Spread of *Aspergillus flavus* by Navel Orangeworm (*Amyelois transitella*) on Almond

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Abstract

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Navel orangeworm (NOW) damage to almond is correlated with increased incidence of aflatoxin contamination caused by *Aspergillus flavus*. However, no reports demonstrate a causative relationship between NOW feeding and *A. flavus* infection. To demonstrate the potential of NOW to act as a vector of *A. flavus* on almond, NOW eggs were dusted with *A. flavus* and incubated in microchambers adjacent to but not touching agar plates or almond kernels. Following egg hatch, *A. flavus* colonies developed on agar along trails left by NOW larvae. Almond kernels damaged with *A. flavus*-carrying NOW showed higher incidence of *A. flavus* colonization and aflatoxin contamination than

control treatments. Interestingly, levels of aflatoxin in NOW-damaged, *A. flavus*-infected almond were significantly higher than control treatments, even in the absence of visible fungal growth. Commercial almond orchards had a relatively low level of contamination with *Aspergillus* section *Flavi* in spring and early summer and a high level during summer, corresponding with the higher level of NOW infestation of the crop. Our study demonstrates that NOW is capable of vectoring *A. flavus* to almond, and that monitoring and sorting of almond kernels for insect damage is warranted to limit aflatoxin contamination potential both before and after harvesting.

Aflatoxin contamination of almond (*Prunus dulcis* (Mill.) D.A. Webb) is the result of colonization by *Aspergillus flavus* Link and *A. parasiticus* Speare. Navel orangeworm (NOW; *Amyelois transitella* (Walker), Lepidoptera: Pyralidae) is a major insect pest of tree nuts in California (9). This insect overwinters in mummy nuts left on the tree or ground during winter and its first moth flight begins in mid- to late March. Depending on the climatic conditions, this lepidopteran pest can complete three to four generations (flights) during the season. However, the highest potential for almond damage occurs when the kernel becomes accessible to the larvae emerging from hatched eggs that were laid on the hull of the almond. Larvae can chew through the soft shell or through the cracked shell (mainly of the soft-shelled cultivars) and then feed on the kernel, thereby creating wounds accessible to fungal invasion. Damaged nuts are associated with increased *Aspergillus flavus* colonization and subsequent aflatoxin contamination of almond and pistachio (5,6,17). The correlation between NOW damage and aflatoxin contamination in almond has been shown in orchard studies (17) and in postharvest sorted nuts (19). These studies, as well as anecdotal evidence, have led to insect control strategies as a means to limit aflatoxin contamination in tree nuts.

Entry of *A. flavus* spores into NOW-damaged nuts may occur when the larvae of NOW feed and damage the almond kernels. The eggs are typically laid in spring and early summer on unharvested nuts (mummies) from the previous season (2,13,23) and from late

June through October on the current nut crop following hull split (23). Both mummy and new-crop nuts may harbor a variety of fungal species, including aflatoxigenic species, and these infected nuts may act as inoculum sources for the spread of *Aspergillus* spp. to uninfected nuts. However, no studies have directly determined whether NOW can play an active role in aflatoxin contamination of almond by either transporting *A. flavus* spores to the nuts or directly contaminating the kernel while feeding.

In order to investigate the possible role of NOW in contaminating almond kernels, we developed in vitro methods to isolate *A. flavus* spores from both growth media and almond, ensuring that fungal inoculation could only occur through larval movement and feeding. In addition, kernels damaged by NOW larvae were analyzed for their aflatoxin content to determine whether the extent of insect damage affected the levels of aflatoxin contamination. Finally, the seasonal incidence of *Aspergillus* section *Flavi* was determined for almond mummies, NOW larvae, and NOW moths collected from commercial almond orchards during the season.

