levels ranging from 1 and 13 ng g^{-1}. However, AFs were not found in 3 almond samples from market in the capital of Bahrain (Musaiger et al., 2008). More recently, in Japan, Sugita-Konishi (2010) demonstrated that AFs occurred in 6 out of 24 almond samples purchased from local retail shops, with maximum value of 1.06 ng g^{-1}. The cultivar is one of the critical factors in the occurrence and level of AFs in almonds. Most likely these analyzed samples are hard shell cultivars that usually are more resistant to mold damage and aflatoxin accumulation and therefore direct comparisons with contamination of Nonpareil almonds cannot be made.

Aflatoxin B_1 and AFB_2 have been detected more than the other AFs. This might be due to both occurrence and invasion of nuts by *A. flavus* rather than *A. parasiticus*. Aflatoxin B_1 comprised most of the aflatoxin found during analysis. The occurrence of AFB_2 in all the samples was 5.2%, ranging from 0.55 to 3.66 ng g^{-1}. While only two almond samples of the 2009 crop that originated from the central California were contaminated with AFG_1, none of the samples contained any AFG_2 **(Table 1)**.

According to our data, the geographic origin of almond kernels has a strong influence on aflatoxin contamination. While none of the almond samples from three different orchards in northern California in both 2008 and 2009 crops contained any aflatoxins (**Figure 2**), 4.2% and 2.1% of the samples originating from central California in 2008 and 2009 crops, respectively were contaminated by aflatoxins (mean of positive samples of 82.8 ng/g). Aflatoxins were found more frequently in almonds originating from southern California. For instance, 20.8 and 14.6% for the 2008 and 2009 samples, respectively, were contaminated with aflatoxins (mean of positive 31.5 ng/g total aflatoxins) **(Table 1).**

It is well-known that climate influences contamination of various crops, in part due to direct effect on the aflatoxin producing fungi. The contamination process is frequently broken down into two phases with the first phase occurring on the developing crop and the second phase affecting the crop after maturation. Rain and temperature influences the phases differently with dry hot conditions favoring the first and warm, wet conditions favouring the second (Cotty and Jaime-Garcia, 2007). California is the most geographically diverse state in the United States and it has generally a Mediterranean climate, with cool, rainy winters and dry summers. Northern parts of the state average higher annual rainfall than central and southern California. The average air temperatures from representative CIMIS stations in Glenn, Stanislaus and Kern Counties were 9.5-25.3^{o}C, 8.4-22.1^{o}C and 9.8-26.1^{o}C during February through August 2008 and 2009, respectively. The levels of mean relative humidity from these stations in

Glenn, Stanislaus and Kern Counties were between 51-71%, 48-79% and 41-67% during February though August 2008, and between 50-77%, 53-81% and 38-68% during February through August 2009, respectively (CIMIS monthly reports).

Our data also showed that the total levels of aflatoxins were not significantly different ($P > 0.05$) between samples collected immediately after shaking the trees and those of samples collected just before removing the nuts from the field. This suggests, at least during the years of this study, that most aflatoxin contamination of almonds probably occurs while the nuts are on the tree as a result of fungal contamination by aflatoxigenic moulds with some additional contamination during the time the nuts are drying on the orchard floor. Infection of tree nuts with aflatoxigenic *Aspergillus* moulds probably occurs most often in the field after hull split before and/or during harvest while the kernels are still moist. Insect feeding damage, especially damage caused by NOW (*Amyelois transitella*) on almonds and other nuts before and/or after harvest might contribute to the invasion and development of aflatoxigenic fungi (Schade et al., 1975). The NOW attacks the almond fruit after hull-split while they are drying on the tree. During drying, high temperatures in the orchard and moisture in the hulls provide an environment for the growth of some fungi. These conditions, in combination with injury caused by the NOW, favor the growth of *A. flavus* and *A. parasiticus*. The activity of NOW may continue after harvest, and this activity may provide moisture that would prolong fungal growth and toxin production (Phillips et al., 1980). Similarly, pistachio kernels infested by the NOW had substantially more infections by *A. niger*, *A. flavus* or *A. parasiticus*, and *A. ochraceus* or *A. melleus* and had 84% of all aflatoxin detected while the nuts not infested by NOW had 16% of all aflatoxins (Doster and Michailides, 1994). In another work, aflatoxin lots averaged 31.7 and 3.47 ng g^{-1} aflatoxin for 100% insect damaged Ne Plus and Nonpareil almonds, respectively (Schatzki and Ong, 2001).

