Almond Stockpile Monitoring for Aflatoxin Potential

Project No.: **08-AFLA2-Lampinen**

Project Leader: Bruce Lampinen
Department of Plant Sciences
UC Davis
One Shields Ave.
. Mail Stop #2
Davis, CA 95616
(530) 752-2588
e-mail: bdlampinen@ucdavis.edu

Cooperating Personnel: Themis Michailides, UC - Davis/Kearney
Jim Thompson, UC - Davis
Samuel Metcalf, UC - Davis
David Morgan, Heraclio Reyes, Y. Luo and B. Kabak, UC - Davis/Kearney

Objectives:

The objectives of this study are to examine temperature and moisture conditions in stockpiled almonds in several production areas in California. The ultimate goal is to develop recommendations for stockpiling that minimize potential for growth of aflatoxigenic *Aspergillus* and aflatoxin contamination of nuts.

Interpretive Summary:

Almond stockpiles in Kern, San Joaquin and Glenn Counties were monitored following the 2007 and 2008 harvests. In 2007, seven stockpiles were outfitted with temperature and relative humidity sensors at four depths in the stockpiles. In 2008, six stockpiles were monitored. Conditions were monitored at 1 to 3 minute intervals in the stockpiles throughout the storage period ranging from 84 to 150 days. Generally, when initial moisture content of nuts was low, mold growth in stockpiles was minimal and accordingly there was no aflatoxin detected. However, stockpiling of nuts with a water activity notably above the recommended 0.65 - 0.70 (= equilibrium relative humidity of 65 - 70%) resulted in significant mold growth near the pile surfaces. The two piles where this was observed in 2007 had initial moisture contents of: 1) hulls 13.1% and kernels 5.2% (total fruit moisture content 9.2%); and 2) hulls 12.0% and kernels 7.3% (total fruit moisture content 9.7%). There was *Aspergillus* growth at the top and bottom edge of these stockpiles and analysis of kernel samples from one pile showed this was associated with aflatoxin production. This growth and aflatoxin production

was associated with wetting of the nuts and hulls resulting from significant condensation of moisture on tarps that was observed in these areas. Significant mold growth and aflatoxin were only associated with these outer portions of the piles, because within the piles, the equilibrium relative humidity came to a steady state below the maximum limits recommended by current storage guidelines. The moisture level in stored nuts is one of the most critical factors in the growth of aflatoxigenic molds and aflatoxin production and contamination. In both years, moisture content tended to stay constant or decrease in the body of the stockpile and increase near the surface as a result of moisture condensation resulting from diurnal temperature fluctuations. High humidity and temperature within the stockpile increase the incidence of aflatoxin contamination. For this reason, moisture content (and temperature) must be carefully controlled during stockpiling so that the water activity (a_w) of nuts is low enough that these fungi cannot grow. In 2008, the only stockpile that had detectable aflatoxin contamination, however, since aflatoxin was detected at the time of stockpiling suggested some production of aflatoxin occurred in the orchard. The nuts from this stockpile came from a heavily shaded portion of the orchard and this may have played a role**.**

Materials and Methods:

Field Data Collection

2007
Dataloggers with relative humidity and temperature sensors (Onset, Pocasset, MA 02559), were installed in stockpiles in Glenn, San Joaquin and Kern County. In 2007, dataloggers were equipped to monitor temperature and relative humidity at four locations in each stockpile. Sensors were placed near the top of the pile, near the bottom of the pile, and at two intermediate distances between the top and bottom of each stockpile. In addition, ambient temperature and relative humidity were monitored at each location. Data were recorded every three minutes.

2008
A total of six almond stockpiles located in Kern, San Joaquin and Colusa Counties were monitored. The stockpiles were outfitted with temperature and relative humidity sensors at two depths in the center of the stockpiles (near the surface and the bottom) and two positions on the side of the pile near the surface. Samples were also taken from other areas where visible mold growth occurred.

At the Kern County site, samples were again taken from the Nonpareil rows in the high water/high nitrogen and moderate water/moderate nitrogen treatments in an irrigation/fertigation trial for stockpiling. However, the moderate water/moderate nitrogen treatment was converted to high water/high nitrogen in 2008 but differential drying due to different canopy sizes may still make the differences significant. Stockpiles and sensors were installed at the Kern County site on 8/25/08. The piles were covered immediately and fumigated with phosphine gas on 8/27/08.

The San Joaquin County stockpile #1 was installed on 9/9/08 and San Joaquin County stockpile #2 was installed on 9/17/08. Both stockpiles were Nonpareil.

For Colusa, dataloggers were installed after stockpiles were formed for Stockpile #1 on Sept. 12, 2008 and Stockpile #2 on Sept. 23, 2008. Both stockpiles were removed on Dec. 5, 2008. Both Colusa County stockpiles were also Nonpareil.

Initial moisture contents of nuts at the time of stockpiling are shown in **Table 1** for all sites and stockpiles.

Samples - 2008

In shell nuts in hulls were sampled at the time sensors were installed at all locations. During the period the tarps were on, samples were taken from the pile using an approximately 2 inch diameter metal tube with a perforation near the end. The tube was inserted into the pile and hooked to a vacuum cleaner to suck the samples out. A subsample (approximately 2.2 lbs) was taken from each location, weighed and separated in the field into hulls and in-shell kernels. These subsamples were then weighed in the field, brought to Davis, dried in an oven to constant weight, and reweighed. In 2008, samples were taken near each sensor at the time the stockpiles were placed (except Kern where one overall sample was taken), once during the period the stockpile was in place and at the time the stockpile was removed.

