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

Project No.: **11-AFLA1-Michailides**

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Objectives:

1. Monitor the toxigenic strains of *Aspergillus flavus* present in almond orchards.
2. Repeat the application of the atoxigenic *A*. flavus strain AF36 in a research almond orchard in order to determine the establishment and survival of AF36 and the displacement of aflatoxin-producing fungi.
3. Determine in which stage of physiological development almond nuts are most susceptible to aflatoxin contamination.
4. Determine aflatoxigenic fungi in almond mummies and contamination of NOW by these fungi.

Interpretive Summary:

1. Aflatoxigenic *Aspergillus* species. Several nut crops including almonds, pistachios, and walnuts occasionally are affected by aflatoxin contamination. In order to assess the degree of contamination and spatial variability of soil populations of *Aspergillus flavus* and *A. parasiticus* in California almonds orchards, *Aspergillus* section *Flavi* was monitored over a four-year period. The aflatoxin-producing ability of soil communities of *Aspergillus* constitutes a risk factor for aflatoxin contamination in almonds. Over 2,700 *Aspergillus* isolates from 28 almond-producing orchards located in the northern, central, and southern Central Valley (California) were isolated from soil samples from 2007 to 2010. Results indicated that the *A. flavus* L-strain was the most common, followed by *A. parasiticus*. The incidence of the L-strain was significantly higher ($P < 0.005$) in the southern region while a lower incidence occurred over this time period in the north. Average aflatoxin-producing potentials of the L-strain fluctuated between years with toxigenic isolates increasing from 64% in 2007 to 82% in 2008 but

decreasing to 52% in 2010. L-strain isolates from northern orchards showed the highest aflatoxin-producing potential in 2007-08 and produced on average 149 µg g^{-1} B_1 aflatoxin. The incidence of *A. flavus* strains not producing aflatoxins (atoxigenic) decreased in all regions from 2007 to 2008 whereas in 2010, the proportion of toxigenic to atoxigenic was almost equal. Orchard soils in the northern and central regions had significantly higher incidences of *A. parasiticus* ($P < 0.005$) than those in the south. *A. parasiticus* incidence in northern and central regions ranged from 16% to 56%, whereas in the southern region incidences ranged only from 1% to 7%. Greatest quantities of aflatoxin B_1 were produced by *A. parasiticus* with 95% of isolates producing >10 µg g^{-1} aflatoxin B_1. Although the incidence of the high aflatoxin-producing *A. flavus* S-strain was low (8%) in 2007, it was almost doubled (15%) in 2010. The results suggest that the levels of the aflatoxin-producing fungi may change from year to year and may affect aflatoxin contamination levels of the crop.

2. 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 biocontrol agent has been very successful in substantially reducing aflatoxin contamination of cottonseed in commercial cotton fields in Arizona and corn in Texas. More recently AF36 has been registered as a biopesticide for pistachio orchards in California and Arizona. 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, and 2011, an application of wheat infected with AF36 (the same commercial product used in cotton fields and pistachio orchards) 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, the most common aflatoxin-producing fungus present in the orchard was *A. parasiticus*, 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 frequency of *A. parasiticus* and of *A. flavus* S strain in the fungal population decreased substantially until in 2009 only 2.3% of the isolates were *A. parasiticus* or *A. flavus* strain S. This demonstrates 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 introducing 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, indicating that the effect of application lasts more than a year (no wheat-AF36 product was applied in 2009) and perhaps the wheat-AF36 product does not need to be applied every year. After further decrease in the level of AF36 in 2010, an additional application of wheat-AF36 was made in 2011. However, the effectiveness

of the application in 2011 was disappointing, perhaps due to applying too early and to the unusually cool and rainy weather in June 2011.

Applying AF36 did not significantly increase the incidence of hull decay of the nuts by *A. flavus*. The percentage of hulls decayed by *A. flavus* in treated areas was never significantly different from the decay in nuts from the untreated areas. By 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 product of AF36 in an almond orchard in a manner similar to that done in commercial cotton fields and in commercial pistachio orchards are 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.

3. Determine in which stage of physiological development almond nuts are most susceptible to aflatoxin contamination. Because the postdoctoral associate left in February and the ongoing search for a new postdoc to continue this project, this objective was initiated late and there are no results to report at this time.

4. Aflatoxigenic fungi in almond mummies. Almond mummies of the cultivars Nonpareil, Butte, and Padre were collected periodically during two consecutive winters in Madera County. The damage of almond kernels by the lepidopteran navel orangeworm (NOW; *Amyelois transitella*), the incidence, and strain distribution of *Aspergillus* section Flavi were recorded. In addition, the potential of the *Aspergillus* isolates from mummies in producing aflatoxins was determined and the amounts of aflatoxins produced were quantified. The incidence of NOW damage was 12.3% in mummies of Nonpareil, 3.6% in those of Butte, and 2% in mummies of Padre cultivars, though these differences were not significant at $P \leq 0.05$. However, the overall incidence of *Aspergillus flavus* and *A. parasiticus* was higher in mummies of Nonpareil (9.5%) than in those of Butte (2.4%) and Padre (2%) cultivars ($P < 0.05$). The incidence of toxigenic strains of *A. flavus* ranged from 12.5 to 41.7% among the isolates recovered from these mummies. The majority of the aflatoxin producing isolates produced more than 100 µg g^{-1} of B1 aflatoxin. Mummies mixed with the crop of subsequent years may contribute to the levels of aflatoxin contamination since isolates recovered from them are able to produce high levels of aflatoxins. In addition, because NOW moths that emerged from mummies were highly contaminated with propagules of *Aspergillus* section *Flavi* (shown in previous studies)*;* removal of mummies during winter (sanitation) should contribute to aflatoxin management (by not causing wounds or carrying spores of *A. flavus* in the wounds).

Materials and Methods:

1. Monitor the toxigenic strains of *Aspergillus flavus* present in almond orchards.
Survey sites. Soil samples were collected from 28 commercial almond orchards in three regions of the Central Valley during September 2007, 2008 and 2010. The number of orchards was distributed as follows: 11 orchards in the northern region (Colusa, Glenn, and Butte Counties), 9 orchards in the central region (Madera County), and 8 orchards in the southern

region (Kern County). The Central Valley is located in the center of the State of California and is characterized by yearly average temperatures ranging from 8.6°C to 24.8°C (CIMIS, 2011), yearly average precipitation ranging from 284 mm to 469 mm (with decreasing precipitation from north to south), average elevation ranging from in the south 36 m to 397 m in the north, and a soil pH ranging from of 4.0 to 8.2 (Soil Survey Staff, 2011).

