Efficacy of AF36 Prevail after Commercial Application, Search for the Best Timing of Application, and Susceptibility of Almond Cultivars to Aflatoxigenic *Aspergillus* Species

Project No.: **AFLA1.Michailides**

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Grantee(s) of the Almond Board are REQUIRED to address sections A through G. These should be **submitted in PDF**, using Arial font size 12 for the main text, and be five to seven pages in length.

A. Summary

Aflatoxins are carcinogenic contaminants produced by the fungi *Aspergillus flavus* and *A. parasiticus.* The almond industry has taken extensive measures and supports pre- and post-harvest research to control aflatoxin and to assure compliance with aflatoxin regulations. Area-wide management programs might be the best option to reduce the risk of aflatoxin contamination in tree nuts, including almonds. Determining the effect of atoxigenic biocontrol effects on area-wide sets requires to increase the number of fields and samples to analyze. Specific primers differentiating *A. flavus* AF36 from other *A. flavus*/*A. parasiticus* were developed and used in a Real Time PCR (SNP-qPCR) program to quantify the proportions of both AF36 and *A. flavus/A. parasiticus* contained in the samples. This method can expedite the analysis of samples, reduce its costs and increase the certainty of the study. In the 2018 timing of application experiment, the first application was made on before hull split (26 June). Sporulation at all times of application was around 50%, with the highest percentages observed in July after hull split started. Aflatoxin was not detected in any of the almond samples. Displacement, measured as % of AF36, in the kernels at all times of application ranged from 47% to 55% and it was significantly higher than the control (5%), except for the latest application in August (22%). This experiment is being repeated in 2019 and currently samples are being processed and analyzed for both displacement and aflatoxin. The influence of soil moisture and temperatures on the sporulation of AF36 Prevail® and a second product, Afla-Guard®, was evaluated. Optimal sporulation occurred at soil moisture between 15 and 18%. Temperatures below 15ºC significantly delayed the sporulation of AF36 Prevail®, regardless of soil moisture. These temperatures are common in the Central Valley until mid-June. In an area where tree nuts in risk of aflatoxin contamination (pistachio and almond) are grown together the effect of AF36 applications on area-wide is currently being evaluated. In one part of the area both pistachio and almond were treated, while in another only pistachio was treated. The

change of the population structure of *A. flavus* between the almond treated and not treated areas are underway and should be finished before the start of next crop season.

B. Objectives *(300 words max.)*

1. To develop a qPCR protocol to evaluate the ratio AF36 / *A. flavus* & *A. parasiticus*
   Milestones and deliverables: In the process to be validated with field samples from almonds.
2. To study the risk of infection during the period that the almond fruit are drying on the ground.
   Milestones and deliverables: Data analyses and manuscript preparation for publication are in process.
3. To determine the optimal period for the AF36 Prevail® application in almond and improve sporulation of this product so that we increase the displacement of the toxigenic *A. flavus/parasiticus*.
   Milestones and deliverables: Experiments to determine the optimal time of application of the biocontrol *A. flavus* AF36 Prevail® in almond orchards were established. Analysis of samples and data is underway. Experiments to determine the optimal conditions for sporulation of the biocontrol products *A. flavus* AF36 Prevail® and Afla-Guard® were also established.
4. To determine the effect of almond cultivars on aflatoxin accumulation.
   Milestones and deliverables: Data analyses and manuscript preparation for publication are in process.
5. To monitor the atoxigenic *A. flavus* AF36 in commercial almond where AF36 is applied and evaluate its effect on area-wide long-term reduction of aflatoxin in almonds.
   Milestones and deliverables: The first area to evaluate and implement area-wide management program were initiated this crop season. We are in the process of analyzing samples to quantify the biocontrol strain AF36 in both treated and not treated almond orchards and determine its effects on area-wide bases.

C. Annual Results and Discussion *(This is the core function of this report)*

1. To develop a qPCR protocol to evaluate the ratio AF36 / *A. flavus* & *A. parasiticus.*

Currently, the biocontrol AF36 is quantified by vegetative compatibility group analysis, which is expensive and time consuming. We developed a quantitative real-time PCR (qPCR) procedure to quantify the proportions of both AF36 and the toxigenic isolates at reduced time and assay costs. Specific primers to target the AF36 strain, and *A. flavus* and *A. parasiticus* were designed. The pair of primers SNP36 Sh2 and SNP36 Cb specifically amplify AF36, while the pair of primers 3Fw-no mut B and 8Rv-no mut-267 amplify any *A. flavus* (including toxigenic and atoxigenic) and *A. parasiticus* isolates, except AF36. The standard curves were generated to quantify the amounts of DNA based on the threshold values (Cq) for each strain of interest and calculate the rate of AF36/ (*A. flavus*-*A. parasiticus*). Standard curves of the quantity of DNA as a function of Cq fit linear regression models for both strain AF36 (y = 11.455 - 0.307X, r2 = 0.988,