Samples were taken from all stockpiles from each of the sensor locations on each sampling date. Each sample was split into 3 sub-samples and refrigerated until use. The samples were examined and all the almonds bearing mold were separated and used for isolation of fungi. Mycelium and sporulation of different species of fungi were picked up from almond hulls by using a sterile inoculating loop and transferring to Si10 medium (a medium that favors the growth of *Aspergillus* fungi). The Si10 plates were incubated at 30^{o}C for 4 weeks. Then, fungal colonies were transferred to Czapex Agar and incubated at 30^{o}C for 4 days. Following incubation, colonies were identified using Klich and Pitt (1988) identification key for *Aspergillus* species.

.All aflatoxin analyses were conducted on kernels.

Results:

See the 2007-2008 Final Research Report, Project 07-AFLA1-Lampinen, and the 2008 Proceedings, Project 08-AFLA2-Lampinen for detailed reports of the 2007 results.

Colusa County Site - 2008

Both Colusa County sites had overall moisture levels in the 6.2 to 7.3% range at the start of the stockpiling period (**Table 1**). Temperature and relative humidity data from the high and moderate canopy cover stockpiles are shown in **Figure 1**.

The numbers of isolates of *Aspergillus* species isolated from hulls of in-shell nuts and hulls sampled from different parts of stockpile 1 in Colusa County are shown in **Figure 5**. A total of 77 *Aspergillus,* 16 *Fusarium,* and 8 *Penicillium* species were isolated from hulls in stockpile in sampling 1. The predominant species was *A. terreus* (50.7% of the *Aspergillus* isolates)

followed by *A. ochraceus* (18.2%), and *A. wentii* (11.7%). However, no *A. flavus* was found at the bottom and edge of the stockpiles; only 4 *A. flavus* isolates were found on the top and 1 isolate in hulls from the valley of the plastic cover of the stockpile, representing only 6.5% of all the *Aspergillus* isolates.

From stockpile 2, a total of 80 isolates of *Aspergillus* species were isolated and represented mainly by *A. niger* (43.8%), *A. ochraceus* (30%), and *A. melleus* (16.3%). In addition, 26 *Penicillium*, 13 *Fusarium*, 6 *Alternaria,* and 4 *Mucor* strains were isolated from hulls of in shell nuts and hullsof nuts sampled from different parts of the stockpile. No *A. flavus* or *A. parasiticus* were isolated from any of these samples from stockpile 2.

No aflatoxin was detected in any kernel samples from the Colusa County stockpiles in 2008.

Kern County Site - 2008

At the Kern County site, samples were taken from the Nonpareil rows in the high canopy cover and moderate canopy cover sections of an orchard that had an irrigation/fertigation treatments imposed from 2001 to 2007. In 2008, all treatments had similar irrigation and nitrogen applications but canopy size/shading difference remained. The nuts on trees from the high canopy cover sections of the orchard received lower levels of sunlight for drying on the orchard floor. Stockpiles and sensors were installed at the Kern County site on 8/20/07 and removed on 1/17/08. The stockpiles were covered with 0.025" clear polyethylene tarps. Initial moisture content of nuts and hulls from the high water/high nitrogen treatment (Stockpile #1) were 3.0 and 5.7% respectively. Initial moisture content of nuts and hulls from the moderate water/moderate nitrogen treatment (Stockpile #2) were 3.0 and 6.1% respectively, with overall moisture content of 4.4%) (**Table 1**).

Temperature and relative humidity data from the high and moderate canopy cover stockpiles are shown in **Figure 2**. Data are shown for one day near each date on which nut samples were taken from the piles. Generally, temperatures at all locations inside the pile tended to be higher than ambient temperatures (**Figure 2**). Temperature at higher positions in the stockpiles tended to be greater and relative humidity lower compared to that in lower positions. As expected, temperatures in the stockpiles decreased as the season progressed and differences in temperature between high and low positions in the piles tended to get less through the storage period (**Figure 2**). Temperatures and relative humidity's were similar in both of the Kern County stockpiles throughout the storage period in 2008 (**Figure 2**). Although some mold growth occurred at most locations in both Kern County stockpiles in 2008 (**Figures 6, 7**) numbers of species isolated from Stockpile #1 (from high canopy cover orchard section) was greater compared to Stockpile #2 (from moderate canopy cover orchard section). This difference in mold growth occurred despite the fact that moisture levels in the two stockpiles was similar in 2008 (**Table 1**), suggesting that there might have been differences in the microflora on the surface of nuts initiated from and based on differences of the orchard canopy cover.

The numbers of isolated *Aspergillus* strains from hulls of the in-shell nuts and hulls in Kern County stockpiles for November 19, 2008 are shown in **Figure 6**. In the high water and nitrogen treatment 1, a total of 87 isolates of *Aspergillus*, 4 isolates of *Fusarium*, and 2

Alternaria and 1 *Penicillium* isolates were isolated. The dominant species present in the almond hulls was *A. terreus* (56.3% of the *Aspergillus* isolates) followed by *A.niger* (26.4%), and *A. flavus* (14.9%). No *A. flavus* was found in the part of the valley of the plastic cover and bottom in stockpiles, while most (76.9%) of the *A. flavus* isolates were found in nuts collected from the top of stockpiles**.**

Fewer *Aspergillus* species (38 isolates) were isolated from hulls of the stockpile from the moderate water and nitrogen and less-canopy cover Treatment 2 as compared to those of samples from the high canopy cover section Treatment 1). A total of 18.2% of *A. flavus* were isolated from hulls collected from the bottom, edge, and edge of the edge of this stockpile. Most of the *A. flavus* (5 isolates) were found in hulls at the bottom of the stockpile**.** In addition, 2 *Alternaria*, 1 *Fusarium* and 1 *Penicillium* species were isolated from almond hulls in the Kern County stockpile.

