Plant Disease • 2021 • 105:1657-1665 • https://doi.org/10.1094/PDIS-05-20-1097-RE

Quantification of the Aflatoxin Biocontrol Strain *Aspergillus flavus* AF36 in Soil and in Nuts and Leaves of Pistachio by Real-Time PCR

M. Teresa Garcia-Lopez,[1,2] Yong Luo,[2] Alejandro Ortega-Beltran,[3] Ramon Jaime,[2] Juan Moral,[1,†] and Themis J. Michailides[2,†]

[1] Department of Agronomy (Maria de Maeztu Excellence Unit), University of Córdoba, Campus de Rabanales, 14071 Córdoba, Spain
[2] Department of Plant Pathology, University of California-Davis Kearney Agricultural Research and Extension Center, Parlier, CA 93648, U.S.A.
[3] International Institute of Tropical Agriculture, Ibadan, Nigeria

Abstract

The species *Aspergillus flavus* and *A. parasiticus* are commonly found in the soils of nut-growing areas in California. Several isolates can produce aflatoxins that occasionally contaminate nut kernels, conditioning their sale. Strain AF36 of *A. flavus*, which does not produce aflatoxins, is registered as a biocontrol agent for use in almond, pistachio, and fig crops in California. After application in orchards, AF36 displaces aflatoxin-producing *Aspergillus* spp. and thus reduces aflatoxin contamination. Vegetative compatibility assays (VCAs) have traditionally been used to track AF36 in soils and crops where it has been applied. However, VCAs are labor intensive and time consuming. Here, we developed a quantitative real-time PCR (qPCR) protocol to quantify proportions of AF36 accurately and efficiently in different substrates. Specific primers to target AF36 and toxigenic strains of *A. flavus* and *A. parasiticus* were designed based on the sequence of *aflC*, a gene essential for aflatoxin biosynthesis. Standard curves were generated to calculate proportions of AF36 based on threshold cycle values. Verification assays using pure DNA and conidial suspension mixtures demonstrated a significant relationship by regression analysis between known and qPCR-measured AF36 proportions in DNA (R^2 = 0.974; $P < 0.001$) and conidia mixtures (R^2 = 0.950; $P < 0.001$). Tests conducted by qPCR in pistachio leaves, nuts, and soil samples demonstrated the usefulness of the qPCR method to precisely quantify proportions of AF36 in diverse substrates, ensuring important time and cost savings. The outputs of this study will serve to design better aflatoxin management strategies for pistachio and other crops.

Keywords: aflatoxins, qPCR, atoxigenic strain, *Aspergillus flavus*, Aspergillus parasiticus

Aflatoxins are secondary metabolites produced by several *Aspergillus* species, and they are the most toxic and carcinogenic among the currently known mycotoxins (Yu et al. 2004). The four major aflatoxins (B_1, B_2, G_1, and G_2) negatively impact crop quality and safety. Because aflatoxins are harmful to humans and livestock, crops exceeding the stringent tolerance thresholds are banned from commercialization and consumption in nations that enforce aflatoxin regulations (Grace et al. 2015; Payne 1998).

In California, tree nut crops are economically important commodities destined for both domestic consumption and export, and their production continues to increase (California Department of Food and Agriculture 2018; Perez et al. 2017). Tree nut crops such as pistachio and almond are occasionally contaminated with aflatoxins (Doster and Michailides 1994; Palumbo et al. 2014). Aflatoxin contamination in pistachio and almond nuts sometimes exceeds the regulatory limits of 20 or 10 μg/kg for total aflatoxins imposed by the U.S. Food and Drug Administration and the European Food Safety Authority, respectively. The low tolerance for aflatoxins is a serious concern for Californian growers, and sometimes their nut lots are denied from entering lucrative markets. During the last 10 years, the Rapid Alert System for Food and Feed put in place by the European Union reported the border rejection of 86 and 92 loads of almond and pistachio, respectively, coming from the United States (Moral et al. 2020). Therefore, growers in California must implement aflatoxin mitigation strategies to prevent rejection of their crops.

Two fungal species, *A. flavus* and *A. parasiticus*, are the major producers of aflatoxins and both are common in nut-growing areas in California (Donner et al. 2015; Doster and Michailides 1994). *A. parasiticus* produces both B and G aflatoxins, whereas the population of *A. flavus* is composed of both toxigenic isolates, which produce B aflatoxins, and nontoxigenic (i.e., atoxigenic) isolates (Amaike and Keller 2011; Donner et al. 2015; Klich 2007). The populations of each of these species can be divided into vegetative compatibility groups (VCGs) (Bayman and Cotty 1993; Horn and Greene 1995). There are several *A. flavus* VCGs composed exclusively of atoxigenic members and those can be used as biocontrol agents to limit crop aflatoxin contamination in the field (Mehl et al. 2012; Ortega-Beltran et al. 2019).

After several years of research during the 1980s and 1990s, the U.S. Department of Agriculture Agricultural Research Service (USDA-ARS) registered the aflatoxin biocontrol product *A. flavus* AF36 with the U.S. Environmental Protection Agency as the first atoxigenic aflatoxin biocontrol product for use in cottonseed in Arizona (Cotty and Mellon 2006). The active ingredient of that biocontrol product is the strain AF36 (referred to hereafter as AF36). AF36 was originally isolated in Yuma Valley, Arizona (Cotty 1989). Subsequently, AF36 was registered for use in maize, pistachio, almond, and fig grown in different U.S. states (Cotty et al. 2007; Doster et al. 2014; Ortega-Beltran et al. 2019).

Competitive exclusion of toxigenic isolates of *Aspergillus* spp. is the main mechanism through which aflatoxin biocontrol agents decrease aflatoxin contamination in treated crops (Abbas et al. 2011; Cotty et al. 1994; Doster et al. 2014; Mehl and Cotty 2010). Hence, the quantification of AF36 before and after treatment is fundamental for understanding its ability to colonize the target ecosystem and to displace native *Aspergillus* toxigenic isolates. Routinely, tracking of the AF36 strain has been done using vegetative compatibility

†Corresponding authors: T. J. Michailides; tjmichailides@ucanr.edu, and J. Moral; juan.moral@uco.es

J. Moral and T. J. Michailides contributed equally to this work.

Funding: M. T. Garcia-Lopez was supported by the FICYT Foundation and the California Pistachio Research Board. J. Moral held a Marie Skłodowska-Curie Fellowship launched by the European Union (contract number 658579).

The author(s) declare no conflict of interest.

Accepted for publication 18 October 2020.

© 2021 The American Phytopathological Society

assays (VCAs) (Doster et al. 2014; Grubisha and Cotty 2015). However, VCA is resource intensive and time consuming. Pyrosequencing is a suitable tool to distinguish *A. flavus* genotypes, including AF36, within diverse matrices (Das et al. 2008; Mehl and Cotty 2010; Shenge et al. 2019) but the corresponding equipment is relatively expensive and not commonly used. Quantitative real-time PCR (qPCR) can distinguish various *Aspergillus* species (Luo et al. 2009; Sardiñas et al. 2011) and could be used to distinguish specific genotypes within mixtures of *Aspergillus* genotypes.

Members of VCG YV36, to which AF36 belongs, bear a single nucleotide polymorphism (SNP) that causes an early stop codon in the polyketide synthase (*aflC*) gene, resulting in the inability to produce aflatoxins (Ehrlich and Cotty 2004). Based on the genetic dissimilarity of AF36 (i.e., having a SNP in *aflC* not present in aflatoxin producers), Ortega-Beltran et al. (2016) honed a multiplex-PCR assay to target the SNP to differentiate AF36 from toxin producers. The assay incorporates an intentional mismatch to destabilize binding by an "erroneous" primer (Kwok et al. 1990) and allows amplification of the PCR product only by isolates containing the SNP. The development of a qPCR assay that builds on the multiplex assay mentioned above would be an asset to cope with the shortcomings of VCAs. Such a method would be valuable to distinguish between crop samples treated with AF36 and those that received no treatment and also to monitor frequencies of the biocontrol agent in any given environment to determine whether the application of the product is necessary.

In this study, we designed a qPCR protocol to quantify proportions of aflatoxin biocontrol strain AF36 within samples that contained toxigenic isolates of *A. flavus* and *A. parasiticus*. Results of this work demonstrate the potential of this specific qPCR assay to be used in a diverse source of substrates (soil, conidial suspensions, fruit, or leaf tissues), giving rise to the continuity of epidemiological and competition studies of AF36 in both the laboratory and field, since these studies can be conducted using any substrate in a time- and cost-effective manner. Shedding additional light on the biology, epidemiology, and ecology of the biocontrol strain will ultimately lead to the design of more efficient aflatoxin mitigation strategies.