P<0.001) and *A. flavus-A. parasiticus* (y = 9.541 - 0.236X, r2 = 0.995, P<0.001). Verification tests of the proportion of AF36 given by the qPCR as a function of known concentration of both spore mixtures (y = -5.82 + 1.05X, r2 = 0.960, P<0.001) and DNA mixtures (y = 9.22 + 1.03X, r2 = 0.912, P<0.001) show a highly significant relationship indicating that the proportion of AF36 given by the qPCR assay are very accurate. Furthermore, the qPCR assay was used to quantify the proportions of AF36 from leaves, nuts, and soil samples, demonstrating its usefulness to accurately quantify the proportion of AF36 in the population of the aflatoxin producing *Aspergillus* fungi. The use this qPCR assay can help to increase the number of samples that can be quantified on time and based on available resources and improve the study of the epidemiology of aflatoxin producing fungi. However, the assay needs to be validated with actual samples from the field and compared to the values given by both VCG analysis and normal PCR analysis, which is underway.

2. To study the risk of infection during the period that the almond fruit are drying on the ground.
   Results from this objective were reported in last year report. Now we are in the process of data analysis to start preparing a manuscript for publication.

3. To determine the optimal period for the AF36 Prevail® application in almond and improve sporulation of this product so that we increase the displacement of the toxigenic *A. flavus/parasiticus*.
   Evaluation for the best timing of application on almond was initiated in the 2018 season. The first application was made on June 26 before hull split and they were repeated every two weeks (July 10th and 24th, and August 7th). Sporulation at all times of application was around 50%, with the highest percentages observed in both applications made in July, after hull split started. Aflatoxin was not detected in any of the almond samples, including the untreated control. Although displacement, measured as % of AF36 in the kernels, was not optimal, it ranged from 47% to 55% and it was significantly higher than the control (5%) at all times of application, except for the latest application in August (22%). This experiment was repeated in 2019 and currently samples are being processed and analyzed for both displacement (percentage of AF36) and aflatoxin content in the kernels. This indicates that the atoxigenic biocontrol product A. flavus AF36 Prevail® has better sporulation on applications made during July. However, the application before almond splitting (late June), even though had lower sporulation, it had similar percentages of displacement than the applications made in July. This indicates that if sporulation at earlier stages is improved, it might improve the overall success of biocontrol applications. Looking to improve sporulation at earlier stages we started experiments to determine the factors influencing sporulation under environmental conditions of almond orchards in the Central Valley. Under this premises we evaluated the influence of soil moisture and temperatures on the sporulation of *A. flavus* AF36 Prevail® biocontrol product and a second product, Afla-Guard® under control environment conditions in a growth chamber. In general, results indicate that sporulation of *A. flavus* AF36 Prevail® is delayed under low night temperatures, especially under

low soil humidity, while Afla-Guard® had good sporulation even under low temperatures. Variance component analysis indicates that temperature is the main factor influencing sporulation of *A. flavus* AF36 Prevail®, while moisture is the main factor influencing sporulation of Afla-Guard®. Water content has a higher effect on the percentage of product grains sporulating (explaining around half of the total variance for both products) than on the amount of spore produced with around a quarter of the variance explained for AF36 Prevail® and around 40% for Afla-Guard®. Temperature is not an important factor for Afla-Guard® on the percentage of sporulation, but it has a higher influence on the amount of spores produced. However, for AF36 Prevail® temperature explains almost half of the variance, which indicates that temperature has a higher effect on the sporulation of AF36 Prevail® than on Afla-Guard®. AF36 Prevail® has acceptable sporulation with over 80% of grain sporulating and a sporulation index over 1.5 only at the warmest temperature regimes and soil water contents of 12% to 21%, while Afla-Guard® had the same levels of sporulation even at the lowest temperatures. At the warmest temperature regimes both products had similar sporulation at any water content. In general, good sporulation at low soil moisture and at lower temperatures, similar to those occurring during May in the Central Valley, could be significant for earlier applications of the biocontrol and consequently, it could solve the problem of not achieving the full potential of the atoxigenic strain technology to control aflatoxin contamination in nut crops in California.

