Research

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Spatial and Temporal Population Dynamics of *Aspergillus flavus* in Commercial Pistachio Orchards in Arizona

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Abstract

Aspergillus flavus infects a wide range of crops, including pistachio, and subsequent aflatoxin contamination results in significant economic losses. Application of biocontrol products based on nonaflatoxigenic (atoxigenic) strains of *A. flavus* is one of the most effective tactics for controlling aflatoxins in crops. Both risk of aflatoxin contamination and effectiveness of biocontrol are influenced by the extent to which *A. flavus* spores move into pistachio tree canopies during periods of nut development. Thus, the purpose of this study was to evaluate spatial and temporal population dynamics of *A. flavus*, including the applied biocontrol strain AF36, in canopies of pistachio orchards in Arizona. Propagule densities of *A. flavus* were quantified on leaf samples collected from lower, middle, and upper canopies from spring through harvest in 2018 and 2019. *A. flavus* propagule densities peaked during periods of high temperature and rainfall in 2018 (up to 600 CFU/g) and 2019 (up to 23 CFU/g), which coincided with nut development and maturation. The applied biocontrol strain AF36 was detected at all canopy heights but overall propagule densities were greater in the upper and middle canopy (mean = 70 CFU/g) compared with the lower canopy (mean = 47 CFU/g). Results suggest that June to August is the period during which *A. flavus* inoculum increases in Arizona pistachio orchards and, to most effectively displace aflatoxin-producing fungi in tree canopies, biocontrol applications should precede this period. In addition, this study demonstrates that soil-applied biocontrol strains can successfully disperse throughout the canopies of commercial tree nut orchards.

Keywords: aflatoxins, *Aspergillus*, biocontrol, biological control, epidemiology, mycology, non-aflatoxigenic, plant pathology, tree nuts

Pistachio nuts are a valuable crop in the United States, with a production of approximately 474,000 metric tons valued at $2.87 billion in 2020 (USDA-NASS 2021). A majority of the U.S. pistachio crop is produced in California which, based on the 2017 agricultural census, comprised 97.7% of the total area in production (USDA-NASS 2021). The remaining pistachio-producing states are all in the Southwest, with approximately 2,000 yielding hectares (2% of total) in Arizona and limited production in New Mexico (197 ha), Nevada (33 ha), Texas (21 ha), and Utah (20 ha) (USDA-NASS 2021). This high-value crop is susceptible to preharvest infection by aflatoxin-producing *Aspergillus* spp. that can result in aflatoxin concentrations in the harvested crop above regulatory limits. The European Union (EU) is a major market for pistachio growers in the United States. However, the EU has stricter regulations for aflatoxins in pistachio at 10 µg/kg (European Commission 2010) compared with the U.S. standard of 20 µg/kg (Food and Drug Administration 2010). Every year, U.S. tree nut exports to the EU are rejected based on the EU Rapid Alert Systems for Food and Feed (RASFF); there were 187 notifications for pistachio rejections based on aflatoxin concentrations from 2010 to 2019 (Alshannaq and Yu 2021). Thirteen border rejections of U.S. pistachio imports into the EU were recorded just in 2020 due to unacceptable levels of aflatoxins (RASFF 2020). Thus, aflatoxin contamination of pistachio is a major trade barrier and results in significant economic losses for U.S. pistachio growers (Bui-Klimke et al. 2014).

Aspergillus flavus and *A. parasiticus* are the causal agents of aflatoxin contamination in pistachio (Doster and Michailides 1994a; Sommer et al. 1986). The primary inoculum for aflatoxin-producing *Aspergillus* spp. in pistachio orchards consists of conidia formed on decaying litter (Doster and Michailides 1994a). Warm temperature

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(>30°C) and high relative humidity (>85%) favor sporulation and dispersal of the fungus (Jones 1979) and, if this coincides with the period of time that pistachio nuts are developing, airborne conidia can move into the canopy and land on the nuts. Direct hull penetration by the fungus is limited (Sommer et al.1976, 1986; Thomson and Mehdy 1978) but openings in the hull caused by early splits (nuts with the shell and hull open), bird and insect feeding (e.g., navel orange worm), or physical cracking caused during harvest create access for colonization by the fungus (Doster and Michailides1994b; Salmon et al.1986; Sommer et al. 1986). Heat and drought stress can lead to increased early shell splitting and, if temperatures >28°C and high humidity occur following colonization of the nuts by aflatoxin-producing fungi, aflatoxin can rapidly accumulate (Doster and Michailides 1994b; Kaminiaris et al. 2020; Sommer et al. 1986). Aflatoxin contamination can also occur postharvest if crops are stored with high moisture content (>6%) (Baazeem et al. 2021).

Biological control of aflatoxins through application of nonaflatoxigenic (atoxigenic) strains of *A. flavus* that outcompete aflatoxin-producing fungi on the crop has been effective for reducing aflatoxin concentrations in crops, including cottonseed, corn, peanut, and tree nuts (Bandyopadhyay et al. 2019; Cotty 1994a; Dorner and Lamb 2006). In the United States, the biocontrol product AF36 Prevail was developed by the United States Department of Agriculture–Agricultural Research Service, and it is manufactured and distributed by the Arizona Cotton Research and Protection Council (ACRPC) (Antilla and Cotty 2004; Cotty et al. 2007). The initial registration of AF36 was for cotton but, by 2012, the Environmental Protection Agency (EPA) had approved expansion of the label to include several additional crops, including the use of the Prevail formulation on pistachio (Picot et al. 2018). Since EPA approval, application of AF36 Prevail has become a standard practice in commercial pistachio orchards for mitigation of aflatoxin contamination (Picot et al. 2018). For example, during the 2020 growing season, a majority of the Arizona pistachio-bearing crop (approximately 1,600 ha out of an estimated 2,000 yielding hectares) was treated with AF36 Prevail (L. Liesner, personal communication). This practice is likely to have increased the frequency of the *A. flavus* genotype that is the active ingredient in AF36 Prevail in pistachio orchards. The active ingredient genotype is endemic to pistachio-growing regions in the United States (e.g., Arizona and California) and is known to be present in nontreated pistachio orchards (Doster et al. 2014; Picot et al. 2018), although at frequencies far below that observed on nontreated cotton or corn.