Soil sampling and strain isolation. Soil samples were obtained from the middle of each orchard by walking along a triangle approximately 100 m on a side. Ten subsamples of 4 to 8 g each were taken at every third tree from the soil surface at a depth of 3 cm by walking along each side of the triangle. The ten subsamples were pooled to make one replicate sample with a total of three replicated pooled samples per orchard. All samples were stored in a cold room at 4°C. After the soil samples were dried in paper bags at room temperature for 7 days, they were homogenized by hand mixing and hammered to break clumps. The density of *A. flavus* and *A. parasiticus* populations in the soil was quantified by sprinkling 0.2 g ground soil evenly on 10 plates of Modified Rose Bengal Agar, MRBA (Cotty, 1994c). For fields with low densities of propagules, plating was repeated until a minimum of 15 colonies were obtained. Plates were incubated in the dark for 3 days at 31°C. Colonies were identified and counted by colony morphology. No more than 25 isolates were subcultured on Czapek yeast agar (Klich and Pitt, 1988) and incubated unilluminated for 7 days at 31°C. *A. flavus* was assigned to S or L-strains based on their colony characteristics, conidial morphology, and size of their sclerotia (Cotty, 1989). *A. parasiticus* was initially characterized by colony and spore morphology (Klich and Pitt, 1988). Identifications were confirmed by the color reaction on AFPA (*A. flavus* and *A. parasiticus* agar, Pitt et al., 1983). The density of *Aspergillus* section *Flavi* in soil was calculated as colony-forming units (CFU) per gram of soil. The isolates were stored as conidia and mycelial plugs in water vials at 4°C.

Determination of isolates producing Aflatoxin. The ability of *Aspergillus flavus* isolates to produce aflatoxins was quantified to assess frequencies of non-aflatoxin and high aflatoxin producing strains across the orchards. Up to 10 *A. flavus* L-strain isolates per orchard were tested each year. In 2010, *A. flavus* S-strain isolates and *A. parasiticus* isolates were also tested.

Isolates were grown in Adye & Mateles medium (A&M, Mateles and Adye, 1965), using 22.4 mM urea as the sole nitrogen source and adjusted to pH 4.7 prior to autoclaving (Cotty and Cardwell, 1999), prepared, and processed for aflatoxin analyses. The mycelia of each isolate were collected on previously weighed Whatman No.4 filter paper, dried in a forced air oven at 46°C for 7days, and weighed to quantify fungal biomass.

Aflatoxins were quantified with a Hewlett Packard 1050 HPLC with an isocratic pump, autosampler, and fluorescence detector (Hewlett Packard 1046A). Aflatoxins were separated on Nova-Pak® C18 column and the column temperature was set at 25°C (Pickering laboratories, CHX650). The mobile phase was a mixture of methanol: water (45:55, vol/vol) with a flow rate of 0.8 ml min^{-1}. A postcolumn photochemical reactor (Aura Industries, Inc., Staten Island, NY) with a knitted reactor coil (0.25 mm ID × 25 m) was used to enhance aflatoxin fluorescence. Light wavelengths of 360 and 440 nm were used for excitation and emission, respectively. The injection volume was 100 µl. Limits of detection (LOD) of aflatoxins were 0.29 ppb for B1 and G1, 0.12 ppb for B2, and 0.15 ppb for G2. Aflatoxin concentrations

were extrapolated from an 8-point calibration curve obtained from standards for all four aflatoxins (Supelco Inc., Bellefonte, Pa).

2. Repeat the application of the atoxigenic *A. flavus* strain AF36 in a research almond orchard in order to determine the establishment and survival of AF36 and the displacement of aflatoxin-producing fungi. The atoxigenic *A. flavus* strain AF36 was applied as steam-sterilized wheat seed that had been colonized by the fungus. This is the same commercial product registered for application in cotton fields and pistachio orchards. The wheat-AF36 product was applied to the soil surface at a rate of 10 pounds of wheat per acre (same application rate as used for cotton fields and pistachio orchards) in a research almond (cv. Nonpareil) orchard at the Nickels Soil Laboratory on 28 June 2007, 2 July 2008, and 3 June 2011 (the wheat-AF36 product was applied to the same areas for 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, and 2 September 2011. 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. Determine in which stage of physiological development almond nuts are most susceptible to aflatoxin contamination. Because the postdoctoral associate left in February and the ongoing search for a new postdoc to continue this project, this objective was initiated late and there are no results to report at this time.

4. Determine aflatoxigenic fungi in almond mummies and contamination of NOW by these fungi.

Mummy sampling and processing. Almond mummies were sampled from December 2010 until February 2011 from commercial orchards of Madera Co. Samples were placed into plastic bags and stored in a cold room at 4^{o}C until examination and processing. We examined 600 mummies of Nonpareil, 400 mummies of Butte, and 500 mummies of the Padre cultivar. Mummies were cracked carefully and kernels separated from the shells in order to quantify the damage by NOW. In addition, a number of kernels were surface sterilized by submerging in 10% solution of sodium hypochlorite (NaClO) for 3 minutes and rinsing with sterile water. Samples of kernels were plated onto Soil Isolation Agar Media – Si10 (agar 15 g/L, sucrose 10 g/L, NaCl 60 g/L, yeast extract 1 g/L, chloramphenical 0.1 g/L, dichloran 10 ml/L and CuSO4 –

ZnSO4 solution 1 ml/L) and incubated for 10 days in darkness at 30^{o}C. Colonies were identified by colony morphology and transferred to Czapek Yeast Agar media – CYA (Czapek solution agar 49 g/L, yeast extract 5 g/L and CuSO4 – ZnSO4 solution 1 ml/L). Plates were incubated for 7 days in darkness at 31^{o}C. Following incubation, the strains ("morphotypes") of *Aspergillus flavus* morphotypes were identified based on the size and abundance of sclerotia (Cotty 1989, Cotty and Cardwell 1999). *A. parasiticus* isolates were identified based on their colony and spore morphology (Klich and Pitt 1988). Identifications of *A. flavus/parasiticus* was confirmed based on the color reaction of their colonies on AFPA (*A. flavus* and *A. parasiticus* agar, Pitt et al., 1983) Conidia and mycelial plugs were stored in 4 ml water vials at 4^{o}C for further toxicological analyses.

