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

Project No.: **07-AFLA1-Michailides**

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Interpretive Summary:

In 2007, we studied the distribution and densities of *Aspergillus* section *Flavi* in 29 commercial almond orchard soils in the north (Sacramento Valley), center (Madera County), and south (Kern County) Central Valley. All three species in the section *Flavi* group (*Aspergillus flavus, A. parasiticus,* and *A. tamarii*), were isolated from the majority of these almond orchard soils. Of these species, *A. flavus* and *A. parasiticus* produce aflatoxins while *A. tamarii* does not. Contamination of some nut samples (kernels, hulls, and shells), with only B, or G, and B and G aflatoxins suggests that both of these fungi (*A. flavus/A. parasiticus*) are involved in infection of almonds. Aflatoxin analyses of the 2007 samples revealed more aflatoxin positive samples collected from central and

southern regions than the samples from the northern region. The higher frequency of positive samples from the central and southern regions can be partially explained by the lower rainfall in these areas, as it was shown in other crops (i.e., corn). We also detected a higher frequency of aflatoxin positive hull and shell samples than aflatoxin positive kernels, suggesting that some of the infections that started while the nuts were on the ground had not progressed enough to reach, infect, and contaminate the kernels with aflatoxins. Therefore, the time and conditions the nuts are lying on the ground to dry may be considered as a risk factor. Another risk factor can be the density of aflatoxigenic fungi in the orchard soil, although the direct relationships between fungal density and aflatoxin contamination levels have not been yet defined.

Another important risk factor can be the kinds and strains of *A. flavus* and *A. parasiticus* present in almond orchards. For instance, it is important to know the ratio of the densities of S and L strains of *A. flavus* in the orchard. Our results showed that the densities of both the S strain of *A. flavus* and *A. parasiticus* are higher in northern region than the other regions. From other studies using isolates collected from pistachio orchards, we found that 57% of the L isolates did not produce aflatoxins while 100% of the S strains produced B aflatoxins. In addition, 100% of the *A. parasiticus* isolates produced both B and G aflatoxins. Although the risk for aflatoxin is high in northern orchards based on the densities and species of aflatoxigenic *Aspergillus* spp., apparently there are other factors (most likely environmental and lower NOW damage), that lead to a lower frequency of aflatoxin positives from this region.

Our 2007 results also showed that there are a lot of atoxigenic isolates of *A. flavus* in almond orchards, and most importantly, the bio-pesticide AF36 (atoxigenic strain of *A. flavus*) ranged from 3.5 to 12.6% in almond orchard soils. The AF36 strain is registered on cotton to reduce aflatoxin contamination of cottonseed and it is conditionally registered in California to treat 3,000 acres of pistachio (under an experimental use permit), for the reduction of aflatoxin contamination. Furthermore, we initiated a study in 2007 by applying AF36 in an experimental almond orchard at Nickels Estates in Colusa County to gather efficacy data and facilitate and expand the registration of AF36 to almonds.

Our results also showed that both larvae and moths of NOW can be heavily contaminated with *A. flavus, A. parasiticus*, and *A. tamarii*, as well as with other species of *Aspergillus* (*A. ochraceous, A. terreus, A. melleus*, etc.). This high incidence of these fungi carried by NOW moths suggests that NOW insect not only will create the wounds for infection but also can introduce the aflatoxigenic pathogens into the right site for infection of hulls, shells, and kernels. This may explain why there is a very strong association between NOW damage of almond nuts and aflatoxin contamination for California nuts. We detected higher levels of contamination of NOW moths emerged from mummies collected from the ground and kept in plastic buckets than those emerged from tree mummies, suggesting that this insect can get contaminated from the soil more so than the moths that emerged from mummies of infested nuts on the trees and trapped on sticky traps in commercial orchards.

The levels of aflatoxins detected in the 2007 samples collected from windrows were very low, and all were below the EU tolerance. However, the multiple rapid alerts for California almonds exceeding the EU tolerance suggests that higher levels of aflatoxins may develop after the nuts are removed form the orchard, perhaps when the nuts are stockpiled and/or during transport to the foreign destination markets.

Objectives:

1. The main objective was to determine the levels of *A. flavus/A. parasiticus* in almond orchards and risk factors in these orchards associated with aflatoxin contamination.

2. Determine the levels of larvae and navel orangeworm moths contaminated with *A. flavus /A. parasiticus* and other *Aspergillus* species. [This objective evolved as we were involved in a cooperative project with Dr. Siegel (USDA, ARS, SJVERC, Parlier, CA). The project began because the initial plating of navel orangeworm moths emerged from mummies on specific agar media developed high levels of *A. flavus*].

Materials and Methods:

Levels of *A. Flavus* and *A. Parasiticus* in Soil of Almond Orchards

Soils samples were collected during April to May 2007 from eleven (#1 to #11), nine (# 12 to #15 & #17 to #21), and eight (# 25 to #32), Nonpareil almond orchards from the northern, central, and southern part of the state where significant almond acreage is located (**Figure 1**). We decided to sample these three regions since they have different rainfall averages. For instance, an average of 2.23 inches (or 5.67 cm) per month in the north (Chico weather station), 0.94 inches (or 2.39 cm) per month in the Central Valley (Madera weather station), and 0.5 inches (or 1.27 cm) per month in the South (Bakersfield weather station).

Each soil sample was a composite of 10 sub-samples collected by walking on a triangular pattern in each orchard. The three composite samples (each of the 10 sub-samples for each side of the triangle) represented the three replicated soil samples per orchard. The soils were transported to the laboratory in an ice chest and kept at the room temperature (23 ± 1°C) for a week to dry. Then, the dried soil was sieved through a #2 sieve and ground using a pestle and mortar.