The effectiveness of aflatoxin biocontrol with nonaflatoxigenic strains is dependent on the ability of the biocontrol genotype to move onto the crop and displace aflatoxin-producing fungi (Cotty and Bayman 1993; Dorner and Lamb 2006; Horn et al. 2000). Furthermore, the risk of crop aflatoxin contamination is influenced by the extent to which aflatoxin-producing fungi move onto and colonize susceptible crop host tissues (Mehl et al. 2012). Most studies on the dispersal and movement of *Aspergillus* spp. onto crops have been for low-canopy crops such as corn, cotton, and peanut (Atehnkeng et al. 2008; Bock et al. 2004; Cotty 1994a; Horn et al. 2001). The effectiveness of AF36 Prevail in reducing aflatoxins in pistachio has been demonstrated both in vitro and in field studies (Doster et al. 2014; Ortega-Beltran et al. 2019) but movement of applied biocontrol strains and other *Aspergillus* propagules into the upper canopy of pistachio trees has not been characterized. Some studies have quantified displacement of aflatoxin-producing fungi on the pistachio crop by the biocontrol strain AF36 but this has only been done postharvest, without considering where the nuts originated from within the tree canopy (Doster et al. 2014; Garcia-Lopez et al. 2021). Canopy architecture for tree nuts such as pistachio differs greatly from other crops that are targets of aflatoxin biocontrol such as corn or cotton. Pistachio trees grow taller than corn or cotton plants and produce more aboveground cover. In addition, due to open spaces between trees, pistachio orchards likely have more air movement below the canopy compared with corn and cotton, which are densely cropped. Canopy cover may affect microconditions such as humidity and air movement, both of which can affect fungal growth and movement (Thomson and Mehdy 1978).

In tree nuts, the extent to which *A. flavus* propagules move into the middle and upper canopy is likely to influence both the risk of aflatoxin contamination and the efficacy of the applied biocontrol strain. Spore densities of *Aspergillus* spp. are expected to peak in the summer due to favorable conditions for sporulation and dispersal but the movement of *Aspergillus* spp. propagules to different heights within the tree canopy during various stages of pistachio crop development has not been studied in detail. The purpose of this study was to evaluate spatial and temporal population dynamics of *A. flavus*, including a commercially applied atoxigenic biocontrol strain (AF36), in a pistachio orchard in Bowie, AZ. Our specific objectives were to (i) develop a method for monitoring *A. flavus* strains and propagule densities in the canopy of tree nuts, (ii) examine changes in *A. flavus* propagule densities within the canopy of pistachio trees over time, and (iii) quantify *A. flavus* propagules and displacement by the applied biocontrol strain in the lower, middle, and upper canopy of pistachio trees over two growing seasons.

Sampling of Pistachio Orchards

Samples were collected in a commercial 'Kerman' pistachio orchard in Bowie, AZ from 2018 to 2019. One field was sampled in 2018 (microsprinkler irrigated) and the same field plus an additional field (drip-irrigated) separated by a 15-m-wide road were sampled in 2019 (Fig. 1). Both fields were irrigated at 72-h intervals. Sampling areas in each field were located 10 rows (approximately 70 m) from the field border and consisted of 5 rows with 7 trees/row. Each row represented a sampling replicate. Both fields were treated with a commercial atoxigenic *A. flavus* biological control product, AF36 Prevail (ACRPC, Phoenix, AZ). The biocontrol product was applied by the grower by ground broadcasting using an all-terrain vehicle at the labeled rate of 11.2 kg/ha. AF36 Prevail was applied on 2 July 2018 and 1 July 2019. Standard agronomic practices for pistachio orchards in the area were followed, and maintenance chemicals applied by the grower are listed in Supplementary Table S1.

To determine the vertical distribution of *A. flavus* within pistachio canopies over time, leaves were collected from seven trees within each of five replicate rows per field at 7- to 14-day intervals for two growing seasons. For the 2018 growing season, samples were collected from 12 June to 30 October and, for the 2019 season, samples were collected from 16 May to 6 November. One leaf (pinnate of three to five leaflets) was collected from each of three sampling heights (3, 4, and 5 m from ground level) within a tree canopy. In each sampled field, leaves from the seven trees within a row that were collected from the same height were combined into one composite sample, creating five replicates per height for each sampling date. Leaf samples were collected into cotton sampling bags and immediately transported to the lab.

Pistachio Leaf Processing and Fungal Characterizations

Leaf samples were placed into a drying oven at 42°C for 24 h. Dried leaf samples were homogenized using a Retsch GM 200

blender (Retsch, Germany) at 10,000 rpm for 20 s. For each sample, ground leaf tissue (0.1 g from an approximate 10 g of total sample weight) was sprinkled evenly on three modified Rose Bengal agar plates (Cotty 1994b). After incubation for 5 days at 31°C, *Aspergillus* colonies were identified by using both macroscopic and microscopic characteristics (Klich and Pitt 1988; Probst et al. 2007), and colonies were counted. Colony counts were averaged for the three plates and converted to CFU per gram of leaf. No more than 5 isolates/plate (15 isolates/sample) were transferred to 5-2 agar (5% V8 juice and 2% Bacto-agar, pH 6.0) and incubated at 31°C for 3 days, and three plugs of each isolate were transferred into a 4-ml water vial containing 3.5 ml of sterile water for storage.

Vegetative compatibility analysis was conducted to determine the proportion of the *A. flavus* population that belonged to the same vegetative compatibility group (VCG) as the active ingredient in the biocontrol product AF36 Prevail (*A. flavus* strain AF36, which belongs to VCG YV36). Briefly, nitrate nonutilizing (*nit*) mutants were generated by seeding spore suspension onto SEL agar (Czapek-Dox broth, 25 g of $KClO_3$, 50 mg of rose Bengal, and 2% Bacto-agar; pH 7.0) and incubating plates at 31°C. Mutants were stabilized on MIT agar (Czapek-Dox broth, 15 g of $KClO_3$, and 2% Bacto-agar per liter; pH 6.5). Complementation tests were conducted on starch agar (3 g of $NaNO_3$, 1 g of K_2HPO, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 36 g of glucose, 20 g of starch [Difco], and 13 g of agar per liter; pH 6) using previously developed *nit* mutant tester pairs of YV36, isolates ATCC 96045 and ATCC 96047 (Cotty 1994a). Formation of prototrophic growth in the zone of hyphal interaction between a *nit* mutant of an unknown isolate and either of the YV36 testers indicated membership in VCG YV36. The proportion of AF36 was calculated by dividing the number of isolates that complemented with YV36 testers by the total number of *A. flavus* colonies tested. The CFU of AF36 were calculated by multiplying the proportion of AF36 by total *A. flavus* CFU. The non-AF36 *A. flavus* CFU were calculated by subtracting AF36 CFU from total *A. flavus* CFU.