Aflatoxin extraction. *Aspergillus* section *Flavi* were grown in Adye & Matales media, (A&M, Mateles and Adye, 1965), using 22.4 mM urea as the sole nitrogen source and adjusted to pH 4.7 (Cotty and Cardwell 1999). A similar protocol as that used to extract and quantify aflatoxins from cultures was used to quantify aflatoxins present in kernels of mummies. Aflatoxins were quantified with a HPLC system (Hewlett Packard 1050) (Donner et al. 2011 unpublished data).

Vegetative Compatibility Groups (VCG). All atoxigenic *A. flavus* isolates were tested to determine if they belong to the VCG of the atoxigenic strain AF36. Nitrate-nonutilizing (*nit*-) mutants were generated from wild types on modified selection media – SEL (35 g/L of Czapeks-Dox Broth, 25 g/L Potassium Chlorite and 10 ml/L Rose Bengal stock solution at pH 7.0) and incubated for 7 days at 30^{o}C (Cotty, 1994). In order to stabilize and purify *nit*$^{-}$ mutants, the fungal isolates were transferred to chlorate media – MIT (15 g/L Potassium Chlorate, 35 g/L Czapeks-Dox Broth and 20 g/L Bacto Agar) and incubated for 3 days at 31^{o}C. Complementary tester mutants (*cnx*$^{-}$ and *niaD*$^{-}$) of AF36 were used on starch complementation medium (Cotty, 1994) to test if the *nit*$^{-}$ mutant had the ability to complement one of the tester mutants within 10 days.

Data analysis. Statistical analyses were performed with SAS (version 9.1.3 SAS Institute Inc., Cary, NC). Analysis of variance was performed on all data with the general linear model (GLM), suitable for unbalanced data. The GLM of SAS uses the least-squares method to fit data to a general linear model. Means were compared at the 5% significance level using Fisher's Least Significant Difference test (LSD). Analyses for percentage values, CFU g^{-1}, and aflatoxin concentrations were performed with data transformed with the arcsine of the square root, the natural logarithm (log), and the log (count +1), respectively. Pearson's correlation coefficients were generated to assess relationships between ecological and biological variables.

Results of mummy processing were also analyzed with the SAS package as above. Status of damage, recovered *Aspergillus* section *Flavi* and strain distribution were quantified by the following formula: *Incidence = (n^{o} of occurrence)/ (total sample size)* x100 adapted by Subramaniam et al. (2006). Analysis of variance (ANOVA) was performed and means were separated by using the LSD Fisher Test. All percentage data were transformed to arcsine square root prior to the analysis.

Results and Discussion:

1. Monitor the toxigenic strains of *Aspergillus flavus* present in almond orchards.
***Aspergillus* populations.** *Aspergillus* species were present in all orchards across California. The most common member of the *Aspergillus* section *Flavi* isolated from soils was the *A. flavus* L-strain followed by *A. parasiticus* and the *A. flavus* S-strain. Total incidences of the L-strain did not change from 2007 to 2008 (56%) but increased in 2010 to 68% (**Table 1**). The L-strain was significantly ($P < 0.05$) more frequent in the south than the north (**Figure 1**). More than 80% of *Aspergillus* section Flavi belonged to the L-strain in the southern region. Incidences of *A. parasiticus* decreased from over 30% in 2007 and 2008 to 17% in 2010. Isolates of *A. parasiticus* were more frequent ($P < 0.05$) in the northern and central regions (ranging from 29% in 2010 to 53% in 2008) than in the southern region (**Figure 1**). Similar to *A. parasiticus*, the S-strain was less common in the south than the north. Overall, the S-strain was least common in the central region, ranging from 1% to 4%. S strain incidences varied widely across years with the highest incidence (15%) observed in 2010 and the lowest (3%) in 2008. Working with isolates of *A. flavus* from pistachio orchards, Doster & Michailides (1994) found that all the S strain isolates were aflatoxin producers while 43% of the L-strain isolates were aflatoxin producers.

The mean CFU (colony-forming units) of *Aspergillus* colonies per gram soil ranged from 11 to 48 CFU g^{-1} In 2008 and 2010, the northern region had significantly fewer CFU than the central and southern regions (**Table 2**). Soil pH did not differ significantly ($P = 0.05$) among years nor region and ranged from 6.7 in the north to 7.5 in the south (**Table 2**). The results indicate that populations of the *A. flavus* fungi can change from year to year probably because of favorable or unfavorable conditions for reproduction (production of spores) of these fungi on litter substrates in the orchard soil.

Frequency of toxigenic and atoxigenic isolates *A. flavus* L-strains. In almost all orchards, the majority of the tested *A. flavus* L-strain isolates produced aflatoxin. The frequencies of toxigenic and atoxigenic L-strains varied among orchards, regions and years (**Table 1; Figure 2**). This is in contrast to what we found in pistachio isolates of *A. flavus* (Doster & Michailides, 1994). Among the regions, the incidences of toxigenic isolates were evenly distributed and ranged in 2007 from 62% in the north to 67% in the south. Overall, incidences of toxigenic isolates increased significantly ($P<0.05$) from 67% in 2007 to 86% in 2008, whereas in 2010 incidences of toxigenic and atoxigenic were more balanced. In 2010, the frequency of toxigenic and atoxigenic *A. flavus* isolates varied widely among the regions compared to previous years. Atoxigenic isolates of the *A. flavus* communities were more prevalent in the northern region (61%) than toxigenic (39%). In contrast, the incidence of toxigenic strains was higher than the atoxigenic strains in the south region (69%) (**Figure 2**).