In the December 29, 2008 sampling from these stockpiles, the numbers of the isolated and identified *Aspergillus* isolates from hulls of the in-shell nuts and hulls collected from different locations of the stockpiles in Kern County California are shown in **Figure 7**. In the high canopy cover, Treatment 1, a total of 91 *Aspergillus*, 12 *Penicillium,* and 11 *Fusarium* isolates were identified in hulls from different parts of the stockpiles. *A. terreus* was found to be predominant species (45%) followed by *A. flavus* (34.1%), and *A. niger* (16.5%). Most of the *A. flavus* were found in nuts collected from the valley of the plastic cover (35.5%) and edge of edge (35.5%) of the stockpile. Additionally, 2 *A. tamarii* strains were isolated from hulls of the edge of edge of this stockpile.

In the moderate canopy cover, treatment 2, a total of 29 *Aspergillus*, 13 *Fusarium*, 2 *Alternaria*, 1 *Penicillium*, 1 *Cladosporium,* and 1 *Mucor* isolates were isolated and identified in hulls from the stockpile. Among the *Aspergillus* isolates, 65.5% were *A. niger* and 10.3% *A. flavus.* The *A. flavus* was isolated only on the top and edge of edge of this stockpile.

For both sampling dates, more *Aspergillus* species were isolated from almond hulls in treatment 1 (high canopy cover) than treatment 2 (moderate canopy cover). This suggests that the canopy cover (and the resulting shading effect) is playing a significant role in the infection of almonds by *Aspergillus,* including *A. flavus*, since both of these treatments received similar levels of water and nitrogen in 2008.

Aflatoxin production in kernels analyzed was limited to samples from the surface / edge of the stockpile from the high canopy cover portion of the Kern County orchard (**Table 2 and 3**). A positive for aflatoxin was detected in this stockpile on the day the stockpiles were formed (**Table 2**), suggesting that aflatoxin production started in the orchard.

San Joaquin County Site – 2008

The initial water content for the nuts and hulls from the San Joaquin County stockpile #1 was 5.1% and 11.5%, 8.7% overall moisture and #2 was 8.8% and 25.9% respectively, with an overall moisture of 16.8% (**Table 1**). Relative humidity during the storage period is shown in **Figure 3**. Despite the particularly high initial moisture content and relative humidity, mold growth in stockpile #2 tended to be relatively low. This is probably due to the fact that temperatures ranged for most of the time below the optimum temperatures for the development of aflatoxigenic fungi and the levels of the *A. flavus.*

The numbers of *Aspergillus* isolates from hulls of in-shell nuts and hulls sampled from the stockpile in San Joaquin County, California) are shown in **Figure 8**. Data in **Figure 8** is a compilation of data from both sampling dates.

In stockpile 1, 91 *Aspergillus* isolates were isolated from hulls of in shell nuts and hulls at the top (8.8% of the *Aspergillus* isolates), the valley of the plastic cover (30.8%), the bottom (22.0%), edge (35.2%), and edge of edge (3.2%) of the stockpile. The main species was *A. niger* (33%), *A. ochraceus* (15.4%), and *A. flavus* (12.1%). When compared to different parts of the stockpile, *A. flavus* was more frequently detected at the bottom. A total of 26 *Fusarium*, 17 *Penicillium*, 2 *Alternaria,* and 2 *Mucor* isolates were also isolated from hulls of in shell nuts and hullsof nuts taken from in the stockpile in the San Joaquin County.

In stockpile 2, a total of only 10 *Aspergillus,* 12 *Eurotium*, 12 *Fusarium*, 6 *Penicillium*, 1 *Alternaria,* and 1 *Mucor* species were isolated from hulls of in shell nuts and hullsof nuts sampled from the bottom and the edge of edge of the stockpile. *A. flavus* were not isolated from these samples; instead 2 isolates (one each from the bottom and the edge of the stockpile) of *A. parasiticus,* representing 20% of the total *Aspergillus* isolates were recovered. Among the other isolated *Aspergillus,* 30% were *A. niger*, 30% *A. terreus,* and 20% *A. wentii*.

No aflatoxin was detected in kernels from either stockpile of the 2008 San Joaquin County study.

Overall

The relationship between relative humidity in the stockpile versus water content of nuts and hulls for Kern (Nonpareil variety) and San Joaquin (Livingston variety) County 2007 stockpiles are shown in **Figure 9**. Since the air in the stockpile is at equilibrium with the nuts and hulls, the water activity in the pile should be equal to the (relative humidity)/100 as shown on the bottom axis of this figure. These data agree well with published recommendations on almond storage in the UC Almond Production Manual, Page 275 (UC Division of Agriculture and Natural Resources, Publication 3364). The levels of relative humidity in the Kern County stockpiles were well below the 65 – 70% relative humidity recommended in the UC Almond Production Manual (Page 275) to balance the mold growth potential with optimal texture, color, flavor and stability. King et.al (1983) found that fungal growth occurred at a water activity of 0.75 (equal to an equilibrium relative humidity of 75%) and above.

Summary

The results from the 2007 and 2008 seasons suggest that a major concern of growers and handlers should be to pay attention to the initial moisture content when stockpiling nuts. In general, conditions at the top and bottom edge of the stockpiles tended to be most problematic. This is likely due to wetting of the nuts and hulls by condensation of moisture that has been observed in these areas.

In 2008, monitoring focused on these problem areas. Although there was substantial growth of *Aspergillus* in these locations, it was not generally associated with production of aflatoxin. Although Aspergillus was present from most sample locations on all stockpiles in 2008, most samples did not show detectable aflatoxin. Only samples from the Kern County Stockpile #1, from the high canopy cover part of the orchard, showed detectable levels of aflatoxin from the top of the stockpile on the first sampling date (**Table 2**) and from the edge of the stockpile on the final sampling date (**Table 3**). The fact that aflatoxin was present at the time of stockpiling suggests that aflatoxin contamination had occurred in the orchard and orchard conditions contributed to the problem. In addition, aflatoxin production occurred in the stockpile even though the overall nut moisture content at the time of stockpiling was only 4.4% (**Table 1**). This again suggests that conditions in the orchard contributed to the problem.