The aflatoxin-producing fungi, *A. flavus* and *A. parasiticus*, are widespread, and occur in the soil of nearly every almond orchard (ABC Aflatoxin Final Report 2008-2009). These fungi can invade and produce aflatoxins before harvest and, during drying and storage. In a previous work by Purcell et al. (1980) *A. flavus* was found to be more common on almonds in warmer areas of California than in cooler areas. In a previous study, Michailides et al. (2009) showed that *A. parasiticus* was more common in northern California orchards than in those of central and southern San Joaquin Valley, although *A. flavus* was recovered from all northern, central, and southern commercial orchards. In almond kernels from California, the *A. niger* group was the most common, but the *A. flavus, A. glaucus, A. ochraceus,* and *A. wentii* groups are also found. Similarly, Bayman et al. (2002) found that California almonds had *A. niger* at the highest incidence followed by *A. ochraceus*, *A. melleus*, *A. fumigatus,* and *A. flavus.* The range of densities of *A. flavus*/*A. parasiticus* in California almond orchards was greater (2-219 CFU g^{-1}) than the 2-36 CFU g^{-1} in pistachio and 1-9 CFU g^{-1} in fig orchards.

Navel orangeworm (NOW) studies.
The 2009 trapping of NOW moths has been completed and shown in **Figure 3**. However, the 2010 NOW trapping in an orchard in Madera County is still in progress.

Clearly, the 2009 data show that NOW moths that most likely emerge from mummies in spring show some small levels of *A. flavus* (3 to 5%). However, the incidence of NOW contamination with *A. flavus* increased two to threefold during August through October (**Figure 3**). The increased contamination of NOW moths late in season can be explained by the fact that this is the time when more propagules of *Aspergillus* are found on the crop and when nuts are infected by the *Aspergillus* fungi.

Transmission of Aspergillus by NOW.
Only the kernels that were exposed to both *A. parasiticus* and larvae developed significantly higher levels of aflatoxins (**Figure 4**). Specifically, in treatment 3, the NOW larvae first attacked the *Aspergillus*-infected fruit, becoming infested with the *Aspergillus* spores, and then infested the healthy kernels, resulting in infections of the almond kernels by A. parasiticus and aflatoxin contamination. In Treatment 4, where the source of inoculum was present but the larvae absent, there was only a very small amount of aflatoxins (**Figure 4**). Thus, NOW larvae get contaminated with spores of *Aspergillus* as they crawl on infected fruit with sporulating *Aspergillus.* Thus these larvae serve a dual role in infection by *Aspergillus* and aflatoxin contamination of nut kernels not only by bringing the propagules of the fungus to the substrate but also and, perhaps most importantly, by creating wounds that are needed for infection. Because the almond seed coat imposes a barrier to infection (Gradziel and Wang, 1994), breaking this barrier is a contributing factor to aflatoxin contamination.

Aspergillus in Almond Mummies.
The almond mummies collected in winter 2009 from an almond orchard in Madera County were highly infected with *Aspergillus* section Flavi fungi. Surprisingly, 96% of the kernels plated produced *Aspergillus* section *Flavi* along with 100% of the section *Nigri*. Furthermore, even the kernels that did not show any NOW damage were highly infected (84%) with *Aspergillus* section *Flavi* along with 100% of the section *Nigri.* Mummies kept at cold storage (near 32ºF) were also highly infected with both *Aspergillus* section *Flavi* (98%) and section *Nigri* (98%). The high incidence of section *Flavi* in mummies explains why NOW moths emerged from mummies were highly contaminated with isolates of the *Flavi* section reported in last year's final report (Almond Board of California, Final Reports 2008-09). All the isolates of *Aspergillus* section *Flavi* from these mummies were stored for further studies to determine the aflatoxigenic strains. In a previous study we showed that 2% of almond mummies had obvious sporulation by *Aspergillus* section Flavi, suggestion infection by *A. flavus*. Active sporulation was not found in the 2009 mummies, however, their kernels had a general "green" appearance which is an indication that *Aspergillus* section Flavi were present on the surface of the kernels ("contaminated" with *Aspergillus*).

