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

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

Project Leader: Themis J. Michailides
UC Kearney
9240 South Riverbend Ave.
Parlier, CA 93648
(559) 646-6546
themis@uckac.edu

Project Cooperators and Personnel:
Peter Cotty, USDA/ARS and University of Arizona, Tucson, AZ
Mark Doster, UC Kearney, Parlier
Joel Siegel, USDA/ARS, SJVERC, Parlier
David Morgan, Yong Luo, Bulent Kabak, Lorene Boeckler-Doster, Ryan Puckett, Jessica Windh, and Heraclio Reyes, UC Kearney, Parlier
Eva Mondini, Ecole Nationale d' Ingénieurs des Travaux Agricoles de Bordeaux, Gradignan Cedex, France
Aminata Diawara, Summer Student Intern, Université de Bretagne Occidentale, Brest, France
Barry Wilk, PCA, Chico

Objectives:

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

Interpretive Summary:

We have made a large collection (about 1,500 isolates) of *Aspergillus flavus* and *A. parasiticus* collected from almond orchards representing three major geographical regions. Analyses of soil samples collected in 2008 revealed slightly different results from those of 2007. Low levels of *A. parasiticus* were detected in the southern region for both years. The density of *A. flavus* ranged from 0 to 16 for the northern, 0 to 14 in central, and 2 to 13 CFU/g of soil. One orchard in central had 62 CFU/g soil and one in southern region had 548 CFU/g soil. As in 2007, soils in northern and central California had high densities of *A. parasiticus* (range 0 to 30 and 0 to 39 CFU/g soil, respectively) while those in the southern region had the lowest (range 0 to 6 CFU/g soil). The presence of *A. flavus* S strain (producing small sclerotia) is considered a major risk factor for aflatoxin contamination since previous research (working with isolates of *A. flavus* from pistachio orchard soils) showed that all the S strains produce aflatoxins typically at high amounts. In contrast, about 50% of the L strains (producing large sclerotia) of *A. flavus* do not produce aflatoxins, while those producing aflatoxins frequently only produce small amounts of aflatoxins. The incidence of the S strain was significantly higher (19% of the total *A. flavus* isolates) in the southern region than in central (2%) and northern (3%) regions of California.

In 2008, we collected two sets of five almond samples of 2 to 4 lbs each from each of nine commercial orchards from northern, central, and southern California as soon as the nuts were shaken from the trees and similar samples as soon as the nuts were swept to windrows and picked up. Almond kernel samples were analyzed to quantify aflatoxin levels in parts per billion (ng /g kernels) per orchard. The levels of aflatoxins were low in 2008 (0.0 to 3.29 ppb). In general, more samples from the northern orchards had aflatoxins than in those from the southern region. Furthermore, all three orchards in the northern region had G1 aflatoxin, which implies that almonds were infected by *A. parasiticus.* This is in agreement with the soil results of 2007 and 2008, which showed that the occurrence of *A. parasiticus* was higher in the northern region than in the central, and it was very low in the southern region. Two sets of samples were also collected in 2009. Samples collected as soon as the nuts were shaken to the ground are being analyzed currently. Information on the aflatoxin levels between the nuts collected as soon as the trees are shaken and those collected 7 to 10 days later when the nuts are swept and removed from the floor in each orchard will be useful to determine how much, if any, aflatoxin develops during the time the nuts are drying while laying on the orchard floor. Another study in cooperation with Dr. Lampinen (Plant Sciences, UC Davis) addresses the risk factors associated with aflatoxin contamination during stockpiling of almonds (Project 09-AFLA2-Lampinen).

Experiments were conducted to determine relative humidity as a risk factor for aflatoxin contamination of almonds. After the initial infection and incubation for 7 days, increasing relative humidity increased the amounts of sporulation for both *A. parasiticus* and *A. flavus,* suggesting that at higher relative humidity the aflatoxigenic fungi will produce higher amounts of spores that can either increase the soil population in almond

orchards or spread to other almonds in proximity (i.e., during stockpiling of the almonds) than at lower relative humidity. Thus drying the nuts sufficiently before placing them in stockpiles and making sure that water does not leak into the stockpiles is essential. If for some reason nuts get wet in a stockpile, relative humidity will result in an explosive growth of aflatoxigenic *Aspergillus* and an increase of the risk for aflatoxin contamination. Interestingly, the maximal aflatoxin contamination level at 95% relative humidity was attained within the first 4 days, although symptoms of infection were not apparent. This may explain why sometimes symptomless nuts indeed may contain high levels of aflatoxins. Incubating the infected kernels longer at 95% relative humidity did not alter the amounts of aflatoxins produced by *A. parasiticus*. This implies that once conditions are favorable for infection, high levels of aflatoxins can develop in relatively short time.