Aflatoxin production. Populations of the *A. flavus* L-strain, the *A. flavus* S-strain, and *A. parasiticus* resident in the North, Central, and Southern portions of the study area all produced concentrations of aflatoxin B_1 that generally exceeded 50 ppm ($\mu g\ g^{-1}$). Mean aflatoxin, production by L-strain populations ranged from 65 to 164 ppm across the three regions from 2007 through 2010 (**Table 3**). Isolates belonging to the S strain of *A. flavus* and originating from the south and north regions produced mean concentrations of aflatoxins 190 and 301

ppm, respectively; as expected. However, only 4 isolates from the central region were evaluated and these produced a range of aflatoxin concentrations between 1 and 27 ppm with a mean of 11 ppm. Overall, 81% of the isolates with S-strain morphology produced >10 μg g^{-1}.On average, *A. parasiticus* produced the greater concentrations of aflatoxins (Table 3). All 35 *A. parasiticus* isolates produced both B and G aflatoxins (mean aflatoxin B_1 = 607 μg g^{-1} (range = 2 to 4086 μg g^{-1}); mean aflatoxin G_1 486 μg g^{-1} (range = 2 to 3274 μg g^{-1})). It is apparent that isolates of *A. flavus* that produce aflatoxins can be commonly found in commercial almond orchards and their levels depend on specific conditions of each year.

2. Repeat the application of the atoxigenic *A. flavus* strain AF36 in a research almond orchard in order to determine the establishment and survival of AF36 and the displacement of aflatoxin-producing fungi. 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. On 3 June, 2011, an additional application of wheat-AF36 was made in this orchard. Unexpectedly, the frequency of AF36 did not increase in 2011 to the extent that was observed in 2007 (**Figure 3**), perhaps due to applying the wheat-AF36 product too early in the year or due to the unusually cool and rainy weather in June 2011. In the untreated areas, AF36 also increased over time from 1.7% before applying AF36 in June 2007 to 74% in September 2008 (**Figure 3**), which suggests the movement of AF36 from the treated areas to the untreated areas.

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 4**). In addition, the density of *A. flavus*/*A. parasiticus* increased further during the second year in the treated areas after an additional application, but showed moderate decreases in 2009 and again in 2010, when no additional AF36 was applied (**Figure 4**). In 2011 the density remained low even though the wheat-AF36 product had been applied (**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 186 and 138 propagules / g soil for the AF36-treated and untreated areas, respectively.

Before applying the wheat-AF36 product in 2007, the most common aflatoxin-producing fungus present was *A. parasiticus* (**Figure 5**), 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 percentage of isolates as the L strain of *A. flavus* (which includes AF36 and many other atoxigenic strains). However, after applying the wheat-AF36 product, the percentage of isolates that were 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 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, the percentage of isolates belonging to *A. parasiticus* and *A. flavus* S strain increased in 2011 (**Figure 5**).

Applying AF36 did not significantly increase (P=0.05) the incidence of hull decay by *A. flavus* for nuts from the treated areas (**Table 4**), 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 4**). 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. Although several different fungi within the *A. flavus* group decayed the nuts (**Table 5**), 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.

In general, the favorable results from this study support the use of the wheat-AF36 product in almond orchards. A single 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. Determine in which stage of physiological development almond nuts are most susceptible to aflatoxin contamination. Because the postdoctoral associate left in February and the ongoing search for a new postdoc to continue this project, this objective was initiated late and there are no results to report at this time.

4. Determine aflatoxigenic fungi in almond mummies and contamination of NOW by these fungi.

Mummy sampling and processing. NOW damage was found in kernels of all tested varieties. Nonpareil almonds showed 12.3% NOW damage while 3.6% and 2% of the Butte and Padre cultivars, respectively, were damaged by NOW. The differences in the levels of NOW damage among the three cultivars, though large, were not significant ($P \leq 0.05$) (**Figure 6**). Isolates of the *Aspergillus* section *Flavi* were recovered from all three varieties. Overall, significantly ($P \leq 0.05$) more isolates of the section Flavi were observed on Nonpareil mummies (9.5%), than on Butte (2.4%) or Padre (2.0%) **(Table 6).** The incidence of *Aspergillus* section *Flavi* isolates on damaged Nonpareil almonds was 12.2% and on Butte 5.6% while no such fungi were found in damaged Padre nuts **(Table 6).** Although the incidences of *Aspergillus* section *Flavi* isolates between damaged and nondamaged kernels of Nonpareil and Butte were not statistically different, this difference was significant ($P \leq 0.05$) in Padre nuts. Overall, the *A. flavus* L-strain was the most common strain (90.9%) isolated from mummies followed by *A. parasiticus* (7.8%) and *A. flavus* S-strain (1.3%) (**Table 7**). Regardless of cultivar, about 90 to 100% of *Aspergillus* isolates belonged in the L-strain. The results correspond well with the high occurrence of the L-strain of *A. flavus* recorded in soil samples for three years (**Figure 1**). *A.*

parasiticus was isolated from 10.5% (six Nonpareil nuts) and the S-strain of *A. flavus* in 1 Butte nut (8.3%) (**Table 7**), suggesting that soft-shelled cultivars can suffer more infection by aflatoxigenic fungi.

Aflatoxin production. Among the isolates of *Aspergillus* section *Flavi* recovered from NOW damaged and non-damaged mummies, the majority of them were atoxigenic (range 63% to 88%) (**Table 8**). Incidences of *Aspergillus* section *Flavi* isolates from Nonpareil, Butte, and Padre producing aflatoxin were 29.4%, 36.4%, and 12.5% (**Figure 7**). The frequency of atoxigenic isolates within *A. flavus* L-strain isolates from Nonpareil, Butte and Padre was 70.6%, 63.6% and 87.5% (**Table 8**). Among the *Aspergillus* isolates that produced aflatoxin, more than 40% of the isolates produced more than 300 µg g^{-1}aflatoxin (**Table 9**). Similarly, some toxigenic strains of *A. flavus* isolated from almond orchard soils had similar or higher levels of aflatoxin (**Table 3**). The results suggest that mummies can harbor high levels of aflatoxins and if they are mixed with the marketable product of the subsequent year's nuts, they may contribute to detectable aflatoxin levels.

The vegetative compatibility group test was performed on all of the atoxigenic isolates of *A. flavus* recovered from mummies to determine whether any of these strains were the atoxigenic strain AF36. None of the atoxigenic isolates belonged to the VCG of the AF36 strain.

Research Effort Recent Publications:

Donner, M. Lichtemberg, P. S., Morgan, D. P., and Michailides, T. J. (2011) Aflatoxin producing potential and community structure of *Aspergillus* section *Flavi* in almond orchards of the Central Valley of California. Phytopathology 101 (Supplement): S44.