In 2009, work on this project is focusing on looking at the impacts of different tarp materials on stockpile conditions and resulting fungal growth potential. In addition, work is being done to identify rapid methods of quantifying nut moisture before harvest operations are started.

References

Kader, Adel A. 1996. “In-Plant Storage”, pp. 274-277. In Almond Production Manual, Warren C. Micke, Technical Editor. University of California Division of Agriculture and Natural Resources, Publication 3364.

King, A.D.Jr., W.U. Halbrook, G. Fuller, and L.C. Whitehand. 1983. Almond nutmeat moisture and water activity and it influence on fungal flora and seed composition. J. Food Sci. 48: 615-617.

Klich, M. A., J. I. Pit.t 1988. A laboratory Guide to Common *Aspergillus* Species and their Teleomorphs. CSIRO, North Ryde, New South Wales, Australia. 116 pp.

Table 1. Summary of stockpile varieties, start dates, removal dates and initial moisture content of hulls and nuts for all locations.

2008

Location	variety	stockpile start date	Start hull % moisture	Start nut % moisture	Overall % moisture	stockpile removal date	total days in stockpile
Colusa County #1	Nonpareil	9/12/08	7.9	3.9	6.2	12/5/08	84
Colusa County #2	Nonpareil	9/23/08	9.8	4.1	7.3	12/5/08	73
Kern County #1 (blue)*	Nonpareil	8/25/08	5.7	3.0	4.4	3/12/09	199
Kern County #2 (pink)**	Nonpareil	8/25/08	6.1	3.0	4.7	3/12/09	199
San Joaquin County #1	Nonpareil	9/9/08	11.5	5.1	8.7	12/29/08	111
San Joaquin County #2	Nonpareil	9/17/08	25.9	8.8	16.8	1/13/09	118

* High canopy cover treatment
** Moderate canopy cover treatment
*** Ending moisture content from top of pile

Table 2. Almond samples (cv. Nonpareil) analyzed for aflatoxin from Kern County Stockpile #1 (high canopy cover) from the first sampling date on 8/28/08.

Location in Stockpile	Aflatoxin levels (ppb)				
	B1	B2	G1	G2	Total
Top	0.3	None Detected	None Detected	None Detected	0.3
Valley	None Detected	None Detected	None Detected	None Detected	None Detected
Bottom	None Detected	None Detected	None Detected	None Detected	None Detected
Edge	None Detected	None Detected	None Detected	None Detected	None Detected
Edge of edge	None Detected	None Detected	None Detected	None Detected	None Detected

Table 3. Almond samples (cv. Nonpareil) analyzed for aflatoxin from Kern County Stockpile #1 (high canopy cover) on the final sampling date on 3/12/09.

Location in Stockpile	Aflatoxin levels (ppb)				
	B1	B2	G1	G2	Total
Top	None Detected	None Detected	None Detected	None Detected	None Detected
Valley	None Detected	None Detected	None Detected	None Detected	None Detected
Bottom	None Detected	None Detected	None Detected	None Detected	None Detected
Edge	108	12.0	None Detected	None Detected	120
Edge of edge	0.3	None Detected	None Detected	None Detected	0.3

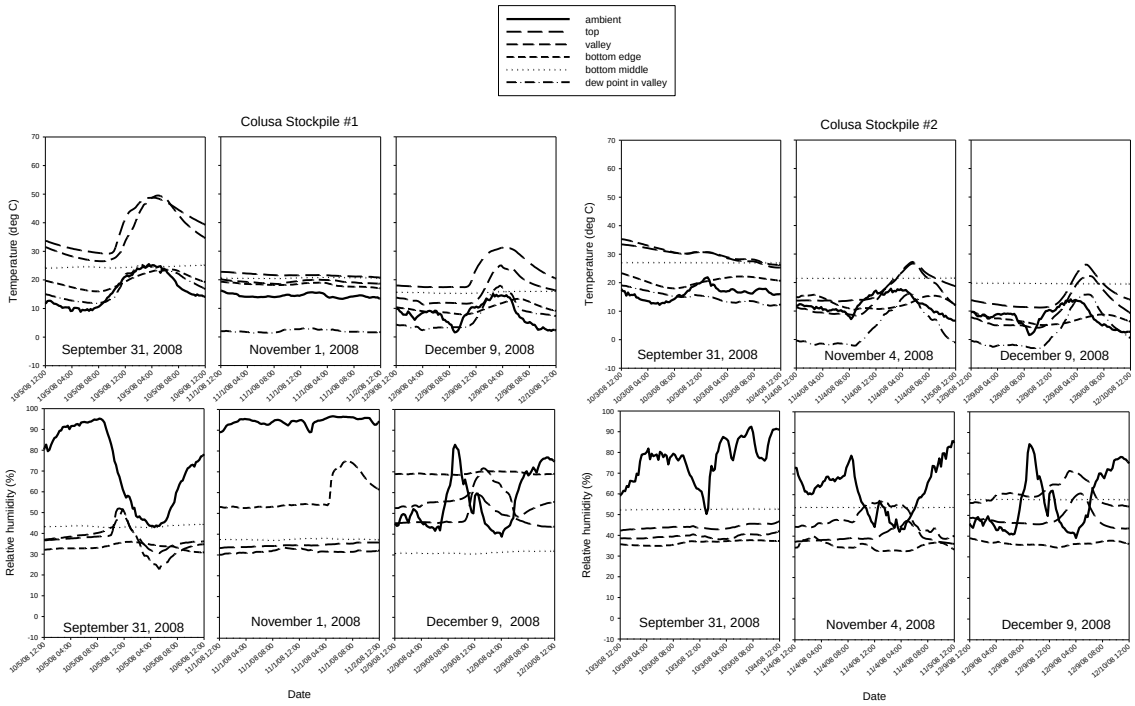

Figure 1. Temperature and relative humidity on three dates in 2008 at various locations in stockpiles from Colusa County stockpile #1 and #2.