To determine the role navel orangeworm (NOW) moths and larvae play in vectoring aflatoxigenic fungi, we continue trapping NOW adults from April to July 2009 in 4 almond orchards. Levels of contamination of NOW with *Aspergillus* section *Flavi* ranged from 0 to 6%, 0 to 4%, 0 to 14%, and 0 to 8%, depending on the date of sampling in the four orchards respectively. In another experiment, clean larvae provided by Dr. Siegel (ARS, USDA, Parlier, CA) were closed in a Petri dish with an *Aspergillus*-infected almond nut for 5 days. Five NOW larvae of 2^{nd} to 3^{rd} instar were then caged with each of 7 healthy Nonpareil kernels for 10 days. Analyses of the kernels revealed that the larvae fed in them, transferred the propagules of *Aspergillus,* and resulted in aflatoxin contamination (up to 10 ppb) of the kernels. The experiment was repeated in the almond orchard, but aflatoxin analyses of these kernels have not been completed yet. The results thus far suggest that there is a close association of aflatoxigenic fungi and the NOW, and this pest not only creates the wounds for infection but also and perhaps most importantly brings the propagules of the pathogen to the right place for infection to occur.

AF36 is the atoxigenic strain of *A. flavus* registered as a biopesticide to reduce aflatoxin contamination in cottonseed in Arizona and Imperial Co., California. A second atoxigenic strain registered under the trade name Aflaguard and used to reduce aflatoxin contamination in peanuts. The strain AF36 was used to treat 3,000 acres of pistachio under an Experimental Use Permit to reduce aflatoxins in pistachios. AF36 is now used in microplots of almonds in Nickels Soil Laboratory orchards to obtain efficacy results and support registration of this strain in almonds. Although the level of the atoxigenic strain AF36 was very low in the soil before applying the wheat with AF36 in June 2007, after applying the wheat almost all of the *A. flavus* isolates were AF36 (**Tables 1 and 2**). This suggests 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 in treated areas from August 2007 to July 2008, indicating that AF36 survived the winter well. Applying AF36 did not seem to significantly increase the level of hull decay of the nuts, suggesting that the application of AF36 in almond orchards show similar effects as those shown in pistachio orchards. Our results from collecting fungal isolates from almond orchards showed that 1) the AF36 strain is widespread among the almond orchards; 2) the AF36 strain occurs

in high levels in commercial orchards; and 3) the strain can compete and survive well under natural conditions. This information can be used to support registration of AF36 strain as a biocontrol agent on almonds for the reduction of aflatoxin contamination. Furthermore, preliminary results show that about 50% of *A. flavus* isolated from NOW trapped in orchards where the AF36 strain was applied were the AF36 strain. This shows that NOW may also play a role in moving the AF36 strains among nut crops.

Publications:

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

Michailides, T. J., Doster, M. A., Morgan, P., Eveillard, H., and Charbaut, T. 2009. Levels of *Aspergillus flavus* and *A. parasiticus* in soils of almond orchards. Phytopathology **99**:S85 (Abstr.).

Table 1. Density of *Aspergillus flavus*/*A. parasiticus* and *A. niger* in soil at Nickels Soil Laboratory experimental orchard (cv. Nonpareil).

		Density (cfu/g soil) of the specified fungus	
Sample date	Treatment	*A. flavus*	*A. niger*
June 2007[z]	AF36	2.8 ns	733.1 ns
	Untreated control	4.3	265.7
August 2007	AF36	45.4 ns	104.2 ns
	Untreated control	4.0	170.2
July 2008	AF36	54.8 ns	34.7 ns
	Untreated control	6.3	217.1
September 2008	AF36	192.3 a	136.0 ns
	Untreated control	5.4 b	144.3
September 2009	AF36	133.1 ns	143.7 ns
	Untreated control	15.1	98.6

[z] These samples were collected prior to applying the AF36-wheat inoculum.

Table 2. Occurrence of the atoxigenic strain AF36 of *Aspergillus flavus* in the soil at Nickels Soil Laboratory Nonpareil experimental orchard.

	Percentage of *A. flavus* isolates belonging to strain AF36			
Treatment	June 2007	August 2007	July 2008	September 2008
AF36	0.0 ns	95.7 a	93.3 a	99.4 ns
Untreated control	1.7	5.6 b	21.8 b	74.4