Donner, M., Lichtemberg, P. S. F., Cotty, P. J., and Michailides, T. J. (2012) Community structure of *Aspergillus flavus* and *A. parasiticus* in major almond-producing areas of California (under review).

Lichtemberg, P. S. F., Donner, M., Siegel, J., Michailides, T. J. (2012) Damage by navel orangeworm, infection by *Aspergillus flavus* and *A. parasiticus*, and potential of aflatoxin contamination of almonds in California (in preparation).

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Cotty P.J. (1994c) Comparison of 4 media for the isolation of *Aspergillus flavus* group fungi. Mycopathologia 125:157-162.

Cotty P.J., Cardwell K.F. (1999) Divergence of West African and North American communities of *Aspergillus* section Flavi. Applied and Environmental Microbiology 65:2264-2266.

Doster, M. A., and Michailides, T. J. (1994). Development of *Aspergillus* molds in litter from pistachio trees. Plant Disease 78:393-397.

Klich M.A., Pitt J.I. (1988) Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. Transactions of the British Mycological Society 91:99-108.

Mateles R.I., Adye J.C. (1965) Production of aflatoxins in submerged culture. Applied Microbiology 13:208-211.

Pitt J.I., Hocking A.D., Glenn D.R. (1983) An improved medium for detection of *Aspergillus flavus* and *A. parasiticus*. Journal of Applied Bacteriology 54:109-114.

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Table 1. Percentage of *Aspergillus* section *Flavi* strains resident in soils of almond orchards, percentages of toxigenic and atoxigenic L-strains, mean of aflatoxin concentration of the L-strains, soil pH, and colony-forming units (CFU) of *Aspergillus* section *Flavi* for three years[a].

Year	N[b]	% *A. flavus* -L	% *A. flavus* -S	% *A. parasiticus*	toxigenic[c]	atoxigenic[c]	aflatoxin B_1[c]	pH	CFU
2007	765	56 a	8 a	36 a	64 b	36 b	112 a	6.95 a	31 a
2008	643	56 a	3 b	41 a	82 a	18 b	105 a	7.04 a	24 a
2010	1334	68 a	15 c	17 a	52 b	47 a	140 b	7.43 a	27 a

[a]Percent data were arcsine square root transformed, the total aflatoxin concentration were log transformed for the analysis, and CFU data were log transformed prior to the analysis. Means not sharing a common letter are significantly different according to Fisher's least significant difference test (α = 0.05).
[b] Total number of isolates per year.
[c] Proportions of *A. flavus* L-strain isolates. The total aflatoxin concentrations are in µg aflatoxin B_1 per gram mycelium (µg g^{-1})

Table 2. Propagule density in colony-forming units (CFU) of *Aspergillus* section *Flavi* per gram of soil and soil pH for three years[a].

		2007				2008				2010			
		CFU g^{-1} soil		soil pH		CFU g^{-1} soil		soil pH		CFU g^{-1} soil		soil pH	
Region	N[b]	mean	range	mean	range	mean	range	mean	range	mean	range	mean	range
North	33	31 a	2-219	6.72 a	4.75-7.98	11 b	4-33	6.93 a	4.91-7.98	11 b	1-32	7.41 a	6.52-7.85
Central	24	25 a	4-70	6.97 a	5.91-7.52	39 a	11-75	7.07 a	6.46-7.73	28 a	5-62	7.38 a	6.92-7.92
South	24	37 a	3-175	7.26 a	6.66-8.22	24 a	4-120	7.07 a	6.52-7.77	48 a	6-153	7.49 a	7.22-8.00
Total	81	31	2-219	6.95	4.75-8.22	24	4-120	7.04	4.91-7.98	27	1-153	7.43	6.52-8.00

[a] Means not sharing a common letter are significantly different according to Fisher's least significant difference test (α = 0.05). CFU were log transformed prior to analyses.
[b] Number of tested soil samples for each year.

Table 3. Mean aflatoxin quantities produced by three aflatoxin-producing taxa of *Aspergillus* section *Flavi* originated from three regions in California and three years.

	2007			2008			2010										
	A. flavus L-strain			*A. flavus* L-strain			*A. flavus* L-strain			*A. flavus* S-strain			*A. parasiticus*				
	B_1 (µg g^{-1})			B_1 (µg g^{-1})			B_1 (µg g^{-1})			B_1 (µg g^{-1})			B_1 (µg g^{-1})			G_1 (µg g^{-1})	
Region	N[a]	mean[b]	range	N[a]	mean[b]	range	N[a]	mean[b]	range	N[a]	mean[b]	range	N[a]	mean[b]	range	mean[b]	range
North	76	148 a	0.4-1285	59	149 a	0.3-2516	47	130 b	0.02-1392	18	301 a	3-1690	16	641 a	4-4086	747 a	9-3274
Central	86	68 b	0.6-492	53	78 ab	0.3-749	40	164 a	2-2402	4	11 b	1-27	16	485 a	4-3270	235 a	2-1655
South	71	105 ab	0.6-947	54	65 b	0.1-484	56	127 b	0.3-955	16	190 a	2-1429	3	80 b	2-319	139 b	3-595
Total	233	112	0.4-1285	166	105	0.1-2516	143	140	0.02-2402	38	237	2-1690	35	672	2-4086	489	2-3274

[a] Number of toxigenic isolates.
[b] Aflatoxin concentration values are µg g^{-1} and were log transformed prior to statistical analysis. Means followed by the same letter in a column are not significantly different by the Fisher's least significant difference test (α = 0.05).

Table 4. 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

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

Table 5. Involvement of specific *Aspergillus flavus* group fungi in the decay of hulls 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				
			A. flavus L strain				
Date	Treatment	Nuts evaluated	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
2010	AF36	9,000	0	0	0	0	0
	Control	9,000	0	0	0	0	0
2011	AF36	9,000	0	0	0	0	0
	Control	9,000	0	0	0	0	0

Table 6. Incidence of *Aspergillus* section *Flavi* isolates isolated from mummies of different almond cultivars.