To estimate leaf weight and surface area, additional leaf samples (n = 74) were collected in 2018 from August to October. Leaf samples were randomly collected from a single tree in each row. Three leaf samples were collected per tree at 3, 4, and 5 m from the ground. The samples were pressed and left to dry for 5 days in a standard plant press. The leaves were weighed on a balance and photographed together with a reference 1 cm scale using an iPhone 6 camera. Leaf surface area was calculated from the leaf images using ImageJ 1.53e (United States National Institutes of Health) calibrated with the photographed reference scale (Rasband 2012). Data for CFU per gram were converted to CFU per leaf using the formula CFU/leaf = (CFU/g) × (average leaf weight) and to CFU/leaf area using the formula CFU/leaf area = [CFU/(g) × (average g/leaf)]/(average cm^2/leaf).

Weather Data

Weather data for Bowie, AZ during the sampling seasons were obtained from the Arizona Meteorological Network (https://cals.arizona.edu/azmet/33.htm). The weather station was located at 32.289° N, −109.472° W, approximately 500 m from the study site. Total daily rainfall was recorded for the site. Temperature data were logged every 10 s and averaged to obtain daily maximum, minimum, and mean temperatures.

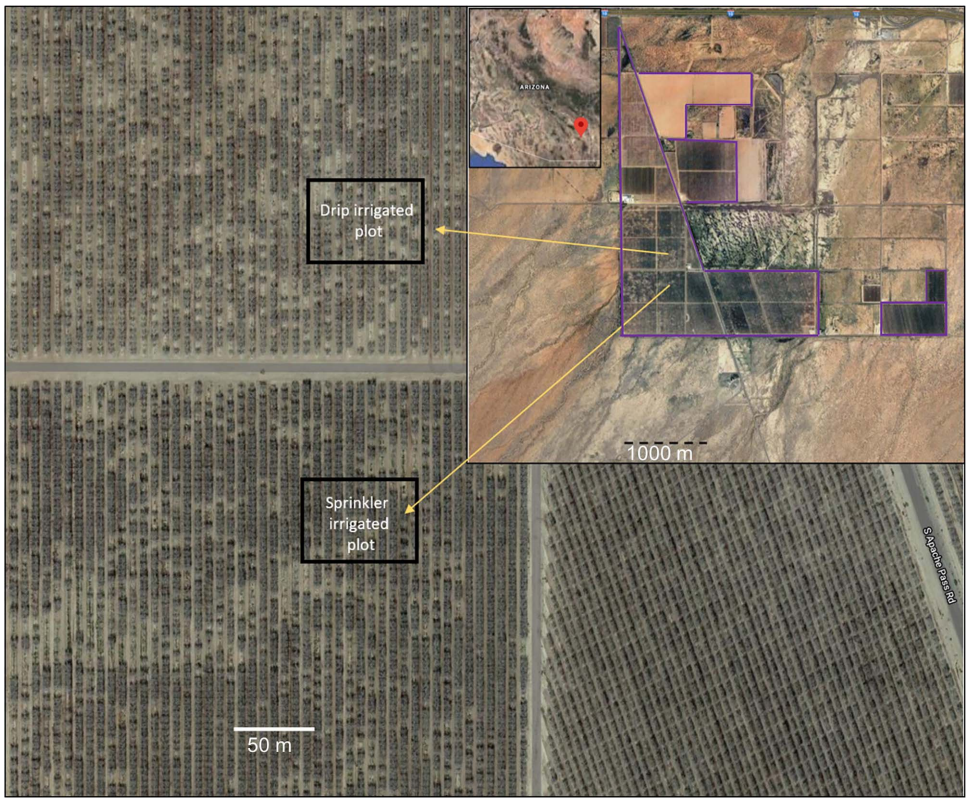

FIGURE 1

Location and size of the two study plots in Bowie, AZ. In 2018, only the plot in the sprinkler-irrigated field was sampled but, in 2019, plots in both the sprinkler- and drip-irrigated field were sampled. Pistachio orchards are highlighted in purple, while all other surrounding fields are uncultivated.

Data Analysis

Data were analyzed using JMP 11.1.1 (SAS Institute, Cary, NC, U.S.A.). CFU data (July to September) were log transformed to normalize variance prior to analysis. Analysis of variance was used to test effects of sampling date, sampling height, and the interaction between the two factors for each site on overall *A. flavus*, AF36, and non-AF36 *A. flavus* population densities. Each site-year was analyzed separately because there were different numbers of sites for each year (one site in 2018 and two sites in 2019). Tukey's honestly significant difference (HSD) was used to compare propagule density means among the three sampling heights. Percent AF36 data were averaged for each month from July to October and arcsine square-root transformed before analysis to normalize variance across sampling days. The influence of sampling height and sampling month on percent AF36 was analyzed using a factorial analysis of variance with the factors of sampling month, height, and interaction between sampling month and height. Means were separated using Tukey's HSD. Significant differences are reported at $\alpha = 0.05$.

Method for Monitoring *Aspergillus* Propagule Densities in the Tree Canopy

Exposure of pistachio nuts to *A. flavus* is an initial step necessary for infection and aflatoxin development. In this study, a nondestructive crop sampling method that utilized the collection of leaf samples from different heights in the canopy where nuts are formed was used. Based on *A. flavus* isolation from leaf tissues, propagule densities in the canopy to which the nuts are exposed throughout development were estimated. CFU per gram of leaf tissue are summarized in Table 1. Average leaf weight was 3.7 g and average leaf surface area (upper and lower area) was 213 cm^2 (n = 74). The average leaf weight and average leaf surface area were used to calculate population density per leaf and per leaf surface area. Estimated population density reached up to 600 CFU/g, equivalent to 10 CFU/cm^2 of leaf area or 2,130 CFU/leaf in the sprinkler-irrigated field in 2018; up to 23 CFU/g, equivalent to 0.4 CFU/cm^2 of leaf area or 84 CFU/leaf in the sprinkler-irrigated field in 2019; and up to 13 CFU/g, equivalent to 0.2 CFU/cm^2 of leaf area or 48 CFU/leaf in the drip-irrigated field in 2019. This approach provides an alternative method to direct sampling of nuts to track potential nut exposure to *A. flavus* inoculum. Direct characterization of fungal populations from nuts potentially gives a more representative assessment of nut exposure to *A. flavus*; however, this method is destructive to the nut crop if large quantities of samples are to be collected. In addition, recovering spores from nut surfaces may be challenging given that dislodging the spores requires either vigorous shaking in a nonionic surfactant solution or homogenization of the nuts. When the nuts are homogenized, *A. flavus* spores, which contain hydrophobins (Shait Mohammed et al. 2019), can be absorbed into the homogenized oils, making recovery on agar media difficult, whereas spores associated with the leaf may easily be dislodged and recovered. Air sampling is another method of tracking nut exposure but this method may be less representative of actual propagules landing in the canopy.

