Bridging the Use of the Atoxigenic *Aspergillus flavus* Strain AF36 from Pistachios to Almonds

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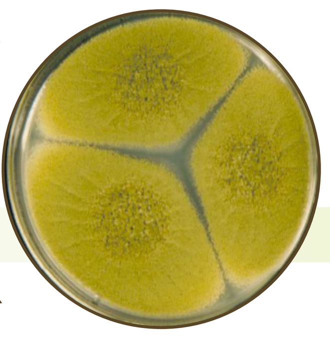

Aspergillus flavus


Aspergillus parasiticus

INTRODUCTION

Aflatoxins, produced by *Aspergillus flavus* and *A. parasiticus*, are the most potent liver carcinogens and are widely regulated by governments who have set very low tolerances for aflatoxins in food and feed. The almond industry has taken extensive successful measures to control aflatoxin. The focus of this research is to provide background for obtaining registration for the atoxigenic *Aspergillus flavus* strain AF36 to use as a biocontrol agent to reduce aflatoxin potential in almond orchards, using a lot of the studies performed with pistachios and widespread application of AF36 in pistachio orchards. We are trying to bridge the data generated from the research on pistachio with the aflatoxin research on almonds.

OBJECTIVE

Using the data generated from pistachio and almond research to register the atoxigenic *Aspergillus flavus* AF36 strain to reduce aflatoxin contamination in almonds.

1. Occurrence and spread of the atoxigenic strain of *Aspergillus flavus* AF36 strain.

Soils were sampled from a number of orchards in various counties where almonds are growing. Soil samples were processed to determine the incidence of AF36 among the other strains.

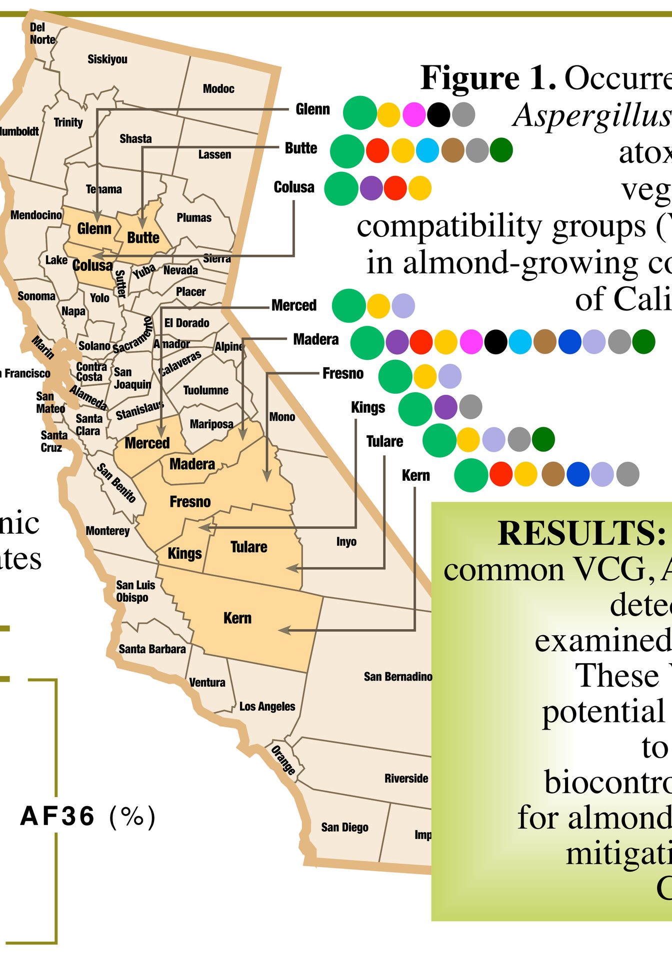


Figure 1. Occurrence of *Aspergillus flavus* atoxigenic vegetative compatibility groups (VCGs) in almond-growing counties of California.

Table 1. Natural occurrence of the atoxigenic strain AF36 among *A. flavus* isolates from orchards in California

County	Almond	Pistachio	Fig
Butte	6.5	---	---
Colusa	3.0	---	---
Fresno	---	3.1	6.1
Glenn	4.4	---	---
Kern	8.5	12.7	---
Madera	5.0	7.2	7.2
Merced	---	15.0	5.8
Tulare	---	2.9	---

AF36 (%)

RESULTS: The most common VCG, AF36, was detected in all examined counties. These VCGs are potential resources to register a biocontrol mixture for almond aflatoxin mitigation across California.