Almond Mummy Status	Nonpareil		Butte		Padre	
	n	mean[a]	n	mean[a]	n	mean[a]
no-damage	48	9.1% a	11	2.3% a	8	2.0% a
damage	9	12.2% a	1	5.6% a	0	0.0% b
Overal Incidence[b]	**57**	**9.5% a**	**12**	**2.4% b**	**8**	**2.0% b**

[a]Percent data were transformed with the arcsine square root transformation prior to analysis. Means with common letters are not significantly different (P<=0.05).
[b]Differences in overall incidence are significantly (P<=0.05) different among the three studied cultivars.

Table 7. Incidences of *Aspergillus flavus* L-strain, S-strain, and *A. parasiticus* isolates recovered from mummies of three different almond cultivars.

Strains	Nonpareil		Butte		Padre		Overall distribution[b]	
	n	mean[a]	n	mean[a]	n	mean[a]	n	mean[a]
L-strain	51	89.5% a	11	91.7% a	8	100.0% a	70	90.9% a
P-strain	6	10.5% b	0	0.0% b	0	0.0% b	6	7.8% b
S-strain	0	0.0% c	1	8.3% b	0	0.0% b	1	1.3% b

[a]Percent data were transformed with the arcsine square root transformation prior to analysis. Means with common letters are not significantly different (P<=0.05).
[b]Means of overall distribution of strains regardless of almond cultivar.

Table 8. Toxigenicity of *Aspergillus flavus* L-strain isolates recovered from mummies of three almond cultivars according to kernel status (damage or no damage).

Almond Variety	Almond Mummy Status	Number of *Aspergillus flavus* "L strain"	Toxigenicity Distribution			
			Atoxigenic Strains		Toxigenic Strains	
			n	(%)	n	(%)
Nonpareil	damage	8	5	62.5%	3	37.5%
	no-damage	43	31	72.1%	12	27.9%
	total	**51**	**36**	**70.6%**	**15**	**29.4%**
Butty	damage	1	-	-	1	100.0%
	no-damage	10	7	70.0%	3	30.0%
	total	**11**	**7**	**63.6%**	**4**	**36.4%**
Padre	damage	0	-	-	-	-
	no-damage	8	7	87.5%	1	12.5%
	total	**8**	**7**	**87.5%**	**1**	**12.5%**

Table 9. Incidence of *Aspergillus* Section *Flavi* from mummies with damage or no damage of three almond cultivars according to the levels of aflatoxin amounts produced.

Almond Variety	Almond Mummy Status	Aflatoxin Concentration Distribution Range[a]							
		1 - 9 µg g^{-1}		10 - 99 µg g^{-1}		100 - 299 µg g^{-1}		> 300 µg g^{-1}	
		n	(%)	n	(%)	n	(%)	n	(%)
Nonpareil	no-damage	2	13.3	2	13.3	3	53.3	8	20
	damage	1	33.3	1	33.3	1	33.3	-	-
	total[b]	**3**	**16.7%**	**3**	**16.7%**	**4**	**22.2%**	**8**	**44.4%**
Butte	no-damage	-	-	1	25	1	25	2	50
	damage	-	-	-	-	1	100	-	-
	total[b]	**-**	**-**	**1**	**20.0%**	**2**	**40.0%**	**2**	**40.0%**
Padre	no-damage	-	-	-	-	-	-	1	100
	damage	-	-	-	-	-	-	-	-
	total[b]	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**100%**

[a]Aflatoxin amounts are in µg g^{-1} (equivalent to ppm) related to 1 g of fungal mycelia.
[b]Distribution of total isolates recovered within a cultivar.

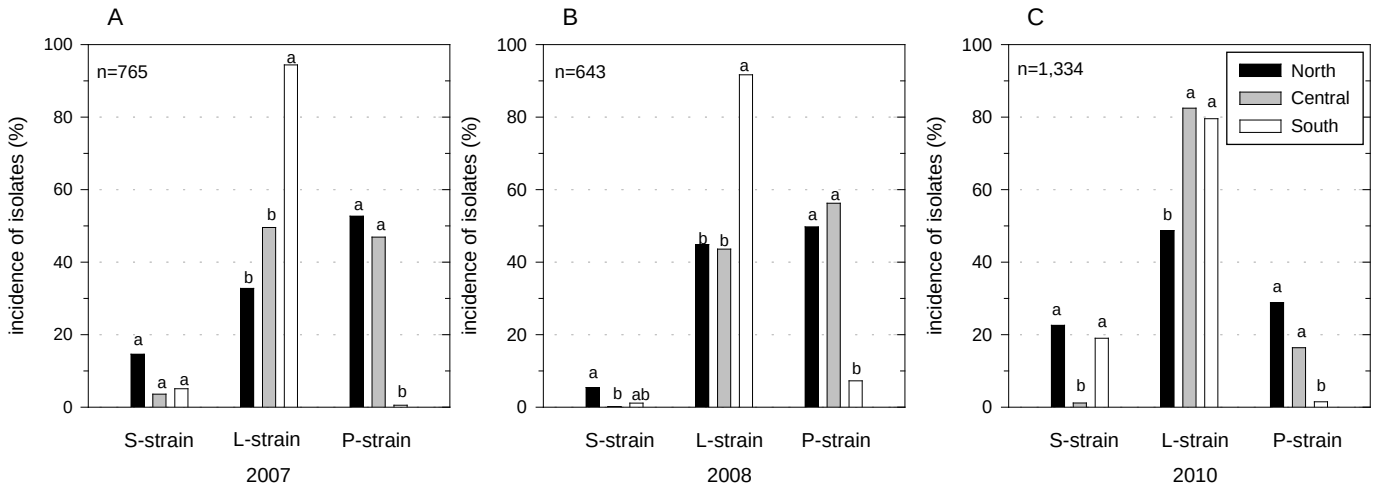

Figure 1. Incidences of *Aspergillus* section Flavi in soils of three major almond growing regions for three years. (**A**) Represents the incidences in 2007, (**B**) in 2008 and (**C**) in 2010. Means not sharing a common letter are significantly different according to the Fisher's least significant difference test (α = 0.05).