Materials and Methods

Spread of *A. flavus* by NOW in vitro. Eggs were laid on paper towels and sanitized by treatment with 10% formalin for 15 min, followed by three 10-min washes with sterile distilled water. Paper towel discs (2 cm in diameter) containing 15 to 20 eggs were used in each treatment. A sterile cotton swab was used to dust the eggs with conidia from a sporulated culture of a toxigenic *A. flavus* strain (NRRL3357; 22) that was grown on potato dextrose agar (PDA) at 28°C for 5 days. Each dusted paper towel disc containing eggs was placed into a sterile 0.5-ml microcentrifuge tube, which was then dipped into 70% ethanol to sterilize the exterior surface of the tube. The bottom of each tube was cut off aseptically, using either a sterilized razor or scissors, and each open-bottom tube was placed onto the surface of a PDA plate. Plates were sealed with parafilm and incubated at 28°C for 48 to 72 h. Control treatments consisted of paper towel discs containing NOW eggs without *A. flavus* spores, and egg-free paper towel discs dusted with *A. flavus* spores using a cotton swab as described above. Each experiment was performed twice, with five replicate plates per treatment.

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Treatments were observed for fungal growth for 72 h after the initiation of each experiment.

Spread of *A. flavus* by NOW on almond kernels. Almond ('Nonpareil') fruit were harvested immediately following hull split and kernels were aseptically removed from the shells. Individual microcentrifuge tubes containing the treatments described above were placed onto sterile 60-mm petri dishes containing single almond kernels with visually intact seed coats. The petri dishes were sealed with parafilm and incubated in sealed 3-liter polypropylene food storage containers for 21 days at 25°C. Treatments consisted of almond kernels incubated with NOW eggs + *A. flavus* spores, NOW eggs only, and *A. flavus* spores only. Untreated almond kernels were included to determine the baseline presence of naturally occurring insect or fungal damage in the harvested nuts. Each experiment contained 20 replicate samples per treatment, and was performed twice.

Aflatoxin determination. Almond samples after incubation were examined visually for insect feeding damage and external fungal growth. Each nut was ground in 20 ml of methanol, and 1 ml of the extract was evaporated to dryness under a stream of nitrogen at 40°C. Samples were then derivatized (11) with 200 µl each of trifluoroacetic acid and hexane for 10 min, then evaporated to dryness under a stream of nitrogen at 40°C. Samples were redissolved in 1 ml of 10% aqueous acetonitrile, and 20-µl aliquots were analyzed for aflatoxins by high-performance liquid chromatography (HPLC) using the protocols described by Rodriguez and Mahoney (18). Samples were injected onto a Vydac 218TP54, 4.6-by-250-mm C₁₈ column (Grace Vydac) and run using water/methanol/acetonitrile/acetic acid (60:35:5:0.5) as the mobile phase. The amount of aflatoxin B1 and B2 per almond was calculated from peak areas relative to standard curves of authentic aflatoxin B1 and B2 (Sigma-Aldrich).

Incidence of *Aspergillus* section *Flavi* on NOW moths trapped in almond orchards. Sticky traps (Suterra) containing three virgin NOW female moths in mesh bags (changed every 3 days) were hung 2 m high in the tree canopy (approximately one trap per 100 ha) and monitored from March to October in two almond orchards (S&J12-16 and S&J07-09 in Madera County and a third orchard (San Joaquin Valley Agricultural Sciences Center [SJVASC], United States Department of Agriculture [USDA]) in Fresno County for 2 years (2008 and 2009) and in two orchards (S&J12-16 and SJVASC) in 2010. A pheromone produced by the female moth attracts the male moths to the traps and the males, in turn, become stuck to a removable glue-covered bottom panel. This bottom panel was replaced at intervals, collected, placed in a clean 19.4-liter plastic bucket sealed with a cover, and brought back to the laboratory, where the moths were counted and removed. In all, 50 moths per panel were randomly selected using sterile forceps, plated on Si10 medium (containing 10 g of sucrose, 60 g of NaCl, 1 g of yeast extract, 0.1 g chloramphenicol, 10 ml of a dichloran solution in 2% ethanol, 1 ml of CuSO₄·ZnSO₄ solution, and 15 g of Bacto agar in 1 liter of deionized water), 10 moths per plate, and the plates were incubated at 30°C for 1 week. When fewer than 50 moths were caught in a trap, all trapped moths were removed and plated. Only colonies of *Aspergillus* spp. originating from the moth's body were recorded; the bodies were examined using a dissecting microscope or, when needed, a compound microscope. Isolates that could not be identified on Si10 plates were transferred to Czapek yeast agar (CYA; containing 49 g of Czapek solution agar, 5 g of yeast extract, 1 ml of CuSO₄·ZnSO₄ solution in 2% ethanol, and 1 liter of deionized water), then identified to species. Only fungi of *Aspergillus* section *Flavi* and section *Nigri* were identified and all remaining isolates were designated as "other *Aspergillus*". The same sampling procedure was repeated in 2012 in the SJVASC and a new almond orchard, AW Felgers (Madera County), after learning from entomologists that, due to the mild winter, there was an increased risk of high levels of NOW damage in these orchards.

