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

Project No.: **10-AFLA1-Michailides**

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Objectives:

1. Identify risk factors and spatial patterns associated with aflatoxin development in California almonds.
2. Determine the spread and survival of the atoxigenic *Aspergillus flavus* strain AF36 previously applied to orchards.

Interpretive Summary:

Spatial patterns of aflatoxigenic fungi. The density of aflatoxin producing species was determined in almond orchards in three major almond producing regions for 2010 and compared to those in samples collected in 2007 and 2008. *Aspergillus* section *Flavi* was resident 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. Incidences of the L-strain increased from 59% in 2007 to 70% in 2010. The highest incidences of L-strain

isolates were found in the south, whereas lower incidences were observed for soils in the north.

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 regions and years.

The S-strain, which is a casual agent for aflatoxin contamination on various crops, was most commonly found in northern soils and the least commonly isolated in the central region. Among the years the incidences varied widely. All tested S-strain and *A. parasiticus* isolates produced high concentrations of aflatoxin in liquid fermentation.

The northern almond orchards have the highest reservoir of highly toxigenic strains. Of these, the *A. flavus* S-strain and *A. parasiticus*, were the most commonly found strains.

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. Currently, AF36 is registered as a biopesticide for cotton in Arizona, Texas, and California and corn in Texas. Because application of this strain in experimental pistachio and fig orchards in California has given promising results, we initiated a project with almonds in 2007 investigating the use of AF36 to reduce aflatoxin contamination in almond orchards. On 28 June, 2007 and on 2 July 2008, wheat infected with AF36 (the same commercial product used in cotton fields) was applied to the ground in a drip-irrigated almond orchard at the Nickels Soil Laboratory in Arbuckle, CA. No AF36 has been applied after July 2008, although nut and soil samples continued to be collected to determine the survival and spread of AF36.

Before applying AF36, 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 with AF36, the frequency of *A. parasiticus* and the S strain of *A. flavus* in the fungal population decreased substantially. And in 2010 only 6.1% of the isolates were *A. parasiticus* or *A. flavus* strain S.

Although very little of the atoxigenic strain AF36 was present in the orchard soil before applying the wheat with AF36 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 level of AF36 has 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 and perhaps the wheat-AF36 does not need to be applied every year. However, by September 2010 the level of AF36 decreased substantially, suggesting that an additional application of wheat –AF36 would be

needed. The results demonstrate that applying wheat with AF36 was very effective in introducing the atoxigenic strain AF36 under the conditions present in this almond orchard.

Applying AF36 did not significantly increase the incidence of hull decay of the nuts. The percentages of hulls decayed by *A. flavus*/*A. parasiticus* in treated areas were only 0.20%, 0.03%, 0.03%, and 0% for nuts harvested in 2007, 2008, 2009, and 2010, respectively. For comparison, the incidence of hull decay caused by *A. niger* was substantially higher (1.0% of the hulls were decayed by *A. niger* in both 2008 and 2009 and 0.065 in 2010).

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 is effective in increasing the level of this atoxigenic strain AF36. The future plan for this study might include applying more AF36 in the same orchard in order to determine any increase of AF36 again in the soil over multiple years. In addition, another area of research interest is to evaluate using sorghum as the substrate for AF36 instead of wheat, which would be desirable because wheat allergies are somewhat common.

Materials and Methods:

1. 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* L-strain 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 *A. flavus* L-strain isolates were randomly picked and over 500 were analyzed twice for aflatoxin content in liquid fermentation.

Isolates were fermented in Adye & Matales medium, (A&M, Mateles and Adye, 1965), using 22.4mM urea as the sole nitrogen source and adjusted to pH 4.7 prior to autoclaving (Cotty and Cardwell, 1999). Vials of 15-ml capacity containing 5 ml of A&M media were inoculated with 50 µl of approximately 2×10^3 conidia, and incubated unilluminated for 7 days at 31^{o}C. The fungal cells lyses after 3 ml acetone was added. After one hour, one volume of water was added and 3 ml of dichloromethane. The dichloromethane containing the aflatoxin was extracted and filtered (0.2µm nylon membrane, VWR International) into 4ml vials. Extracts were evaporated to dryness and redissolved in 1 ml methanol. Subsequently, 100 µl of the extract was transferred to autosampler vials containing 1.9 ml of HPLC mobile phase consisting of methanol:water (1:1, v/v). Highly aflatoxin concentrated extracts were diluted as appropriated. The

mycelia were collected on previously weighted Whatman No.4 filter paper, dried in a forced air oven (46^{o}C for 7 days), and weighted to quantify fungal biomass.