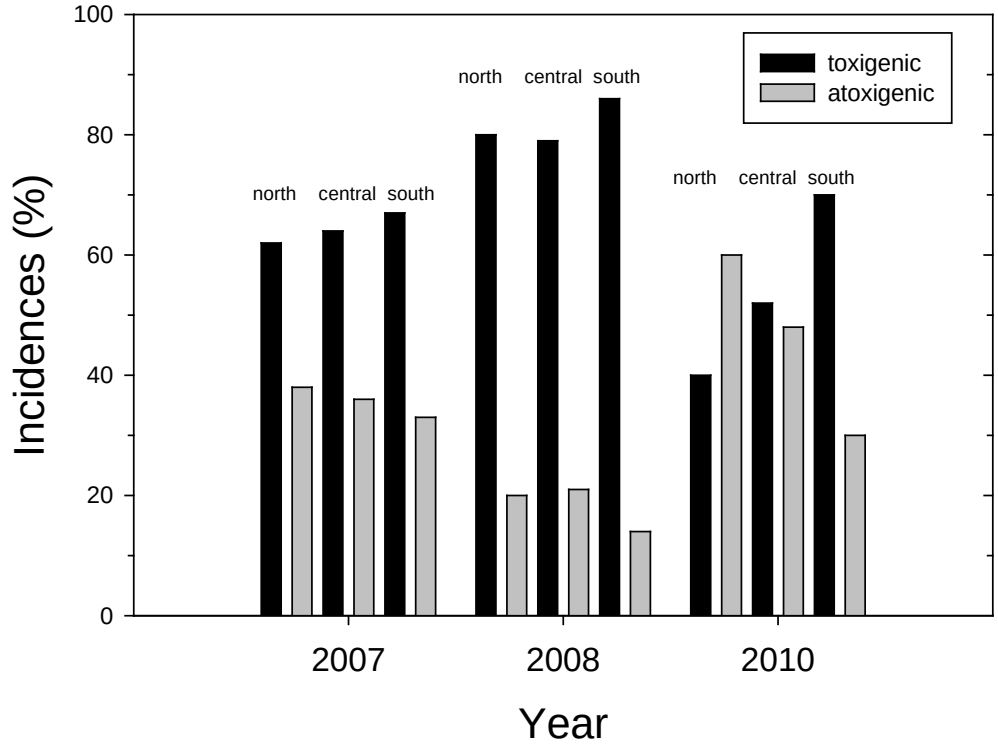

Figure 2. Distribution of aflatoxin-producing and atoxigenic *Aspergillus flavus* L-strain isolates among three regions of California and for three years.

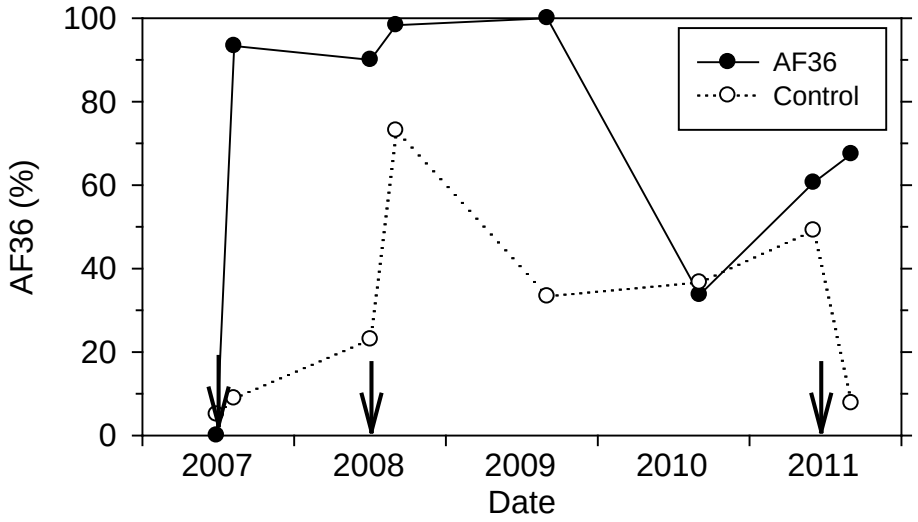

Figure 3. Percentage of *Aspergillus flavus* isolates belonging to the atoxigenic strain AF36 for isolates from soil collected from the areas treated with the wheat-AF36 product or from untreated areas in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product was applied on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

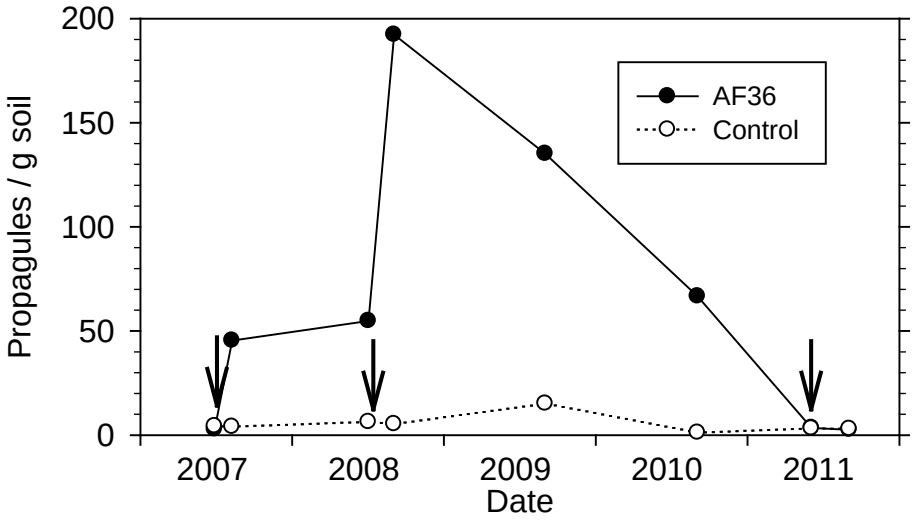

Figure 4. Density of *Aspergillus flavus*/*A. parasiticus* in soil collected from areas treated with the wheat-AF36 product or from untreated areas in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product was applied on 28 June 2007, 2 July 2008, and 3 June 2011 (arrows).