Relationship Between Environmental Variables and Population Densities of *Aspergillus flavus* in Pistachio Tree Canopies

During the two sampling seasons, *A. flavus* was the only aflatoxin-producing species isolated from pistachio canopies. No *A. parasiticus* was detected. Total *A. flavus* densities associated with pistachio leaves were greatest in 2018 (Table 1). Several factors, including agronomic practices, may have contributed to the differences in *A. flavus* population density between years; however, variable environmental conditions were likely the largest contributor (Fig. 2). Although the sampled fields were irrigated in both years and temperature patterns were similar, total rainfall was greater during the 2018 season (275 mm) compared with the 2019 season (92 mm) (Table 1). In addition, there were more precipitation events during the 2018 growing season (from 1 June to 30 September) (26 precipitation events) compared with the 2019 growing season (20 precipitation events) (Table 1; Fig. 2). Moisture from rainfall is an important factor supporting fungal growth and sporulation on soil surfaces outside the range reached by irrigation water (Giorni et al. 2012). In addition, the formulated biocontrol product AF36 Prevail requires hydration by high humidity, dew, irrigation water, rainfall, or other source for the biocontrol strain to grow and sporulate. Seasonal variation in *A. flavus* populations indicates that yearly variations in risk of *A. flavus* infection and aflatoxin contamination may be caused by local weather conditions such as rainfall or high humidity during susceptible stages of crop development (Battilani et al. 2016; Leggieri et al. 2020).

Aspergillus flavus Population Dynamics within Pistachio Tree Canopies over Time

Temporal changes in *A. flavus* population density in 2018 and 2019 in relation to different stages of crop development are summarized in Figure 3. In 2018, *A. flavus* population density in the sprinkler-irrigated field was influenced by the interaction between sampling date and height (P = 0.0005) (Fig. 3, left panel). For example, *A. flavus* population density was highest in the upper and middle canopies during the month of July; however, *A. flavus* population density in the lower canopy was less in September. In contrast, 2019 *A. flavus* population density in the sprinkler-irrigated field was influenced by sampling date ($P < 0.0001$) but

TABLE 1
***Aspergillus flavus* population density in pistachio tree canopies and environmental conditions in a sprinkler-irrigated field and drip-irrigated field during the 2018 and 2019 growing seasons (June to September)[z]**

	A. flavus density (CFU/g)						Weather conditions				
	Sprinkler-irrigated			Drip-irrigated			Temperature (°C)			Rainfall	
Year	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Total (mm)	Events (*n*)
2018	0	600	93.5	–	–	–	12	41	26	275	26
2019	0	66.7	3.2	0	53.3	2.3	11	40	26	92	20

[z] *A. flavus* density was estimated by counting *Aspergillus* CFU per weight of pistachio leaf tissue sprinkled on modified Rose Bengal agar plates. Min = minimum, Max = maximum, and Avg = average. One field (sprinkler-irrigated) was sampled in 2018 and two fields (sprinkler and drip-irrigated) were sampled in 2019.

not by height ($P = 0.2282$) or the interaction between the two factors ($P = 0.4470$) (Fig. 3, middle panel). Regardless of height, *A. flavus* population rose steadily from mid-June, peaked by mid-July, and began to decline in mid-August. In the drip-irrigated field in 2019, *A. flavus* population density was influenced by sampling date ($P = 0.0003$) and height ($P = 0.0066$) but not by the interaction between the two factors ($P = 0.0898$) (Fig. 3, right panel). Similar to the pattern observed with the sprinkler-irrigated field, *A. flavus* population density rose steadily beginning in mid-June, peaked in July and August, and began to decline in mid-August. July and August are typically hot and humid due to monsoon rainfall (Fig. 2). The *A. flavus* peak period was also associated with a period of high temperatures and increased precipitation events, suggesting that favorable environmental conditions played a significant role in growth and dispersal of *A. flavus* spores. In another study, Bock et al. (2004) also reported increases in *A. flavus* CFU in the air associated with high-temperature conditions in cotton fields of southern Arizona. Temporal changes in population densities of AF36 and other *A. flavus* in the canopy followed a similar pattern (Fig. 3). Even though AF36 was applied to fields in this study, it also is endemic to the region (Cotty 1989) and, therefore, its response to the environment is expected to be similar to the non-AF36 resident population. AF36 detection prior to application (Fig. 3) may reflect recovering overwintering inoculum from material colonized following application in previous years.

The initial increase in both AF36 and non-AF36 *A. flavus* propagules in the pistachio tree canopies coincided with the period when pistachio nut shells began to harden, and populations peaked when the crop was most susceptible to *A. flavus* infection due to shell splitting during nut maturation (Fig. 3). Because pistachio shells naturally split during nut development and maturation, the only layer of nut protection is the outer hull, which sometimes also splits, exposing the nut and allowing easy fungal entry (Doster and Michailides 1994b). Increases in *A. flavus* density suggest that risk of infection and aflatoxin contamination is high during this period and signifies a critical stage for aflatoxin management using biological control. During the same period of nut maturation, an increase in AF36 was observed across all fields following AF36 treatment in early July (Fig. 3). AF36 is applied to the soil surface when favorable environmental conditions such as moisture from irrigation and rainfall and high temperature favor reproduction and dispersal. However, because total *A. flavus* on the crop started to increase about 2 weeks before AF36 application (Fig. 3), it would be beneficial to shift the biocontrol product application date to late-May through early-June to increase displacement of aflatoxin-producing fungi during the phase of rapid

