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

Project No.: **08-AFLA1-Michailides**

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

Project Cooperators and Personnel:
Peter Cotty, USDA/ARS & University of Arizona, Tucson, AZ
Mark Doster, UC - Kearney Agricultural Center, Parlier, CA
Joel Siegel, USDA/ARS, SJVERC, Parlier, CA
Yong Luo, Bulent Kabak, Lorene Boeckler-Doster, David Morgan, Jessica Windh, and Heraclio Reyes, all at UC - Kearney Agricultural Center, Parlier, CA
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, CA

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 (up to 900 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 mean density of *A. flavus* was 3, 11, and 5 propagules per gram soil for the northern, central, and southern regions, respectively. As in 2007, soils in northern California had the lowest *A. tamarii* density (2 propagules/g soil) while central and southern had 18 to 20 propagules per 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 strain (producing large sclerotia) of *A. flavus* does not produce aflatoxins, while those producing aflatoxins frequently only produce small amounts of aflatoxins. The density of the S strain was significantly higher in the southern region than in central and northern California.

Because it makes better sense to determine the levels of *Aspergillus flavus /A. parasiticus* during the time when trees are shaken and the nuts come in close contact with the soil (where the aflatoxin-producing fungi reside), we plan to collect a third set of soil samples during harvest in 2009. For these samples, we will determine the levels of *A. flavus* and *A. parasiticus,* and the ratio of the S and L strains of *A. flavus.* The levels of *A. flavus* and *A. parasiticus* during harvest may represent a more realistic risk factor for aflatoxin contamination of the nuts, which will be stored in stockpiles for several months.

In 2008, we collected five almond samples of 2 to 4 lbs each from 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. Five almond samples (each of 2 to 4 pounds of kernels per sample) were analyzed to quantify aflatoxin levels in parts per billion (ng /g kernels) per orchard. The range of aflatoxins was low in 2008 (0.0 to 3.29 ppb). In general, more aflatoxins were detected in orchards in the northern than in the southern region. Furthermore, all three orchards in the northern region had G1 aflatoxins, which imply that almonds were infected by *A. parasiticus.* This is in agreement with the 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. Samples collected as soon as the nuts were shaken to the ground are being analyzed currently, and more samples will be collected in 2009 as soon as the trees are shaken and on the day of picking the nuts from the ground.

Inoculation experiments with aflatoxin-producing *Aspergillus parasiticus* strain (A408) and *A. flavus* stain (A224) were conducted using almond kernels to study the effects of relative humidity on infection and aflatoxin development. A real-time polymerase chain reaction (PCR) assay was used to quantify the infection level (spores / kernel) and high performance liquid chromatography (HPLC) was used to quantify the aflatoxin levels.

Sporulation of *A. flavus* and *A. parasiticus* on almond kernels increased with increasing relative humidity (RH), while no relationship was found between the extent of infection and aflatoxin levels. Aflatoxins were detected in kernels before any apparent infection symptoms (visible fungal mycelia and sporulation). Aflatoxins of *A. parasiticus* were produced earlier, faster, and reached higher levels than aflatoxins produced by *A. flavus*. For both fungi, aflatoxins accumulated faster at 95% than at 75% RH. For *A. parasiticus,* it took 3 days after inoculation and 1 day at 95% RH to reach the maximum aflatoxin level and 17 days at 75% RH after inoculation and incubation to reach such level. However, huge variations in aflatoxin levels were found. These results suggest that it is essential to lower the water content of nuts and the relative humidity in storage (during stockpiling) in order to reduce aflatoxin levels.

Although the level of the atoxigenic strain AF36 was very low in the soil of the Nickels Soil Laboratory orchard before applying the wheat with AF36 in June 2007, after applying the wheat almost all of the *A. flavus* isolates were AF36. This suggests that applying wheat with AF36 was very effective in introducing the atoxigenic strain AF36 under the conditions 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. And in 2008 after applying AF36 for a second time, 99% of the *A. flavus* isolates in the treated areas belonged to the AF36 strain.

For a second year, the naturally occurring levels of the atoxigenic AF36 strain of *A. flavus* were determined. Among the 643 isolates tested 28 were AF36, a 4.2% incidence. The results suggest that AF36 strain is widespread among almond orchards. Determining the levels of natural occurrence of the AF36 strain will help obtain the registration of this atoxigenic strain, which can then be used to reduce aflatoxins in almonds.