Aflatoxins were quantified with a HPLC (high pressure liquid chromatography) system (Hewlett Packard 1050) consisting of an isocratic pump, an autosampler and fluorescence detector (Hewlett Packard 1046A). Aflatoxins were separated on Nova-pak C18 column (length of 150 mm, inner diameter of 3.9 mm and particle size of 4 µm) supplied by Waters (Massachusetts, USA) and the column temperature was set to 25^{o}C (Pickering laboratories, CHX650). The mobile phase was a mixture of methanol:water (45:55, v/v) with a flow rate of 0.8 ml min-1. A postcolumn photochemical derivatization with a photochemical reactor (Aura Industries, Inc., Staten Island, NY) containing a knitted reactor coil (0.25mm ID x 25 m) was used to enhanced the fluorescence of aflatoxin B_1 and G_1. Detection of aflatoxins was carried out using 360 and 440 nm as wavelengths for excitation and emission, respectively. A volume of 100µl was injected into the HPLC for each sample. The limit of detection and the limit of quantitation for aflatoxins B_1, B_2, G_1, and G_2 were all below 1.0 ng g^{-1}.The aflatoxin concentrations were calculated based on a 8-point calibration curve obtained from standards for all four aflatoxin (Supelco Inc., Bellefonte, Pa).

Statistical analyses

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 (LSD). Analyses for percentage values and aflatoxin concentrations were preformed with data transformed, using the arcsine of the square root and the natural logarithm (log), respectively.

2. Determine the spread and survival of the atoxigenic *Aspergillus flavus* strain AF36 previously applied to orchards.

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 drip-irrigated 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, 1 September 2009, and 2 September 2010. 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. Strain identification was done using the Vegetative Compatibility Group procedure.

Results and Discussion:

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

Toxigenicity of Aspergillus populations

The density of aflatoxin producing species was determined in almond orchards in three major almond producing regions for the years 2007 and 2008. *Aspergillus* section *Flavi* was resident 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. Therefore, isolates of the most common strain resident in the valley were tested. 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 regions and years. In 2008, the proportion of toxigenic isolates increased 14% to 19% in all tested regions. Among the regions, the incidences of toxigenics were nearly the same and ranged in 2007 from 62% in the north to 67% in the south **(Figure 1)**. In 2008, the frequency of toxigenic isolates was higher and ranged from 80% in the north to 86% in the south **(Figure 1)**. Overall, the average incidence of toxigenic isolates increased significantly ($P<0.05$) from 67% in 2007 to 86% in 2008 **(Figure 2)**.

Variability in productions of aflatoxin across regions, especially among *A. flavus* isolates, has often been reported (Cotty et al., 1994; Mahoney and Rodriguez, 1996). Results also indicated that within a single field a wide range of *A. flavus* isolates were found varying in potential to produce aflatoxin (Abbas et al., 2004). Studies in Argentina (Vaamonde et al., 2003) and Iran(Razzaghi-Abyaneh et al., 2006) revealed that less than 30% of the *A. flavus* isolates produce aflatoxin while in the southern USA the majority of *A. flavus* isolates produce aflatoxin. The relative distribution of toxigenic to atoxigenic isolates is modulated by many factors including plant species present, soil composition, cropping system, crop management, and environmental conditions, including rainfall and temperature (Horn et al., 1995; Zablotowicz et al., 2007). Interestingly, in California the incidence of toxigenic isolates was relatively constant across the valley in any given year.

Aflatoxin quantification

In both years 2007 and 2008, the highest average aflatoxin concentration, close to 140 µg g^{-1} B_1, was produced by isolates originating in the north while the lowest aflatoxin producing average varied among the years **(Figure 3)**. In 2007, the L-strains isolates of the central region produced the lowest aflatoxin and averaged 68 µg g^{-1} B_1, whereas in 2008 the isolates of the southern region produced the least concentrations of aflatoxin and averaged 65 µg g^{-1} B_1 **(Figure 3)**. Interestingly, the total aflatoxin producing average of the regions overall for both years was almost equal with an average of 112 µg g^{-1} B_1 in 2007 with 166 *A. flavus* L-strain isolates averaged, and 105 µg g^{-1} B_1 in 2008, with 226 isolates averaged (**Table 1**). Nevertheless, the aflatoxin-producing potential varied widely among the orchards and years (**Figure 4**). The highest average aflatoxin concentration was in orchard #11, located in the north, with 500 µg g^{-1} B_1 in 2008, while the least average aflatoxin concentration was also measured in the north in orchard #9 with 3 µg g^{-1} B_1. (**Figure 4**).