4. To determine the effect of almond cultivars on aflatoxin accumulation.
   Results from this objective were reported in last year's report. Now we are in the process of data analysis to start preparing a manuscript for publication.

5. To monitor the atoxigenic *A. flavus* AF36 in commercial almond where AF36 is applied and evaluate its effect on area-wide long-term reduction of aflatoxin in almonds.
   In the previous season (2018) AF36 was applied in a commercial almond orchard. The persistence and sporulation of the product was monitored two weeks after application. Although the product sporulated, it was at a low percentage and some product was lost and partially eaten. However, the pieces of product left were still viable and capable of sporulating. All almond samples were free of aflatoxins. However, since aflatoxin contamination is occasional, the best indicator of the success of the applications is the displacement of toxigenic strains by the biocontrol. The percentage of the applied AF36 before application and after harvest indicates a successful application with an average of the applied AF36 strain over 80%, compared to a 6.6% incidence prior to the application. The untreated control orchard had a 26% average incidence of the AF36 and the area next to the treated orchard had 40% after harvest, indicating the cross effects of applications. The high percentage of displacement achieved in the treated orchard indicates a successful application of the biocontrol AF36 and that it is a viable option for reducing the risk of aflatoxin contamination in almonds in California.

A successful control of aflatoxin will benefit the growers by avoiding the loss of income with lower risks of crop rejections and the public in general by consuming almond products free of aflatoxins. The increase of AF36 incidence in the untreated orchard indicates the capability of the fungus to spread over considerable distances and the cross effects between treated and untreated orchards. Therefore, the implementation of area-wide long-term aflatoxin control programs, as discussed above, might be the best strategy to lower the risks of aflatoxin contamination in tree nut crops, including almonds. In an area where tree nuts in risk of aflatoxin contamination (pistachio and almond) are grown together the effect of AF36 applications on area-wide is currently being evaluated. In one part of the area both pistachio and almond were treated, while in another only pistachios were treated. Soil samples from the orchard (pistachio and almond) of this study were taken both before application and after harvest. Samples taken before application will serve as a base line of the population of the aflatoxin-producing *Aspergillus* fungi in both areas. Samples after harvest will indicate the change of the population structure and will serve to calculate the percentage of displacement of toxigenic isolates by the applied biocontrol AF36. Comparing the population structure of *A. flavus* between the almond treated and not treated areas after harvest will indicate the influence of the treatments in an area-wide basis and currently these studies are underway and should be finished before the start of next crop season.

D. Outreach Activities

Please describe outreach activities including the event description (date, location, topic of the presentation, aprox number of participants and type of audience)

1. Michailides .T.J. Aflatoxin and Band canker pathogens in young almond trees. 46th Almond Conference, 6 December 2018, Sacramento, Ca. (300 attendees).

2. Jaime R. and Michailides .T.J. Efficacy of AF36 Prevail after commercial application, search for the best time of application. 47th Almond Conference, 12 December 2019, Sacramento, California (25-35 attendees).

3. Michailides T.J. and Jaime R. Discussion on Aflatoxins of almond. Summit Meeting. Quality and Safety Committee, Almond Board of California (ABC), 28 February 2019, Modesto, Ca. (15+ attendees).

4. Michailides, T. J. Aflatoxin understanding and control – All the tools in the shed. Almond Quality & Food Safety Symposium held on 13 June 2019 at Wine & Roses in Lodi, California, Almond Board of California. There were approximately 235 attendees (almond growers, pest control advisers, processors and marketers (exact numbers were not kept).

E. Materials and Methods:

1. Factors affecting sporulation of aflatoxin biocontrol products. The effect of both low temperatures and soil moisture were evaluated in soil pots under controlled environment in a growth chamber. Water content in the soil was determined

based on the field capacity (FC) of the soil. Temperature regimes were according to day and night temperatures in May through July in the Central Valley. Sporulation was recorded at four days and repeated after one and two weeks of incubation. Sporulation was determined as percent of grain sporulating and quantity of spores produced per product grain, estimated by a sporulation index graded according to the percent of the grain covered with spores as follow: 0, no sporulation, 1, visible sporulation to 10%, 2, 10% to 40%, 3, 40% to 70%, and 4, 70% to 100%.