Materials and Methods:

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

In 2008 we repeated the soil sampling from 28 almond orchards: 11 located in northern, 10 in central, and 8 in southern California. The main difference among these three regions is the rainfall which amounts to 2.23 inches/month in the northern, 0.94 inches in the central, and 0.5 inches in the southern (based on 2007 data). Sampling was done in a similar way as in 2007, by collecting three composite samples per orchard consisting of 10 sub-samples each taken from the upper 0.5 to 1 inch soil in each orchard. To quantify the *Aspergillus* section *Flavi,* we followed similar procedures as those in 2007 (which are routinely used in our laboratory). We used Si10, a 3% salt medium agar that also has enough dichloran to inhibit certain *Aspergillus* spp. (such as *A. niger*) but select *Aspergillus* section *Flavi.* Identification of *Aspergillus* species was done by transferring the *Aspergillus* section *Flavi* isolates on CYA media and observing the microscopic characteristics and coloration of the three species (*A. flavus, A. parasiticus, & A. tamarii*).

We also used the soil samples from the 2007 collection to develop a more efficient and less expensive technique to quantify the levels of *A. flavus /parasiticus* in soils collected from almond soils. The technique used is called Real-time PCR (polymerase chain reaction) and was performed by extracting DNA of *A. flavus* from soils. More details on this approach are given in the publication entitled "Quantification of conidial density of *Aspergillus flavus* and *A. parasiticus* in soil from almond orchards using real-time PCR" (Luo et al., 2009). Presently, although this is an efficient and quick technique, its sensitivity is not high enough to detect the low levels of *A. flavus/parasiticus* propagules encountered in almond orchard soils in California. Therefore, we will continue quantifying the density of *A. flavus/parasiticus* in orchard soils using the traditional dilution plating technique until a more efficient method is developed.

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

Three Nonpareil almond orchards were selected in each of three geographical regions of California: Arbuckle (Colusa County), Ripon (San Joaquin County), and Bakersfield (Kern County) located in northern, central, and southern California, respectively. Collection of almond samples was done twice: a) on the same day of or the day following tree shaking and b) on the same day of or one day before the nuts were picked up. In each sampling, 8 to 10 rows were randomly identified and about 15 to 20 pounds of almond nuts were collected from the floor of each of these rows. The samples were dried further under the sun, cracked, and the kernels were evaluated for insect damage. Sub-samples of 2 to 4 pounds of kernels were analyzed for aflatoxin contamination. A total of 300 kernels were randomly selected and the number of kernels damaged by insects or infected with fungal species was recorded. These kernels were further ground and 25 g of ground kernels per sample was extracted and processed to obtain the aflatoxin contamination level (ng/g kernel = ppb) with a High Performance Liquid Chromatography (HPLC). Extracted aflatoxins were cleaned-up using an immunoaffinity column (Vicam, MA). For the HPLC analyses the parameters used were: liquid phase of 45% methanol + 55% water; pump flow rate of 0.8 ml/min; injection value of 20 or 100 µL sample depending on aflatoxin contamination level; excitation at 360 nm and emission at 450 nm of a florescence detector (HP 1046A).

Analyses of the samples from the first sampling time are still in progress. Mean values of the aflatoxin contamination level for the second sampling time were calculated from five random samples in each orchard. The corresponding standard deviation was also calculated with the function in Microsoft Excel.

Relative humidity (RH) as a risk factor for aflatoxin contamination of almonds.
To determine the effect of relative humidity on infection and aflatoxin accumulation in almond kernels we performed several inoculation experiments as follows. Dry Nonpareil kernels were placed in sealed containers (32 × 24 × 10 cm) on screens over saturated salt solutions to create an atmosphere of 70, 75, 80, 85, 90, 95, and 100% RH by using saturated NaCl + KCl, NaCl, $(NH_4)_2SO_4$, KCl, KNO_3, K_2SO_4, and H_2O at 30ºC, respectively (Winston and Bates, 1960). A sterilized needle was used to make a wound of 6 to 10 mm^2 on the surface of sterilized kernels. Kernels then were inoculated with a

drop of a 10,000 spores /ml solution, and the containers were incubated at 30ºC for 3 to 7 days and the kernels then transferred under the various levels of RH for various lengths of time, depending on the experiment. To seal the container, the perimeter of the container cover was painted with Vaseline. Kernels were collected then at specified times and analyzed for aflatoxins. Extracted aflatoxins were cleaned-up using an immunoaffinity column (Vicam, MA) and quantified with the HPLC method (given above).

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.

The atoxigenic *A. flavus* strain AF36 was grown on wheat seed and applied to the soil at a rate of 10 pounds of wheat per acre (same rate as used for commercial cotton fields) in a research almond (cv. Nonpareil) orchard at the Nickels Soil Laboratory on 28 June 2007 and 2 July 2008 (wheat was applied to the same areas for both years). The experimental design was a randomized complete block design with 3 replications. After applying the wheat 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 AF36-wheat inoculum. Nuts and additional soil samples were collected during the period of commercial harvest on 9 August 2007 and 2 September 2008. 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 the closely related fungi in *Aspergillus* sect. *Nigri*), 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*. 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 (Cotty, 1994). Nut samples are in the process of being analyzed for aflatoxin contamination. Additional soil and nut samples will be collected during the period of commercial harvest in 2009.