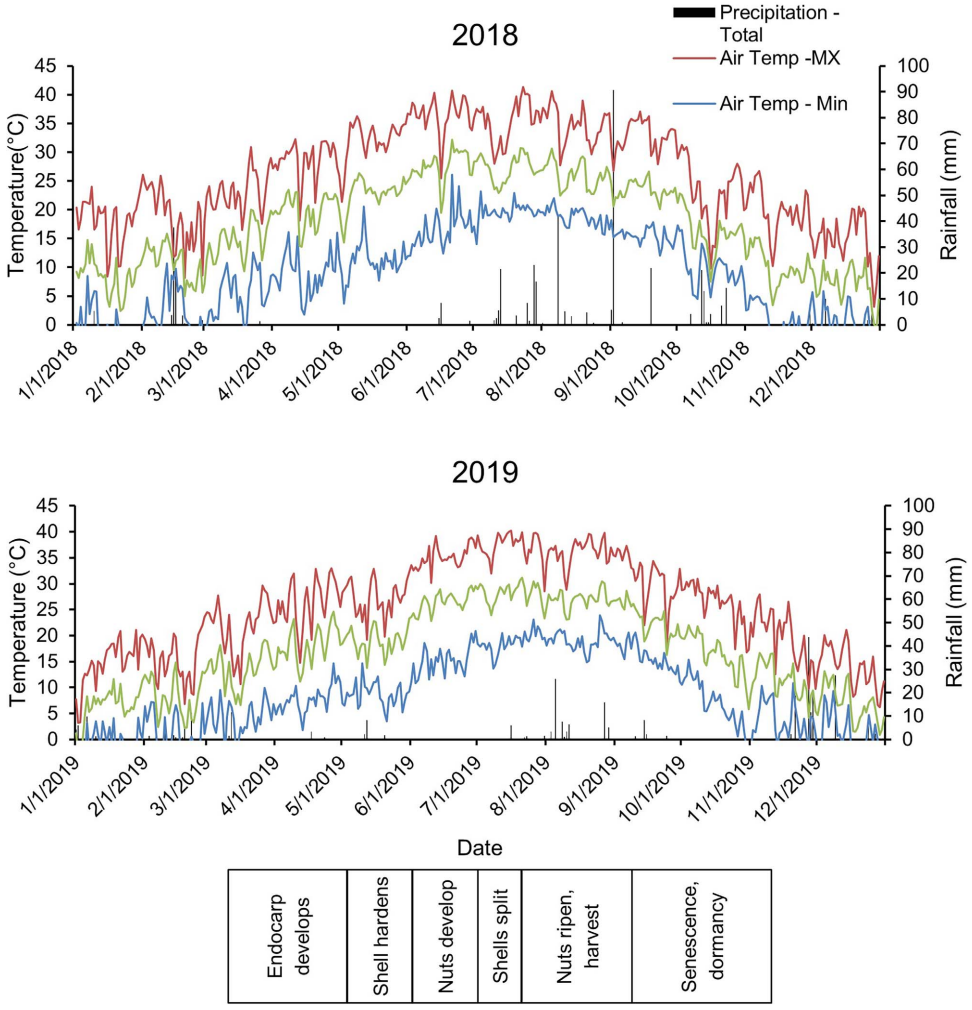

FIGURE 2

Weather data (temperature and rainfall) for Bowie, AZ in 2018 (top) and 2019 (bottom).

A. flavus reproduction and dispersal. According to the label, application of AF36 Prevail is recommended between late May and early July but data from the current study suggest late June and early July may be less ideal for the Bowie region. In both fields and years, AF36 and other *A. flavus* propagules in the canopy decreased starting from late August to late November, when trees go into senescence and dormancy. The decline in *A. flavus* population densities corresponded to declines in temperature between August and November (Fig. 2).

Proportions of the *A. flavus* Population Composed of the Biocontrol Strain AF36 in Pistachio Tree Canopies over Time

Because a goal of aflatoxin biocontrol is to displace aflatoxin-producing fungi on the crop with a nonaflatoxigenic biocontrol strain, proportions of the *A. flavus* population in the canopy comprising AF36 were calculated (Table 2). The proportion of AF36 detected in the canopy was influenced by the interaction between sampling month and height in all fields ($P < 0.001$). When

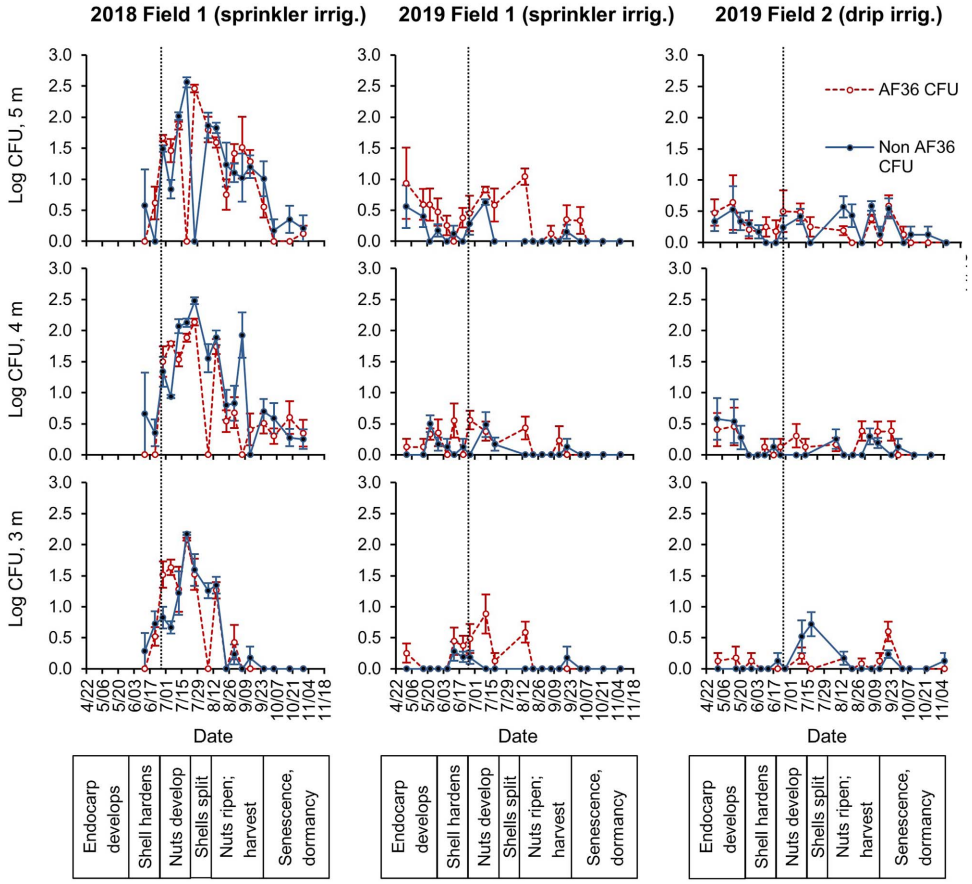

FIGURE 3

Distribution of *Aspergillus flavus* AF36 and non-AF36 CFU per gram in the upper (5 m), middle (4 m), and lower canopy (3 m) of pistachio trees in a sprinkler-irrigated field in 2018 and a sprinkler-irrigated and drip-irrigated field in 2019. Vertical dashed lines indicate AF36 Prevail application dates. Estimated crop stages were adapted from Mosz (2002).