RESULTS: The AF36 strain ranged from 3.0% to 8.5%; this incidence of atoxigenic AF36 is the highest not only in almond orchards (also **Figure 1**) but also in pistachio and fig orchards as shown in **Table 1.**

2. Displacement of toxigenic strains of *A. flavus*/*A. parasiticus* in an experimental almond orchard.

Soils were sampled from an experimental almond plot set at Nickels Soil lab Estates orchards (**Fig. 2**) from treated with the AF36 strain and the untreated control. Soils were analyzed in our laboratory for the incidence of AF36 and the levels of toxigenic stains of *A. flavus*/*A. parasiticus* (**Figure 3**).

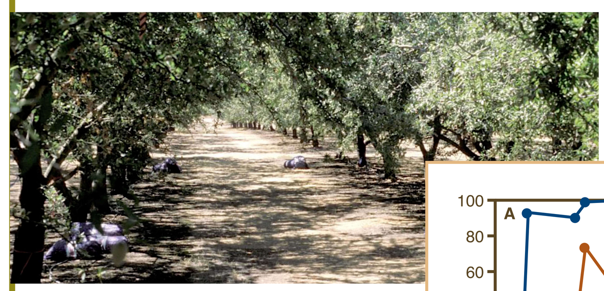


Figure 2. Almond orchard at Nickels Soil Laboratory Estates (Colusa Co.) used for the initial biocontrol of aflatoxins study during 2007 to 2014. Three replicated plots of 9 trees each were treated with AF36 (10 lbs per acre) and three plots of 9 trees each were not treated and served as controls.

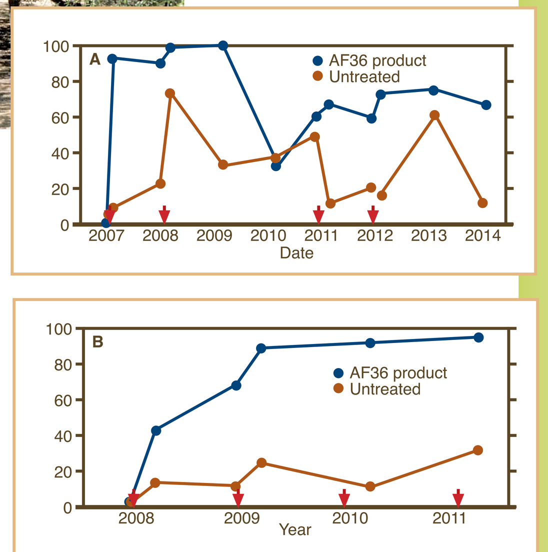


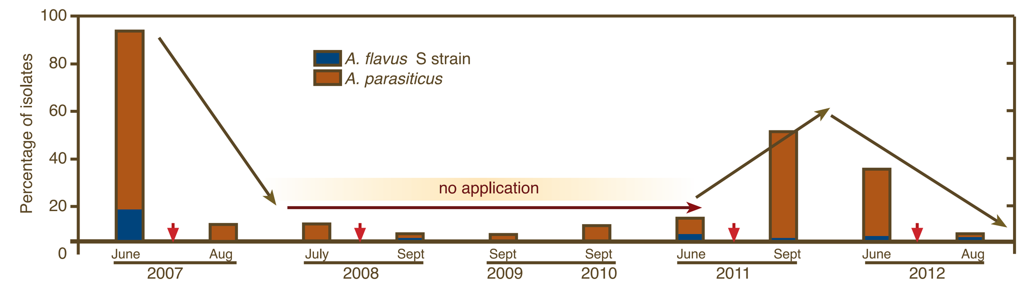
Figure 3. Percentage of *Aspergillus flavus* isolates from soil collected from almond orchard (arrow indicates application).

RESULTS: Notice that AF36 becomes the predominant strain that is recovered from soils in the treated areas and maintains high levels with subsequent applications. Notice also an increase of AF36 in the untreated areas (especially in the almond experimental orchard due to small plots and not so much in the commercial pistachio orchards), an indication that the AF36 could move from the areas where it was applied to areas where it was not applied. That is why we expect even better results with applications of AF36 year after year and on an area-wide application.

3. Persistence of the atoxigenic strain AF36 of *A. flavus* after discontinuing application.

Following the two years 2007 and 2008 when the biopesticide AF36 was applied in the Nickels Soil Lab Estates almond orchard, in 2009 and 2010 AF36 was not applied. However, soil samples were collected to study the survival and persistence of AF36 in soil.

Figure 4. Reduction of aflatoxin-producing *Aspergillus flavus*/*A. parasiticus* isolates in areas of the almond orchard treated with the AF36 product.