Materials and Methods

Isolates. The AF36 strain was obtained from the USDA-ARS Aflatoxin Reduction in Crops Laboratory in Tucson, Arizona. The strains *A. flavus* 2A1L-11 and *A. parasiticus* 4C1P-11, native to California, were used as toxigenic strains (Ortega-Beltran et al. 2019). Both toxigenic strains are part of the fungal collection maintained at the University of California Kearney Agricultural Research and Extension Center (KARE).

DNA extraction from pure cultures. The three strains (2A1L-11, 4C1P-11, and AF36) were grown separately in potato dextrose broth liquid media (Difco Laboratories Inc., Detroit, MI) in Parafilm-sealed volumetric flasks at 25°C for 4 days. Under aseptic conditions, mycelia of each strain were washed with sterile water, air dried, harvested, and transferred into FastDNA tubes containing garnet matrix and a 0.25-inch ceramic sphere (as shipped). The FastDNA extraction kit (MP Biomedicals, Irvine, CA) was used for DNA extraction following the method described by Luo et al. (2009). The DNA extracted from each sample was diluted in 35 µl of nucleotide-free water and stored at −20°C for later use.

DNA extraction from conidia grown on plates and collected from leaves. Conidia of each strain were scraped and harvested from 5-day-old potato dextrose agar (PDA) cultures grown at 30°C to obtain conidial suspensions. In subsequent experiments, conidial suspensions were also obtained from the surface of pistachio leaves by washing them with sterile water. DNA extraction and dilution was conducted as described above.

DNA extraction from pistachio nuts. Pistachio nuts were intentionally chosen and picked manually from cultivar Kerman trees prior to harvest of a pistachio orchard at KARE. The nuts were split by kernel and hulls and cut into fine pieces using a sterile scalpel; 0.3 g was placed into a FastDNA extraction tube with 250 µl of protein precipitation solution and 900 µl of cell lysis/DNA solubilizing solution for vegetation (MP Biomedicals). Samples were ground twice with a homogenizer (MP Biomedicals) for 40 s. DNA extraction and dilution was conducted as described above.

DNA extraction from soil. Soil samples were collected from treated and nontreated orchards at KARE. Several well-distributed soil subsamples were taken from the first 2 cm of the surface layer to obtain 1 kg of soil. Soil clods were pulverized using a rubber hammer. Then soil was passed through a sieve (No. 20, ATSM E-11; disinfected with 10% bleach, rinsed, and dried between samples) and stored in clean paper bags at 22 to 30°C until use. To extract genomic DNA of *Aspergillus* spp., 500 mg of sieved soil samples was added into the Lysing Matrix E tube (MP Biomedicals). The DNA extraction was performed as described above and DNA of each sample was diluted in 40 µl of nucleotide-free water.

Specific primer design. We designed two pairs of primers to quantify the proportion of AF36 with respect to toxigenic strains of *A. flavus* and *A. parasiticus*. AF36 contains a naturally occurring mutation conferring atoxigenicity. The single-site mutation (G→A) is located at the 591 nucleotide in the *aflC* gene (Ehrlich and Cotty 2004). We used the primer-BLAST designing tool of NCBI. The *aflC* reference sequence for the first pair was that of AF36 and the second was that of toxigenic *A. flavus* strain NRRL3357. Those sequences are publicly available in NCBI and deposited in GenBank as accessions GCA_000006275.2 and GCA_012897275.1, respectively. The first pair of specific primers, SNP36 Sh2 (5′-GCCTATCGCTGTA CAAACTG-3′) and SNP36 Cb (3′-GCTGGGGATCCAGAACTCA-5′; the letter in bold indicates the *aflC* mutation site found in AF36), was used to target AF36 DNA. The SNP36 Cb primer, previously designed by Ortega-Beltran et al. (2016) to identify AF36 using a conventional PCR multiplex assay, incorporates an intentional mismatch at the 593 nucleotide position in *aflC* (fifth nucleotide of the primer from the 5′ end). The second nucleotide of the SNP36 Cb primer binds to the *aflC* SNP but not to DNA of strains that do not contain the SNP (Fig. 1). The combination of SNP36 Cb and the new primer SNP36 Sh2 amplifies a 137-bp amplicon.

Likewise, we designed a new primer pair comprising Fw-nomutB (5′-CTTGGTCTACCATTGTTTGG-3′), in which the first nucleotide from the 3′ end binds at the 591 nucleotide in *aflC* of isolates lacking the AF36 SNP, and Rv-nomut267 (5′-GGTAGGCGTCGTGTC TAG-3′). Isolates lacking the AF36 SNP amplify a 284-bp amplicon.

Conventional PCR. The PCR amplifications were performed in a 25-µl volume containing 5 µl of PCR Master Mix (Promega, Madison, WI), 0.8 µl of forward and reverse primers (4 µmol/liter each), 2 µl of template DNA (20 ng), and 16.4 µl of water. The following conditions were used: an initial preheat at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. Gradient PCR revealed 64°C as the optimal annealing temperature. PCR products were examined in 1.5% agarose gels in Tris-acetate-EDTA (TAE) buffer with a reference ladder.

Real-time qPCR. qPCR amplifications were performed with a CFX96 Touch instrument (Bio-Rad, Hercules, CA) using SYBR Green I fluorescent dye. Two amplifications for each sample (one for each primer pair) were conducted in a 25-µl volume containing 12.5 µl of Brilliant II SYBR Green QRT-PCR Master Mix (Stratagene Corp., La Jolla, CA), 2 µl of template DNA extracted from any source described above, 0.25 µl of both forward and reverse primers (4 µmol/liter each), and 10 µl of water. The conditions used

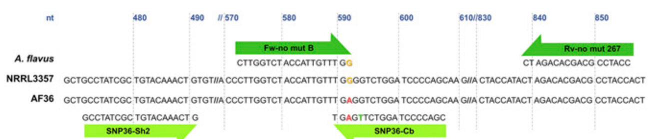

Fig. 1. Comparison of portions of the polyketide synthase gene (*aflC*) between highly toxigenic *Aspergillus flavus* strain NRRL3357 and biocontrol strain *A. flavus* AF36 showing the single-site mutation (G→A) at the 591 nucleotide that confers atoxigenicity to AF36 and the regions targeted by the two sets of primers used in this study.

were the same as described above. After the amplifications were completed, melting curves were obtained based on a standard protocol of the qPCR system (CFX Maestro Software) and used to confirm that the signal of the melting curve peak was from the expected target product. The qPCR products were also examined in 1.5% agarose gels in TAE buffer.

Development of standard curves for DNA and conidia number quantification (qPCR assay calibration). The primer pair, SNP36 Sh2/SNP36 Cb (for AF36) and Fw-nomutB/Rv-nomut267 (for 2A1L-11 and 4C1P-11), plus six 10× serial dilutions of DNA of each strain (from 14×10^5 pg to 14 pg) were utilized to generate the standard curves for quantitative detection of the three strains used in this study. The qPCR conditions described previously were used and the threshold cycle (Cq) values versus the corresponding log quantities (in picograms) of DNA from two replicates were employed to generate the corresponding standard curve for each strain. The standard curves gave rise to two equations used to calculate the DNA concentration of atoxigenic and toxigenic strains (standard curve generated for AF36 [SCAF36] and standard curve generated for toxigenic *A. parasiticus* and *A. flavus* [SCAFP] for 2A1L-11 and/or 4C1P-11). We compared samples containing unknown amounts of DNA to our standard curve to calculate DNA concentrations of each strain.

Similarly, a standard curve (SCAF36-conidia) was generated by plotting the Cq values obtained from qPCR assays conducted in duplicate with the primer pair SNP36 Sh2/SNP36 Cb versus the log number of conidia, which was obtained by extracting DNA from serial conidial dilutions of AF36 (from 2.4×10^6 to 24 conidia/ml). A second standard curve (SCAFP-conidia) was obtained by plotting the Cq values from the qPCR assay performed twice with the primer pair Fw-nomutB/Rv-nomut267 versus the log number of conidia, from the extraction of DNA of six serial conidial dilutions of 2A1L-11 and 4C1P-11 (from 3.4×10^6 to 34 conidia/ml). The equations defining these curves allowed estimation of the number of conidia per milliliter of each genotype present in a certain sample.

Determination of the proportion of AF36 in a sample. After qPCR amplification, two Cq values were obtained, one for each of the two primer pairs used in the study. By inserting the Cq value in the pertinent standard curve equation, the amount of DNA for the corresponding genotype present in the sample could be calculated, and the proportion of AF36 was obtained as follows (equation 1):

$$\text{AF36 }(\%) = \frac{\text{A}}{\text{A}+\text{B}} \times 100 \quad (1)$$

where A is the DNA quantity (in picograms) calculated from SCAF36 using the primers SNP36 Sh2/SNP36 Cb, and B is the DNA quantity (in picograms) calculated from SCAFP by using the primers Fw-nomutB/Rv-nomut267.