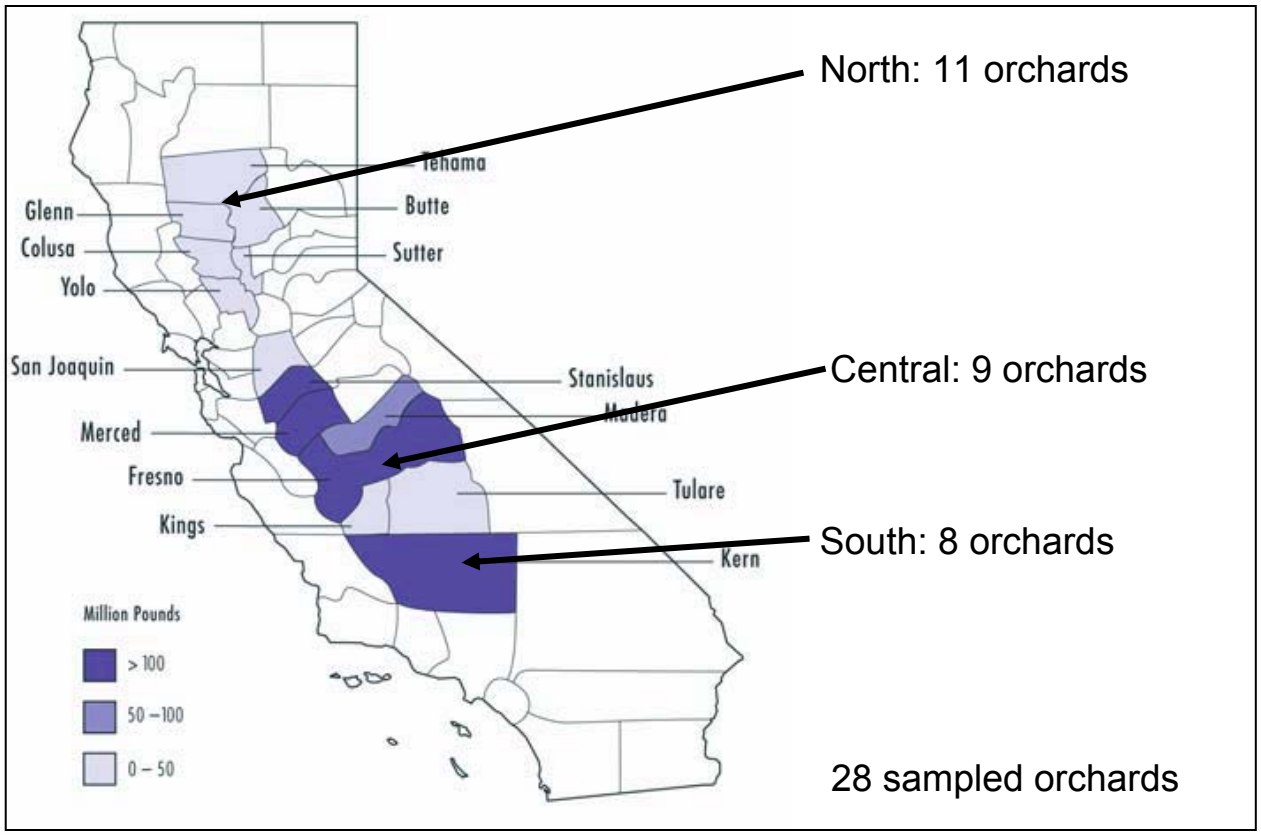

Figure 1. Location of almond orchards in California where soil samples were collected in 2007.

Procedures used to recover and enumerate the isolates have been reported earlier for soils collected from pistachio and fig orchards. For instance, to isolate the *A. flavus* propagules, 0.2 grams from each replicated soil sample were spread as evenly as possible over the surface of the isolation culture media (Si10 medium). The Si10 media contains enough dichloran (Botran) to reduce substantially the growth of *Aspergillus niger.* For each replicate, ten Si10 plates were used (i.e., a total of 30 Si10 plates per orchard).

The cultures were incubated at 35°C (95°F) for 1 week when *Aspergillus* colonies were counted. After incubation, all the putative colonies *A. flavus*, *A. parasiticus,* or *A. tamarii* were transferred twice onto Czapek Yeast Extract Agar (CYA) media (to make sure that pure culture were obtained), and the colonies were identified to species as *A. flavus, A. parasiticus,* and *A. tamari* based on macroscopic features in culture and microscopic characteristics of their spore producing structures (coniodiophores) and conidia spores. The results are presented as colony forming units (cfu) per gram of soil by orchard. In addition, isolates were identified by whether they produced small (S), medium (M), or large (L) size sclerotia to the respective **S, M,** and **L** strains, (see Appendix I). Ten *A. flavus* per replicate soil sample (i.e., 30 *A. flavus* per orchard), were placed each into 3 ml of sterile, deionized water and stored in a refrigerator for future use. Apparently, *A. flavus* can be stored in the refrigerator for 1 year into these water vials. If a replicate soil sample did not produce 10 *A. flavus* colonies, more soil was spread for a second or

third time onto more Si10 plates until 10 isolates of *A. flavus* were obtained per replicate sample. The 30 isolates from each orchard used to determine the strain composition of *A. flavus.*

Because there is a major interest in a common atoxigenic strain of *A. flavus* (AF36 strain), that has been registered for control of aflatoxin in cotton and will be used in treating 3,000 acres of California pistachios in 2008 (EUP granted on 7 Jan 2008), we also wanted to determine the natural incidence of this strain in almond orchards. This strain is determined based on published procedures by using the compatibility test (Vegetatative Compatibility Grouping (VCG)), and with the use of two tester strains (see Appendix II). The two *A. flavus* AF36 tester strains used were provided to our laboratory by our collaborator, Dr. Peter Cotty, USDA, ARS, and University of Arizona, Tucson, AZ. Complementation occurred within 10 days at 30°C.

To determine the levels of aflatoxin contamination of almonds related to specific orchard conditions, 250 mummies were collected in March to April from 20 of these orchards (not all the orchards whose soil samples were taken had mummies), and one sample of about 15 lbs of nuts from each orchard taken from the windrow. The nuts were examined macroscopically for signs of *A. flavus* or *A. parasiticus* infection (i.e., green sporulation and or sclerotia), isolations of putative *A. flavus/A. parasiticus* were made, and finally, each sample was analyzed for aflatoxins.