***Aspergillus* section *Flavi* prevalence in almond mummies.** Five hundred mummies were collected from a Nonpareil almond orchard in Madera County. The mummies were shelled and the

kernels were classified as healthy or moldy. The moldy kernels were classified according to their macroscopic color as "black," "green," or "white." Green kernel fragments (without surface sterilization) were plated on CYA media, which favors the growth of *Aspergillus* spp. Plates were incubated at 30°C for 1 week and the *Aspergillus* spp. present were recorded. The *Aspergillus* isolates were classified as section *Flavi* (*A. flavus*, *A. parasiticus*, and *A. tamarii*) and section *Nigri* (*A. niger*, *A. japonicus*, and *A. carbonarius*) based on morphological characteristics of the colonies and shape, size, and ornamentation of the species conidia (10).

Prevalence of *Aspergillus* section *Flavi* on moths that emerged from almond mummies. Approximately 350 to 400 mummies were placed in 19.3-liter painter's buckets and covered with a mesh with 2-mm² openings. The buckets were incubated at 25°C, and the moths emerged and gathered on the inner surface of the mesh. Moths were individually collected from the mesh using sterilized forceps and containers, killed by freezing, then plated on CYA media, 10 moths per plate. Each sample consisted of 10 plates incubated at 30°C for 1 week.

Statistical analyses. Data on the relationship between the number of NOW feeding sites and aflatoxin levels were analyzed by regression using the SAS REG statistical program (SAS Institute). These relationships were graphed using Excel (Microsoft).

Results

Spread of *A. flavus* by NOW in vitro. Larvae typically emerged from the open-bottom microfuge tubes within 24 h. Treatments containing NOW eggs and *A. flavus* spores showed growth of *A. flavus* in larval track patterns, indicating that NOW larvae had picked up *A. flavus* spores inside the microcentrifuge tube and then deposited them onto the agar surface (Fig. 1A). Following sporulation of *A. flavus* colonies on these plates, NOW larvae crawling over the colonies were observed to accumulate conidia upon their setae (Fig. 1B). In contrast, no fungal growth occurred on plates containing NOW larvae in the absence of *A. flavus*, indicating that the larvae did not harbor *A. flavus* spores. There was also no fungal growth on plates with microcentrifuge tubes containing only *A. flavus* spores, demonstrating that the assay method provided spatial separation between the *A. flavus* spore inoculum and the medium.

Spread of *A. flavus* to almond kernels by NOW. Almond kernels exposed to NOW larvae for 21 days showed extensive feeding damage. Almond kernels treated with NOW + *A. flavus* showed feeding damage in 37 of 40 samples (93%), and kernels treated with NOW only (without *A. flavus*) showed feeding damage in 34 of 40 samples (85%). Almond kernels treated with *A. flavus* and untreated kernels (both without NOW) showed no insect feeding damage. Visible, external *A. flavus* growth was observed in 4 of the 37 damaged almond kernels exposed to NOW + *A. flavus* (11%), as well as in a single intact kernel exposed to *A. flavus* only. Because nonsterilized almond kernels were used, there is the possibility that the growth of *A. flavus* on the *A. flavus*-treated nut originated from ambient field contamination of the nut. No *A. flavus* growth was seen in damaged kernels exposed to NOW only or in intact, untreated kernels.