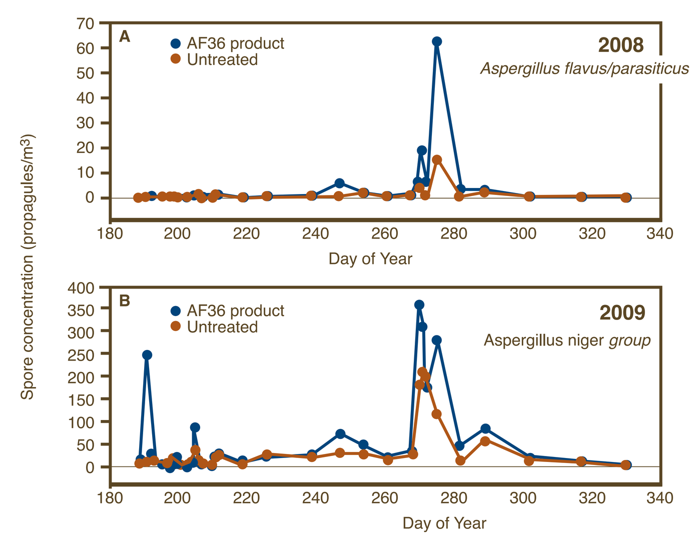


RESULTS: The bar on June 2007 (before the application of AF36 shown with a vertical arrow) shows that more than 90% of the isolates recovered from soil in this orchard were *A. flavus* S strain and *A. parasiticus*. It is well known that the majority of S strains are highly toxigenic and almost all the *A. parasiticus* are toxigenic. After application of the AF36 (shown with the vertical arrow) the % of the toxigenic S strain and *A. parasiticus* was reduced to less than 10%. This low level was maintained during 2008 after the second application of AF36 in July 2008 and during 2009, 2010, until 2011, when a third application was done. Population of the toxigenics increased in September 2011 to June 2012 but after the fourth application of AF36 in June 2012, these populations started to degrease again (Aug 2012, Aug 2013, and Aug 2014).

4. Spore trapping of *Aspergillus flavus* /*A. parasiticus* in orchards.

We presented the spore trapping data submitted for the registration of AF36 on pistachio, and the EPA and the CDPR have accepted the spore trapping data from pistachio (**Figure 5**). So, no additional spore trapping data in an almond orchard were required.

Figure 5. Concentration of fungal spores in the air in two commercial pistachio orchards.



RESULTS: There were no differences in the densities of *Aspergillus* sp. (*A. flavus*/*parasiticus*, or *A. niger* group during the growing season, except during shaking the trees at harvest when treated areas with AF36 showed highter density of spores/m volume orchard air.

5. Incidence of nut decay after application of AF36 strain in the orchard.

Large numbers of almonds were collected at harvest time from AF36-treated and non-treated plots. The almonds were examined with a dissecting scope to determine the incidence of decay and recorded.

Table 2. Percentage of almond hulls decayed by *Aspergillus* fungi

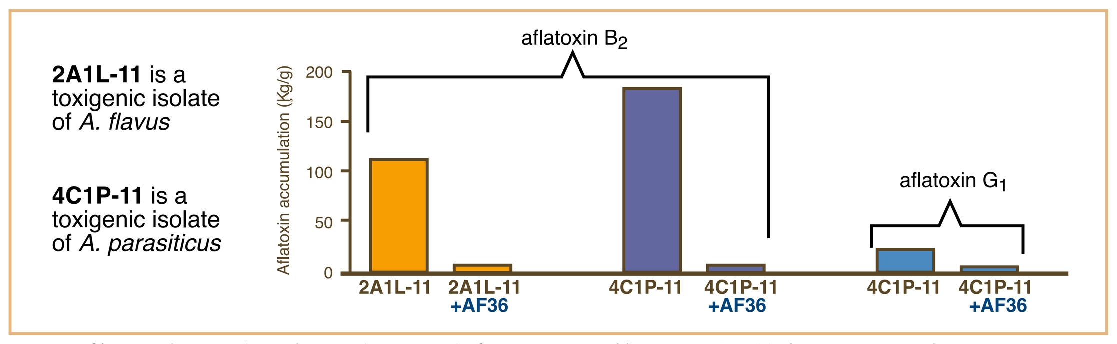
Year	Treatment	*A. flavus*	*A. niger*
2007	AF36	0.197 ns	---
	Control	0.000	---
2008	AF36	0.028 ns	1.028 ns
	Control	0.007	0.262
2009	AF36	0.028 ns	1.008 ns
	Control	0.004	0.614
2010	AF36	0.000 ns	0.059 ns
	Control	0.000	0.059
2011	AF36	0.000 ns	0.138 ns
	Control	0.000	0.132
2012	AF36	0.000 ns	0.431 ns
	Control	0.000	0.280

AF36 product applied 2007, 2008, 2011, and 2012.
At least 9,000 nuts examined for each treatment in each year.