Navel Orangeworm (*Amyelois transitella*) as a Vector of *A. flavus/A. Parasiticus*

It is known that for both almond and pistachio there is a strong association between navel orangeworm (NOW) damage and aflatoxin contamination. Obviously, larvae of NOW can create wounds as they feed on the nut kernel, allowing the *Aspergillus* fungi to infect the kernel. However, it is not known whether NOW can carry *A. flavus* and *A. parasiticus* to almonds (and pistachios). To determine whether NOW adults can contribute to *A. flavus* spread, moths were collected on sticky traps set in almond and pistachio orchards and collected periodically. NOW moths were plated on Si10 or Si6 without surface disinfestation (though the forceps used to transfer the moths were sterilized between each transfer), and the *Aspergillus* spp. that developed from them was recorded. Identification of the *Aspergillus* species was done after transferring each isolate on CYA plates. NOW collected from both almond and pistachio orchards were included in this study, since this is a major pest of these two crops, as well as a limited number of NOW larvae.

Results:

Levels of *A. Flavus* and *A. Parasiticus* in Soil of Almond Orchards

Aspergillus flavus was more common in the southern almond orchards and *A. parasiticus* in the northern area orchards while the incidence of both was about the same in orchards of the central area (Table 1). The range of propagules was greater for *A. tamari >A. parasiticus>A. flavus. A. tamari* was very common in the south and central and less common in the northern California (Table 1). The range of densities of *A.*

flavus/A. parasiticus in California almond orchards was greater (2-219 cfu/g soil), than pistachio orchards (2-36 cfu/g soil), and fig orchards (0.1-9 cfu/ g soil). However, the densities in almond orchards represent numbers lower than the densities in other crops such as cotton fields in Arizona (greater than 1,000 cfu/g soil), corn and peanut fields in Georgia (30-3,600 cfu/g soil), and corn in Iowa and Missouri (103-396 cfu/g soil). Three strains of *A. flavus* were recovered: the S, the L, and the "M" [similar morphologically to Dr. Cotty's T strain (Coty et al, 2007), although it has not been confirmed yet]. Nut samples from most of these orchards were collected and analyzed for aflatoxins.

Although *A. flavus* was isolated from most of the almond orchards sampled it was more common in the southern San Joaquin Valley than the Central Valley and Sacramento Valleys. In contrast *A. parasiticus* was more common in the Sacramento and the Central Valley than in the southern Valley. In fact, the density of *A. parasiticus* was very low in the Southern San Joaquin Valley (Figures 3 and 4).

Table 1. Summary of *Aspergillus* propagules in soil collected from almond orchards in three regions of California.

		Sacramento Valley (# 1 to 11)	**Central SJV (#12 to 21)**	**Southern SJV (#25 to 32)**
Aspergillus flavus	%	29.8	27.8	**45.4**
	Range	0.16 to 26.1[z]	0.53 to 25.53	0.5 to 28.54
Aspergillus parasiticus	%	**46.5**	25.9	0.3
	Range	0 to 30,7	0.31 to 42.18	0 to 0.15
Aspergillus tamarii	%	23.7	46.3	**54.3**
	Range	0 to 7.97	1.57 to 16.60	0.65 to 166.73

[z] The range of propagules is given as colony forming units (cfu) per gram of soil.

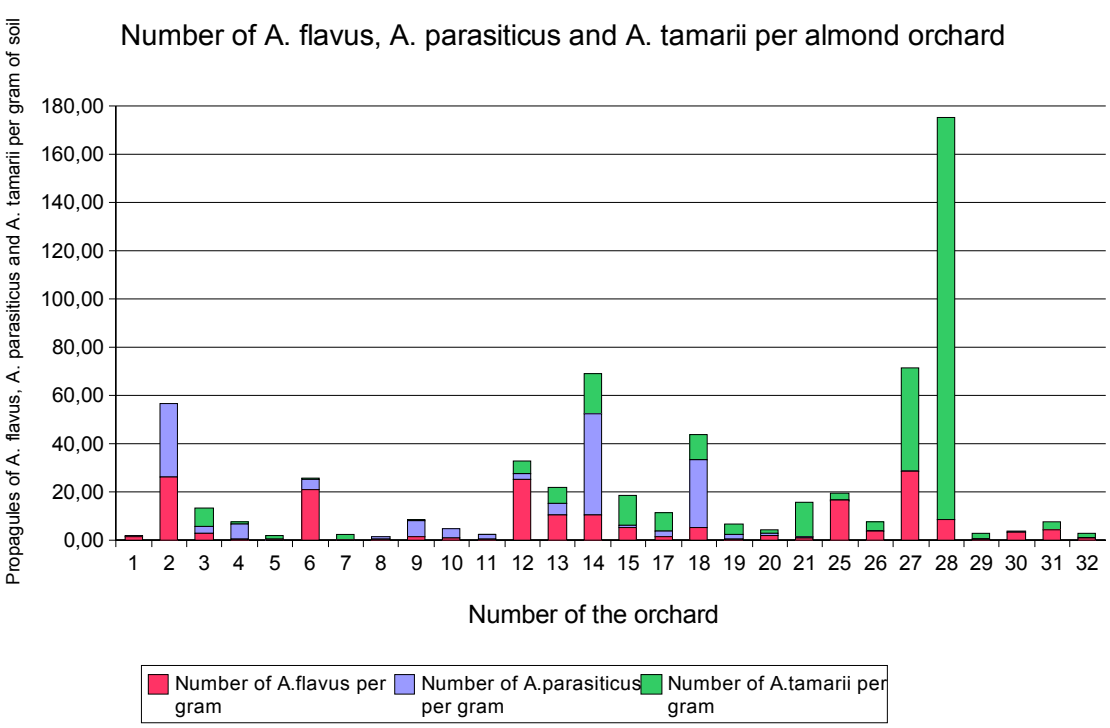

Figure 2. Levels of *Aspergillus flavus, A. parasiticus,* and *A. tamari* propagules (cfu/g) in soils collected from almond orchards in California.