Quantification of the proportion of AF36 (qPCR assay verification). The designed qPCR was verified using (i) known mixtures of DNA from AF36 and 2A1L-11 or AF36 and 4C1P-11 and (ii) known mixtures of conidial suspensions from the same three strains.

DNA mixtures. Different DNA mixtures were prepared by using 14×10^3 pg of DNA of AF36 and 2A1L-11 or 4C1P-11 from pure cultures, comprising 1, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 99% (wt/wt) of AF36's DNA.

Conidial suspension mixtures. Different conidial mixtures of AF36 and 2A1L-11 or 4C1P-11 were prepared with a final density of 10^7 conidia/ml to further confirm the applicability of the qPCR assay. Nine mixtures with various proportions of AF36 were made of 10, 20, 30, 40, 50, 60, 70, 80, and 90% of AF36's conidia. Then 200 μl of each mixture was added into a 2-ml FastDNA tube, ground twice with a homogenizer (MP Biomedicals) for 40 s each time, and centrifuged at 14,000 rpm for 10 min, and the supernatant was discarded. Next, the DNA of each mixture was extracted as previously described for conidial suspensions.

The qPCR assay was conducted twice to obtain Cq values according to both pairs of primers (SNP36 Sh2/SNP36 Cb and Fw-nomutB/Rv-nomut267) for each DNA or conidial mixture. SCAF36 and SCAFP equations were used to calculate DNA (in picograms) with each primer pair for each combination of strains and equation 1 to determine the proportion of AF36 in the mixture. Linear regression through the origin between the known proportions of AF36 DNA in mixtures and those quantified with qPCR assays was calculated. In all linear regression analyses, the following were determined: significance of the regression, coefficient of determination (R^2), coefficient of determination adjusted for degrees of freedom (Ra^2), and the pattern of residuals. All data analysis was conducted using Statistix 10.0 Analytical Software (Tallahassee, FL).

Pistachio leaves. Leaves of pistachio cultivar Kerman were collected from a 15-year-old experimental pistachio plot located at KARE. The leaves were placed in plastic humid chambers (10 leaves/chamber). Subsequently, the leaves were inoculated using 15 ml of a conidial suspension (10^6 conidia/ml) combining AF36 and 2A1L-11 as 5:95, 50:50, and 95:5 (AF36 conidia/2A1L-11 conidia). Inoculated leaves were air-dried overnight at 18 to 22°C. Each group of 10 leaves was then placed into a plastic bag with 25 ml of 0.1% Tween 80. The plastic bags were vigorously shaken for 1 min; conidial suspensions were collected in 50-ml Falcon tubes and centrifuged for 10 min (2,300 rpm). Then 5 ml of the precipitated conidia was separated in four 1.25-ml vials, ground twice with a homogenizer (MP Biomedicals) for 40 s, and centrifuged for 5 min at 14,000 rpm. The supernatant was removed and 500 μl of cell lysis/DNA solubilizing solution for fungi was added to each vial; the vials were vortexed for 20 s. DNA extraction and dilution were conducted as described above. qPCR was conducted twice and the proportion of AF36 DNA in each mixture was calculated using equation 1 as previously described, based on the quantity of DNA of each strain according to SCAF36 and SCAFP.

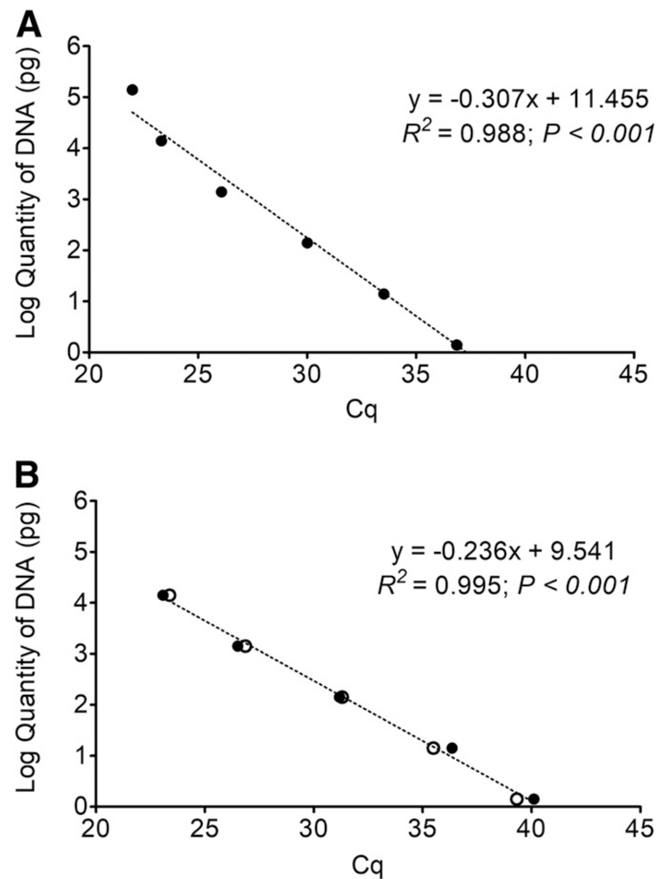

Fig. 2. Percentage of each genotype calculated by quantitative real-time PCR assay using natural soil samples infested with different mixtures of four fungal discs (mycelia and conidia) on potato dextrose agar of **A,** only nontoxigenic *Aspergillus flavus* AF36 (0:4) or **B,** AF36 combined with the toxigenic genotypes (*A. flavus*, 2A1L-11 or *A. parasiticus*, 4C1P-11) in different proportions.

Soil samples. Manually infested soil samples were also used to verify the qPCR assay. For this, 1 kg of soil from the 2-cm top surface was acquired by subsampling a non-AF36-treated almond plot located at KARE and sieved to be separated in seven 2-g samples as described above. Different combinations of 5-mm-diameter discs of PDA with 1-week-old growing colonies (mycelia and conidia) of AF36, 2A1L-11, and 4C1P-11 were properly mixed with 2-g soil samples. Then 500 mg of each soil sample was employed for DNA extraction according to the procedure indicated above. Similarly, qPCR was conducted twice for each sample and the proportion of AF36 DNA was calculated using equation 1 as described previously.

Application of the qPCR assay to determine the proportion of AF36 in nut and soil samples collected from pistachio fields. After verifying that the qPCR performed as expected, the equations from standard curves were also used to compare the proportion of AF36 versus *A. flavus* and *A. parasiticus* present in nuts and soil samples collected from fields.

Nut samples. Thirty early split pistachio nuts—atypical nuts with split hulls, in which the kernel is exposed to insect and mold invasions, including *Aspergillus* spp. (Doster and Michailides 1994)—were harvested from an AF36-treated plot (treated with *A. flavus* AF36 Prevail, the new formulation, applied at a rate 10 kg/ha) located at KARE. The early split nuts were divided into two groups of 15 nuts each: those with rough and shriveled hulls, showing a dark and stained suture (symptomatic) of *Aspergillus* spp. contamination, and those with smooth hulls and without the stained suture (asymptomatic) (Doster and Michailides 1994). In each group, DNA extraction was conducted as described for pistachio nuts, independently for kernels and hulls, and qPCR assays were performed to obtain the Cq values that allow calculation of DNA quantities of each genotype using the equations derived from the standard curves (SCAF36 and SCAFP). Based on the results, AF36 incidence (percent) and AF36 molecular severity (MS) were calculated according to equations 2 and 3:

$$\text{AF36 incidence } (\%) = \frac{\text{AF36 samples } (n)}{\text{N}} 100 \quad (2)$$

$$\text{AF36 molecular severity} = \text{Log}\frac{\text{AF36 DNA (pg)}}{\text{Plant weight (g)}} \quad (3)$$

where AF36 samples is the number of samples (n) with the presence of AF36 (i.e., samples in which a certain amount of AF36 DNA was detected using the qPCR assay, with respect to the total number of samples [N]; equation 2). In equation 3, AF36 DNA is the quantity of DNA according to SCAF36, whereas plant weight (in grams) corresponds to the amount of tissue used for the DNA extraction. In this latter equation, if AF36 DNA < 5 pg, we considered AF36 MS equal to 0.