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

Application of the wheat-AF36 product to the orchard floor resulted in a moderate increase in *A. flavus*/*A. parasiticus* in the soil in 2007, the first year of the study (**Figure**

5). Approximately the same density of *A. flavus*/*A. parasiticus* in the soil occurred in August 2007 and July 2008 (prior to applying AF36 in 2008), suggesting that the fungus survived well through the winter and spring. In addition, the density of *A. flavus*/*A. parasiticus* increased further during the second year in the treated areas, but showed a moderate decrease in 2009 when no additional AF36 was applied (**Figure 5**). For comparison, the density of *A. niger* in the soil during the period of the study ranged from 121 to 499 propagules / g soil (depending on the sample date) with mean values of 230 and 179 propagules / g soil for the AF36-treated and untreated areas, respectively.

Although the level of the atoxigenic strain AF36 was very low in the soil before applying the wheat-AF36 product in 2007, after application almost all of the *A. flavus* isolates were AF36 (**Figure 6**). This suggests that applying the wheat-AF36 product was very effective in introducing the atoxigenic strain AF36 under the conditions present in this almond orchard. The level 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 final application) the level of AF36 in soil remained high in treated areas, indicating that the effect of application lasts more than a year. The increase of AF36 in the untreated areas over time from 1.7% before applying AF36 in June 2007 to 74% in September 2008 demonstrates the movement of AF36 from the treated areas to the untreated areas.

Before applying the wheat-AF36 product, the most common aflatoxin-producing fungus present was *A. parasiticus* (**Figure 7**), which consistently produces aflatoxins at a high level. In addition, the S strain of *A. flavus* (which also tends to produce high levels of aflatoxins) was at approximately the same level as the L strain of *A. flavus* (which includes AF36 and many other atoxigenic strains). However, after applying the wheat-AF36 product, the level of the L strain of *A. flavus* increased until almost none of the *A. flavus*/*A. parasiticus* isolates in the treated areas belonged to the aflatoxin-producing *A. parasiticus* or *A. flavus* S strain (**Figure 7**). In the untreated areas, the percentage of *A. flavus*/*A. parasiticus* isolates belonging to the L strain of *A. flavus* increased from 20% in June 2007 to 92% in September 2009, which was probably due to movement of fungi from the treated areas.

Applying AF36 did not significantly increase the level of hull decay by *A. flavus* for nuts from the treated areas (**Table 2**). Substantially more nuts were decayed by fungi of the *A. niger* group than by those of the *A. flavus* group. For example, 1.03% of the nuts had hulls decayed by *A. niger* group in 2008 compared to only 0.03% by *A. flavus* group (**Table 2**). Nuts from treated areas were more likely to have hulls decayed by the various *Aspergillus* groups than those from untreated areas (**Table 2**). Because the treatment is unlikely to have caused more decay by *A. niger* group or *A. ochraceus* group, the higher levels of decay were probably due to factors other than applying AF36. Although many different fungi within the *A. flavus* group decayed the nuts (**Table 3**), only certain species and strains produce aflatoxins. The *A. flavus* S strain and *A. parasiticus* consistently produce aflatoxins (frequently at high levels), while the atoxigenic *A. flavus* strain AF36 and *A. tamarii* never do.

4. Determine the incidence of the atoxigenic strain AF36 among *A. flavus* isolates occurring in commercial almond orchards.

Strain determination among 727 *A. flavus* isolates analyzed in 2007 and 680 isolates in 2008 showed that 6.9% and 4.7% of isolates, respectively, belonged to the atoxigenic strain AF36. This is good news because this is the strain that is expected to be registered as a biopesticide for combating aflatoxin contamination of pistachios. It is our hope it will then be registered for almonds.

Research Effort Recent Publications:

Luo, Y., W. Gao, M. Doster, and T. J. Michailides. 2009. Quantification of conidial density of *Aspergillus flavus* and *A. parasiticus* in soil from almond orchards using real-time PCR. J. of Applied Microbiology 106:1649-1660.

Kabak, B., Luo, Y., Reyes, H., Michailides, T. J. 2010. Preharvest contamination of California almonds with aflatoxins. J. of Food Protection (in press).