Nearly 70% of the L-strain isolates from both years produced >10 µg of aflatoxin per g mycelium and 8-11% of the isolates produced even more than 300 µg (**Figure 5**). Interestingly, the pattern of aflatoxin production for those isolates producing >10µg was almost the same for both years. However, the percentage of toxigenic isolates producing less than 3 µg varied among the years. The study showed that an increase of toxigenic isolates in the fields from one year to another does not necessary increase the aflatoxin producing potential within the fungal communities of toxigenic isolates. Nevertheless, the study revealed a dominance of highly toxigenic L-strains in the fields. Regional difference in production of aflatoxin B_1 by soil isolates of the *A. flavus* L-strain was evident along the Central Valley. Surveys done in peanut-growing, cotton-growing and corn-growing regions also revealed geographic differences in production of aflatoxin B_1 by *A. flavus* (Cotty, 1997; Donner et al., 2009; Horn and Dorner, 1999). Soil serves as the primary habitat for *Aspergillus* species and from there the fungus can infect susceptible crops if the conditions are favorable. Therefore, high incidences of toxigenic *A. flavus* isolates in soil which are capable of producing high concentrations of aflatoxins can increase the risk of crop contamination. Applications of native atoxigenic *A. flavus* strains to agricultural soil has been used successfully to control aflatoxin contamination (Atehnkeng et al., 2008; Ehrlich and Cotty, 2004). Aflatoxin reduction has been reported for crops like peanuts, cotton, pistachios, and corn (Atehnkeng et al., 2008; Cotty, 1994; Dorner et al., 1998). The results of the present study suggest which regions might benefit most from soil application of an atoxigenic L-strain isolate of *A. flavus*. A frequently applied atoxigenic strain like AF36 would decrease the toxigenic proportion of the *A. flavus* isolates in the long-term and with it the risk of contaminated nuts.

2. 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.

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 level 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 6**). 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 and perhaps the wheat-AF36 does not need to be applied every year. However, by September 2010 the level of AF36 decreased substantially (**Figure 6**), suggesting that an additional application of the wheat-AF36 would be needed. On 3 June, 2011, an additional application of wheat-AF36 was made in this orchard. In addition, 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.

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 7**). 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 further in 2010 when no additional AF36 was applied (**Figure 7**). For comparison, the density of *A. niger* in the soil during the period of the study ranged from 78 to 499 propagules / g soil (depending on the sample date) with mean values of 213 and 155 propagules / g soil for the AF36-treated and untreated areas, respectively.

Before applying the wheat-AF36 product, the most common aflatoxin-producing fungus present was *A. parasiticus* (**Figure 8**), 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 8**). 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 8**). These results provide evidence of the potential for the atoxigenic strain AF36 to displace aflatoxin-producing fungi.

Applying AF36 did not significantly increase the incidence of hull decay by *A. flavus* for nuts from the treated areas (**Table 2**), indicating that applying the fungus AF36 to the orchard floor did not result increased fungal decay of the crop. Furthermore, 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. 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.

Research Effort Recent Publications:

Abstracts

Donner, M., Lichtemberg, S. F. P., and Michailides, T.J. 2011. Physiological almond development associated with preharvest aflatoxin contamination. Tropentag, October 5-7, 2011, Bonn.

Donner, M., Lichtemberg, S. F. P., 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 Supplement (in press and poster presented at the 2011APS-IPPC Join Meeting, Aug 6-10, Honolulu, Hawaii).