TABLE 2
Average frequency of the aflatoxin biocontrol strain *Aspergillus flavus* AF36 in pistachio tree canopies during the growing season in a sprinkler-irrigated field in 2018 and a sprinkler-irrigated and drip-irrigated field in 2019

	AF36 (%) (SE)[z]				
Year, field	June	July	August	September	October
2018, sprinkler-irrigated	54 (6) a	53 (4) a	34 (3) b	41 (7) ab	39 (10) ab
2019, sprinkler-irrigated	80 (6) ab	74 (6) b	100 (0) a	61 (20) b	100 (0) ab
2019, drip-irrigated	75 (14) a	45 (11) ab	27 (8) b	62 (6) ab	57 (7) ab

[z] AF36 frequency was calculated by dividing the number of isolates that complimented with YV36 testers by the total number of *A. flavus* colonies tested ($n = 433$ for the sprinkler-irrigated field in 2018, $n = 127$ for the sprinkler-irrigated field in 2019, and $n = 106$ for the drip-irrigated field in 2019). SE = standard error. Means followed by the same letter among the months are not significantly different ($P > 0.05$) according to Tukey's honestly significant difference.

averaged across all canopy heights, AF36 comprised from 25 to 100% of the *A. flavus* population from June through October in both years (Table 2). Although biological control strains of *A. flavus* have been shown to be effective at reducing aflatoxin contamination even when their proportion in the *A. flavus* population was as little as 25 to 50% (Cotty 1990; Cotty and Bhatnagar 1994; Mauro et al.2015), a higher displacement (>50%) is desired to maximize aflatoxin reduction in the crops. In both years, average proportions of the *A. flavus* population consisting of AF36 were generally high (>50%) in the month of June prior to treatment and in July, the same month the fields were treated with the biocontrol product AF36 Prevail (Table 2). The high proportions of AF36 before treatment in June could be a result of overwintering inoculum. For instance, just prior to senescence of the leaves, a large proportion of the *A. flavus* population in the canopy was AF36, and leaf debris in the field will contribute to the next year's inoculum. Although the proportion of AF36 was high before treatment in June, total fungal density was relatively low before treatment, and it is not guaranteed that high AF36 proportions observed before treatment will be maintained during the growing season because the aflatoxigenic inoculum can move from nearby untreated sites. Therefore, a year-to-year application would be required to maintain the desired high proportion of the biocontrol during the growing season.

Vertical Distributions of *A. flavus* Propagules Within Pistachio Tree Canopies

A. flavus propagules, including both AF36 and other *A. flavus* strains, were recovered from the lower, middle, and upper canopy in both seasons (Fig. 4). Although distribution of *A. flavus* in the canopies fluctuated with sampling date for both seasons and fields (Fig. 3), *A. flavus* density was generally greater in the upper canopy than in the lower canopy (Fig. 4). For example, total numbers of *A. flavus* CFU were greater in the upper and middle canopy than in the lower canopy in the sprinkler-irrigated field in 2018 and in the drip-irrigated field in 2019. Similarly, in 2018, average AF36 CFU were more abundant in the upper canopy (50 CFU/g) compared with the lower canopy (20 CFU/g) (P = 0.0014). Numbers of non-AF36 CFU followed a similar trend and were significantly greater in the upper (51 CFU/g) and middle canopy (64 CFU/g) compared with the lower canopy (19 CFU/g) (P = 0.0005 and 0.0029, respectively). In addition, in the sprinkler-irrigated field in 2019, AF36 CFU were more abundant in the upper canopy (8 CFU/g) compared with the lower canopy (2 CFU/g) (P = 0.0333). Although the reason for this disparity in distribution is not known, dust storms originating from active and abandoned farmland and disturbed desert land are a common occurrence in Bowie (Cowherd et al. 1997; Field et al. 2010). Dust may have been deposited together with *A. flavus* spores on the top of the canopies, which would result in a gradient of greater to less concentrated spore densities from the upper to lower canopy. Overall distributions of AF36 in the canopies did not differ compared with other *A. flavus* propagules. AF36 is endemic to the region and appears to respond equally to environmental conditions in the region. However, endemic proportions of AF36 in *A. flavus* communities associated with pistachio production in California (Ortega-Beltran et al. 2019; Picot et al. 2018) and crop production in Arizona (Cotty 1989, 1994a) are far lower than the proportions of AF36 observed in canopy populations in the current study. The population densities observed in both seasons suggest that *A. flavus* inoculum readily moves throughout the lower, middle, and upper canopy, thus increasing the likelihood of nut exposure to inoculum throughout the tree canopy.

Conclusions and Implications

The leaf sampling technique developed in this study is a valuable tool that can be utilized to monitor densities of *A. flavus* and other fungi in canopies of tree crops and, when combined with techniques to specifically identify active ingredients, track movement of applied biocontrol fungi onto and within target nut crops. Even though the current study only included sampling from three site-years (one field in 2018 and two fields in 2019) in a small