Soil samples. Nine 10-g soil samples were taken from the first 2 cm of the surface layer in the same AF36-treated almond plot at KARE. For comparisons, nine additional samples from AF36 non-treated fields contiguous to the almond plot were collected adopting the same criteria. Samples were dried in paper bags at room temperature (21 to 25°C) for 1 week and then sieved; DNA was extracted as described for soil samples. The qPCR assay was conducted three times. From the results obtained, statistical analyses were conducted to compare AF36 incidence (percent), as indicated in equation 2. Since the presence of *A. flavus* and *A. parasiticus* in soil samples without plant debris taken from the upper centimeter of the first soil layer was mainly composed of scattered conidia (propagules) into the soil matrix (Abbas et al. 2009; Horn 2003; Luo et al. 2009), we also calculated the conidial density (percent) of each plot as follows:

$$\text{Conidial density} = \text{Log}\frac{\text{Conidia } (n)}{\text{Soil weight (g)}} \quad (4)$$

Conidia (n) were calculated based on equations, SCAF36-conidia and SCAFP-conidia resulted from the qPCR calibration test performed using conidial suspensions of AF36 and 2A1L-11 or 4C1P-11 used for Cq values obtained by conducting the qPCR assay as described previously.

In both the nut and soil assays, the different treatments were compared according to the Kruskal-Wallis test ($\alpha = 0.05$). qPCR assays for both nuts and soil samples were performed three times.

Evaluation of the competition between AF36 and 2A1L-11 in culture media. Five conidial suspensions (10^8 conidia/ml) were prepared by combining AF36 and 2A1L-11 conidia at proportions of 95:5, 80:20, 50:50, 20:80, and 5:95 (AF36/2A1L-11). Subsequently, 100 µl of each conidial suspension was transferred to 9-cm Petri dishes with PDA and incubated at 30°C to begin the first generation of competition between both strains. After 3 days of incubation, 1 ml of sterile 0.1% Tween 80 was added to each plate with the *Aspergillus* colonies, and the conidia were scraped with a sterile plastic rod. From this new conidial suspension, 500 µl was used for DNA extraction, and the proportion of AF36 in each sample was calculated as described above. The remaining 500 µl from the conidial suspension of each treatment was adjusted to 10^8 conidia/ml using a hemocytometer, and 100 µl was transferred to a new PDA plate, which was incubated again as described above to be considered as the second conidia generation. This process (culture-conidia and wash-culture) was repeated six times to obtain six conidia generations. The dynamic in the proportions of AF36 over generations was used to study the competitive ability of AF36 and the toxigenic strain over several generations; this may provide clues to AF36 behavior in nature after being released in the field. For each generation and treatment, the mean and standard deviation were calculated using the Summary Statistic of Statistix 10.

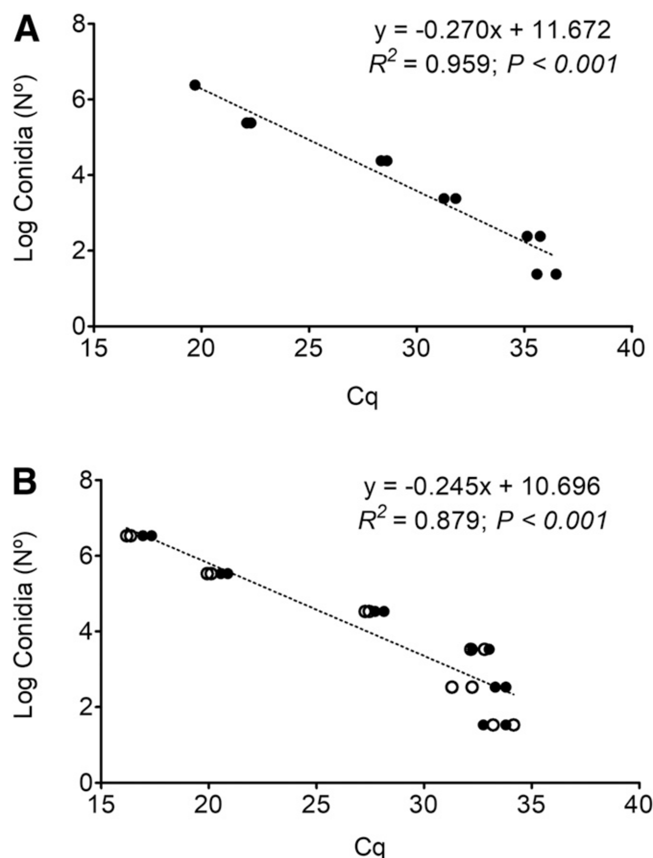

Fig. 3. Standard curves from quantitative real-time PCR (qPCR) by plotting the threshold cycle (Cq) versus the log conidia (N°) using DNA extracted from serial conidial suspensions of *Aspergillus flavus*, AF36 strain, by using **A,** SNP36 Sh2/SNP36 Cb primers and *A. flavus* (2A1L-11, white dots) and *A. parasiticus* (4C1P-11, black dots) by using **B,** Fw-no mut B/Rv-no mut-267 primers.

Results

Primer specificity test. Primer pair SNP36 Sh2/SNP36 Cb successfully distinguished AF36 from the toxigenic *A. flavus* and *A. parasiticus* used in the qPCR assay. No amplification from AF36 was obtained using primer pair Fw-nomutB/Rv-nomut267, while this pair was amplified by both *A. flavus* and *A. parasiticus* toxigenic strains.

Development of standard curves for DNA and conidia number quantification (qPCR calibration). Figure 2 shows the standard curve generated with primer pair SNP36 Sh2/SNP36 Cb by using six serial dilutions of AF36 DNA, from pure culture. Figure 2B shows the standard curve generated with primer pair Fw-nomutB/Rv-nomut267 by using five serial DNA dilutions of 2A1L-11 and 4C1P-11 DNA.

The standard curves for conidia quantification, shown in Figure 3A, were generated with primer pair SNP36 Sh2/SNP36 Cb by using DNA extracted from five serial conidia dilutions of AF36 (SCAF36-conidia) as y = −0.270x + 11.672, where y is the log of the number of conidia (conidia/milliliter) of AF36 and x is the Cq value from qPCR (R2 = 0.959; P < 0.001). Figure 3B shows the curve for primer pair FwnomutB/Rv-nomut267 by using DNA extracted from four serial dilutions of 2A1L-11 and 4C1P-11 as y = −0.245x + 10.696, where y is the log of the number of conidia (conidia/milliliter) of the toxigenic strain and x is the Cq value from qPCR (R2 = 0.879; P < 0.001).

Quantification of the proportion of AF36 (qPCR assay verification). *DNA mixtures.* A highly significant (R^2 = 0.974; P < 0.001) regression was obtained between the percentage of DNA of AF36 existent in 11 known pure DNA mixtures and those quantified with the qPCR assay (Fig. 4A). Regression was forced through the origin to increase the meaning of the relationship between both variables. The adjusted equation was $y = 1.163x$, where x is the percentage of DNA of AF36 present in the DNA mixture and y is the same value calculated by using the qPCR assay.

Conidial suspension mixtures. Similarly, nine known proportions of conidial suspensions as mixtures of AF36 with toxigenic strains were confronted with those calculated using the qPCR assay (Fig. 4B). The linear regression was again forced through the origin; the relation among variables $y = 1.016x$ was highly significant (R^2 = 0.950; P < 0.001), where x is the percentage of conidia of AF36 present in the conidial mixture and y is the same value calculated by using the qPCR assay.

Pistachio leaves. When we inoculated pistachio leaves using different combinations of conidia of AF36 and other *Aspergillus* strains, a highly significant (R^2 = 0.924; P < 0.001) linear regression (y = $1.061x$) was obtained between the inoculated AF36 conidia proportions, x, and the AF36 proportions quantified from the qPCR assay, y.

Soil samples. In the inoculated soil with various known mixtures of the *Aspergillus* genotypes, we detected the presence of 100% of each genotype when added individually to the soil sample and it was possible to identify distinctive proportions when AF36 was mixed in a large (3:1) or a small (1:3) fraction with any of the other genotypes. In the control treatments, the qPCR assay did not detect another *Aspergillus* strain other than those used to infest the soil samples (Fig. 5).

Application of qPCR to determine the proportion of AF36 on various sources of samples collected from fields. *Nut samples.* Application of the qPCR assay over early split pistachio nut samples collected from commercially treated pistachio plots resulted in similar AF36 incidence (percent; equation 2) and AF36 MS (equation 3) values among kernel and hulls tested, but dissimilar values (P = 0.021) for AF36 MS between symptomatic and asymptomatic hulls. No other *Aspergillus* genotype distinct from AF36 was found in the samples. The incidences of AF36 contaminating early split pistachio nuts ranged from 0 to 60% in both kernel and hull tissues, and the corresponding MS values of AF36 were in a log scale range from 0 to 1.7 pg/g (Fig. 6).