Ackowledgments

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Table 1. Incidences of aflatoxin-producing and atoxigenic *A. flavus* isolates among the years and regions.

						aflatoxin concentration B_1 (μg g^{-1})		
Year	Region	Number of orchards	Number of isolates	Atoxigenic	Toxigenic	Mean	Minimum	Maximum
2007	North	11	89	34	55	**148**	0	1285
2007	Central	9	90	32	58	67	0	493
2007	South	8	79	26	53	105	0	947
Total		**32**	**258**	**92**	**166**	**112**	**0**	**1285**
2008	North	11	107	21	86	147	0	2517
2008	Central	9	90	19	71	88	0	749
2008	South	8	80	11	69	65	0	171
Total		**32**	**277**	**51**	**226**	**105**	**0**	**2517**

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
2010	AF36	0.000 ns	0.059 ns	0.072 ns	0.006 ns
	Untreated control	0.000	0.059	0.043	0.000

[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				
			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

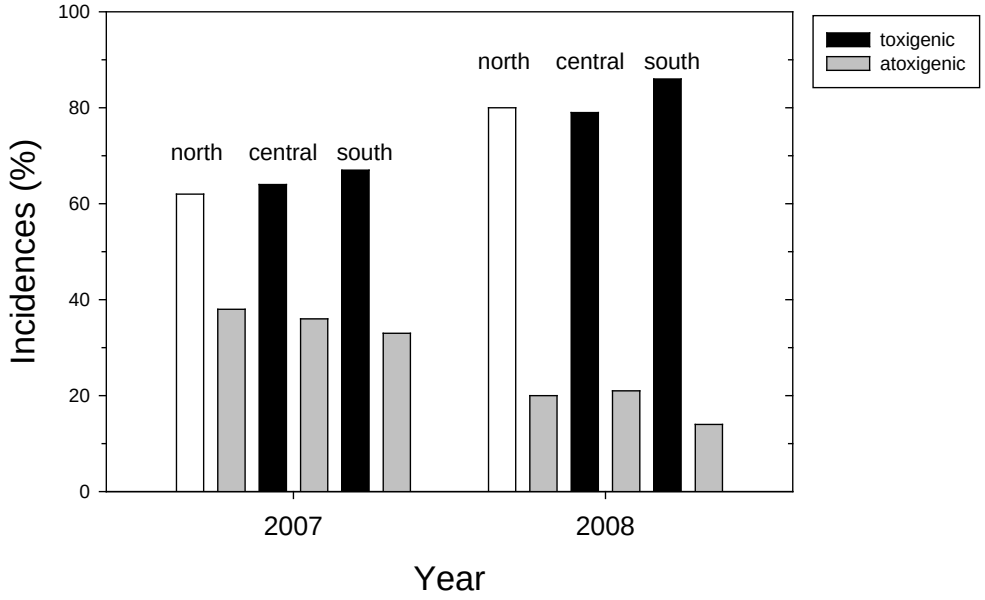

Figure 1. Distribution of aflatoxin-producing and atoxigenic *A. flavus* L-strain isolates among the years and regions of Central Valley.

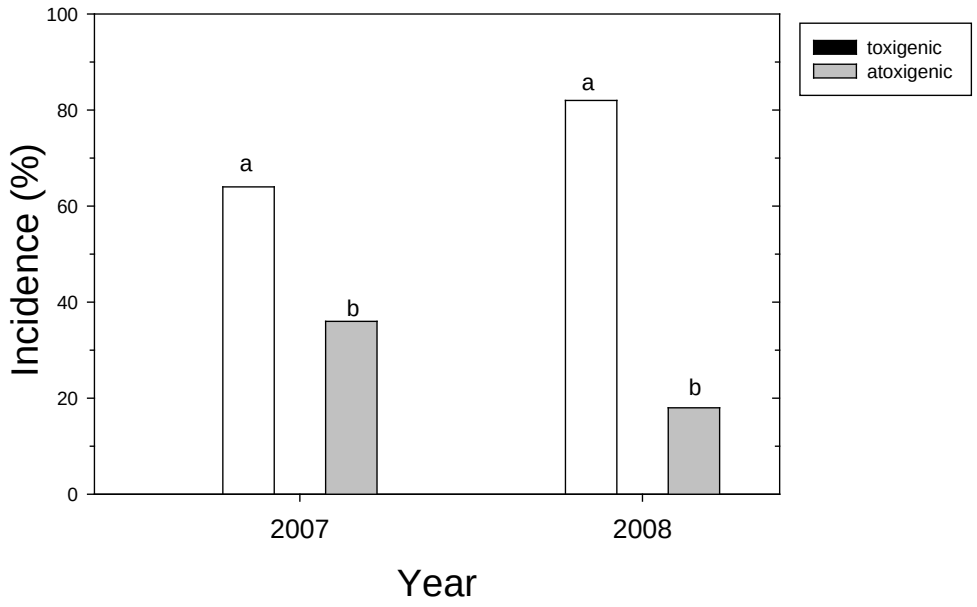

Figure 2. Total incidences of aflatoxin-producing and atoxigenic *A. flavus* L-strain isolates of the year 2007 and 2008. Means not sharing a common letter are significantly different according to Fisher's LSD test (α=0.05).