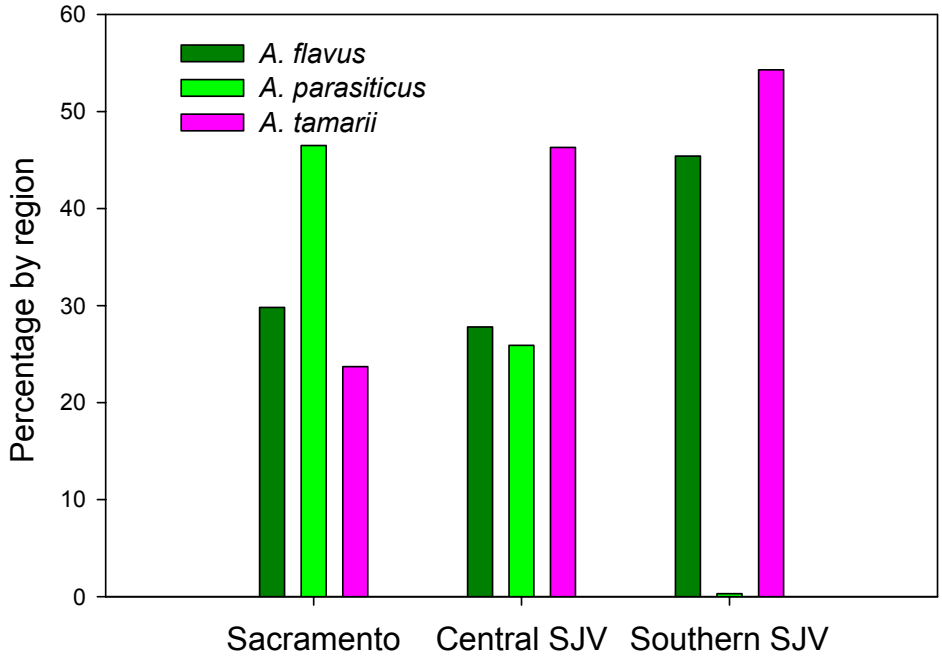

Figure 3. Incidence of *Aspergillus flavus, A. parasiticus,* and *A. tamarii* in soils collected from 28 almond orchards from three different regions in California.

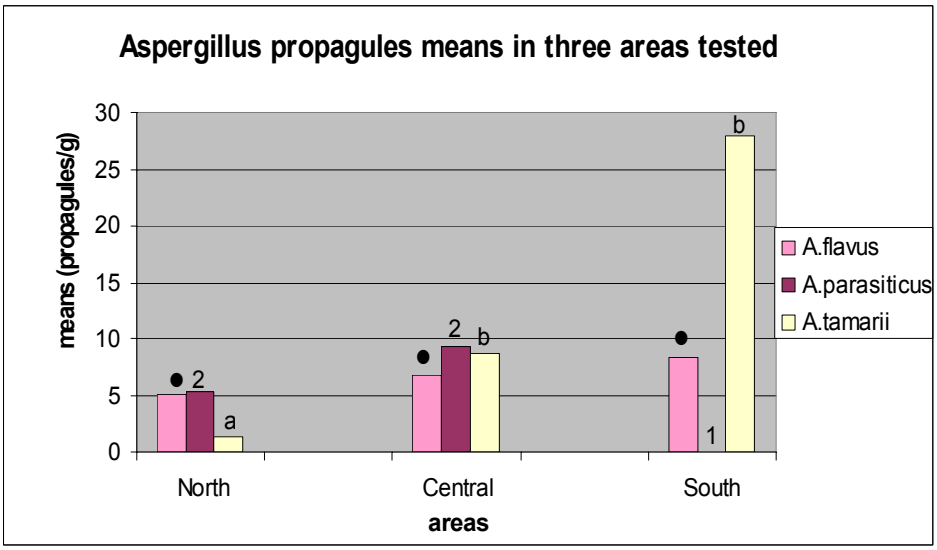

Figure 4. Average regional densities of *Aspergillus flavus, A. parasiticus,* and *A. tamarii* in soils collected from 28 almond orchards from three different regions in California. (Different numbers or different letters over the same color bars show significant differences in the density of *Aspergillus* spp. Propagules.):

The distribution and densities of *A. flavus* S and L strain isolates based on the size of sclerotia are shown in Figure 5 and Table 2. In general, the S strain was more predominant in the Sacramento Valley while the L strain more predominant in the southern region. The distribution of S strain is important because it was shown in previous research that the amounts of aflatoxins are directly related with the frequency of the S strain. Only occasionally strain M isolates of *A. flavus* were isolated (Appendix I).

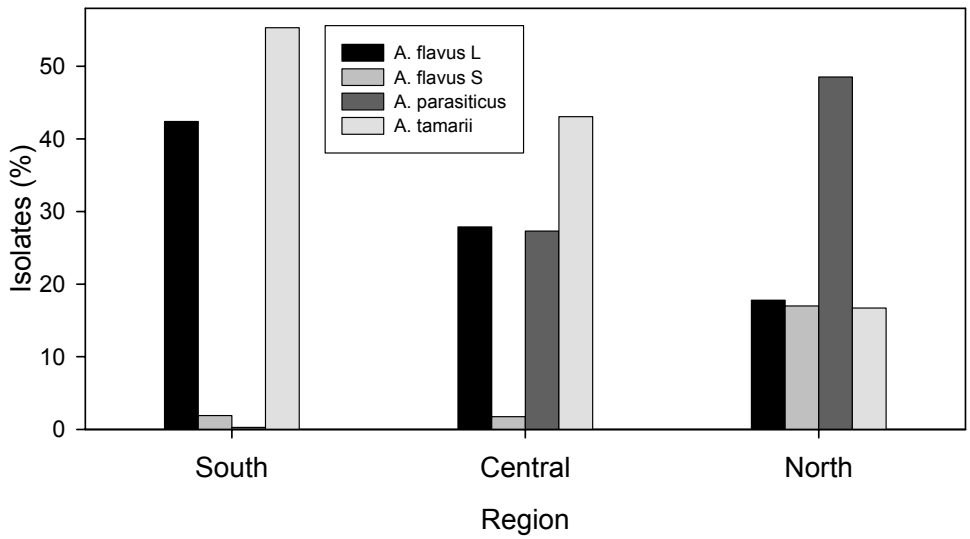

Figure 5. Percentage of *Aspergillus* section *Flavi* isolates belonging to the various species and strains from soil from commercial almond orchards in California.

Table 2. Densities of *Aspergillus flavus* S strain isolates in soil from almond orchards in three different regions of California.