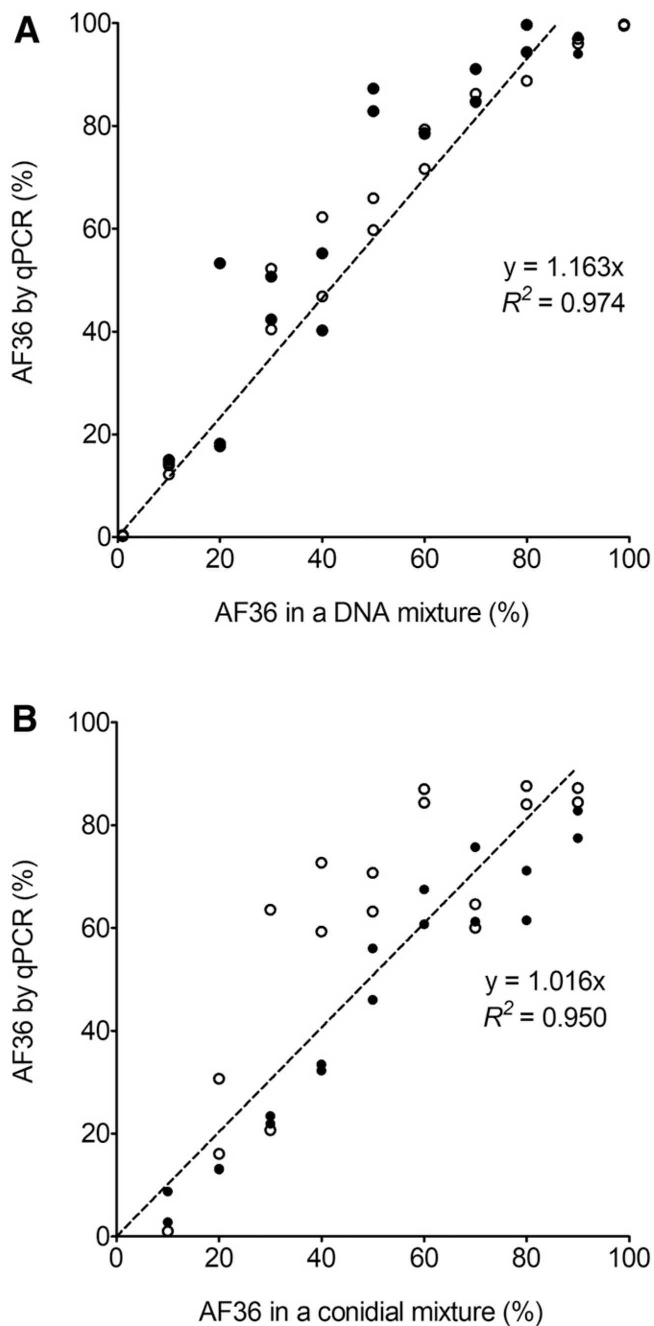

Fig. 4. Linear regression between the percentage of *Aspergillus flavus* AF36 in **A,** known mixtures of DNA and **B,** known conidial mixtures and the percentage of AF36 in the mixture calculated by quantitative real-time PCR (qPCR). White dots represent values of AF36 mixed with *A. flavus* 2A1L-11 and black dots represent values of AF36 mixed with *A. parasiticus* 4C1P-11.

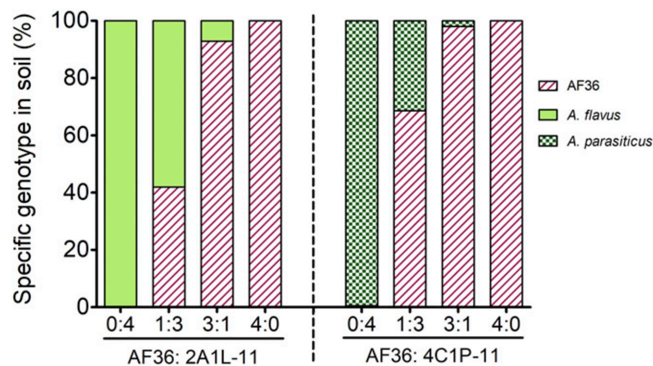

Fig. 5. Percentage of each genotype calculated by quantitative real-time PCR assay using natural soil samples infested with different mixtures of four fungal discs (mycelia and conidia) on potato dextrose agar of only nontoxigenic *Aspergillus flavus* AF36 (0:4) or AF36 combined with the toxigenic genotypes (*A. flavus*, 2A1L-11 or *A. parasiticus*, 4C1P-11) in different proportions.

Soil samples. In treated soils, AF36 had an incidence of 100% among the examined *Aspergillus* communities and that incidence was higher ($P = 0.007$) than that of AF36 in untreated soils (62%; Fig. 7). In addition, the conidial density (equation 4) in the soil of the treated plot (2,598 conidia/g) was significantly higher ($P = 0.021$) than in the untreated plot (39 conidia/g) (Fig. 7).

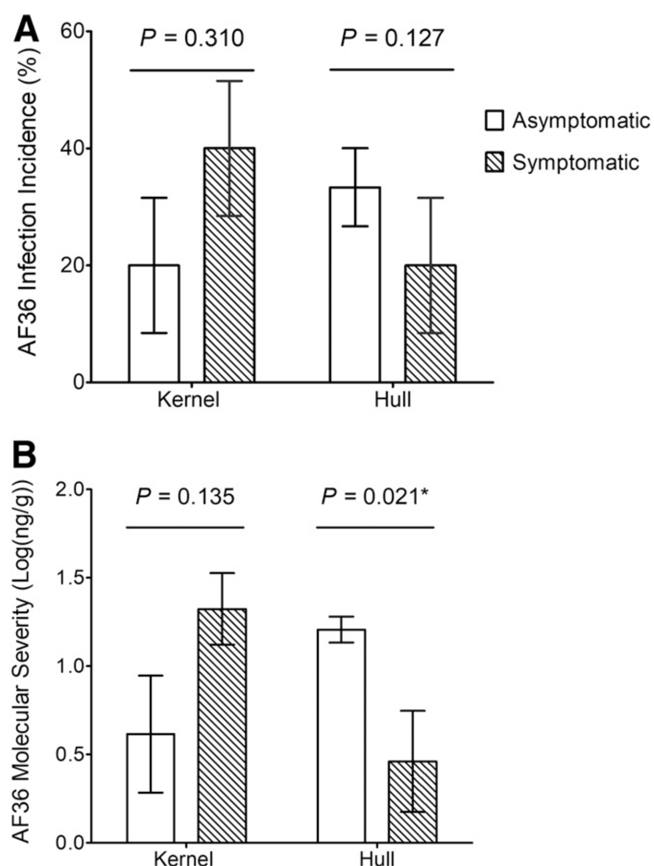

Fig. 6. Infection incidence (in percentages) and log molecular severity (in picograms/gram) of the *Aspergillus flavus* AF36 strain quantified in early split nuts collected from a commercially AF36-treated pistachio plot. Kernels and hulls were tested independently. $*P < 0.05$, significant differences between asymptomatic (nuts with smooth hulls and without the stained suture) or symptomatic (nuts with rough and shriveled hulls, showing a dark and stained suture) kernels or hulls according to the Kruskal-Wallis test.

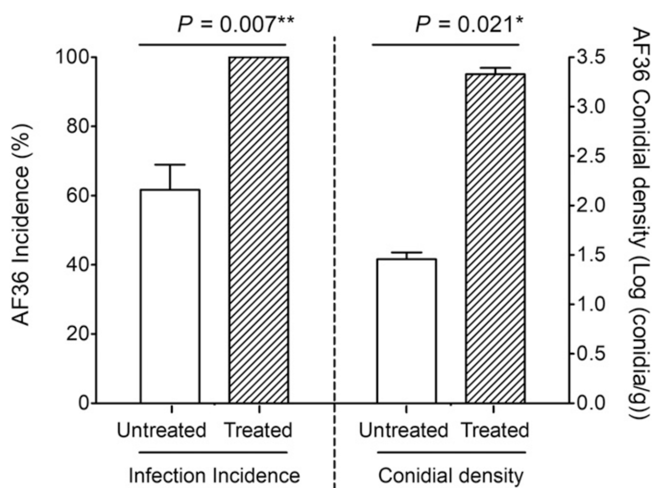

Fig. 7. Left, *Aspergillus flavus* atoxigenic AF36 strain incidence (in percentages) and right, conidial density (log (conidia/gram) quantified in soil samples collected from AF36 commercially treated and contiguous untreated pistachio fields. $**P < 0.01$ and $*P < 0.05$, significant differences between AF36-treated and untreated soils according to the Kruskal-Wallis test.

Evaluation of the competition between AF36 and 2A1L-11 in culture media. The results of the competition experiments demonstrated that after six generations, the proportion of AF36 remained relatively stable when the starting point was at high concentration (close to 100%) or in a low concentration at approximately 5% in the mixture with 2A1L-11. However, from 80% at the beginning, the percentage of AF36 varies around 60 to 80%, with a final decline to 20% at the sixth generation. Conversely, from 50 and 20% as the starting point, the population of AF36 ended up with an increase of 20% (Fig. 8).