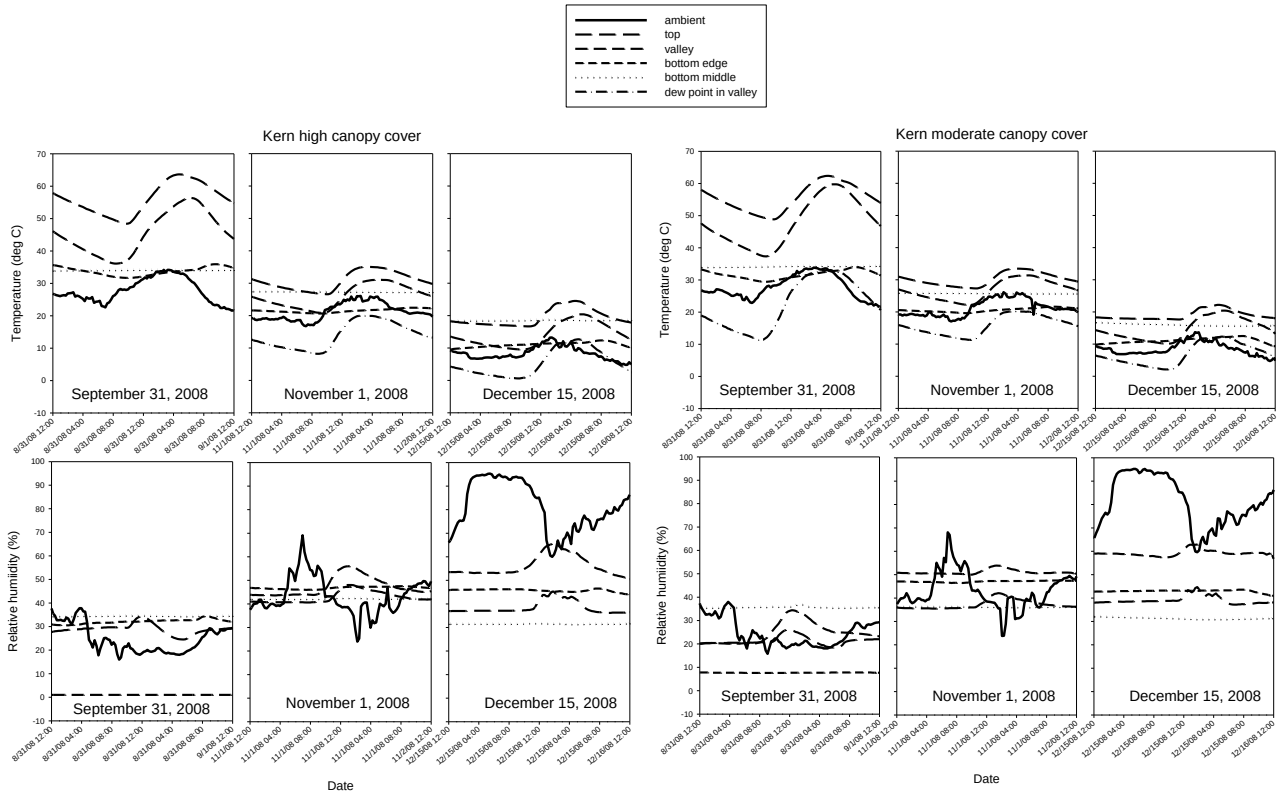

Figure 2. Temperature and relative humidity on three dates in 2008 at various locations in stockpiles from the high canopy cover and moderate canopy cover treatments in Kern County.