	***Aspergillus flavus* S strain**		
	Sacramento Valley	**Central SJV**	**Southern SJV**
Number of *A. flavus* isolates	129	152	163
Number of *A. flavus* which produce sclerotia and percentage	74 (56.4%)	96 (63.1%)	106 (63.0%)
Number of "S" strain isolates	63	9	7
Percentage of "S" strain among all the *A. flavus* isolates tested	48,8%	5,9%	4,3%
Means (*A. flavus* S strain/g of soil)	2.76	0.29	0.18

Incidence of the Atoxigenic *Aspergillus Flavus* Strain AF36

Because this strain is registered as a bio-pesticide to reduce aflatoxin in other crops, and since we were granted an Experimental Use Permit (EUP) to apply the AF36 strain in 3,000 acres of pistachio in 2008, we wanted to determine if this strain occurs naturally in almond orchards. Thus far, of a total of 900 isolates of *A. flavus* collected as of August 2007 we analyzed 372 isolates and determined that among isolates from the northern, central, and southern regions, 3.4%, 7.2%, and 12.3% were AF36 strain, respectively (Table 3). As of January 2008, we analyzed up to 637 isolates and the corresponding incidence of AF36 for the northern, central, and southern regions were 3.5%, 6.4%, and 12.6%, respectively. It is apparent that this strain occurs naturally in almond orchards and as an average at higher levels in almond than in pistachio orchards. In addition, it is more common in almond orchards of the southern than the northern region. Therefore, if efficacy data showed reduction of aflatoxins in pistachio after applying this strain in the field, it would be reasonable to expect similar effects in almonds, and efforts will be directed towards expanding the label of this bio-pesticide to include almonds.

Table 3. Summary of the Vegetatative Compatibility Group assays to determine levels of natural occurrence of the bio-pesticide atoxigenic *A. flavus* strain AF36.

	Sacramento Valley	Central Valley region	Southern region
Number of isolates determined/total isolates obtained by 08/24/2007	146/330	15/270	211/240
Percentage of *Aspergillus flavus* AF36 by 08/24/2007	3.42 %	7.18 %	12.32 %

Aflatoxin Analyses of Almonds Collected From Windrows

We collected a total of 28 almond samples from the majority of the orchards where soil samples were taken, but for some of the orchards samples were not collected. The almonds were shelled, the hulls were examined for presence of decay by putative *A. flavus* or *A. parasiticus,* and the samples were analyzed separately as kernels, hulls, and a few nuts with putative decay (0.1 – 5.0 g kernels and 1.95 – 12.0 g hulls). Among the kernel samples, only 5 were positive with amounts of aflatoxin ranging from 0.06 to 0.74 ppb. Two samples had only B1; one sample had B1 and B2, and one sample B2 and G1 from central and southern regions, and one sample G1 and G2 from the northern region (Table 4). The presence of G aflatoxins in this last sample indicates that the infection was by *A. parasiticus*, since *A. flavus* does not produce G aflatoxins. Interestingly, the sample with only G aflatoxins was from the northern region where *A. parasiticus* was the predominant aflatoxigenic fungus (Figure 1). For the nuts with obvious decay by *Aspergillus,* only one kernel sample had 13.3 ppb G2 aflatoxin, suggesting that was infected by *A. parasiticus,* and this sample was also from an orchard in northern region.

There were 10 positive hull samples, seven samples contaminated with B1 and B2 aflatoxins while three samples had B and G aflatoxins, suggesting infection by *A. parasiticus* also (Table 5). There were 12 aflatoxin positive shell samples. One shell sample had only B1, five had B1 and B2 aflatoxins, 4 samples had B1 or B2 and G1 aflatoxins, and only one sample had G1 and G2. Aflatoxin levels ranged from 0.03 to 1.91 ppb (Table 6). The fact that there were more positive samples of shells and hulls than kernel contamination indicates that in about half of the instances the infections on shell and hull had not progressed enough to infect and contaminate the kernel, which may explain why there were fewer positive kernel samples than hull and shell samples. Among all the samples, only two samples from orchards #20 and #27 had all kernels, shells, and hulls contaminated with aflatoxins (Tables 4 - 6).

Navel Orangeworm (*Amyelois transitella*) as a Vector of *A. Flavus/A. Parasiticus* –

NOW adults emerged in the spring from masses of mummies kept in plastic buckets were heavily contaminated with *Aspergillus* fungi, including *A. flavus* and *A. parasiticus.* In general levels of contamination of moths with *A. flavus/A. parasiticus* were around 40%, but a sample collected on 28 February from Tulare County had more than 65% contamination (Figure 6). The levels of *Aspergillus niger* in these samples ranged from 45 to 95%. These results suggest that NOW moths emerged from mummies from the orchard floor will have high levels of contamination with aflatoxigenic fungi. In an almond orchard in Madera County, we collected periodically NOW moths on sticky traps and plated them on Si10 media. The levels of contamination of these moths were much lower (up to 8% of moths with *A. flavus /A. parasiticus*) than the contamination of moths that emerged from mummies in plastic buckets. In addition, the infestation of moths was the highest during June and July (Figure 7). Levels of *A. niger* were higher than those of *A. flavus/A. parasiticus* on the moths and ranged from about 45% to 95% (Figure 6). Similar results were obtained with NOW moths trapped in commercial pistachio orchards.

To determine if larvae of NOW get contaminated with *A. flavus/A. parasiticus*, NOW larvae were extracted from infested mummies and plated on agar media. Larvae extracted from kernels with no obvious decay signs did not produce any *Aspergillus* colonies on Si10 agar media. However, 60% of the larvae extracted from mummies with symptoms of *Aspergillus* species infections produced colonies of *A. flavus /A. parasiticus* on Si10 media. Similarly, plating a larger number of NOW larvae from infested pistachios revealed up to 42% contamination with *A. flavus/A. parasiticus* and 50 to 90% with *A. niger.* These results suggest that both the larvae and the moths of NOW are suited well to pick up and carry propagules of *Aspergillus* to almonds. Larvae of NOW not only will create feeding wounds in the kernel to facilitate infection by aflatoxigenic fungi, they will also carry the propagules of the aflatoxigenic fungi to the right substrate. This may be another reason why there is a very close association of NOW damage and aflatoxin contamination in almonds and also in pistachios.