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.

Determining the incidence of AF36 in soils of almond orchards is of major importance since this is now being used as a biopesticide reducing aflatoxin contamination of cottonseed and corn. We anticipate getting it registered first on pistachio to reduce aflatoxins and then on almond. In 2008 we analyzed 643 isolates of *A. flavus* to determine the incidence of AF36 using the Vegetative Compatibility Group testing. In these analyses, two known deficient testers of the AF36 strain are paired in Petri plates

with the unknown strain. Fluffy growth along the lines where the known and the unknown strains meet results from complementation, indicating that the unknown strain is AF36 strain. No fluffy growth indicates that the unknown strain belongs to another VCG strain different from the AF36. Data on the incidence of AF36 in soils of commercial orchards will be used to expand the registration label of the biopesticide AF36 to almonds.

Results and Discussion:

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

We have saved about 1,500 isolates of *A. flavus* and *A. parasiticus* collected from almond orchards in 2007 and 2008. The analyses of soil samples collected in 2008 revealed only slightly different results from those of 2007. In 2008, all 28 orchards had variable levels of *A. flavus* and *A. tamarii* while two orchards each in the northern and central regions and four orchards in the southern region did not have any *A. parasiticus* (**Figure 1**). The mean density of *A. parasiticus* was 6 to 7 propagules per gram soil for orchards of northern and central California, while that of southern California was 1 propagule per gram soil (**Figure 2**). As in 2007, the soils in the south had the lowest density of *A. parasiticus.* The mean density of *A. flavus* was 3, 11, and 5 propagules per g soil for the northern, central, and southern regions. As in 2007, soils in the northern California had the lowest *A. tamarii* density (2 propagules/g soil) levels while central and southern had 18 to 20 propagules per g soil. The presence of *A. flavus* S strain (producing small sclerotia) is a major risk factor for aflatoxin contamination since it was shown in previous research (working with isolates of *A. flavus* from pistachio orchard soils) that all the S strains produce aflatoxins and some of them produce high amounts. In contrast about 50% of the L strain (producing large sclerotia) of *A. flavus* do not produce aflatoxins and indeed produce smaller 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 the central (2%) and northern (3%) regions of the Central Valley.

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

Table 1 lists the average aflatoxin levels for each of the 9 orchards in the three regions. The overall total aflatoxin levels were from 0 to 3.29 ppb with corresponding standard deviations ranging from 0 to 4.97. The aflatoxin G2 was not found in any of the orchards while G1 was detected in all three orchards in northern region. The 2007 and 2008 research showed that the incidence of *A. parasiticus* is higher in the northern region, a fact that may explain the contamination of almonds with G1 aflatoxins. (*A. parasiticus* produces all four B1, B2, G1, and G2 aflatoxins while *A. flavus* produces only B1 and B2.) The B2 aflatoxin was detected only in one orchard in the northern region. In general, aflatoxins B1 and G1 were detected more frequently than aflatoxins B2 and G2. In general, the aflatoxin levels were at very low levels in the southern, moderate level in the central and higher in northern region based on the 2008 field samples

(**Figure 3)**. More almond samples will be collected during the 2009 harvest to confirm the results of 2008.

Table 2 lists the average percentages of damaged kernels by insects and of nuts infected by molds. With the exception of one orchard in the south, insect damage was very low (<0.4%) and fungal infection below 0.55% (**Table 2**). Further analyses will be conducted to determine possible difference, if any, in aflatoxin contamination between the two sampling times (as soon as the trees are shaken and 7 to 10 days later when the nuts are swept and removed from the orchard floor) in each orchard. The information 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 Bruce Lampinen addresses the risk factors associated with aflatoxin contamination during stockpiling of almonds. (See project 08-Afla2-Lampinen of this 2008-2009 Final Research Report.)

Relative humidity (RH) 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* (**Figure 4**), suggesting that at higher relative humidity the aflatoxigenic fungi will produce higher amounts of spores that can either enrich the soil of 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 increase and the risk of having an explosion of *Aspergillus* infection, growth, and sporulation could be unavoidable.

Interestingly for the maximal aflatoxin contamination level under 95% RH attained within the first 4 days (**Figure 5**), 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 under 95% RH did not alter the maximum level of aflatoxins produced by *A. parasiticus*. This implies that once conditions are favorable for infection and maximum aflatoxin development during the first 3 to 4 days after infection, almonds could be contaminated the most and the conditions that will follow after that time will not have a major effect on either increasing or decreasing the levels of aflatoxins. However, these conditions (i.e., during stockpiling or other type of storage) could have a major effect in preventing or favoring more initial infections. In contrast to the 95% RH incubation, at 75% RH the development of aflatoxin was delayed and reached the maximum level after a total of 17 days incubation. For *A. flavus,* again the maximum level of aflatoxin was reached within 4 days at 95% RH and showed a trend of reduction with incubation time while at 75% RH; there was a delay over time (**Figure 5**). These findings suggest that lower relative humidity will delay the development of aflatoxins by either *A. parasiticus* or *A. flavus* in almonds.