Discussion

Crops such as almond or pistachio are occasionally contaminated with aflatoxins and this can cause severe economic loss (Bayman et al. 2002; Campbell et al. 2003; Doster 1996; Doster and Michailides 1994; Ortega-Beltran et al. 2019; Palumbo et al. 2014). Overall, an increase in aflatoxin contamination has occurred in the last decade in temperate zones driven by the changing patterns in climate (Battilani et al. 2016; Cotty and Jaime 2007; Medina et al. 2017). These events have bolstered the research and delivery work to mitigate the increment of aflatoxin contamination in regions where susceptible crops have great economic value or are staple foods, such as in many African nations (Bandyopadhyay et al. 2016). Diverse control strategies have extensively been sought to prevent and reduce aflatoxin contamination in crops; however, biocontrol of aflatoxins using endemic atoxigenic strains has become a useful tool in several parts of the world, since it is efficient in preventing toxin accumulation and safeguards the quality of food and feed before and after harvest (Bandyopadhyay et al. 2016; Camiletti et al. 2017, 2018; Mauro et al. 2015; Moral et al. 2020; Ortega-Beltran et al. 2019; Savić et al. 2020). The atoxigenic biocontrol technology was developed in the United States, where it continues to be used and AF36 is currently the most widely used biocontrol strain to reduce aflatoxin contamination (Moral et al. 2020).

Successful aflatoxin biocontrol is revealed by both aflatoxin reductions in treated crops and displacement of aflatoxin producers (Agbetiameh et al. 2019; Atehnkeng et al. 2014; Camiletti et al. 2018; Dorner 2009; Mauro et al. 2015; Weaver et al. 2015; Zhou et al. 2015). Understanding how the populations of nonaflatoxigenic strains change over time after being released into the environment allows for determination of whether treatment is effective (Abbas et al. 2009; Donner et al. 2015; Horn 2003; Jaime-Garcia and Cotty 2004; Mauro et al. 2013; Moore et al. 2017). It is also important to monitor how the atoxigenic strains survive and compete with populations of aflatoxin-producing species (Cotty et al. 2007; Cotty and Bayman

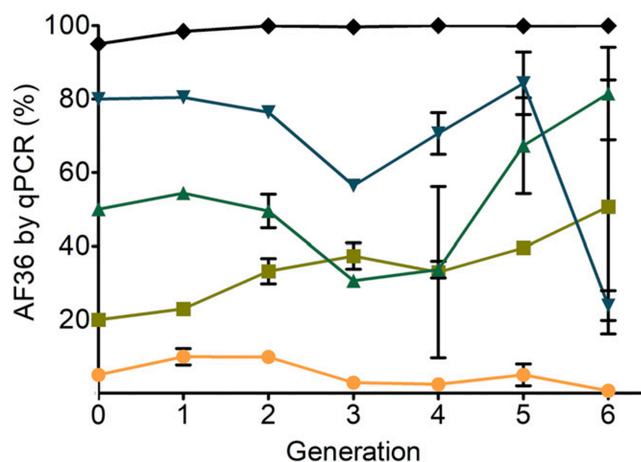

Fig. 8. Dynamic in the proportions of *Aspergillus flavus* atoxigenic AF36 strain quantified by the quantitative real-time PCR (qPCR) assay over six 3-day-long generations in culture media, starting from different initial proportions of AF36 in the mixture with the toxigenic *A. flavus* 2A1L-11 strain.

1993; Mehl and Cotty 2010). It is difficult to answer these questions by using traditional approaches based on fungal culturing methods, such as VCAs. However, VCAs are extensively used despite being a time-, resource-, and labor-intensive task (Atehnkeng et al. 2016; Bayman and Cotty 1991, 1993; Camiletti et al. 2018; Horn and Greene 1995; Ortega-Beltran and Cotty 2018; Ortega-Beltran et al. 2018; Probst et al. 2011). The tediousness of VCAs provided the necessary impetus to reconsider the way in which epidemiological studies of AF36 are conducted in tree nut orchards. Here we developed a qPCR assay to quantify the proportion of AF36 versus toxigenic genotypes of *A. flavus* and/or *A. parasiticus* from a diverse source of samples, including mycelia from pure cultures, conidial suspensions, soil, and plant tissues. The assay can be used to work with 48 samples at a time, obtaining quality results in <2 h for a reasonable cost (<$30 USD) (M. T. Garcia-Lopez and T. J. Michailides, *unpublished data*). In comparison, it can take months to conduct VCAs for the same number of samples.

Strain AF36 belongs to VCG YV36, which has spread from California to Georgia and is also endemic to México (Ehrlich and Cotty 2004; Ortega-Beltran et al. 2016). All YV36 members are atoxigenic because of a SNP in *aflC*, although members of the VCG contain additional degeneration in *aflC* and in other genes necessary for aflatoxin production. However, the *aflC* SNP can be found in atoxigenic isolates belonging to other VCGs (Ehrlich and Cotty 2004; Grubisha and Cotty 2015). Thus, the assay would also quantify other atoxigenic isolates carrying the SNP, in addition to YV36 members that are native to the area. YV36 is one of the most common atoxigenic VCGs across California tree nut orchards, with frequencies of up to 7% of the *Aspergillus* communities (Ortega-Beltran et al. 2019; Picot et al. 2018). However, when applied in orchards, the strain AF36 will most likely dominate the communities. Detecting the SNP in treated samples is a reasonable estimate of the presence of AF36. There are hundreds of VCGs in a single area and not all of them are atoxigenic; among those that are atoxigenic, not all of them contain the *aflC* SNP (Bayman and Cotty 1991; Ehrlich and Cotty 2004; Ortega-Beltran and Cotty 2018).

The proportion of AF36 in various sources was accurately quantified using established standard curves. Highly significant regressions between known and detected proportions of AF36 using the qPCR assay were obtained from various sources. This demonstrates that the qPCR assay can efficiently quantify AF36 proportions at the population level from different sources and distinguish AF36 from other strains of *A. flavus* or *A. parasiticus*. The standard curves for each primer pair were generated and the R^2 coefficients proved to be good indicators of robust and reproducible assays of this study. Because of the equal conditions of qPCR protocols for both primer pairs, the qPCR can be conducted at the same time with the two primer sets, reducing the time of analysis and allowing simultaneous calculation of proportions of AF36/*A. flavus* and/or *A. parasiticus*. Based on the standard curves, the normal range from 20 to 35 of the Cq values permits detection from 5 to 200,000 pg of AF36 DNA and from 19 to 66,000 pg of *A. flavus* and/or *A. parasiticus* DNA that might be present in a sample from pure culture DNA. This sensitivity is appropriate to accurately quantify a target genotype in a given sample. Likewise, our method allows quantification of the number of conidia of each strain in a sample with limits varying from 170 to 2×10^6 conidia of AF36 and from 250 to 1×10^6 of *A. flavus* and/or *A. parasiticus*. Higher accuracy occurred at densities >2,000 conidia/g in both cases, while lower accuracy occurred at densities <250 conidia/g. Use of larger field samples, bearing consequently higher target DNA content, can increase accuracy, as proposed by Luo et al. (2009) and Wang et al. (2006). Maintaining consistency of the methodology in sample processing and quantification is important to systematically guarantee the high accuracy of unknown sample quantification.

The discrimination power for quantification of AF36 versus *A. flavus* and/or *A. parasiticus* was also confirmed using samples containing mixtures of DNA at different proportions. Moreover, the accuracy of the method to quantify these fungi in artificially contaminated matrices was authenticated by an adequate regression coefficient when extracting DNA from washes of inoculated leaves. In addition, the results confirmed the applicability of the method in controlled studies using soil samples. Quantifying fungal levels in field matrices is essential to continue with epidemiological studies for understanding the biocontrol strain behavior in nature.

The methods to extract *Aspergillus* DNA from a diverse source of samples were successful to obtain the required DNA quality for qPCR. Extraction of fungal DNA directly from soil is more difficult compared with extractions from pure fungal cultures, plant tissues, and some restricted environments by using a commercial kit, especially when the density of the pathogen's propagules in soil is very low. Previous reports described protocols to quantify *A. flavus* and *A. parasiticus* (Carbone et al. 2007; Frisvad et al. 2005; Luo et al. 2009), which helped as references for the DNA extraction method from soil samples used in this study.

Using the designed primers and the DNA extraction method, a qPCR assay was generated to quantify the proportion of AF36 with respect to *A. flavus* and/or *A. parasiticus* contained in soil samples from AF36-treated versus untreated contiguous plots. As expected, treated fields were dominated by AF36. Since the untreated fields were adjacent, most likely the AF36 strain moved from the treated plot, although AF36 incidence and its conidial density were significantly lower in the untreated plots.