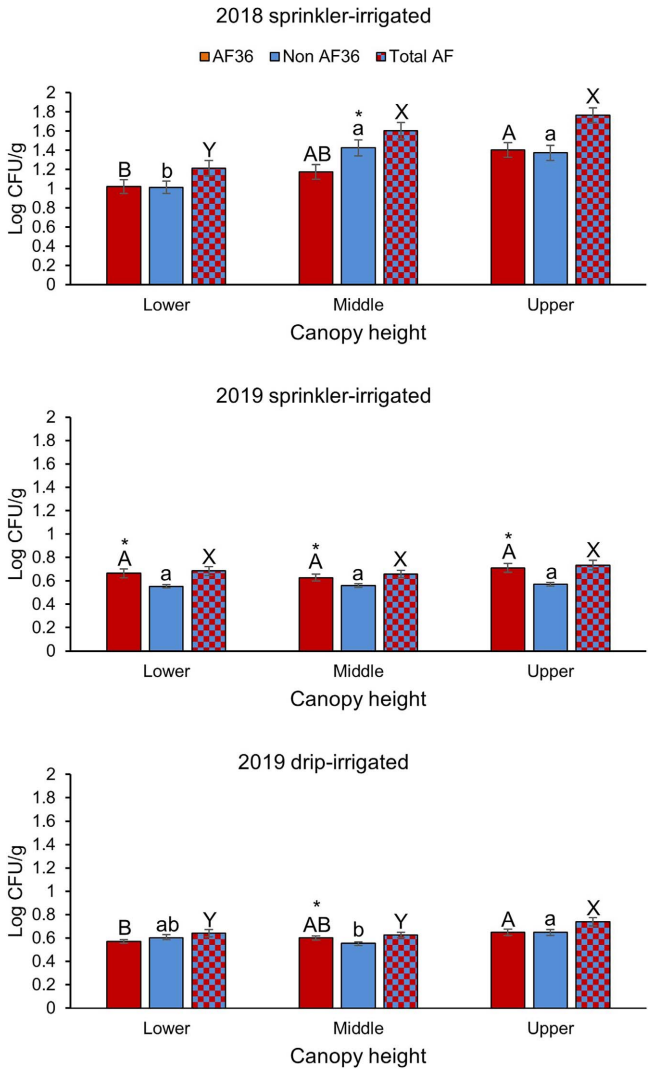

FIGURE 4

Overall distribution of AF36 (red bars), non-AF36 CFU (blue bars), and total *A. flavus* (red + blue bars) at different pistachio tree canopy heights during the growing season (June to September). Leaves were sampled at different heights in a sprinkler-irrigated field in 2018 (top) and 2019 (middle) and a drip-irrigated field in 2019 (bottom). For each site-year, bars with the same letter are not significantly different (P > 0.05) according to Tukey's honestly significant difference. Differences in quantities of AF36 among canopy heights are indicated with uppercase A and B; differences in quantities of non-AF36 propagules are indicated with lowercase a and b; and differences in total *A. flavus* are indicated with X ad Y. Asterisks denote significant difference between AF36 and non-AF36 CFU at the designated canopy height (*t* test, P < 0.05).

geographic area, results generated using the leaf sampling technique provide some insights into the population dynamics of *A. flavus*, including an applied atoxigenic biocontrol strain in pistachio orchards of eastern Arizona. Populations of *A. flavus* within pistachio tree canopies increased with favorable environmental conditions, including maximum temperatures above 30°C and rain events. Increased densities of *A. flavus* propagules in the tree canopy coincided with critical periods in crop development and nut formation. The results suggest that risks of aflatoxin contamination are high during warm, high-rainfall periods; therefore, it is recommended that the biological control product be applied so that the *A. flavus* biocontrol strain population density will also peak during this period. Thus, proper application timing of the biocontrol strain is critical to achieve optimal reductions in the risk of aflatoxin contamination. Because the population of *A. flavus* started to peak in mid-June, it is recommended that the application period of AF36 Prevail be shifted to late May through early June. The applied biocontrol strain and other *A. flavus* strains were recovered at all canopy heights. Although the formulated aflatoxin biocontrol product is applied on soil, this study demonstrates that ground application of AF36 Prevail results in effective displacement of aflatoxin producers by the biocontrol strain in all portions of the tree canopy where the nuts form. This indicates that aflatoxin biocontrol products already developed and registered for low-canopy crops such as maize, cotton, and peanut in other regions may also be useful for tree nuts susceptible to aflatoxin contamination. Although additional sampling over space and time is needed to verify the consistency of the trends observed in the current study and the pistachio industry in Arizona is relatively small compared with that in California, results from this study offer valuable insights on the movement of *A. flavus* propagules, including a ground-applied biocontrol strain, in commercial pistachio orchards throughout the growing season.

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Literature Cited

Alshannaq, A., and Yu, J. H. 2021. Analysis of EU Rapid Alert System (RASFF) notifications for aflatoxins in exported US food and feed products for 2010–2019. Toxins 13:90.

Antilla, L., and Cotty, P. J. 2004. Advances in utilization of atoxigenic strain technology to manage aflatoxin in commercial cotton. Mycopathologia 157:448.

Atehnkeng, J., Ojiambo, P. S., Ikotun, T., Sikora, R. A., Cotty, P. J., and Bandyopadhyay, R. 2008. Evaluation of atoxigenic isolates of *Aspergillus flavus* as potential biocontrol agents for aflatoxin in maize. Food Addit. Contam. 25:1264-1271.

Baazeem, A., Garcia-Cela, E., Medina, A., and Magan, N. 2021. Interacting abiotic factors affect growth and aflatoxin B1 production profiles of *Aspergillus flavus* strains on pistachio-based matrices and pistachio nuts. Front. Microbiol. 11. https://doi.org/10.3389/fmicb.2020.624007

Bandyopadhyay, R., Atehnkeng, J., Ortega-Beltran, A., Akande, A., Falade, T., and Cotty, P. J. 2019. "Ground-truthing" efficacy of biological control for aflatoxin mitigation in farmers' fields in Nigeria: From field trials to commercial usage, a 10-year study. Front. Microbiol. 10:2528.

Battilani, P., Toscano, P., Van der Fels-Klerx, H. J., Moretti, A., Leggieri, M. C., Brera, C., Rortais, A., Goumperis, T., and Robinson, T. 2016. Aflatoxin B 1 contamination in maize in Europe increases due to climate change. Sci. Rep. 6:24328.

Bock, C. H., Mackey, B., and Cotty, P. J. 2004. Population dynamics of *Aspergillus flavus* in the air of an intensively cultivated region of southwest Arizona. Plant Pathol. 53:422-433.

Bui-Klimke, T. R., Guclu, H., Kensler, T. W., Yuan, J. M., and Wu, F. 2014. Aflatoxin regulations and global pistachio trade: Insights from social network analysis. PLoS One 9:e92149.

Cotty, P. J. 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79:808-814.

Cotty, P. J. 1990. Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. Plant Dis. 74:233-235.

Cotty, P. J. 1994a. 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.

Cotty, P. J. 1994b. Comparison of four media for the isolation of *Aspergillus flavus* group fungi. Mycopathologia 125:157-162.

Cotty, P. J., Antilla, L., and Wakelyn, P. J. 2007. Competitive exclusion of aflatoxin producers: Farmer-driven research and development. Pages 241-253 in: Biological Control: A Global Perspective. C. Vincent, M. S. Goettel, and G. Lazarovits, eds. CABI, Wallingford, U.K.

Cotty, P. J., and Bayman, P. 1993. Competitive exclusion of a toxigenic strain of *Aspergillus flavus* by an atoxigenic strain. Phytopathology 83:1283-1287.

Cotty, P. J., and Bhatnagar, D. 1994. Variability among atoxigenic *Aspergillus flavus* strains in ability to prevent aflatoxin contamination and production of aflatoxin biosynthetic pathway enzymes. Appl. Environ. Microbiol. 60:2248-2251.

Cowherd, C., Jr., Grelinger, M. A., Blackburn, R., and Karimvand, R. 1997. Strategy Development for Dust Control and Prevention on I-10 Rep. No. FHWA-AZ97-448. Arizona Department of Transportation. https://apps.azdot.gov/files/ADOTLibrary/publications/project_reports/pdf/az448.pdf

Dorner, J. W., and Lamb, M. C. 2006. Development and commercial use of afla-Guard®, an aflatoxin biocontrol agent. Mycotoxin Res. 22:33-38.

