Title: **Needed Detailed information in Comparing the Efficacy of AF36 Prevail and the Afla-Guard Biocontrol Products Used to Reduce Aflatoxin Contamination**

Principal Investigator: Themis J. Michailides

Major collaborators: Post-doc to be hired.

Other collaborators: Drs. Ramon Jaime and Pummi Singh; Victor Gabri, and John Lake; all with the Department of Plant Pathology, UC Davis/ Kearney Agricultural Research and Extension Center, in Parlier, CA 93648-9774.

Summary:

The main goal of this proposal is to make sure as soon as possible whether the Afla-Guard can be an additional (alternative to AF36 Prevail) product for the biocontrol of aflatoxins and to confirm the initial advantages and disadvantages detected in laboratory experiments with actual field experiments.

Background information

I would like to share the information below with the pistachio industry (represented by the Calif. Pistachio Research Board), since this information was presented to a small group of pistachio industry people in a gathering in early April 2023. My project scientist, Dr. Ramon Jaime who is involved in the long term area wide aflatoxin control project and I met with representatives of the company involved in providing sites for the biocontrol experiments. Because this company makes the biocontrol applications on a commercial scale and provides to us hundreds of library samples from the commercial/experimental sites its members who are involved in these trials wanted to be updated on the progress and findings of this project. The same company (Wonderful Orchards Co.) in addition to providing all the relevant library samples for aflatoxin and ochratoxin analyses, also gives us access, after coordination with them, to sample soils of their fields before application of AF36 Prevail and/or Afla-Guard and after harvest for determining the levels of the applied biocontrol strains and those of the displaced toxigenic strains.

After the registration of Afla-Guard, in 2022, we decided to do additional experiments beyond the lab experiments the registrant had requested. In these additional experiments where we compared the two products side by side in an orchard here at Kearney, we found major differences in sporulation. Although the Afla-Guard product under laboratory conditions sporulated better than AF36 Prevail and in drier soil, in the field did not sporulate as well and it was more sensitive to direct sunlight than the AF36 Prevail product. For instance, no seeds of Afla-Guard sporulated after 1 day exposure to direct sunlight, while the AF36 Prevail sorghum seeds produced spores after 4 days expose to the same direct sunlight. Furthermore, additional tests of co-inoculation of toxigenic and the atoxigenic strains (AF36 and/or Afla-Guard) were

performed. In these co-inoculation studies, we found that although AF36 prevented aflatoxin production by the toxigenic *Aspergillus flavus* strain, the Afla-Guard in one experiment reduced aflatoxin by only a small degree, while in another experiment increased the level of aflatoxin production by the toxigenic *Asp. flavus* of co-inoculated pistachio kernels.

Therefore, these contradicted results and the reduced sporulation of Afla-Guard in comparison with AF36 Prevail in the field prompted the submission of this off-season proposal to take advantage of the 2023 season and resolve these issues as soon as possible. The inability of Afla-Guard in reducing aflatoxin in co-inoculation experiments with the toxigenic species (*A. flavus* and *A. parasiticus*) is puzzling and of major economic importance for the pistachio and almond industries at a time when their members are investing so much now in buying Afla-Guard product in addition to the AF36 Prevail for treating pistachio orchards. In other words, in order to do these studies in the field as soon as possible, I think it is wise and cost effective at this time to have a postdoctoral associate devote his time on this problem and solve it as soon as possible. If the project is funded, I will have one individual working exclusively on comparing the two products carefully under field conditions to determine once and for all whether Afla-Guard is worthwhile to use in pistachio orchards.

Objectives

1. Perform more co-inoculation experiments on pistachio kernels by applying AF36 or Afla-Guard along with the toxigenic strain of *Aspergillus flavus*, 1 day ahead, simultaneously, or 1 day after inoculation with the toxigenic strain.
2. Same as the #1 but using the toxigenic *Aspergillus parasiticus*.
3. Determine the effect of soil moisture levels in the field on the sporulation of AF36 Prevail and Afla-Guard in side by side replicated plots.
4. Effect of pre-wetting each product in the ability to sporulate and the effect under direct sunlight in the field.

Methods

Objective 1: **Co-inoculation experiments using the toxigenic *Aspergillus flavus.***

A. Inoculate the biocontrol strain one day in advance of the toxigenic *A. flavus.*
B. Inoculate the biocontrol strain one day after the inoculation of the toxigenic *A. flavus.*
C. Inoculate the biocontrol strain simultaneously with the inoculation of the toxigenic *A. flavus.*

General procedure: Glass petri dishes will be used with 30 kernels of Kerman pistachio each. The kernels will be autoclaved to kill secondary microorganisms, cooled, and inoculated with 25 µl of each suspension, toxigenic and/or atoxigenic strain. Spore suspensions of fresh cultures of the toxigenic *A. flavus* and each AF36 and Afla-Guard strain, *Aspergillus flavus* (2A1L-11) and *Asp. parasiticus* (4C1P-11) will be prepared and adjusted to 50,000 spores/ml using the hematocytometer under the compound microscope. Inoculations with the toxigenic strain will

be done with 25 µl spore suspension in three sites on the kernels in each petri plate. The biocontrol (25 ul) will be added to the same sites. After inoculation, all the plates will be incubated at 30°C for 1 week. Then, each sample will be prepared for aflatoxin analyses using our HPLC. Comparisons of the various treatments will be done using ANOVA and the LSD test.