Tests were also conducted with early split nuts collected from a commercially AF36-treated plot, allowing the comparison of AF36 infection incidence and its MS in both kernels and hulls with fungal infection symptoms or not. Symptomatic early split kernels (i.e., showing a dark and stained suture) had higher AF36 MS in the kernel than in the hull. Conversely, the healthy-looking (asymptomatic) early split kernels had significantly higher AF36 MS in the hull, indicating that the colonization process was at an early stage. This research also involved a preliminary study on competition between AF36 and other strains, which demonstrated the potential applicability of the qPCR assay to track AF36 survival after being released into the environment as a biocontrol agent. Bayman and Cotty (1993) suggested that an atoxigenic strain was able to compete effectively at the same inoculum proportion (50:50) or with even less atoxigenic inoculum on cotton balls or in liquid fermentation systems. Similar findings were reported by Mauro et al. (2015) when examining atoxigenic strains native to Italy. They suggested that atoxigenic strains used two mechanisms of action: by exclusion of the toxigenic strain from the niche, and by competing for nutrients destined for aflatoxin biosynthesis. The mechanisms of AF36 in reducing aflatoxin contamination still need to be intensively studied. An efficient quantification method is needed to determine the ability of AF36 to compete with other individuals for infection sites and to elucidate how much strength AF36 could express to reduce the aflatoxin production process. When AF36 is released into the environment, many factors may affect its survival, growth, and reproduction (Michailides et al. 2018). It is important to quantify such effects so the faithfulness of biocontrol strains in the environment after release can be accurately modeled and predicted (Abdel-Hadi et al. 2012; Marín et al. 2012). Understanding the dynamics of AF36 under different scenarios is important to design the best strategies of application of a biocontrol agent to reduce the risk of aflatoxin contamination in nut crops (Ortega-Beltran et al. 2018). Determining how AF36 competes with native aflatoxigenic strains under various conditions could greatly help the decision-making process on better and more efficient use of the biocontrol agent. All of the above issues rely on an efficient and accurate method to rapidly process samples from various sources to obtain valuable information timely. Our established qPCR assay could be used to handle such difficult, time-sensitive tasks to accelerate aflatoxin management while improving food safety.

In addition, the formulation of AF36 needs to be improved to increase the efficiency of displacement, and the qPCR assay developed here can be useful for monitoring subsequent experiments without the need to conduct the laborious VCA. Another possibility is to monitor the residual effect of AF36 in treated orchards to determine whether partial or complete yearly or biyearly treatment is needed. This can considerably save costs to farmers and would provide more farmers the opportunity to access the product. Furthermore, it is expected that mixtures of atoxigenic strains will be used to

complement the efficacy of AF36. Assays to quantify the proportions of those strains need to be developed in the future.

Conclusion. A qPCR protocol to quantify proportions of AF36 accurately and efficiently was developed for use in diverse substrates (soil, conidial suspensions, fruit, or leaf tissues). This assay will serve to conduct epidemiological and competition studies of AF36 in both laboratory and field studies in a time- and cost-effective manner. Increased knowledge of the biology, epidemiology, and ecology of the biocontrol strain will ultimately lead to the design of more efficient aflatoxin mitigation strategies.

Acknowledgments

The authors thank all members of the Michailides Laboratory for their skillful technical assistance in the laboratory and field assays. This work is part of M. T. Garcia-Lopez's Ph.D. thesis and was supervised by Dr. J. Moral and Professor T. J. Michailides. M. T. Garcia-Lopez is conducting her Ph.D. between Kearney Agricultural Research and Extension Center in Parlier, California, and the Universidad de Córdoba in Spain.

Literature Cited

Abbas, H. K., Weaver, M. A., Horn, B. W., Carbone, I., Monacell, J. T., and Shier, W. T. 2011. Selection of *Aspergillus flavus* isolates for biological control of aflatoxins in corn. Toxin Rev. 30:59-70.

Abbas, H. K., Wilkinson, J. R., Zablotowicz, R. M., Accinelli, C., Abel, C. A., Bruns, H. A., and Weaver, M. A. 2009. Ecology of *Aspergillus flavus*, regulation of aflatoxin production, and management strategies to reduce aflatoxin contamination of corn. Toxin Rev. 28:142-153.

Abdel-Hadi, A., Schmidt-Heydt, M., Parra, R., Geisen, R., and Magan, N. 2012. A systems approach to model the relationship between aflatoxin gene cluster expression, environmental factors, growth and toxin production by *Aspergillus flavus*. J. R. Soc. Interface 9:757-767.

Agbetiameh, D., Ortega-Beltran, A., Awuah, R. T., Atehnkeng, J., Islam, M.-S., Callicott, K. A., Cotty, P. J., and Bandyopadhyay, R. 2019. Potential of atoxigenic *Aspergillus flavus* vegetative compatibility groups associated with maize and groundnut in Ghana as biocontrol agents for aflatoxin management. Front. Microbiol. 10:2069.

Amaike, S., and Keller, N. P. 2011. *Aspergillus flavus*. Annu. Rev. Phytopathol. 49:107-133.

Atehnkeng, J., Donner, M., Ojiambo, P. S., Ikotun, B., Augusto, J., Cotty, P. J., and Bandyopadhyay, R. 2016. Environmental distribution and genetic diversity of vegetative compatibility groups determine biocontrol strategies to mitigate aflatoxin contamination of maize by *Aspergillus flavus*. Microb. Biotechnol. 9:75-88.

Atehnkeng, J., Ojiambo, P. S., Cotty, P. J., and Bandyopadhyay, R. 2014. Field efficacy of a mixture of atoxigenic *Aspergillus flavus* Link: FR vegetative compatibility groups in preventing aflatoxin contamination in maize (*Zea mays* L.). Biol. Control 72:62-70.

Bandyopadhyay, R., Ortega-Beltran, A., Akande, A., Mutegi, C., Atehnkeng, J., Kaptoge, L., Senghor, A. L., Adhikari, B. N., and Cotty, P. J. 2016. Biological control of aflatoxins in Africa: Current status and potential challenges in the face of climate change. World Mycotoxin J. 9:771-789.

Battilani, P., Toscano, P., Van Der Fels-Klerx, H. J., Moretti, A., Camardo Leggieri, M., 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.

Bayman, P., Baker, J. L., and Mahoney, N. E. 2002. *Aspergillus* on tree nuts: Incidence and associations. Mycopathologia 155:161-169.

Bayman, P., and Cotty, P. J. 1991. Vegetative compatibility and genetic diversity in the *Aspergillus flavus* population of a single field. Can. J. Bot. 69:1707-1711.

Bayman, P., and Cotty, P. J. 1993. Genetic diversity in *Aspergillus flavus*: Association with aflatoxin production and morphology. Can. J. Bot. 71:23-31.

California Department of Food and Agriculture. 2018. California Agricultural Statistics Review 2017-2018. https://www.cdfa.ca.gov/statistics/PDFs/2017-18AgReport.pdf

Camiletti, B. X., Moral, J., Asensio, C. M., Torrico, A. K., and Enrique, I. 2018. Characterization of Argentinian endemic *Aspergillus flavus* isolates and their potential use as biocontrol agents for mycotoxins in maize. Phytopathology 108:818-828.

Camiletti, B. X., Torrico, A. K., Fernanda Maurino, M., Cristos, D., Magnoli, C., Lucini, E. I., and De la Paz Gimenez-Pecci, M. 2017. Fungal screening and aflatoxin production by *Aspergillus* section *Flavi* isolated from pre-harvest maize ears grown in two Argentine regions. Crop Prot. 92:41-48.

Campbell, B. C., Molyneux, R. J., and Schatzki, T. F. 2003. Current research on reducing pre- and post-harvest aflatoxin contamination of U.S. almond, pistachio, and walnut. J. Toxicol. Toxin Rev. 22:225-266.

Carbone, I., Jakobek, J. L., Ramirez-Prado, J. H., and Horn, B. W. 2007. Recombination, balancing selection and adaptive evolution in the aflatoxin gene cluster of A*spergillus parasiticus*. Mol. Ecol. 16:4401-4417.

Cotty, P., and Mellon, J. 2006. Ecology of aflatoxin producing fungi and biocontrol of aflatoxin contamination. Mycotox. Res. 22:110-117.

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

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. CABI, Wallingford, UK.

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., Bayman, P., Egel, D. S., and Elias, K. S. 1994. Agriculture, aflatoxins and *Aspergillus*. Pages 1-27 in: The genus *Aspergillus*. K. Powell, ed. Plenum Press, New York, NY.

Cotty, P. J., and Jaime, R. 2007. Influences of climate on aflatoxin producing fungi and aflatoxin contamination. Int. J. Food Microbiol. 119:109-115.