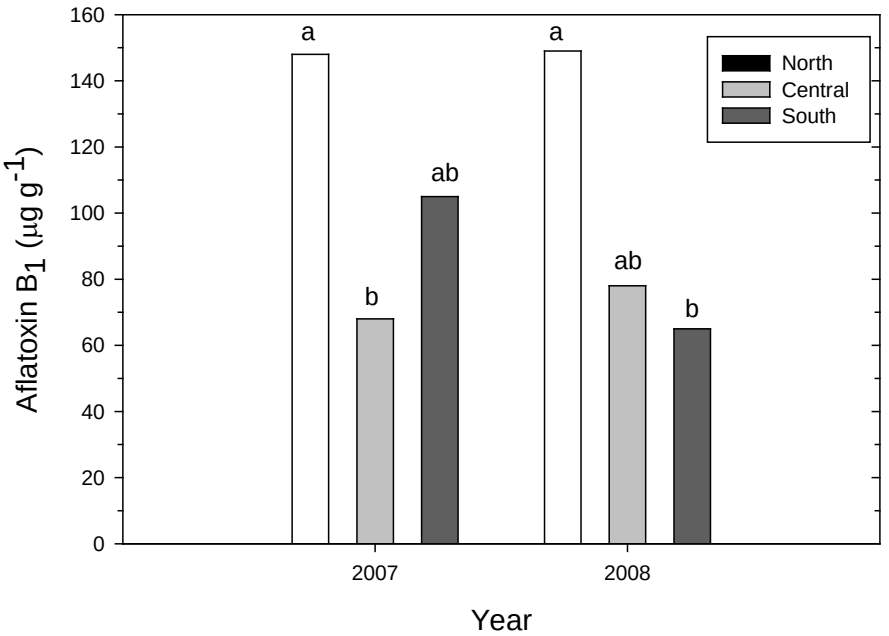

Figure 3. Mean aflatoxin quantities produced by *A. flavus* L-strains among different years and regions of the Central Valley. Means not sharing a common letter are significantly different according to Fisher's LSD test (α=0.05).

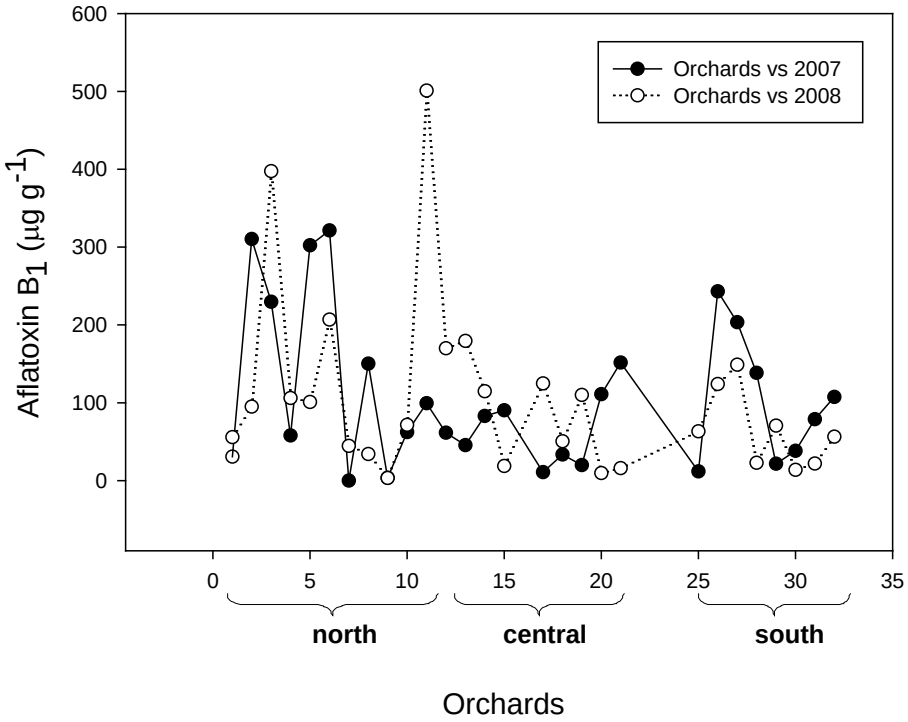

Figure 4. Average aflatoxin concentrations of the aflatoxin producing *A. flavus* L-strain isolates from orchards across the Central Valley of the year 2007 and 2008.