3. Application of the atoxigenic *A. flavus* strain AF36 in a research almond orchard.

Application of the atoxigenic *A. flavus* strain AF36 resulted in a moderate increase in *A. flavus*/*A. parasiticus* in the soil in 2007, the first year of the study (**Table 3**).

Approximately the same density of *A. flavus*/*A. parasiticus* in the soil occurred in August 2007 and July 2008 (prior to applying AF36 in 2008), suggesting that the fungus survived the winter well. In addition, the density of *A. flavus*/*A. parasiticus* increased further during the second year in the treated areas. The density of *A. flavus*/*A. parasiticus* in the treated areas was less than that of *A. niger* in 2007. However, in 2008 the levels of *A. flavus*/*A. parasiticus* was approximately the same or slightly higher than that of *A. niger*, which is an indication of how substantial the increase in *A. flavus*/*A. parasiticus* was.

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 (**Table 4**). This suggests that applying wheat with AF36 was very effective in introducing the atoxigenic strain AF36 under the conditions 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. Also in 2008 after applying AF36 for a second time, 99% of the *A. flavus* isolates in the treated areas belonged to the AF36 strain. The increase of AF36 in the untreated areas over time from 1.7% before applying AF36 in June 2007 to 74% in September 2008 demonstrates the movement of AF36 from the treated areas to the untreated areas.

Before applying AF36, the most common aflatoxin-producing fungus present was *A. parasiticus* (**Table 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 level as the L strain of *A. flavus* (which includes AF36 and many other atoxigenic strains). However, after applying the wheat with AF36, the level of the L strain of *A. flavus* increased until almost all of the *A. flavus*/*A. parasiticus* isolates in the treated areas belonged to the L strain.

Applying AF36 did not seem to significantly increase the level of hull decay of the nuts because only 0.17% was decayed by *A. flavus* in the treated areas (**Table 6**). For the nuts harvested in 2007, no decay of the hulls by *A. parasiticus* was observed, and 42% of the *A. flavus* isolates from decayed hulls were the S strain (the rest being the L strain). Of the *A. flavus* strain L isolates, 55.6% belonged to the atoxigenic strain AF36. The nuts harvested in 2008 are still being evaluated.

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

The atoxigenic strain AF36 has been found to be naturally occurring in California. For instance, we have found earlier that AF36 makes up 5.3% of the *A. flavus* isolates from commercial pistachio orchards and occurs throughout the pistachio-growing regions of California. The occurrence of the atoxigenic strain AF36 in soil from commercial almond orchards was 7.8% after evaluating 637 isolates out of a total of 900 *A. flavus* collected in 2007. AF36 was more common in the southern region (12.6%) than the central and northern regions. Additional isolates were tested in 2008. The mean incidence of AF36 was 4.21% among these additional 643 isolates tested with a range of 2.1% to 7.0%. The results suggest that the AF36 is widespread among the almond orchards. Finding

that the AF36 strain occurs in high levels in commercial orchards, and in fact, this strain is at higher levels than other strains (determined for isolates of *A. flavus* obtained from pistachio soils), indicates that this strain can compete and survive well under natural conditions. This information can be used to support registration of AF36 strain on almonds for the reduction of aflatoxin contamination.

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. J. 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.).

Cotty, P. J. 1994. Influence of field application of an atoxigenic strain of *Aspergillus flavus* on the populations of *A. flavus* infecting cotton bolls and on the aflatoxin content of cottonseed. *Phytopathology* 84: 1270-1277.

Table 1. Aflatoxin levels and standard deviations of almond kernel samples collected in 2008 from orchards in three geographic regions. Samples were collected on the day of or 1 day before harvest.

Region		B1	B2	G1	G2	Total	Total for the three orchards
Northern California	Mean[1]	0.28	0.00	0.13	0.00	0.41	
Orchard #1	S. D.	0.32	0.00	0.29	0.00	0.57	
Orchard #2	Mean	0.53	0.00	0.23	0.00	0.76	
	S. D.	0.50	0.00	0.32	0.00	0.78	
Orchard #3	Mean	1.80	0.02	1.47	0.00	3.29	1.49
	S. D.	2.74	0.04	2.20	0.00	4.97	2.48
Central California							
Orchard #1	Mean	0.04	0.00	0.00	0.00	0.04	
	S. D.	0.10	0.00	0.00	0.00	0.10	
Orchard #2	Mean	0.14	0.00	0.00	0.00	0.14	
	S. D.	0.13	0.00	0.00	0.00	0.13	
Orchard #3	Mean	0.00	0.00	0.00	0.00	0.00	0.06
	S. D.	0.00	0.00	0.00	0.00	0.00	0.07
Southern California							
Orchard #1	Mean	0.00	0.00	0.00	0.00	0.00	
	S. D.	0.00	0.00	0.00	0.00	0.00	
Orchard #2	Mean	0.00	0.00	0.07	0.00	0.07	
	S. D.	0.00	0.00	0.15	0.00	0.15	
Orchard #3	Mean	0.00	0.00	0.00	0.00	0.00	0.02
	S. D.	0.00	0.00	0.00	0.00	0.00	0.09