RESULTS: The application of AF36 does not increase the incidence of the decay caused by *Aspergillus flavus* or *A. niger*. No significant hull decay differences were recorded on almond nut samples collected for 6 years from treated vs. non-treated (control) areas in the research almond orchard (**Table 2**). (*Aspergillus niger* does not produce aflatoxin but it is used here mainly as an example for comparison since it occurs in much higher levels in the soils of almond orchards.)

6. Effect on amounts of aflatoxins after co-inoculation of the AF36 atoxigenic strain with toxigenic strains of *A. flavus* or *A. parasiticus* on aflatoxin reduction with almonds

Figure 6. Aflatoxin reduction ability of atoxigenic AF36 strain when co-inoculated with highly toxigenic isolates of *Aspergillus flavus and* A. parasiticus on viable almond kernels under laboratory conditions.



Aflatoxin reductions by AF36 were well over 94% in comparison to those levels accumulated with toxigenic isolates alone.

RESULTS: This slide shows what happens when the atoxigenic AF36 strain and a toxigenic *A. flavus* or *A. parasiticus* infect almond kernels together. When almond kernels were inoculated with each toxigenic strain alone, about 100 ₭g/g for *A. flavus* and about 175 ₭g/g aflatoxin B1 and about 20 ₭g/g aflatoxin G1 for *A. parasiticus* developed. However when the almond kernels were co-inoculated with each the toxigenic *A. flavus* or *A. parasiticus* and AF36 the levels of B1 were reduced to miniscule amounts and the G1 was not detected. This is evidence that in cases when co-infection by a toxigenic strain and the AF36 strain occur on an almond kernel, it is likely that the amounts of aflatoxin will be greatly be reduced. Aflatoxins were quantified using an HPLC Hewlett Packard 1050 as previously described in other studies by our laboratory.

7. Comparison of displacement of toxigenic strains by using AF36 in an almond orchard with those in commercial pistachio orchards.

These comparisons are presented in **Figures 7 and 8.**

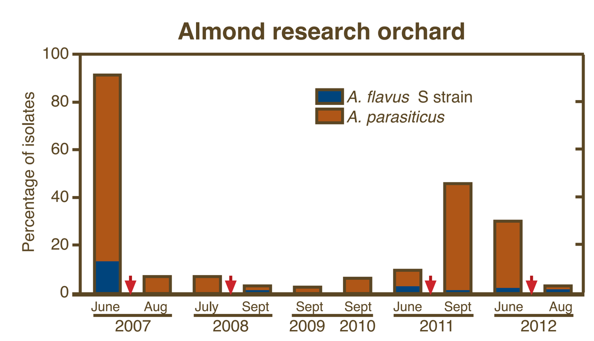


Figure 7

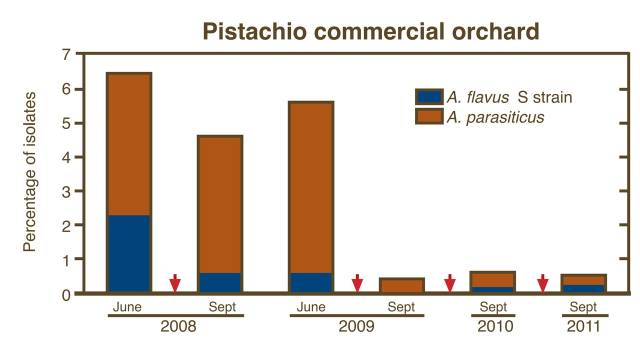


Figure 8

RESULTS: The displacement of the toxigenic strains in the almond orchard were more dramatic than those in the commercial pistachio orchards. Although some reduction occurred by September 2008 and June 2009 after the June 2008 application of AF36, a major displacement of the toxigenics was achieved in September 2009 after the second application of AF36 in June 2009. The levels of toxigenic strains were maintained in very low levels (<1%) after the 3 and the 4 applications of AF36. (Note: the initial displacement was not so great probably because the total incidence of the toxigenics before the first application of AF36 was only 6.5%, while the rest was 93.5%) of the recovered strains were the L strain of *A. flavus*. However, the displacement of the toxigenic strains in the almond orchardwas dramatic (**Figure 7**).

CONCLUSIONS

Research data in applying the atoxigenic strain AF36 of *Aspergillus flavus* in an almond orchard and displacement studies were similar to those in pistachio orchards. Also co-inoculation of toxigenic strains with atoxigenic AF36 strain on almond resulted in a tremendous decrease of aflatoxin production. All these and additional data that bridge the use of the atoxigenic strain AF36 from pistachios to almonds have been submitted to EPA. We expect registation of AF36 for use in almond in 2017.

Aspergillus flavus AF36
(Wheat - inoculum AF36)

Aspergillus flavus AF36 Prevail
(Sorghum - inoculum AF36)