Treatments for each (A), (B), and (C) above will involve:

a) Inoculation with the toxigenic *Asp. flavus* only.
b) Inoculation with the atoxigenic AF36 only.
c) Inoculation with the Afla-Guard strain only.
d) Inoculation with the toxigenic *Asp. flavus* + AF36.
e) Inoculation with the toxigenic *Asp.* + Afla-Guard strain.
f) Inoculation of kernels with sterile water (negative control).

Objective 2: **Co-inoculation experiments using the toxigenic *Aspergillus parasiticus.***

This will be done similarly for time of inoculation of biological control strain as in (A), (B), and (C) above and the same treatments (a), (b), (c), (d), (e), and (f) as in #1 objective, but instead of *A. flavus* the toxigenic **Aspergillus parasiticus** will be used.

Objective 3: **Determine the effect of soil moisture levels on sporulation of AF36 and Afla-Guard in the orchard side by side in replicated plots.**

Previous **laboratory** experiments indicated that in general Afla-Guard sporulated better under lower and drier conditions. However, initial experiments in the **field** showed that these laboratory findings do not agree with the laboratory results. Therefore, the following experiments will be conducted:

A) In a pistachio orchard at Kearney Agric. Res. & Extension Center, after micro-sprinkler irrigation runs for 2 days (saturation condition), sorghum seeds of AF36 and barley seeds of Afla-Guard will be placed in five replicated plots at the end of the day to take advantage of the moist soil overnight. Sporulation on the seeds will be observed after 3 days, and micro-sprinklers will be turn on again for 24 hours. A second recording of the sporulating seeds will be done 2 days after the second cycle of micro-sprinklers. A third micro-sprinkler cycle will run for 12 hours and a third recording of sporulation will be done 2 days later. Comparisons of sporulation will be recorded at each time when micro-sprinklers are stopped and the two products will be compared. <u>This experiment should provide information if short irrigation cycles promote sporulation; also which product sporulates better between the two.</u>
B) In this experiment, the seeds of AF36 and the Afla-Guard will be placed on the dry soil first in five replicated plots and then the micro-sprinkler irrigation will start a) immediately, 1 day, and 2 days later (exposure to dry environment and direct sunlight), the micro-sprinkler system will be turned on and run for 24 hours and then turned off. Seeds will be observed

for sporulation 2 days later. then the micro-sprinkler system will be turned on again, run for 24 hours and then will be turned off. The seeds will be recorded for sporulation and AF36 Prevail will be compared with Afla-Guard. This experiment should provide information on whether irrigation should not be delayed after application of the biocontrol products, and/or the sensitivity of the two biocontrol products staying in the field dry and some under direct sunlight for 1 or 2 days.

C) In this experiment, after the micro-sprinkler irrigation has run for 2 days (saturation condition), sorghum seeds of AF36 and barley seeds of Afla-Guard will be placed in 5 replicated plots, and sporulation will be examined in 2, 4, 6, and possibly 8 days after the micro-sprinkler is turned off. No additional cycles of irrigation will be done in these plots. This experiment should provide information on the longevity of seeds to sporulate after they get wet in the field with the irrigation following the application of the seeds in the field.

4. Effect of pre-wetting each seed product in the ability to sporulate and the effect of exposure under direct sunlight in the field.

A) In the lab, seeds of each product will be soaked for 1 or 2 days. Then they will be placed in the field in saturated soil (after running the micro-sprinkler system for 24 hours). Treatments in 5 replicated plots will include: a) dry seeds of each product; b) 1 day soaking in water; c) 2 days soaking in water. Sporulation will be observed in 2, 4, and 6 days. In all experiments in the field more than needed seeds will be placed to compensate for the losses due to insect predation.

B) Effect of direct sunlight on seeds of AF36 and Afla Guard soaked in water for 1, or 2 days. After soaking the seeds will be placed in non shaded areas to be subjected to direct sunlight in saturated soil (after running the micro-sprinkler system for 24 hours). Treatments in 5 replicated plots will include: a) dry seeds of each product; b) 1 day soaking in water; c) 2 days soaking in water. Sporulation will be observed in 2, 4, and 6 days. In all experiments in the field more than needed seeds will be placed to compensate for the losses by insect predation.

Budget: (1 July 2023 to 30 June 2024)

Postdoc (80%)	54,960
Benefits	11,607
Sub total:	66,567
Supplies	3,500
Travel	3,500
Subtotal:	7,000
Total:	**$73,567**