2. Determination displacement by AF36. To quantify the density and strain determination of *Aspergillus* section *Flavi*, samples were plated on selective isolation medium and incubated at 30°C. From each sample, around 20 *A. flavus* isolates were obtained for strain determination. The incidence of AF36 was determined by vegetative compatibility group (VCG) and PCR analyses. Isolates were identified as belonging to AF36 either by complementation tests with known testers for the VCG YV36 or by PCR with specific primers for AF36. The proportion of AF36 indicates the percentage of displacement of toxigenic *Aspergillus* fungi.
3. Real time PCR for quantification of AF36. Specific primers differentiating *A. flavus* AF36 from other *A. flavus* and *A. parasiticus* in a real time PCR (qPCR) were designed using previously identified single nucleotide polymorphism (SNP) in the aflatoxin gene cluster. These primers were used in a Real Time PCR (SNP-qPCR) program to generate standard curves for the quantitation of DNA of AF36 and *A. flavus/A. parasiticus* as a function of the quantitation cycles (Cq) for each specific set of primers. To verify the qPCR standard curves the relationship of the proportions of AF36 DNA given by the SNP-qPCR were analyzed by regression analysis as a function of the known DNA mixtures. This same procedure was also used to verify the standard curves with DNA obtained from spore suspensions with known proportions of AF36 spores and quantify the proportions of AF36 and *A. flavus/parasiticus.*
4. Aflatoxin analysis. Almond samples were analyzed for aflatoxins at the Kearney Agricultural Center. After the nuts were ground finely and mixed thoroughly, 50 g of the ground nuts were blended with 5 g NaCl and 200 ml methanol-water (60:40; vol/vol) in a blender. After filtering the extract, 25 ml of the diluted filtrate was passed through an AflaTest□ immunoaffinity column, then washed with 20 ml deionized water. Aflatoxins were eluted with 1 ml methanol. Aflatoxins were quantified by HPLC with a C18 column and a fluorescence detector (360 nm excitation and 440 nm emission wavelengths) and a mobile phase of methanol-water (45:55, vol/vol) at 0.8 ml min-1 flow-rate. A photochemical reactor was used to increase the sensitivity and selectivity of the fluorescence detector. The limit of detection for aflatoxins were below 1.0 ng g-1 (ppb).

F. Publications that emerged from this work

1. García-López, M. T., Luo, Y., Ortega-Beltrán, A., Jaime, R., Moral, J., and Michailides, T. J. 2020. Quantification of a non-aflatoxigenic strain of *Aspergillus flavus* (AF36) from various sources using real-time PCR - in preparation for peer review publication (attached).

2. Garcia-Lopez, M. T., Moral, J., Jaime, R., Puckett, R., Doster, M. A., and Michailides, T. J. 2018. Sporulation and dispersal of the biological control agent *Aspergillus flavus* AF36 under field conditions in nut crops in California. (Abstr.) Phytopathology 108:S1.206. - Presented at the 11th International Congress of Plant Pathology, Boston, MA, 29 July – 3 August 2018. (Attached).
3. García López, M. T., Luo, Y., Ortega-Beltran, A., Jaime, R., Moral, J., Michailides, T. J. 2019. Quantification of an atoxigenic strain of *Aspergillus flavus* (AF36) using real-time PCR. (Abstr.) Phytopathology 109:S2.136-137. - Presented at the 2019 APS Annual Meeting. Cleveland, OH, August 3-7, 2019. (Attached)
4. Jaime, R., García López, M. T., Moral, J., Puckett, R., Michailides, T. J. 2019. Effect of soil moisture on sporulation of two aflatoxin biocontrol products at different temperature regimes under controlled environments. (Abstr.) Phytopathology 109:S2.180-181. - Presented at the 2019 APS Annual Meeting. Cleveland, OH, August 3-7, 2019. (Attached)
5. Moral, J., García López, M. T., Jaime, R., Puckett, R., Doster, M. A., and Michailides, T. J. 2017. Sporulation and dispersal of the biological control agent *Aspergillus flavus* AF36 under field conditions. - Poster presented at the 45th Almond Conference, Almond Board of California (ABC), Sacramento, CA. December 2017. (Attached)
6. Jaime, R., García López, M. T., Moral, J., Luo, Y., Puckett, R., Doster, L., and Michailides, T. J. 2018. Efficacy of the biocontrol *Aspergillus flavus* AF36 Prevail® to prevent aflatoxin contamination in almond. Poster presented at the 46th Almond Conference, Almond Board of California (ABC), Sacramento, CA. December 2018. (Attached)
7. Jaime, R., Gabri, V., Doster, L., García, M. T., Moral, J., Puckett, R., and Michailides, T. J. 2019. Efficacy of AF36 Prevail after commercial application, search for the best time of application. Poster presented at the 47th Almond Conference, Almond Board of California (ABC), Sacramento, CA. December 2019. (Attached)