[1] Five random samples were analyzed using the HPLC method.

Table 2. Average incidence of insect and mold of almond samples collected in 2008 from orchards located in three geographic regions.

	Nuts infested by insects (%)[1]	Nuts infected by molds (%)[1]
Northern region		
Orchard #1	0.07	0.13
Orchard #2	0.07	0.33
Orchard #3	0.00	0.07
Central region		
Orchard #1	0.00	0.00
Orchard #2	0.17	0.08
Orchard #3	0.00	0.13
Southern region		
Orchard #1	1.60	3.60
Orchard #2	0.27	0.27
Orchard #3	0.40	0.53

[1] 300 random nuts per sample were evaluated visually for insect infestation and infection by molds and analyzed for aflatoxins; five replicated samples were used for each orchard

Table 3. 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							
	June 2007		August 2007		July 2008		September 2008	
Treatment	*A. flavus*	*A. niger*	*A. flavus*	*A. niger*	*A. flavus*	*A. niger*	*A. flavus*	*A. niger*
AF36	2.8 ns	733.1 ns	45.4 ns	104.2 ns	54.8 ns	34.7 ns	192.3 a	136.0 ns
Untreated control	4.3	265.7	4.0	170.2	6.3	217.1	5.4 b	144.3

Table 4. 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

Table 5. Percentage of isolates that belong to *Aspergillus flavus* (strain L or strain S) *or A. parasiticus* for isolates obtained from soil at Nickels Soil Laboratory experimental orchard (cv. Nonpareil).

Treatment	Percentage of isolates that belong to specified fungus					
	June 2007			August 2007		
	A. flavus L	*A. flavus* S	*A. parasiticus*	*A. flavus* L	*A. flavus* S	*A. parasiticus*
AF36	8.1	13.5	78.4	93.1	0.0	6.9
Untreated control	19.6	8.9	71.4	62.5	6.3	31.3

Treatment	Percentage of isolates that belong to specified fungus					
	July 2008			September 2008		
	A. flavus L	*A. flavus* S	*A. parasiticus*	*A. flavus* L	*A. flavus* S	*A. parasiticus*
AF36	92.8	0.0	7.2	96.7	0.6	2.8
Untreated control	31.1	3.3	65.6	79.2	4.2	16.7

Table 6. Incidence of hulls and external surface of shells of Nonpareil almonds decayed by *Aspergillus flavus* and *A. ochraceus*.

Treatment	Percentage of hulls/shells with decay by specified fungus			
	2007		2008	
	A. flavus	*A. ochraceus*	*A. flavus*	*A. ochraceus*
AF36	0.17	0.08		
Untreated control	0.00	0.02		