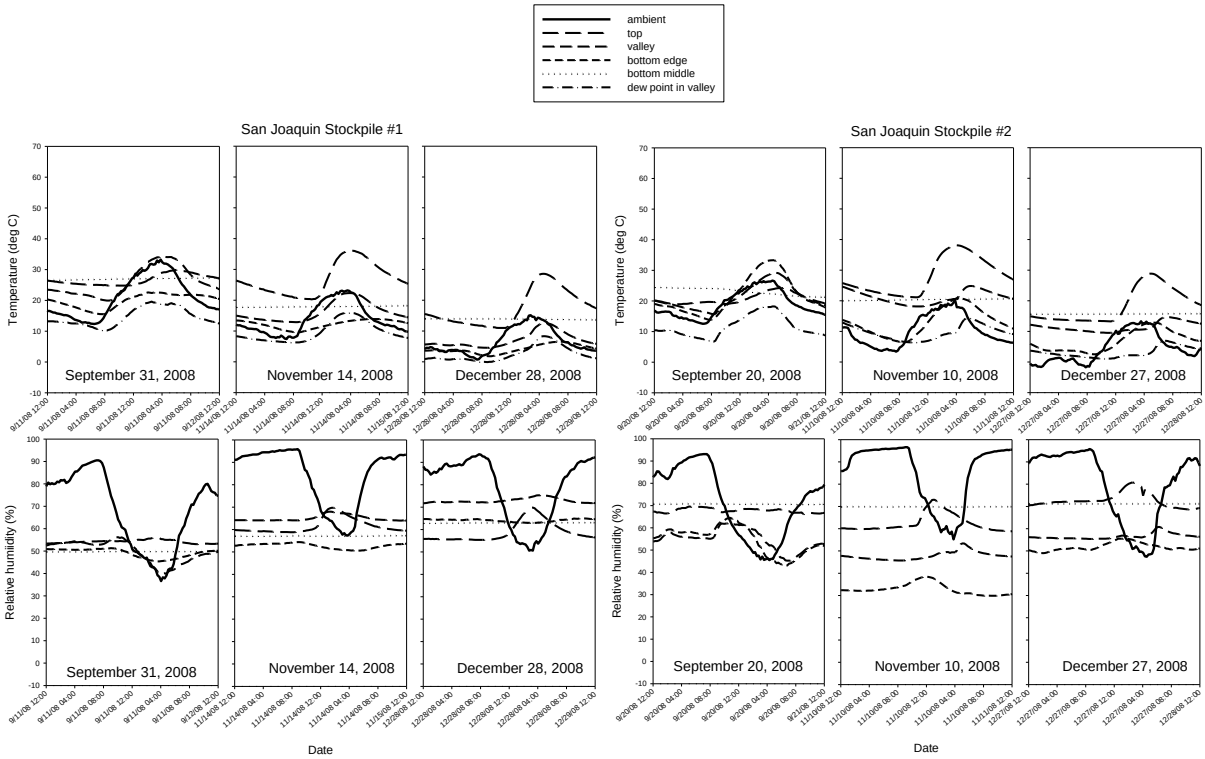

Figure 3. Temperature and relative humidity on three dates in 2008 at various locations in stockpiles from San Joaquin County stockpile #1 and #2.

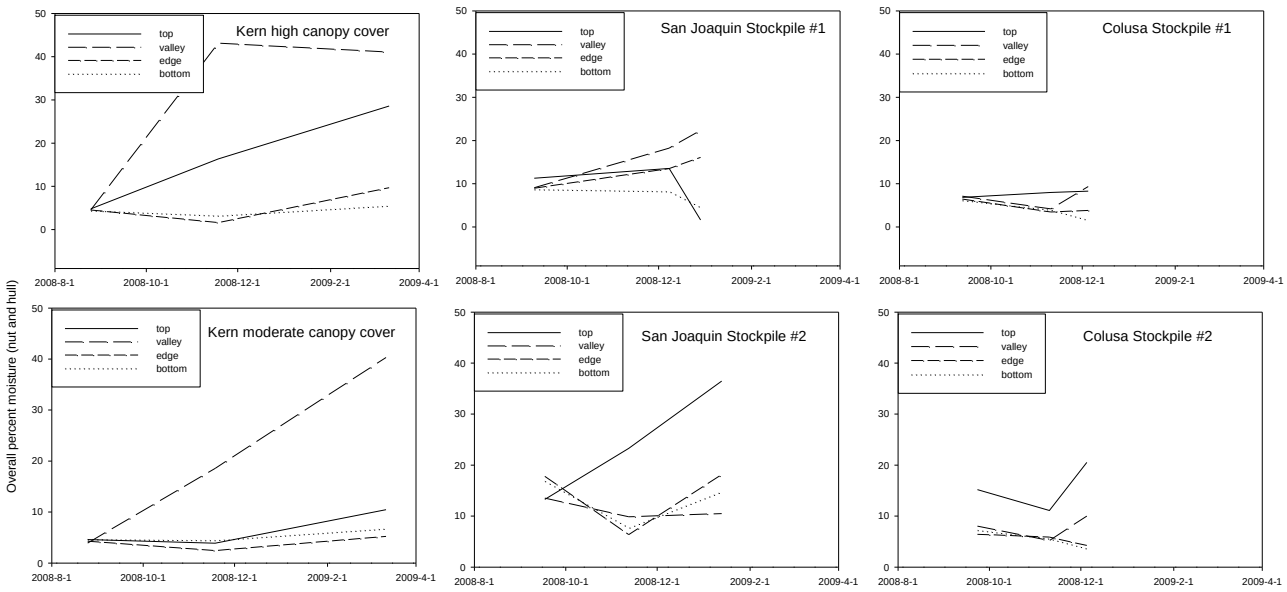

Figure 4. Overall moisture content (nut plus hull) for different positions in stockpiles from all locations and sampling dates for 2008-2009 stockpiles. Top position is about 6 inches below surface near upper center of pile. Valley is on west side of pile where water condenses on tarp. Edge is from west edge of pile below area where water condenses. Bottom is in the center of pile about 6 inches from the ground.

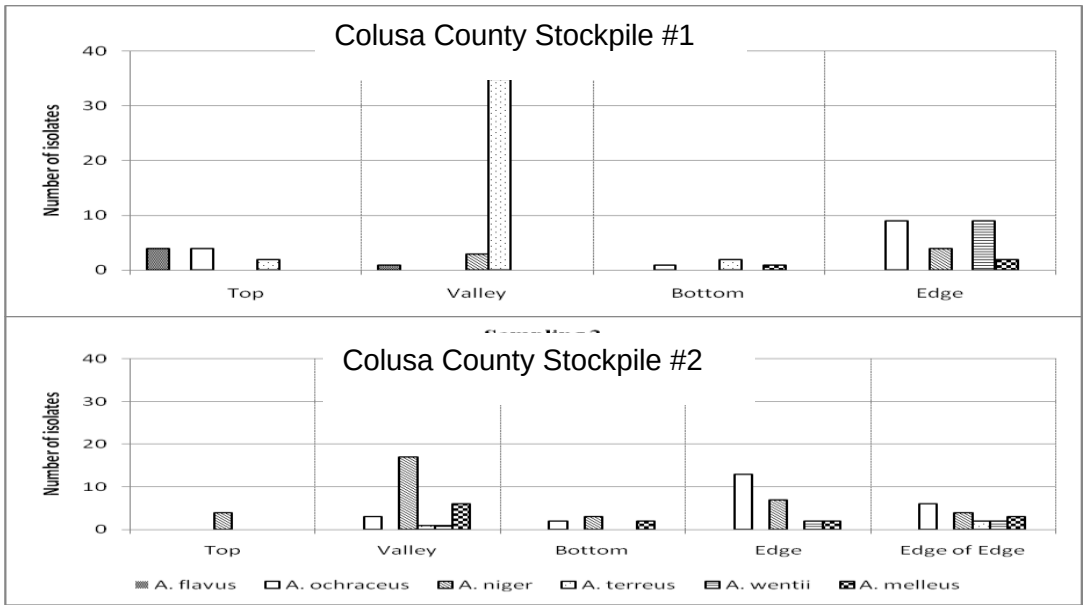

Figure 5. Numbers of various *Aspergillus* species isolated from hulls of in-shell nuts and hulls taken from different parts of stockpiles 1 and 2 in 2008, Colusa County, California.