Table 4. Aflatoxin levels in kernels of almond windrow samples collected from various orchards in northern, central, and southern regions in 2007.

	Aflatoxin in ppb					
Grower#	**B1**	**B2**	**G1**	**G2**	**Total**	**Comments**
1	0	0	0	0	0	neg
2	0	0	0	0	0	neg
3	0	0	0	0	0	neg
4	0	0	0	0	0	neg
5	0	0	0	0	0	neg
6	0	0	0	0	0	neg
7	0	0	0	0	0	neg
8	0	0	0.079	0.054	**0.13**	**pos**
9	0	0	0	0	0	neg
10	0	0	0	0	0	neg
11	0	0	0	0	0	neg
12	0	0	0	0	0	neg
13	0	0	0	0	0	neg
14	0	0	0	0	0	neg
15	0	0	0	0	0	neg
16	0	0	0	0	0	neg
17	0	0	0	0	0	neg
18	0	0	0	0	0	neg
19	0	0	0	0	0	neg
20	0.061	0	0	0	**0.06**	**pos**
21	0	0.045	0.175	0	**0.22**	**pos**
25	0	0	0	0	0	neg
26						
27	0.163	0	0	0	**0.16**	**pos**
28	0.688	0.055	0	0	**0.74**	**pos**
29	0	0	0	0	0	neg
30	0	0	0	0	0	neg
31	0	0	0	0	0	neg

Table 5. Aflatoxin levels in hulls of almond windrow samples collected from various orchards in northern, central, and southern regions in 2007.

	Aflatoxin in ppb					
Grower#	**B1**	**B2**	**G1**	**G2**	**Total**	**Comments**
1	0	0	0	0	0	neg
2	0	0	0	0	0	neg
3	0	0	0	0	0	neg
4	0	0	0	0	0	neg
5	0.083	0.039	0	0	**0.12**	**pos**
6	0	0	0	0	0	neg
7	0	0.041	0	0	**0.04**	**pos**
8	0	0	0	0	0	neg
9	0	0	0	0	0	neg
10	0	0	0	0	0	neg
11	0	0	0	0	0	neg
12	0	0	0	0	0	neg
13	0.073	0	0.103	0	**0.18**	**pos**
14	0	0	0	0	0	neg
15	0	0	0	0	0	neg
16	0	0.043	0.084	0	**0.13**	**pos**
17	0	0	0	0	0	neg
18	0	0	0	0	0	neg
19	0.073	0	0	0	**0.07**	**pos**
20	0	0.032	0.082	0	**0.11**	**pos**
21	0	0	0	0	0	neg
25	0.09	0	0	0	**0.09**	**pos**
26						
27	0.398	0.03	0	0	**0.43**	**pos**
28	0	0	0	0	0	neg
29	0.074	0.041	0	0	**0.11**	**pos**
30	0	0	0	0	0	neg
31	0.85	0	0	0	**0.09**	**pos**

Table 6. Aflatoxin levels in shells of almond windrow samples collected from various orchards in northern, central, and southern regions in 2007.

	Aflatoxin in ppb					
Grower#	**B1**	**B2**	**G1**	**G2**	**Total**	**Comments**
1	0	0	0	0	0	neg
2	0.075	0	0	0	**0.07**	**pos**
3	0	0	0	0	0	neg
4	0	0	0	0	0	neg
5	0	0	0	0	0	neg
6	0	0	0	0	0	neg
7	0	0	0.067	0.052	**0.12**	**pos**
8	0	0	0	0	0	neg
9	0.063	0	0	0	**0.06**	**pos**
10	0	0	0	0	0	neg
11	0	0	0	0	0	neg
12	0	0	0	0	0	neg
13	0	0	0	0	0	neg
14	0	0	0	0	0	neg
15	0.111	0	0.086	0	**0.2**	**pos**
16	0.398	0.021	0	0	**0.42**	**pos**
17	0	0	0	0	0	neg
18	0	0	0	0	0	neg
19	0	0	0	0	0	neg
20	0.206	0.045	0.075	0	**0.33**	**pos**
21	0.073	0	0.063	0	**0.14**	**pos**
25	0.73	0	0	0	**0.07**	**pos**
26						
27	0	0.033	0	0	**0.03**	**pos**
28	1.781	0.134	0	0	**1.91**	**pos**
29	0.666	0.063	0	0	**0.73**	**pos**
30	0.132	0.042	0.083	0	**0.26**	**pos**
31	0	0	0	0	0	neg

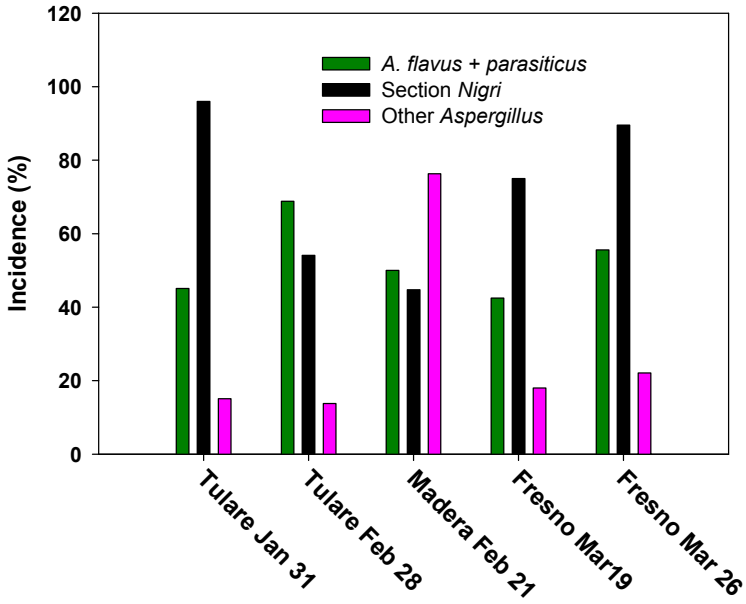

Figure 6. Incidence of *Aspergillus flavus* and *A. parasiticus* and other *Aspergillus* spp. on NOW moths emerged from almond mummies collected from commercial orchards in 2007 and kept in plastic buckets. Incidence was determined after plating NOW moths on Si10 agar media that were incubated at 35°C for 1-2 weeks and then recorded.