Aflatoxin content of NOW-damaged almond kernels. Individual nuts from all treatments were analyzed for aflatoxin content. Of the 40 almond kernels exposed to NOW + *A. flavus* (30%), 12 contained measurable amounts of aflatoxin B1 (Fig. 2A). In the four samples of this treatment that had visible external *A. flavus* growth, aflatoxin B1 content was 584 to 498,000 ng/almond. Total aflatoxin (B1 + B2) content in these samples was 609 to 551,800 ng/almond. More importantly, aflatoxin was found in NOW + *A. flavus*-treated samples that did not show external *A. flavus* growth. In these samples, aflatoxin B1 content was 10 to 9,330 ng/almond, and total aflatoxin (B1 + B2) content was 10 to 10,050 ng/almond. The aflatoxin B1 content was positively related with the extent of NOW feeding damage on almond kernels ($y = 0.89x - 0.30$, where y is the log of nanograms of aflatoxin B1 and x is the number of feeding sites per almond caused by NOW; $r^2 = 0.79$, $P < 0.05$) (Fig. 2B). In the *A. flavus*-only treatment, there was a single al-

mond kernel which showed external *A. flavus* growth and contained 18 ng of aflatoxin B1. In almond samples exposed to NOW-only treatment, a single kernel contained 1 ng of aflatoxin B1, which was at the detection limit of the HPLC assay. No aflatoxins were detected in the untreated nuts.

Prevalence of *Aspergillus* spp. on NOW moths captured in almond orchards. In the first orchard sampled, *A. flavus*, *A. parasiticus*, and *A. tamarii* were recovered from NOW moths at levels ranging from 3 to 7% of the moths in 2008, peaked at 4% in 2009 and at 5% in 2010 from the March to 15 June trapping (Fig. 3A–C). In the second orchard sampled, between 2008 and 2010, there were even higher levels of *Aspergillus* section *Flavi* in the moths sampled (Fig. 3D–F). In both of these orchards, the prevalence of *Aspergillus* section *Flavi* increased after 15 June and peaked in

mid-September. The third orchard was only sampled for 2 years (2008 and 2009) but showed similar trends (Fig. 3G and H). Overall, the prevalence of *Aspergillus* section *Flavi* was 2 to 10% of the plated moths, and reached 35% on some sample dates. However, there were also cases where no *Aspergillus* section *Flavi* was recovered. Recovery of moths that did not carry *Aspergillus* section *Flavi* was more frequent earlier in the season (Fig. 3).

In 2012, the levels of NOW moths trapped with *Aspergillus* section *Flavi* was high initially, in April to May (3 to 10%), and reached an average of 35% in one of the orchards in July. A third peak occurred close to the end of August, with the prevalence reaching 30% in the same orchard (Fig. 4).

***Aspergillus* spp. in almond mummies.** The almond mummies collected in winter 2009 from an almond orchard in Madera

Fig. 1. A, Trails of *Aspergillus flavus* colonies, resulting from navel orangeworm (NOW) transport of conidia from microcentrifuge tube to potato dextrose agar medium. **B,** Accumulation of *A. flavus* conidia on setae (arrows) of NOW larva after crawling across plates containing sporulating *A. flavus* colonies.

Fig. 2. A, Distribution of aflatoxin content in almond exposed to navel orangeworm (NOW) larvae + *Aspergillus flavus* spores. Black bars represent samples with visible *A. flavus* growth on the surface of the almond; gray bars represent samples with no obvious *A. flavus* growth. **B,** Aflatoxin content in almonds from A relative to feeding damage produced by NOW larvae. Black circles represent samples with visible *A. flavus* growth on the surface of the almond; gray circles represent samples with no obvious *A. flavus* growth. Linear regression equation and coefficient for all data are indicated.

County were highly infected with *Aspergillus* section *Flavi* fungi. Surprisingly, 96% of the kernels plated yielded *Aspergillus* section *Flavi* and 100% of the kernels yielded *Aspergillus* section *Nigri*. Even the kernels that did not show any NOW damage were heavily infected with *Aspergillus* section *Flavi* (84%) and *Aspergillus* section *Nigri* (100%). Mummies kept in cold storage (0 to 1°C) were also highly infected with both *Aspergillus* section *Flavi* (98%) and *Aspergillus* section *Nigri* (98%).