Doster, M. A., Cotty, P. J., and Michailides, T. J. 2014. Evaluation of the atoxigenic *Aspergillus flavus* strain AF36 in pistachio orchards. Plant Dis. 98:948-956.

Doster, M. A., and Michailides, T. J. 1994a. Development of *Aspergillus* molds in litter from pistachio trees. Plant Dis. 78:393-397.

Doster, M. A., and Michailides, T. J. 1994b. *Aspergillus* molds and aflatoxins in pistachio nuts in California. Phytopathology 84:583-590.

European Commission. 2010. Commission regulation (EU) No 165/2010 of 26 February 2010 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. Off. J. Eur. Union 50:8-12.

Field, J. P., Belnap, J., Breshears, D. D., Neff, J. C., Okin, G. S., Whicker, J. J., Painter, T. H., Ravi, S., Reheis, M. C., and Reynolds, R. L. 2010. The ecology of dust. Front. Ecol. Environ. 8:423-430.

Food and Drug Administration. 2010. Guidance for industry: Action levels for poisonous or deleterious substances in human food and animal feed. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-action-levels-poisonous-or-deleterious-substances-human-food-and-animal-feed

Garcia-Lopez, M. T., Luo, Y., Ortega-Beltran, A., Jaime, R., Moral, J., and Michailides, T. J. 2021. Quantification of the aflatoxin biocontrol strain *Aspergillus flavus* AF36 in soil and in nuts and leaves of pistachio by real-time PCR. Plant Dis. 105:1657-1665.

Giorni, P., Leggieri, M. C., Magan, N., and Battilani, P. 2012. Comparison of temperature and moisture requirements for sporulation of *Aspergillus flavus* sclerotia on natural and artificial substrates. Fungal Biol. 116:637-642.

Horn, B. W., Greene, R. L., and Dorner, J. W. 2000. Inhibition of aflatoxin B1 production by *Aspergillus parasiticus* using nonaflatoxigenic strains: Role of vegetative compatibility. Biol. Control 17:147-154.

Horn, B. W., Greene, R. L., Sorensen, R. B., Blankenship, P. D., and Dorner, J. W. 2001. Conidial movement of nontoxigenic *Aspergillus flavus* and *A. parasiticus* in peanut fields following application to soil. Mycopathologia 151:81-92.

Jones, R. K. 1979. The epidemiology and management of aflatoxins and other mycotoxins. Pages 381-392 in: Plant Disease, An Advanced Treatise, Vol. IV: How Pathogens Induce Disease. J. G. Horsfall and E. B. Cowling, eds. Academic Press, New York, NY, U.S.A.

Kaminiaris, M. D., Camardo Leggieri, M., Tsitsigiannis, D. I., and Battilani, P. 2020. AFLA-pistachio: Development of a mechanistic model to predict the aflatoxin contamination of pistachio nuts. Toxin. 12:445.

Klich, M. A., and Pitt, J. I. 1988. Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. Trans. Br. Mycol. Soc. 91:99-108.

Leggieri, M. C., Lanubile, A., Dall'Asta, C., Pietri, A., and Battilani, P. 2020. The impact of seasonal weather variation on mycotoxins: Maize crop in 2014 in northern Italy as a case study. World Mycotoxin J. 13:25-36.

Mauro, A., Battilani, P., and Cotty, P. J. 2015. Atoxigenic *Aspergillus flavus* endemic to Italy for biocontrol of aflatoxins in maize. BioControl 60:125-134.

Mehl, H. L., Jaime, R., Callicott, K. A., Probst, C., Garber, N. P., Ortega-Beltran, A., Grubisha, L. C., and Cotty, P. J. 2012. *Aspergillus flavus* diversity on crops and in the environment can be exploited to reduce aflatoxin exposure and improve health. Ann. N. Y. Acad. Sci. 1273:7-17.

Mosz, N. 2002. Pistachio Timeline. https://ipmdata.ipmcenters.org/documents/timelines/CApistachio.pdf

Ortega-Beltran, A., Moral, J., Picot, A., Puckett, R. D., Cotty, P. J., and Michailides, T. J. 2019. Atoxigenic *Aspergillus flavus* isolates endemic to almond, fig, and pistachio orchards in California with potential to reduce aflatoxin contamination in these crops. Plant Dis. 103:905-912.

Picot, A., Doster, M., Islam, Md-S., Callicott, K., Ortega-Beltran, A., Cotty, P., and Michailides, T. 2018. Distribution and incidence of atoxigenic *Aspergillus flavus* VCG in tree crop orchards in California: A strategy for identifying potential antagonists, the example of almonds. Int. J. Food Microbiol. 265:55-64.

Probst, C., Njapau, H., and Cotty, P. J. 2007. Outbreak of an acute aflatoxicosis in Kenya in 2004: Identification of the causal agent. Appl. Environ. Microbiol. 73:2762-2764.

Rasband, W. S. 2012. ImageJ: Image processing and analysis in Java. Astrophysics Source Code Library, ascl-1206. https://ui.adsabs.harvard.edu/abs/2012ascl.soft06013R/abstract

RASFF. 2020. RASFF Portal. Rapid Alert Systems for Food and Feed. https://ec.europa.eu/food/safety/rasff-food-and-feed-safety-alerts/rasff-portal_en

Salmon, T., Crabb, A., and Marsh, R. 1986. Bird damage to pistachios. Calif. Agric. 40:5-8.

Shait Mohammed, M. R., Balamurugan, M., Amrathlal, R. S., Kannan, P., Jayapal, J. M., Namperumalsamy, V. P., Prajna, L., and Kuppamuthu, D. 2019. Identification of the proteoforms of surface localized Rod A of *Aspergillus flavus* and determination of the mechanism of proteoform generation. J. Proteomics 193:62-70.

Sommer, N. F., Buchanan, J. R., and Fortlage, R. J. 1976. Aflatoxin and sterigmatocystin contamination of pistachio nuts in orchards. Appl. Environ. Microbiol. 32:64-67.

Sommer, N. F., Buchanan, J. R., and Fortlage, R. J. 1986. Relation of early splitting and tattering of pistachio nuts. Phytopathology 76:692-694.

Thomson, S. V., and Mehdy, M. C. 1978. Occurrence of *Aspergillus flavus* in pistachio nuts prior to harvest. Phytopathology 68:1112-1114.

USDA-NASS. 2021. Quick Stats. United States Department of Agriculture–National Agricultural Statistics Service. https://quickstats.nass.usda.gov/