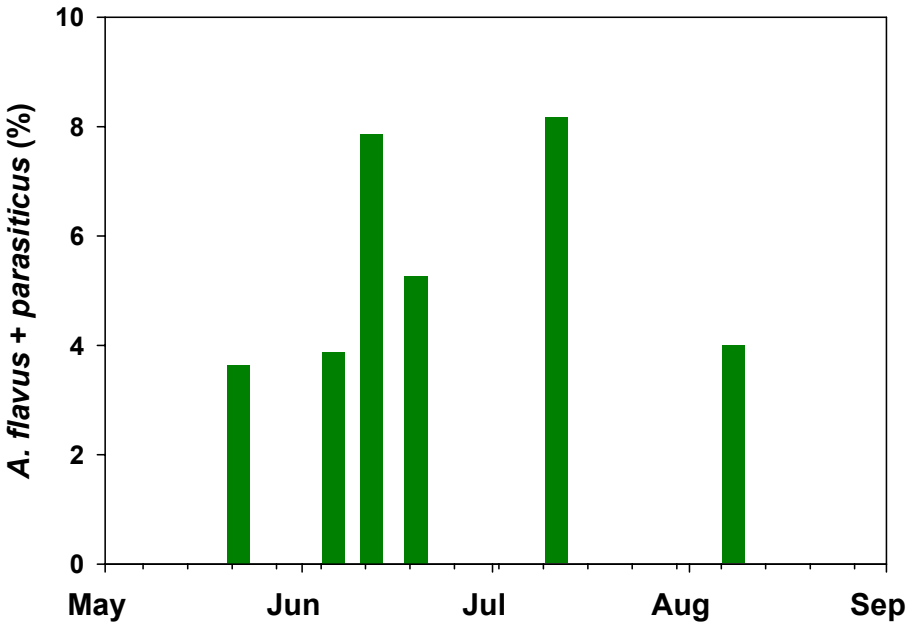

Figure 7. Incidence of *Aspergillus flavus* and *A. parasiticus* on NOW moths trapped periodically on sticky traps placed in an almond orchard in Madera Co. in 2007. Incidence was determined after plating NOW moths on Si10 agar media that were incubated at 35°C for 1-2 weeks and then recorded.

Discussion – Conclusions:

Growth of *Aspergillus* species and aflatoxin contamination are the consequence of interactions among the fungus pathogen, the host, and the environment. The right combination of these factors determines the infestation and colonization of the

substrate, and the type and amount of aflatoxin produced. The precise factors affecting aflatoxin contamination of almonds are not well understood. In general, water stress, high-temperature stress, and insect damage of the host are major determining factors in preharvest mold infestation and aflatoxin toxin production; however, postharvest aflatoxin contamination is favored by warm temperatures and high humidity. Similarly, the growth stage of almond (pre hull split, hull split, hull opening, etc.), the high density of newly planted orchards, and the presence of weeds in the orchards and type of irrigation that may affect humidity can contribute to increased mold growth and aflatoxin contamination of almond. Aflatoxin formation is also affected by associated growth of other microorganisms.

We studied the distribution and densities of *Aspergillus* section *Flavi* in 29 commercial almond orchard soils in three different regions of California, northern (cities of Chico, Orland, Durham, and Williams), central valley (Madera Co.), and southern (Wasco, Lost Hills, Bakersfield, and other surrounding small towns). All three species in the section *Flavi* group (*Aspergillus flavus*, *A. parasiticus,* and *A. tamarii*) were isolated from the majority of these almond orchard soils. Of these species *A. flavus* and *A. parasiticus* produce aflatoxins while *A. tamarii* does not. Contamination of nut samples (kernels, hulls, and shells) with only B, B and G, or only G aflatoxins suggests that both of these fungi are involved in infection of almonds. This is similar to infection and contamination of pistachios with aflatoxins and different from other crops where either *A. flavus* (i.e., in cotton), or *A. parasiticus* (i.e., peanuts), are involved. Aflatoxin analyses of the 2007 samples revealed more aflatoxin positive samples collected from central and southern regions than the samples from the northern region. Previous research in corn has shown that aflatoxin contamination is greater in regions with below average rainfall (Payne, 1992). The higher frequency of positive samples from the central and southern regions can be partially explained by the lower rainfall in these areas. Another explanation could be the deficit irrigation practices that some almond growers on the west side of the San Joaquin Valley were forced to carry out because their surface water deliveries were reduced in 2007. Conditions of higher temperatures and lower humidity in these regions are more favorable for *A. flavus* infection but less than ideal for other microorganisms that are typically present in soils during the time the nuts are drying on the ground. The higher frequency of aflatoxin positive hull and shell samples than aflatoxin positive kernels suggests that some of the infections that started while the nuts were on the ground had not progressed enough to reach and infect the kernels and produce aflatoxins. Therefore, the time and conditions the nuts are lying on the ground to dry may be considered as a risk factor. Another risk factor can be the density of aflatoxigenic fungi in the orchard soil. It is expected, with all other factors equal, the higher the density of the aflatoxigenic fungi the higher the risk for aflatoxin contamination. Therefore, knowing the densities of the aflatoxigenic fungi in soils is an important indication of the magnitude of the risk for aflatoxin contamination.

Another important factor can be the kinds and strains of *A. flavus* and *A. parasiticus* present in almond orchards. For instance, it is important to know the ratio of the densities of S and L strains of *A. flavus* in the orchard. After checking isolates of *A. flavus* from pistachio orchards, we found that 57% of the L isolates did not produce aflatoxins while 100% of the S strains produced B aflatoxins. In addition, 100% of the

A. parasiticus isolates produced both B and G aflatoxins. Our results showed that the densities of both the S strain of *A. flavus* and *A. parasiticus* are higher in northern region than the other regions. Although the risk for aflatoxin is high in northern orchards based on the densities and species of aflatoxigenic *Aspergillus* spp., apparently, due to other factors (most likely environmental and lower NOW damage) almond samples from northern region had the lowest frequency of aflatoxin positives.