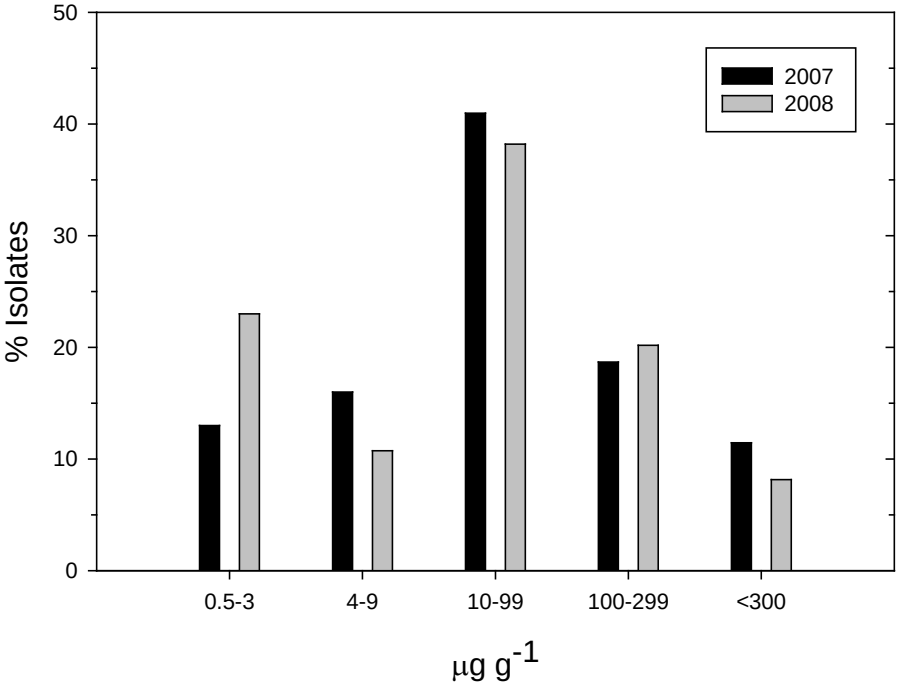

Figure 5. Production of aflatoxin B1 by the L-strain isolates of *A. flavus* from 2007 (n=166) and 2008 (n=226).

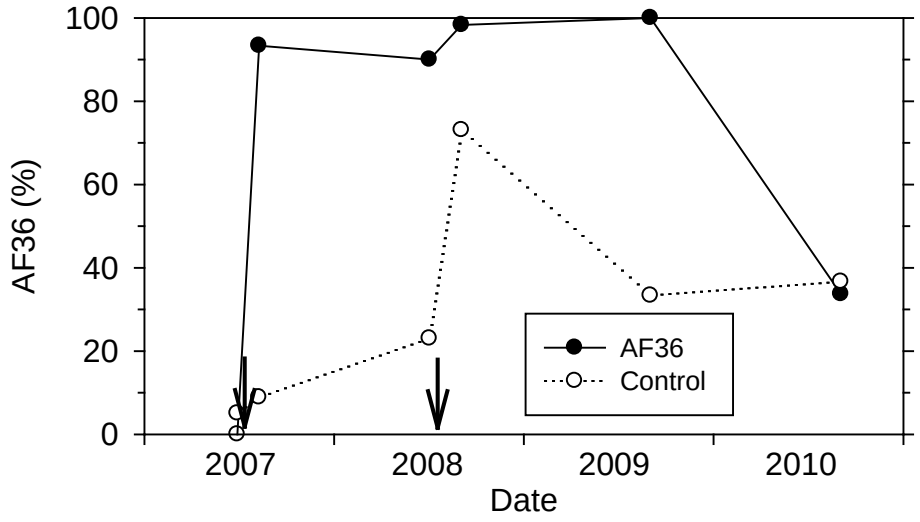

Figure 6. Percentage of *Aspergillus flavus* isolates belonging to the atoxigenic strain AF36 for isolates from soil collected from the areas treated with the wheat-AF36 product or from untreated areas in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product was applied on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