Abstracts:

Luo, Y., H.C. Reyes, D.P. Morgan, and T.J. Michailides. 2009. Effect of relative humidity on infection of almond kernels by *Aspergillus flavus* and *A. parasiticus* and levels of aflatoxin contamination. Phytopathology, July Supplement, APS Meeting Abstracts 99(6):S77.

Michailides, T.J., M.A. Doster, D.P. Morgan, H. Eveillard, and T. Charbaut. 2009. Levels of *Aspergillus flavus* and *A. parasiticus* in soils of almond orchards. Phytopathology, July Supplement, APS Meeting Abstracts 99(6):S85.

Michailides, T. J. 2009. Aflatoxin Contamination of Almonds – almond course, Western Farm Press (online course).

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Luo, Y., Gao, W., Doster, M., and Michailides, T. J. 2009. Quantification of conidial density of *Aspergillus flavus* and *A. parasiticus* in soil from almond orchards using real-time PCR. Journal of Applied Microbiology Vol. 106:1649-1660.
Michailides, T. J., Doster, M. A., Morgan, P., Eveillard, H., and Charbaut, T. 2009. Levels of *Aspergillus flavus* and *A. parasiticus* in soils of almond orchards. Phytopathology 99:S85 (Abstr.).
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Table 1. Occurrence of AFs in California almonds collected from commercial orchards in 2008 and 2009.

Origin of samples	Year	Samples positive/total (%)				AFs concentration (min-max, ng g^{-1})[a]				Total AFs in positive samples (mean±sD, ng g^{-1})
		AFB_1	AFB_2	AFG_1	AFG_2	AFB_1	AFB_2	AFG_1	AFG_2	
Northern California	2008	0/48 (0)	0/48 (0)	0/48 (0)	0/48 (0)	<LOD[b]	<LOD	<LOD	<LOD	<LOD
	2009	0/48 (0)	0/48 (0)	0/48 (0)	0/48 (0)	<LOD	<LOD	<LOD	<LOD	<LOD
Central California	2008	2/48 (4.2)	2/48 (4.2)	2/48 (4.2)	0/48 (0)	68-89.3	<LOD-0.6	5.6-17.3	<LOD	91.5±5.7
	2009	1/48 (2.1)	1/48 (2.1)	0/48 (0)	0/48 (0)	<LOD-70.6	<LOD-3.5	<LOD	<LOD	74.1
Southern California	2008	10/48 (20.8)	6/48 (12.5)	0/48 (0)	0/48 (0)	2.4-102	0.7-2.2	<LOD	<LOD	37.6±45.9
	2009	7/48 (14.6)	6/48 (12.5)	0/48 (0)	0/48 (0)	3.8-69.5	0.6-3.7	<LOD	<LOD	25.3±25.0

[a] There were no differences ($P > 0.05$) in the aflatoxin positive samples between the two times of sampling: immediately after shaking the trees and at the time when the nuts were removed from the orchard, suggesting that aflatoxin can develop on the tree and also during the time the nuts are drying on the orchard ground.

[b]LOD=Limit of detection (0.29 ng g^{-1} for AFB_1 and AFG_1, 0.12 ng g^{-1} for AFB_2, and 0.15 ng g^{-1} for AFG_2).

Table 2. Incidence of hulls and external surface of shells 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/shells 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

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

Table 3. Involvement of specific *Aspergillus flavus* group fungi in the decay of hulls and external surface of shells of Nonpareil almonds 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.

			Number of nuts decayed by specified *Aspergillus* fungus				
Date	Treatment	Nuts evaluated	*A. flavus* L strain				
			AF36	Non-AF36	*A. flavus* S strain	*A. parasiticus*	*A. tamarii*
2007	AF36	15,835	10	8	13	0	7
	Control	14,477	0	0	0	0	0
2008	AF36	9,000	0	1	0	3	0
	Control	9,000	0	1	0	1	0
2009	AF36	9,000	0	2	0	2	0
	Control	9,000	0	1	0	0	0