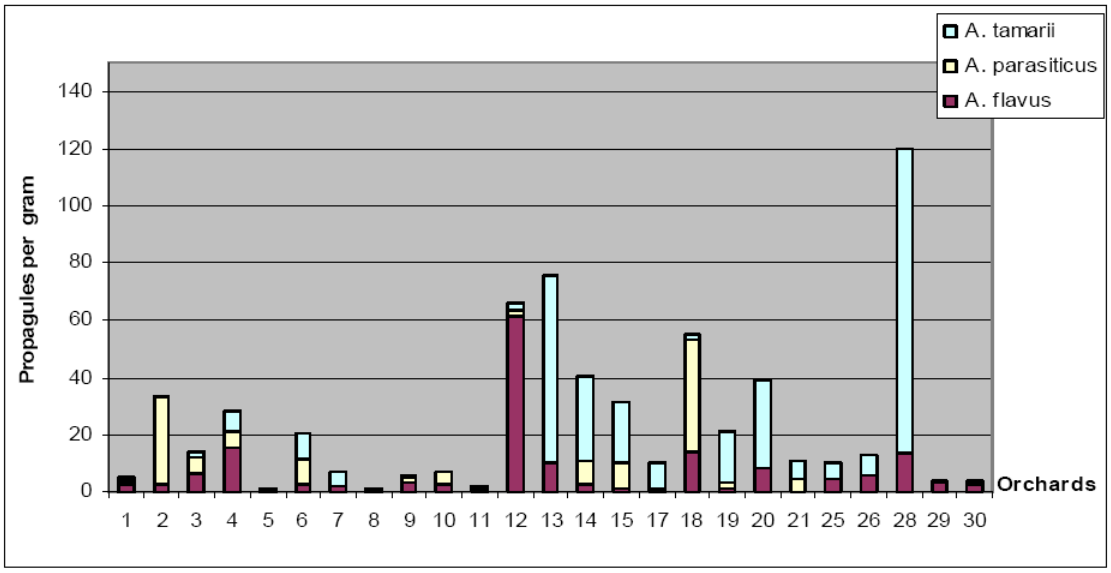

Figure 1. Density of propagules of *Aspergillus flavus, A. parasiticus,* and *A. tamarii* in soils collected from 11 northern (#1 to #11) orchards, 9 central (#12 to #21), and 8 southern (# 25 to #32) almond orchards in California in 2008.

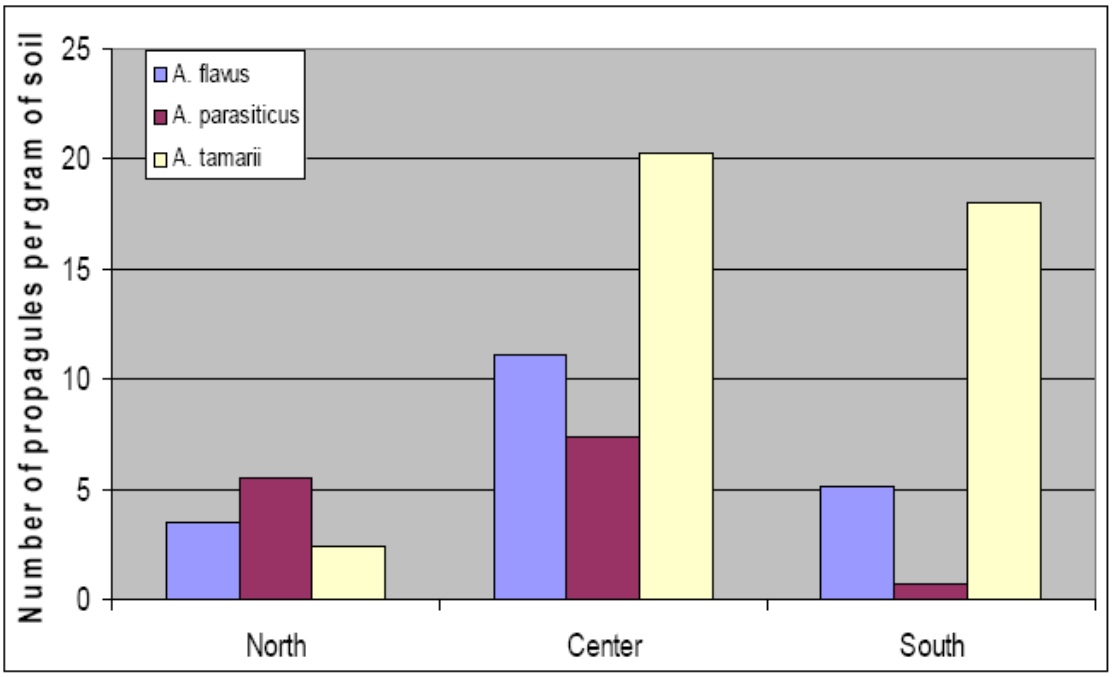

Figure 2. Mean densities of propagules of *Aspergillus flavus, A. parasiticus,* and *A. tamarii* in soils collected from 11 northern, 9 central, and 8 southern almond orchards in California in 2008.