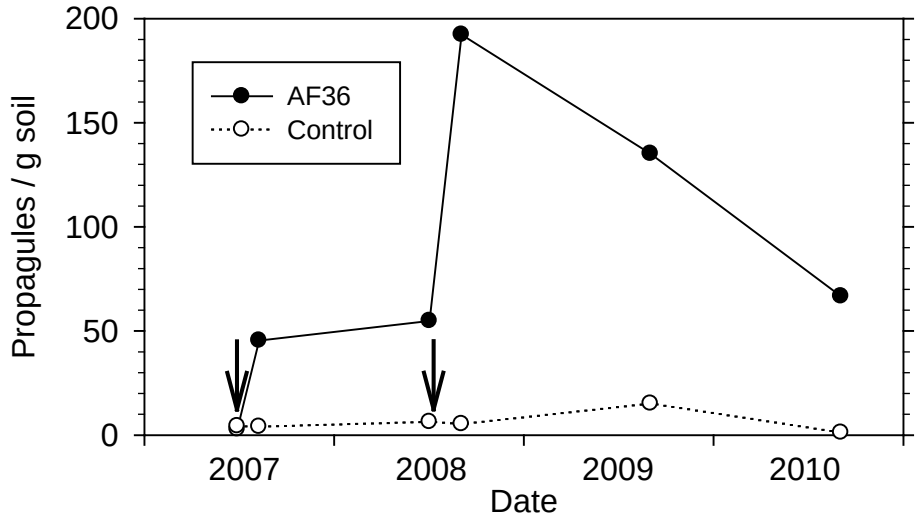

Figure 7. Density of *Aspergillus flavus*/*A. parasiticus* in soil collected from areas treated with the wheat-AF36 product or from untreated areas in a research almond orchard at the Nickels Soil Laboratory. The wheat-AF36 product was applied on 28 June 2007 and 2 July 2008 (arrows).

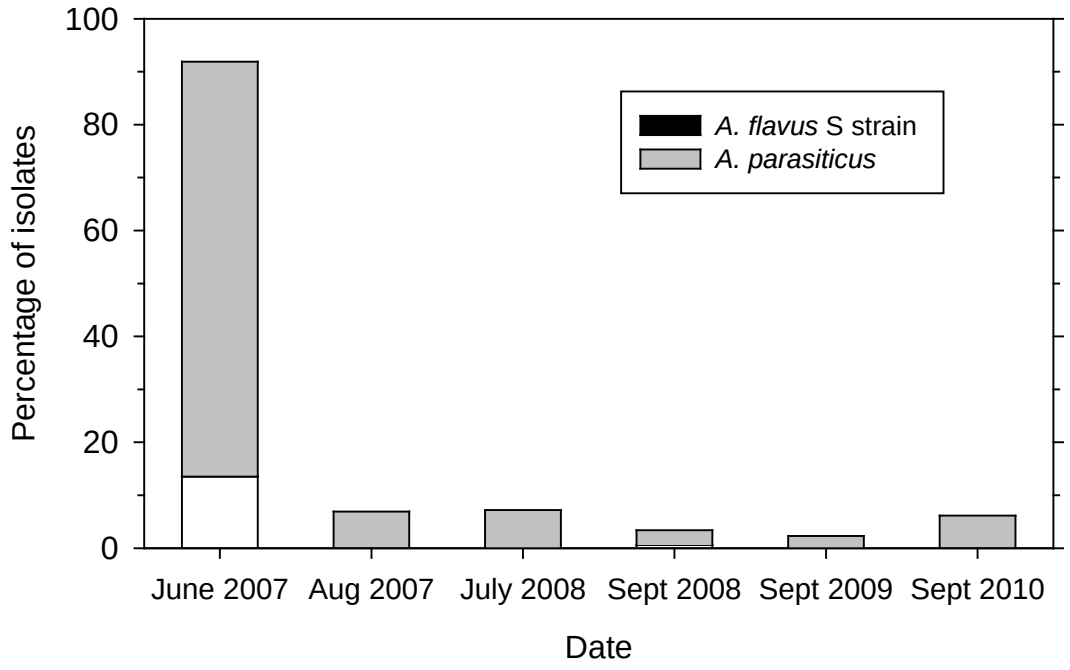

Figure 8. Percentage of *Aspergillus flavus*/*A. parasiticus* isolates that are the aflatoxin-producers *A. parasiticus* and *A. flavus* S strain for isolates from soil collected from areas treated with the wheat-AF36 product in a research almond orchard at the Nickels Soil Laboratory.