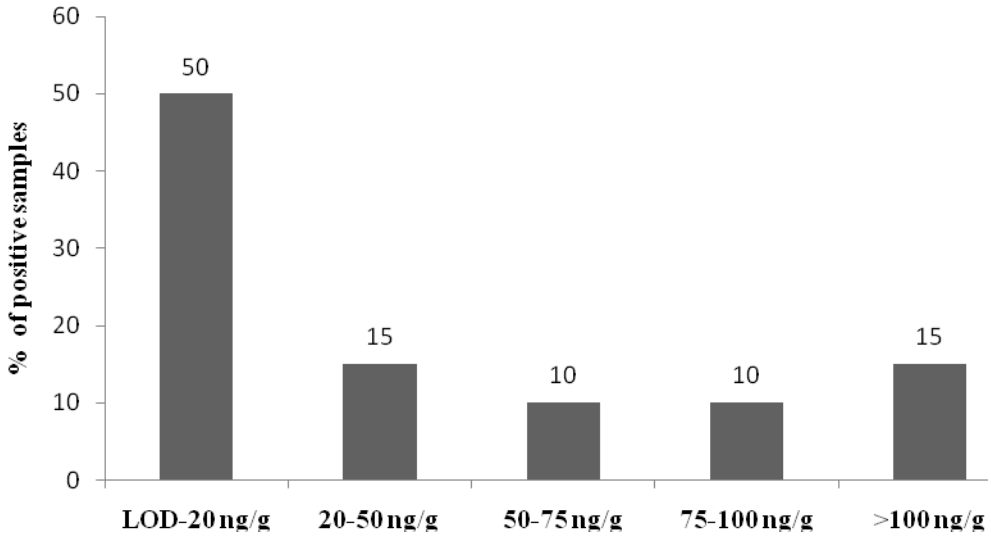

Figure 1. Distribution of aflatoxins in contaminated almond samples collected from commerical orchards in 2008 and 2009. LOD = limit of detection

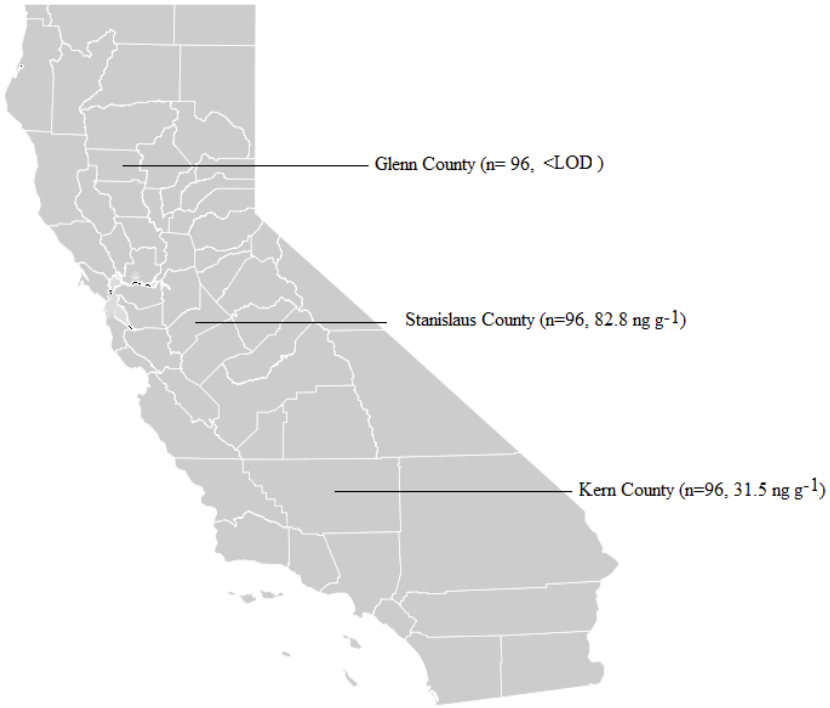

Figure 2. A California map showing the regions where almond samples in different region of California state (number of analyses samples, and mean (ng g^{-1}) of positive samples in parentheses, LOD = limit of detection)