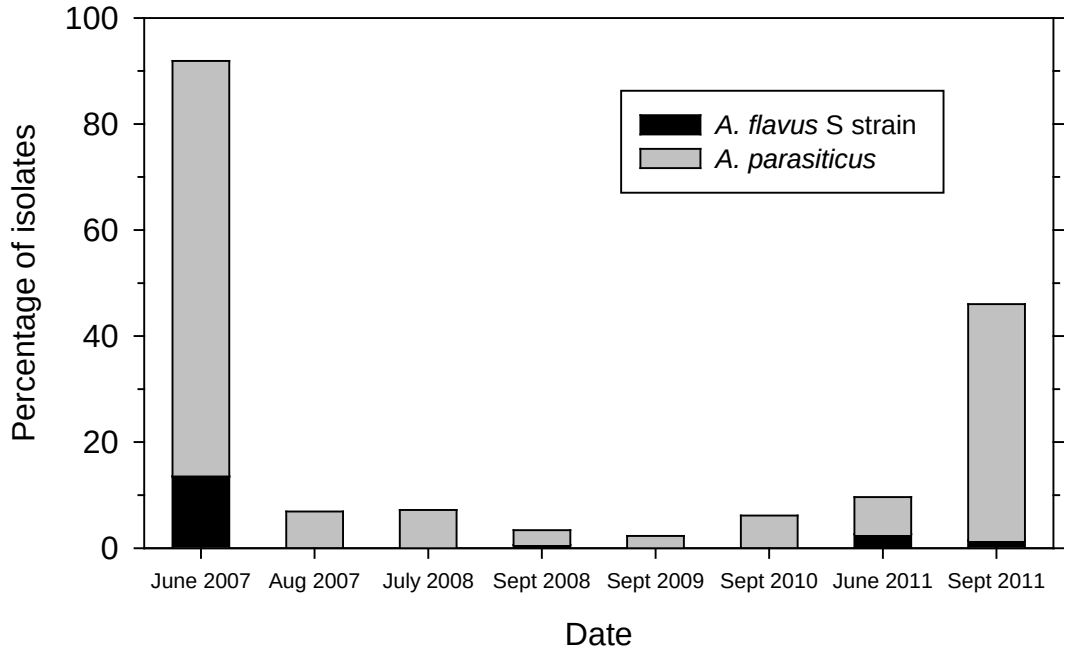

Figure 5. Percentage of *Aspergillus flavus*/*A. parasiticus* isolates that are the aflatoxin-producers *A. parasiticus* and *A. flavus* S strain for isolates from soil collected from areas treated with the wheat-AF36 product in a research almond orchard at the Nickels Soil Laboratory.

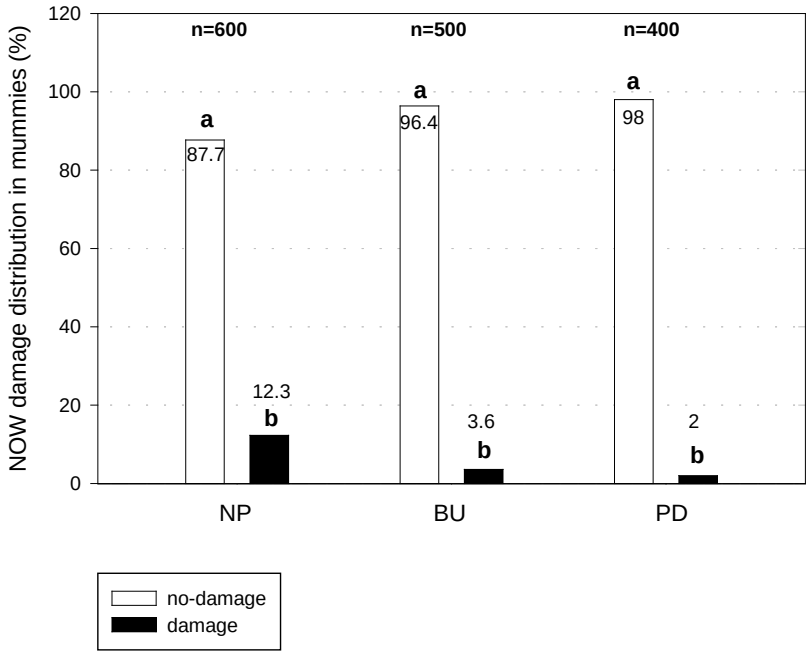

Figure 6: ANOVA of NOW damage distribution found in almond mummies varieties. [a] Percent data were arcsine square root transformed prior to analysis. [b] Means with common letter are not significantly different (*P*<=0.05). NP = Nonpareil, BU = Butte and PD = Padre.

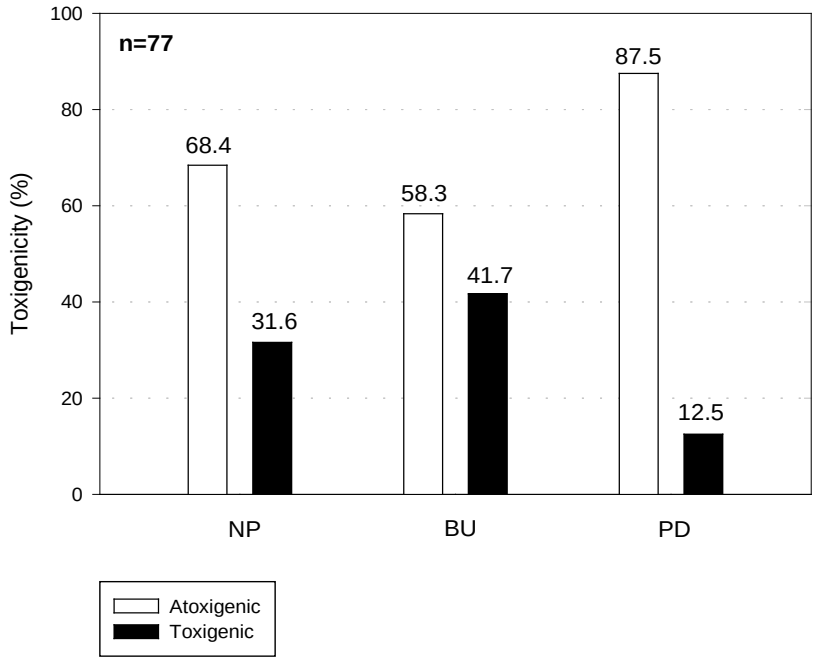

Figure 7. Overall toxigenicity distribution of *Aspergillus* Section *Flavi* isolates within three studied varieties. NP = Nonpareil, BU = Butte, and PD = Padre.