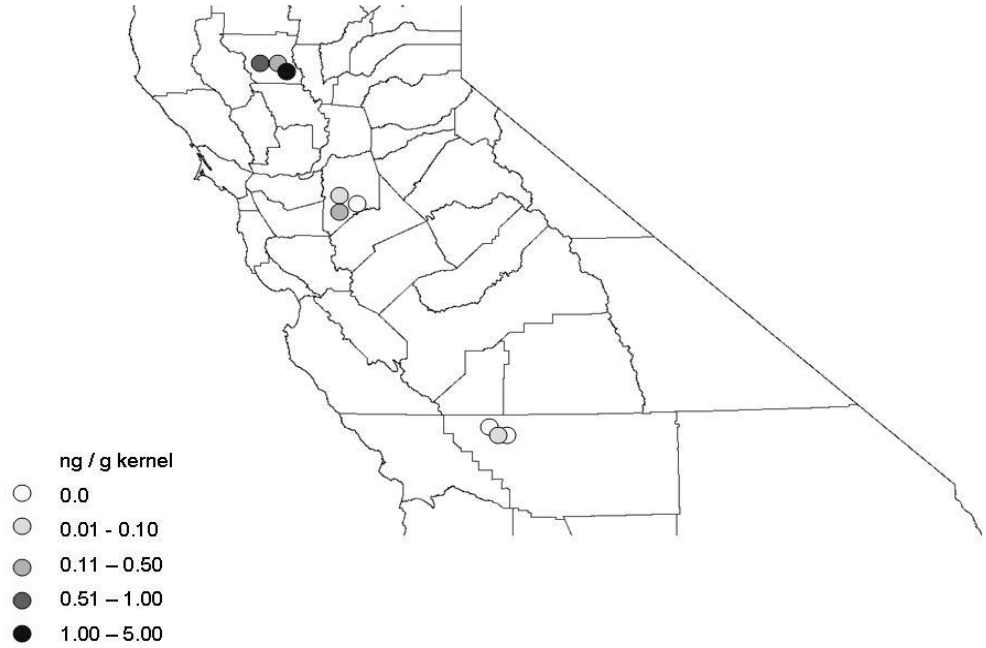

Figure 3. Average aflatoxin levels of five samples collected from 9 almond orchards, three each in southern, central, and northern regions of California in 2008.

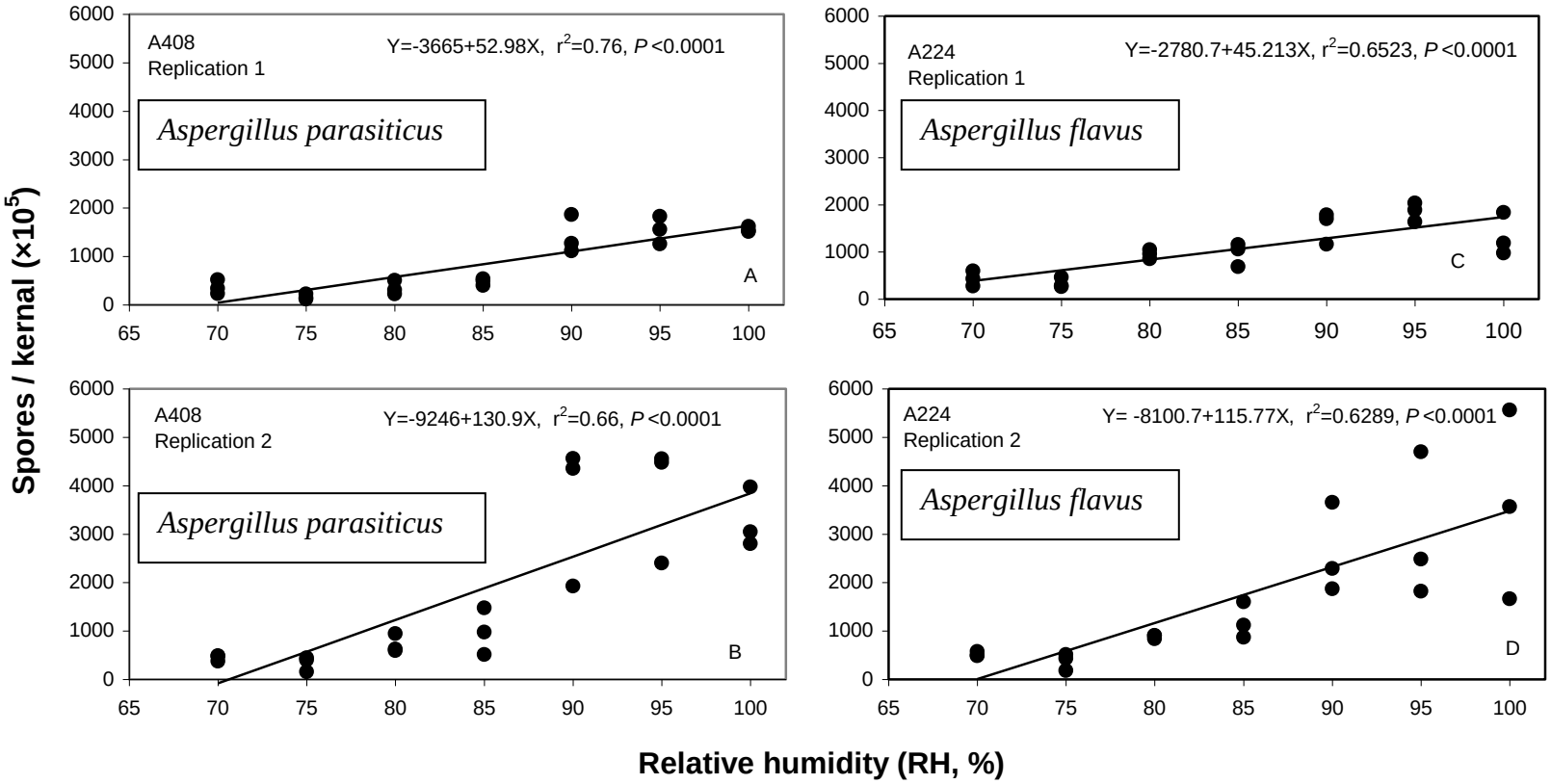

Figure 4. Relationship between relative humidity (RH) and number of spores produced per kernel after inoculation with *Aspergillus parasiticus* (A408) and *A. flavus* (A224), incubation at 30ºC for 7 days, and then transferring the kernels under the different levels of RH. Data of two replications in two experiments (only data of Experiment 1 is shown).