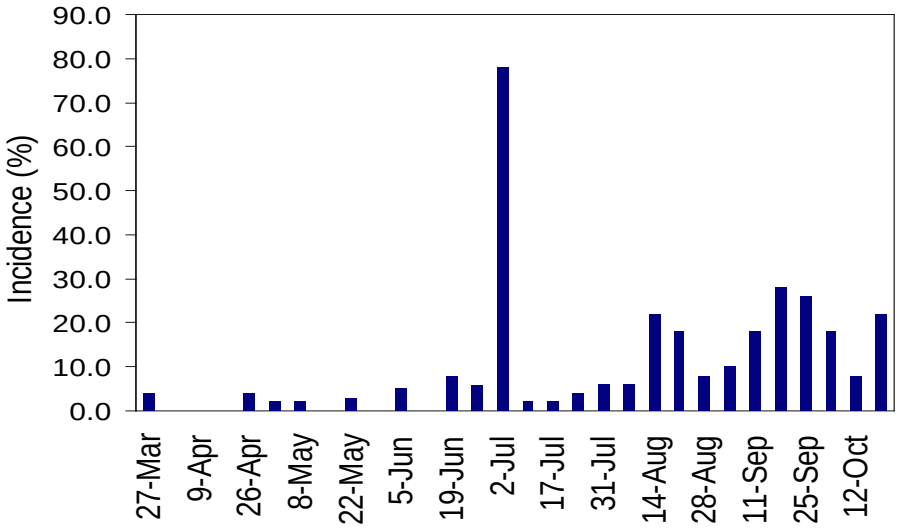

Figure 3. Incidence of *Aspergillus* section *Flavi recovered* from NOW moths trapped on sticky traps in an almond orchard in Madera County during March to October 2009.

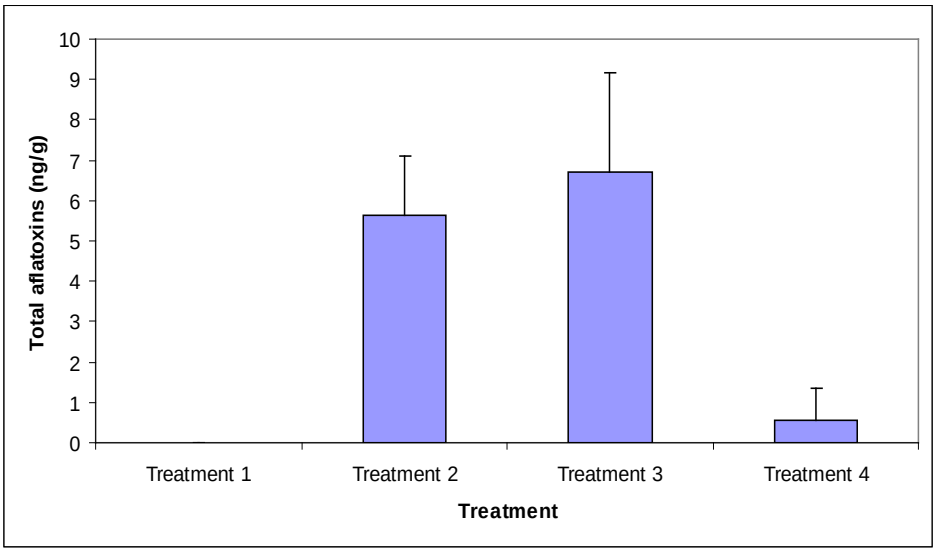

Figure 4. Amounts of aflatoxins produced in healthy almond kernels caged with and without larvae of navel orangeworm in Petri dishes. Treatment 1 = healthy kernels without *Aspergillus parasiticus* or NOW larvae; Treatment 2 = with NOW larvae contaminated with spores of *A. parasiticus*; Treatment 3 = with NOW and almond kernels infected entirely by *A. parasiticus*: and Treatment 4 = with an almond kernels infected entirely by *A. parasiticus* (lines on top of the bars indicate standard error).