Das, M. K., Ehrlich, K. C., and Cotty, P. J. 2008. Use of pyrosequencing to quantify incidence of a specific *Aspergillus flavus* strain within complex fungal communities associated with commercial cotton crops. Phytopathology 98:282-288.

Donner, M., Lichtemberg, P. S. F., Doster, M., Picot, A., Cotty, P. J., Puckett, R. D., and Michailides, T. J. 2015. Community structure of *Aspergillus flavus* and *A. parasiticus* in major almond-producing areas of California, United States. Plant Dis. 99:1161-1169.

Dorner, J. W. 2009. Development of biocontrol technology to manage aflatoxin contamination in peanuts. Peanut Sci. 36:60-67.

Doster, M. A. 1996. Species and mycotoxins in figs from California orchards. Plant Dis. 80:484.

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. 1994. *Aspergillus* molds and aflatoxins in pistachio nuts in California. Phytopathology 84:583-590.

Ehrlich, K. C., and Cotty, P. J. 2004. An isolate of *Aspergillus flavus* used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase gene. Appl. Microbiol. Biotechnol. 65:473-478.

Frisvad, J. C., Skouboe, P., and Samson, R. A. 2005. Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B_1, sterigmatocystin and 3-O-methylsterigmatocystin, *Aspergillus rambellii* sp. nov. Syst. Appl. Microbiol. 28:442-453.

Grace, D., Mahuku, G., Hoffmann, V., Atherstone, C., Upadhyaya, H. D., and Bandyopadhyay, R. 2015. International agricultural research to reduce food risks: Case studies on aflatoxins. Food Secur. 7:569-582.

Grubisha, L. C., and Cotty, P. J. 2015. Genetic analysis of the *Aspergillus flavus* vegetative compatibility group to which a biological control agent that limits aflatoxin contamination in USA crops belongs. Appl. Environ. Microbiol. 81: 5889-5899.

Horn, B., and Greene, R. 1995. Vegetative compatibility within populations of *Aspergillus flavus*, *A. parasiticus*, and *A. tamarii* from a peanut field. Mycologia 87:324-332.

Horn, B. W. 2003. Ecology and population biology of aflatoxigenic fungi in soil. J. Toxicol. Toxin Rev. 22:351-379.

Jaime-Garcia, R., and Cotty, P. J. 2004. *Aspergillus flavus* in soils and corncobs in South Texas: Implications for management of aflatoxins in corn-cotton rotations. Plant Dis. 88:1366-1371.

Klich, M. A. 2007. *Aspergillus flavus*: The major producer of aflatoxin. Mol. Plant Pathol. 8:713-722.

Kwok, S., Kellogg, D. E., McKinney, N., Spasic, D., Goda, L., Levenson, C., and Sninsky, J. J. 1990. Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies. Nucleic Acids Res. 18:999-1005.

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

Marín, S., Ramos, A. J., and Sanchis, V. 2012. Modelling *Aspergillus flavus* growth and aflatoxins production in pistachio nuts. Food Microbiol. 32: 378-388.

Mauro, A., Battilani, P., Callicott, K. A., Giorni, P., Pietri, A., and Cotty, P. J. 2013. Structure of an *Aspergillus flavus* population from maize kernels in northern Italy. Int. J. Food Microbiol. 162:1-7.

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.

Medina, A., Akbar, A., Baazeem, A., Rodriguez, A., and Magan, N. 2017. Climate change, food security and mycotoxins: Do we know enough? Fungal Biol. Rev. 31:143-154.

Mehl, H. L., and Cotty, P. J. 2010. Variation in competitive ability among isolates of *Aspergillus flavus* from different vegetative compatibility groups during maize infection. Phytopathology 100:150-159.

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.

Michailides, T. J., Puckett, R. D., Moral, J., Garcia-Lopez, M. T., Felts, D., Doster, L., and Jaime, R. 2018. Factors affecting the efficacy of AF36 improvement of the biocontrol agent and monitoring commercial applications. California Pistachio Research Board, Fresno, CA.

Moore, G. G., Olarte, R. A., Horn, B. W., Elliott, J. L., Singh, R., O'Neal, C. J., and Carbone, I. 2017. Global population structure and adaptive evolution of aflatoxin-producing fungi. Ecol. Evol. 7:9179-9191.

Moral, J., Garcia-Lopez, M. T., Camiletti, B. X., Jaime, R., Michailides, T. J., Bandyopadhyay, R., and Ortega-Beltran, A. 2020. Present status and perspective on the future use of aflatoxin biocontrol products. Agronomy (Basel) 10:491.

Ortega-Beltran, A., and Cotty, P. J. 2018. Frequent shifts in *Aspergillus flavus* populations associated with maize production in Sonora, Mexico. Phytopathology 108:412-420.

Ortega-Beltran, A., Grubisha, L. C., Callicott, K. A., and Cotty, P. J. 2016. The vegetative compatibility group to which the US biocontrol agent *Aspergillus flavus* AF36 belongs is also endemic to Mexico. J. Appl. Microbiol. 120: 986-998.

Ortega-Beltran, A., Moral, J., Picot, A., Puckett, R., Cotty, P., and Michailides, T. 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.

Ortega-Beltran, A., Moral, J., Puckett, R., Morgan, D. P., Cotty, P. J., and Michailides, T. J. 2018. Fungal communities associated with almond throughout crop development: Implications for aflatoxin biocontrol management in California. PLoS One 13:e0199127.

Palumbo, J. D., Mahoney, N. E., Light, D. M., Siegel, J., Puckett, R. D., and Michailides, T. J. 2014. Spread of *Aspergillus flavus* by navel orangeworm (*Amyelois transitella*) on almond. Plant Dis. 98:1194-1199.

Payne, G. A. 1998. Aflatoxin-producing fungi and their impact on crops. Page 279 in: Mycotoxins in agriculture and food safety. K. K. Sinha and D. Bhatnagar, eds. Marcel Dekker, New York, NY.

Perez, A., Ferreira, G., and Minor, T. 2017. Fruit and tree nuts outlook: Economic insight. USDA Econ. Res. Serv. 1494-2:1-6.

Picot, A., Doster, M., Islam, M. 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., Bandyopadhyay, R., Price, L. E., and Cotty, P. J. 2011. Identification of atoxigenic *Aspergillus flavus* isolates to reduce aflatoxin contamination of Maize in Kenya. Plant Dis. 95:212-218.

Sardiñas, N., Vázquez, C., Gil-Serna, J., Gonzalez-Jaen, M. T., and Patiño, B. 2011. Specific detection and quantification of *Aspergillus flavus* and *Aspergillus parasiticus* in wheat flour by SYBR® Green quantitative PCR. Int. J. Food Microbiol. 145:121-125.

Savić, Z., Dudas, T., Loc, M., Grahovac, M., Budakov, D., Jajic, I., Krstovic, S., Barosevic, T., Krska, R., Sulyok, M., Stojsin, V., Petres, M., Stankov, A., Vukotic, J., and Bagi, F. 2020. Biological control of aflatoxin in maize grown in Serbia. Toxins (Basel) 12:162.

Shenge, K. C., Adhikari, B. N., Akande, A., Callicott, K. A., Atehnkeng, J., Ortega-Beltran, A., Kumar, P. L., Bandyopadhyay, R., and Cotty, P. J. 2019. Monitoring *Aspergillus flavus* genotypes in a multi-genotype aflatoxin biocontrol product with quantitative pyrosequencing. Front. Microbiol. 10:2529.

Wang, Y., Zhang, W, Wang, Y., and Zheng, X. 2006. Rapid and sensitive detection of *Phytophthora sojae* in soil and infected soybeans by species-specific polymerase chain reaction assays. Phytopathology 96:1315-1321.

Weaver, M. A., Abbas, H. K., Falconer, L. L., Allen, T. W., and Pringle, H. C. L. 3rd., and Sciumbato, G. L. 2015. Biological control of aflatoxin is effective and economical in Mississippi field trials. Crop Prot. 69:52-55.

Yu, J., Chang, P., Ehrlich, K. C., Cary, J. W., Bhatnagar, D., Cleveland, T. E., Payne, G. A., Linz, J. E., Woloshuk, C. P., and Bennett, J. W. 2004. Clustered pathway genes in aflatoxin biosynthesis. Appl. Environ. Microbiol. 70:1253-1262.

Zhou, L., Wei, D. D., Selvaraj, J. N., Shang, B., Zhang, C. S., Xing, F. G., Zhao, Y. J., Wang, Y., and Liu, Y. 2015. A strain of *Aspergillus flavus* from China shows potential as a biocontrol agent for aflatoxin contamination. Biocontrol Sci. Technol. 25:583-592.