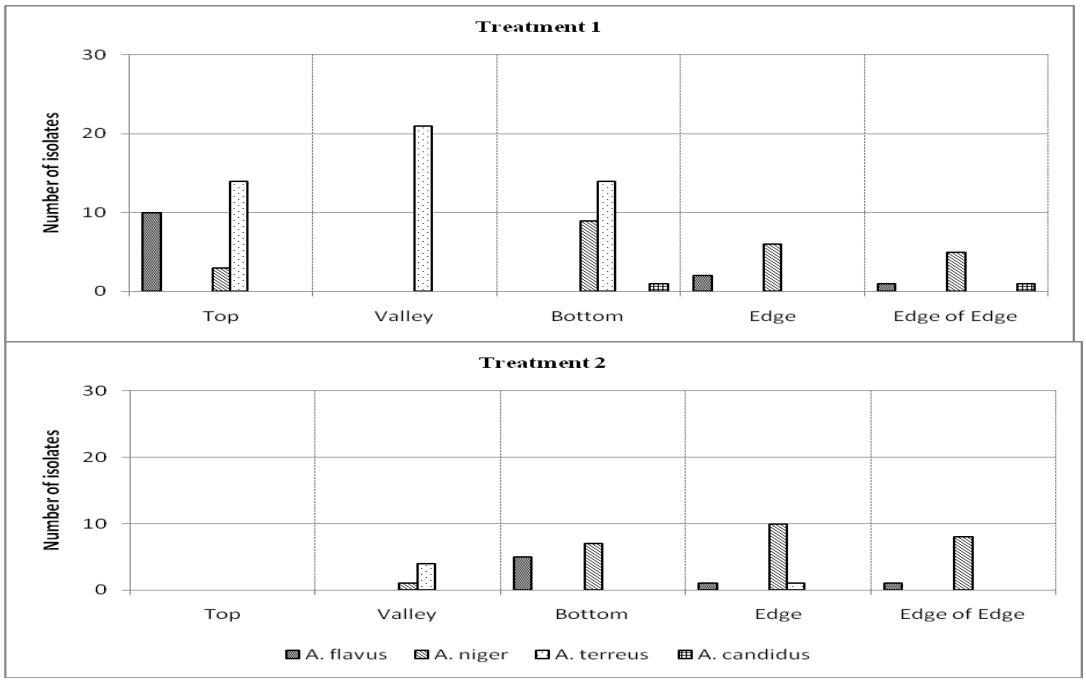

Figure 6. Number of *Aspergillus* species isolated from hulls of in-shell nuts and hulls taken from different locations of the high canopy cover stockpile (Treatment 1) and the moderate canopy cover stockpile (Treatment 2) sections of the same orchard in Kern County, California (sampling date 19 November 2008).

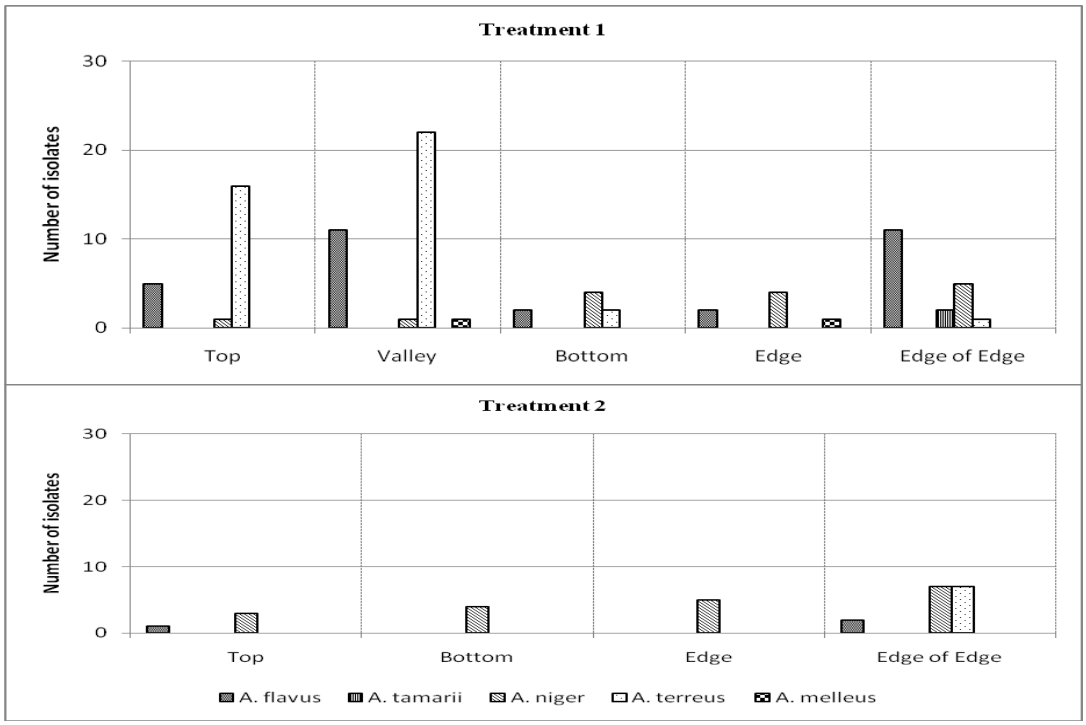

Figure 7. Number of *Aspergillus* species isolated from hulls of in shell nuts and hulls taken from different locations of the high canopy cover stockpile (Treatment 1) and the moderate canopy cover stockpile (Treatment 2) sections of the same orchard in Kern County, California (sampling date 29 December 2008).