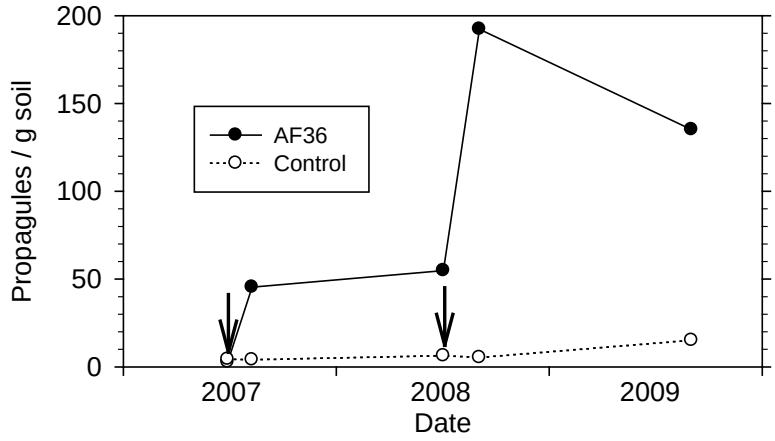

Figure 5. Density of *Aspergillus flavus*/*A. parasiticus* in soil collected from areas treated with the wheat-AF36 product or from untreated areas in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product was applied on 28 June 2007 and 2 July 2008 (arrows).

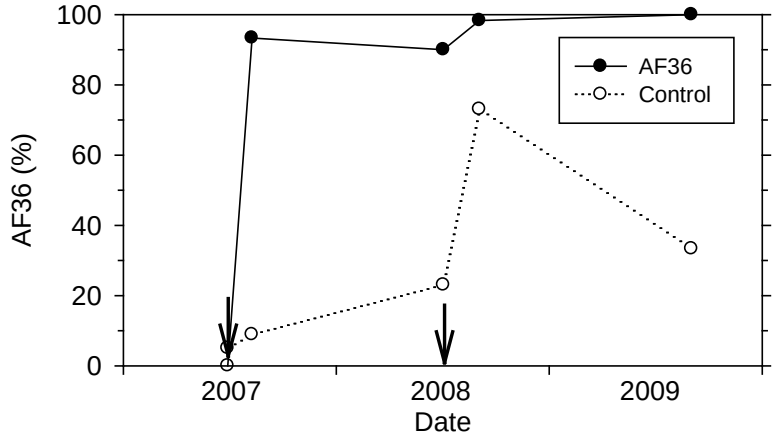

Figure 6. Percentage of *Aspergillus flavus* isolates belonging to the atoxigenic strain AF36 for isolates from soil collected from the areas treated with the wheat-AF36 product or from untreated areas in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product was applied on 28 June 2007 and 2 July 2008 (arrows).

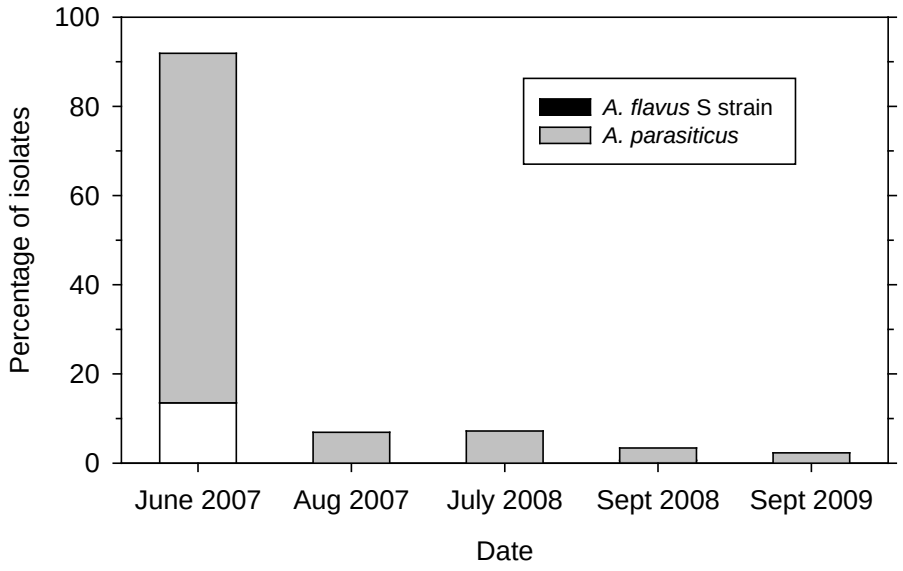

Figure 7. Percentage of *Aspergillus flavus*/*A. parasiticus* isolates that are the aflatoxin-producers *A. parasiticus* and *A. flavus* S strain for isolates from soil collected from areas treated with the wheat-AF36 product in a research almond orchard at the Nickels Soil Laboratory.