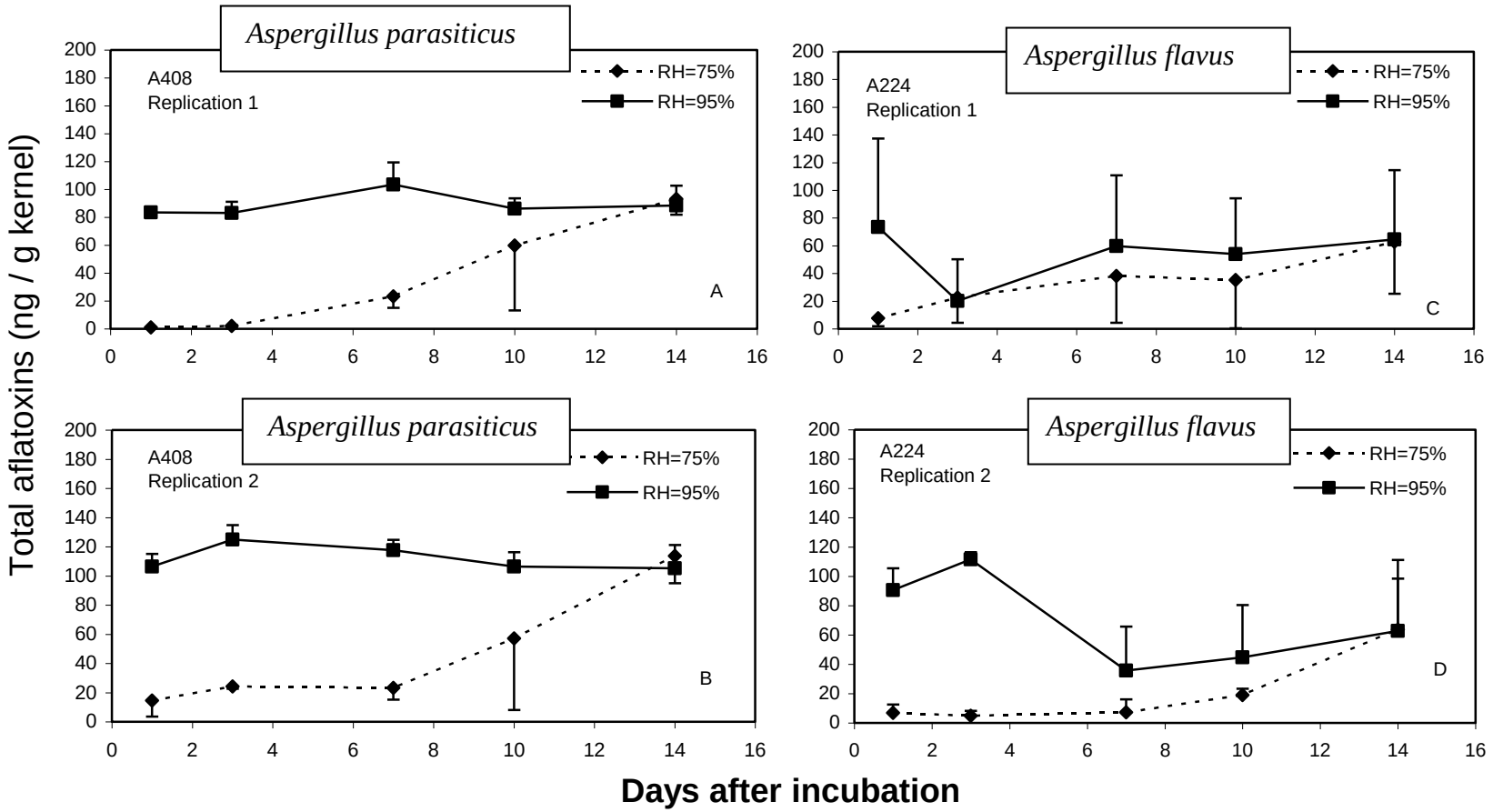

Figure 5. Dynamics of aflatoxin levels in almond kernels inoculated with *Aspergillus parasiticus* (A408) and *A. flavus* (A224), incubated at 30ºC for 3 days, and then transferred under 75% and 95% RH.