***Aspergillus* in NOW moths collected directly from almond mummies.** NOW moths that emerged from almond mummies collected from three orchards located in the central San Joaquin Valley (one each in Fresno, Madera, and Tulare Counties) were heavily contaminated with propagules of *Aspergillus* spp. (Fig. 5). The prevalence of *A. flavus* and *A. parasiticus* was 45 to 70%, while *Aspergillus* section *Nigri* was 46 to 95% (Fig. 5). In addition, other *Aspergillus* spp. (*A. ochraceus*, *A. terreus*, *A. melleus*, and *Emericella* spp.) were observed at levels of 13 to 75%. In many instances, more than one species of *Aspergillus* developed from the same moth. Other fungi recovered included common contaminants of agricultural products coming in contact with the soil (i.e., *Alternaria*, *Fusarium*, *Penicillium*, and *Cladosporium* spp.; T. J. Michailides, personal observations; 4).

Discussion

This is the first study to demonstrate that NOW larvae can act as a vector for the transport and introduction of *A. flavus* onto almond kernels. Kernels damaged by NOW are rendered more susceptible to *A. flavus* growth and aflatoxin contamination because insect feeding disrupts the pellicle, which functions as both a physical and biochemical barrier to fungal establishment. In our study, increased aflatoxin levels were directly proportional to increased

numbers of NOW feeding sites per almond, presumably due to extensive pellicle breaches. This positive relationship demonstrated the strong association between NOW feeding damage and an increased likelihood of aflatoxin contamination. Interestingly, whereas all nuts showing *A. flavus* signs (mycelia and sporulation) contained high aflatoxin levels, aflatoxin was also detected in insect-damaged nuts where *A. flavus* growth was not apparent. This finding suggests that insect feeding may alter the kernel in a manner that favors aflatoxin production if *A. flavus* colonizes the nut; therefore, a combination of NOW vector activity and feeding dam-

Fig. 4. Incidence of *Aspergillus* section *Flavi* on navel orangeworm (NOW) moths trapped in two almond orchards (SJVASC and AW Felgers) in 2012 and plated on Si10 media in the laboratory. Each point represents the average of five plates with 10 NOW moths plated per plate.

Fig. 3. Incidence of *Aspergillus* section *Flavi* on navel orangeworm (NOW) moths trapped in three almond orchards: A–C, S&J12-16; D–F, SJVASC; and G and H, S&J07-09; in A, D, and G, 2008; B, E, and H, 2009; and C and F, 2010 and plated on Si10 media in the laboratory. Each point represents the average of five plates with 10 NOW moths plated per plate.

age may lead to higher levels of aflatoxin contamination than found in almond kernels where *A. flavus* became established due to poor shell seal.

A. flavus is introduced into almond kernels by a variety of means, including wind (especially when there is a crack in the shell of the soft-shelled cultivars) or secondary pests (ants, birds, and so on) preceding or following damage caused by NOW. However, in commercial orchards, although kernels can be damaged by birds, rodents, and other agents, NOW damage is more significant and the single most important biotic factor involved in *Aspergillus* contamination of almond. NOW feeding facilitates *Aspergillus* sp. infection of almond by two means: transport of the propagules of the pathogen, and by creating favorable sites for infection at the feeding wounds. Our finding that almond kernels damaged by NOW are contaminated with aflatoxin are in agreement with the results of Whitaker et al. (21), who showed a strong correlation between aflatoxin contamination and various USDA-grade categories of shelled almond, including NOW-damaged nuts. Furthermore, Doster and Michailides (5) reported that damage of pistachio kernels by NOW leads to higher levels of aflatoxin contamination than in undamaged nuts.