.

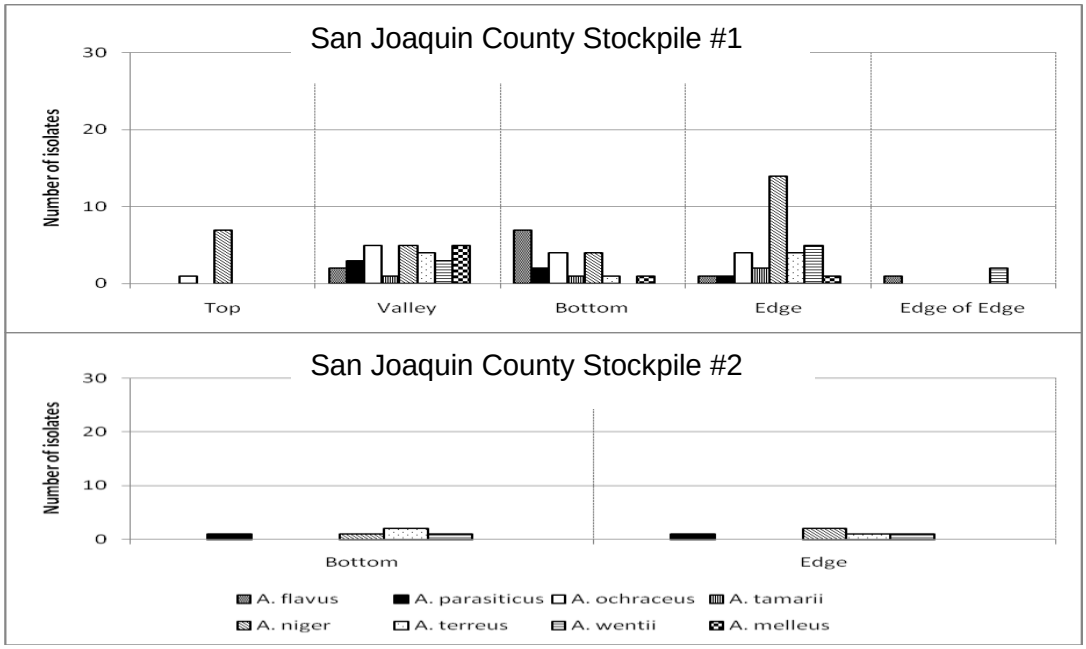

Figure 8. The numbers of various *Aspergillus* species in hulls of in-shell nuts and hulls taken from different parts of stockpiles 1 and 2 in 2008, San Joaquin County, California.

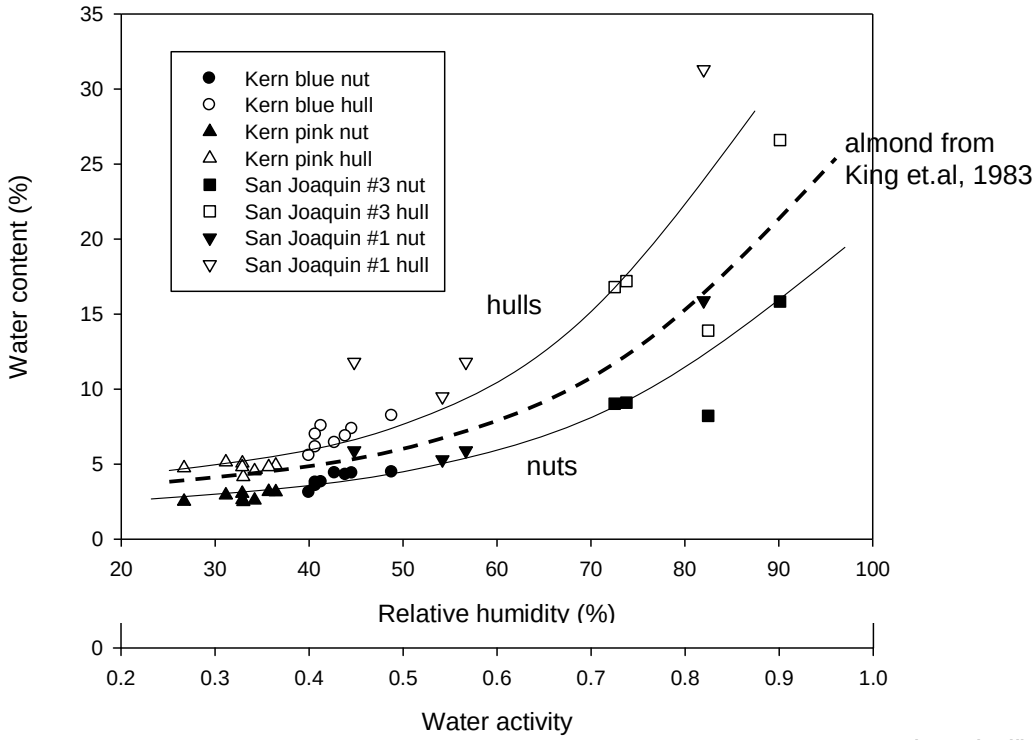

Figure 9. Relative humidity and water activity versus water content for nuts (including shell) and hulls from the Kern and San Joaquin County stockpiles in 2007. Nuts and hulls labeled as blue and pink are from the high water, high nitrogen treatment and moderate water, moderate nitrogen treatments respectively. Data include Nonpareil from Kern County as well as stockpile #1 and stockpile #3 from San Joaquin County. Dashed line is approximate curve for almond kernels from King et. al, 1983.