The levels of aflatoxins detected in the 2007 samples collected from windrows were very low and all below the EU tolerance. However, the multiple rapid alerts for California almonds exceeding the EU tolerance suggests that higher levels of aflatoxins may develop after the nuts are taken form the orchard, perhaps when they are stockpiled and/or during transport to the foreign destination markets.

Our 2007 results also showed that there are a lot of the atoxigenic isolates in almond orchard (a portion of L strain of *A. flavus* and all the *A. tamarii* isolates). Interestingly, the bio-pesticide atoxigenic *A. flavus* strain AF36 ranged from 3.5% to 12.6% in almond orchard soils. Of the 637 isolates of *A. flavus* tested thus far, an average of 7.5% belonged to AF36 strain. This density is higher than the density of AF36 recorded in pistachio and fig orchards. We were granted in 2008 an Experimental Use Permit (EUP CA REG. #71693-550001-EX conditional registration on 01/23/08 (ID#220986)) to treat 3,000 acres of pistachio with AF36 to study its effects in displacing the toxigenic isolates and reduce aflatoxins in pistachios. This bio-pesticide is registered and used in cotton fields in Arizona and California and corn in Texas. Natural occurrence of the AF36 strain in soils of almond orchards will facilitate the registration of this strain for reducing aflatoxins in nut crops after the completion of the EUP period. In general, the public prefers to see applications of bio-pesticides that are encountered in nature than an introduced microorganism from another location or a foreign country. Moreover, we initiated a study in 2007 by applying AF36 in an experimental almond orchard at Nickels Estates in Colusa County to gather efficacy data and facilitate and expand the registration of AF36 to almonds.

Our results also showed that both larvae and moths of NOW can be contaminated with *A. flavus, A. parasiticus*, and *A. tamari,* as well as with other species of *Aspergillus* (*A. ochraceus, A. terreus, A. melleus,* etc.). This high incidence of these fungi carried by NOW moths suggests that NOW insect not only will create the wounds for infection but also will introduce the aflatoxigenic pathogens into the right site for infection of hulls, shells, and kernels. This may be why there is a very strong association between NOW damage of almond nuts (also pistachio nuts) and aflatoxin contamination for California nuts. The high levels of contamination of NOW moths emerged from mummies collected from the ground and kept in plastic buckets suggests that this insect can get contaminated from the dirt more so than the moths that emerge from mummies of infested nuts on the trees and trapped on sticky traps. We noticed an increase in the incidence of contamination with *Aspergillus* species of NOW moths in June and July (Figure 6). This may be because during that time hull split increased, there are overlapping cycles in nut infestation, and higher incidence of nuts infected by *A. flavus /A. parasiticus*, resulting in higher incidence of NOW larvae and adult contamination with these fungi.

Recent Publications:

Doster, M. A., Michailides, T. , Cotty, P., Felts, D., Eveillard, H., Charbaut, T., Boeckler, L., Morgan, D., Reyes, H., and Windh, J. 2007. Aflatoxin control in figs and almonds using the atoxigenic strain AF36. Pages 56-57 in: Proceedings 2007 Annual Multicrop Aflatoxin /Fumonisin Elimination & Fungal Genomics Workshop, Atlanta, GA Oct. 22-24.

Michailides, T. J., Siegel, J., Doster, M. A., Morgan, D., Boeckler, L., Eveillard, H., Charbaut, T., Felts, D., and Reyes, H. 2007. *Aspergillus flavus/A. parasiticus* in almond orchards and on navel orangeworm (a major pest of almonds and pistachios. Page 67 in: Proceedings 2007 Annual Multicrop Aflatoxin /Fumonisin Elimination & Fungal Genomics Workshop, Atlanta, GA Oct. 22-24.

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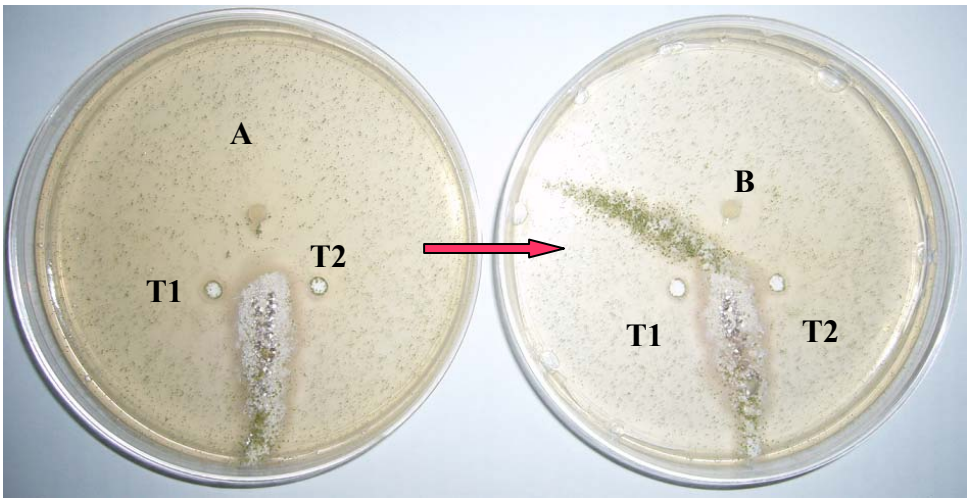
APPENDIX I

Aspergillus flavus S (left plate), M (middle plate), and L (right plate) strains based on the size of sclerotia and isolated from soils of almond orchards in California

(**Note:** *Aspergillus flavus* S strain isolates generally produce more aflatoxin than the L strain isolates. That is why the percentage of *A. flavus* S strain isolates among the *A. flavus* community in the orchard soil may be an indication of the risk for aflatoxin contamination.)

APPENDIX II

Complementation among *Nit* mutant of *Aspergillus flavus* to determine strains (this assay is used to determine if a strain is or is not AF36)



As a result of complementation, dense hyphae grow (± sclerotia) between the two tester strains (T1 and T2) at the zone where the mycelia meet. If the unknown strain of *A. flavus* isolated from almond orchard soil is an AF36, the complementation occurs between the unknown isolate and one or the two tester strains. For example, the *A. flavus* strain **A** (left photo) is not an AF36 (A is not in the VCG of T1 and T2); however, the *A. flavus* strain **B** (right photo) is an AF36 since it formed a complementation zone with the T1 tester).