A variety of associations between insect pests and host contamination have been described for maize, cotton, and peanut, but, for many insects, their vectorial capacity has not been demonstrated in the laboratory (7). Nitidulid sap beetle species, including *Carpophilus lugubris* Murray, *C. freemani* Dobson, and *Glischrochilus quadrisignatus*, have been described as *A. flavus* vectors in maize (14,16), and the European corn borer (*Ostrinia nubilalis*) can move *Fusarium verticillioides* spores from plant surfaces to the ears (20). Insect-mycotoxin associations were also described for pink bollworm (*Pectinophora gossypiella*) on cotton and lesser cornstalk borer (*Elasmopalpus lignosellus*) on peanut. Given the frequency of the association between insect damage and aflatoxin contamination in these other annual crops, it is not surprising that, in a perennial crop (almond), aflatoxin contamination is closely related to NOW infestation and damage.

The strength of the association between NOW damage and aflatoxin contamination of almond may be due, in part, to the biology of this insect. NOW overwinters as a larva or pupa (9,23), and can spend as long as 8 months in an almond mummy. These mummies, in turn, persist in the environment for a longer time than the harvested crop. Many mummies fall to the ground and are in contact with the soil, where they can be heavily infested or infected by

Aspergillus spp., including *A. flavus* or *A. parasiticus*. In our study, when mummies were plated on specific media, a high incidence of *Aspergillus* spp. was detected, suggesting that almond kernels serve as a good substrate for the development of *Aspergillus* spp. The high prevalence of *Aspergillus* section *Flavi* detected on moths that emerged from mummies enclosed in buckets (Fig. 5) also suggests that there is cross contamination when moths touch each other, because the prevalence of *Aspergillus* spp. on moths captured in the orchards early in March only occasionally exceeded the 5% level (Fig. 3). The increased contamination of moths late in the season may occur because more propagules of *Aspergillus* are found on the crop at this time, and nuts infected earlier in the season show sporulation later in the season (15). Empirical experience shows that, in years when high NOW damage occurs, aflatoxin contamination occurs more frequently than in low-NOW-damage years. In 2006, there was unusually high NOW damage in almond followed by an unusually high number of Rapid Alerts for aflatoxin contamination in almond loads exported to Europe; consequently, in 2007, the European Union placed almond imports from California under the Special measures protocol (1).

In all years of NOW trapping, *Aspergillus* section *Nigri* was more frequently recovered than any other *Aspergillus* spp. Other *Aspergillus* spp. isolated included *A. tamaris*, *A. ochraceus*, *A. melleus*, and *Emericella*. *A. niger* and *Alternaria* spp. showed the highest incidence in the species composition of fungi in washings from leaves of asymptomatic male and female pistachio trees and fruit clusters collected from three orchards (3). The high prevalence of *Aspergillus* section *Flavi* in mummies can help explain our finding that NOW that emerged from mummies were highly contaminated with *Aspergillus* section *Flavi*. In a previous study (T. J. Michailides, unpublished), we showed that 2% of almond mummies had obvious sporulation by *Aspergillus* section *Flavi*, suggesting infection by *A. flavus*.

Aflatoxin contamination of almond is of considerable economic and health importance because of stringent limits set for aflatoxin B1 contamination of crops, including tree nuts, in both the United States (15 ppb) and the European Union (8 ppb). The almond industry has taken a number of measures to reduce pre- and postharvest aflatoxin contamination. Efforts to reduce crop damage caused by NOW include improved orchard sanitation practices to remove mummy nuts and other crop debris (23), the development of new almond varieties with improved insect resistance (8), and the introduction of new insecticide chemistries to improve NOW control (9). Additionally, postharvest sorting of insect-damaged almond kernels during processing and insect control strategies during almond storage can further improve product quality (12,19). This study strongly supports the previous associations observed between NOW damage and increased *A. flavus* exposure and aflatoxin contamination in almond. As such, control of NOW populations and removal of damaged product continue to be important for maintaining almond quality and safety.

Acknowledgments

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Fig. 5. Incidence of *Aspergillus* sections *Flavi* and *Nigri* and other *Aspergillus* spp. recovered from navel orangeworm moths emerged in the spring from almond mummies that were enclosed in buckets for three almond orchards, one each in Tulare, Fresno, and Madera Counties.

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