

## DEVELOPMENT OF *ASPERGILLUS FLAVUS* AF36

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Aflatoxins are highly toxic cancer causing fungal metabolites known to cause immune-system suppression, growth retardation, liver disease, and death in both humans and domestic animals. Human exposure to aflatoxins is limited by regulations that prohibit the use of crops containing excess quantities of aflatoxins for foods and feeds. Aflatoxins are regulated in part per billion (ppb) ranges with the maximum allowable level varying with country and intended use of the commodity. The quantity permitted in U.S. foods ranges from 0.5 ppb to 20 ppb. *Aspergillus flavus*, the asexual species responsible for most aflatoxin contamination of many crops, is composed of many genetic groups, called vegetative compatibility groups, that vary widely in several characteristics. *Aspergillus flavus* is not sufficiently aggressive as a pathogen to cause meaningful losses in yield. However, infection of crop components predisposed by stress, insect damage or the environment can result in high aflatoxin levels. Relatively small proportions of a crop infected with highly toxic isolates can result in unacceptable crop aflatoxin content. Isolates of *A. flavus* belonging to different vegetative compatibility groups may produce widely different quantities of aflatoxins and fungal communities resident in different areas frequently vary in average aflatoxin-producing potential. Some naturally occurring isolates of *A. flavus* produce no aflatoxins and are called atoxigenic strains. Certain atoxigenic strains have the ability to competitively exclude aflatoxin-producing strains during crop infection and thereby reduce aflatoxin contamination. One of these, AF36, has been registered as a biological control for the competitive exclusion of aflatoxin producing fungi from cottonseed. The registration process for AF36 that began in 1993 and extended over a decade succeeded in facilitating treatment of over 100,000 acres (Figure 1). The process was greatly facilitated by the IR-4 which served as a liaison and helped prepare and file submissions to EPA.

Many atoxigenic strains are effective at reducing contamination in vitro. However, fewer are effective at reducing aflatoxin contamination during crop infection and in vitro activity does not predict in vivo activity. Laboratory, greenhouse and field plot tests indicated high efficacy of AF36 in competitively excluding aflatoxin producers and reducing aflatoxin contamination of cottonseed and corn (Figure 2). However, it was not until entire commercial cotton fields were treated under an experimental use registration that the full potential of atoxigenic strain technology became apparent. For the registration process the dynamics of population shifts in *A. flavus* communities was monitored carefully. The proportion of *A. flavus* communities composed of the highly toxigenic S strain and of AF36 were monitored prior to treatment, on the crop, and annually after treatment. AF36 was monitored by characterizing numerous *A. flavus* isolates by vegetative compatibility analyses. Treatments caused large reductions in the incidence of aflatoxin producers on treated crops and in soils one year after application and these changes to the *A. flavus* community structures were achieved without increasing the quantity of overall *A. flavus* present (Figure 4). These changes to the structure of *A. flavus* communities influence not only crop aflatoxin content, but the environment as a whole. Propagules (i.e. spores, sclerotia and mycelial fragments) of aflatoxin-producing *Aspergilli* contain large concentrations of aflatoxins. Thus as incidences of atoxigenic strains increase and aflatoxin-producers decrease, and incidences and concentrations of aflatoxins in the soil, air, and throughout the environment also decrease. Thus, use of the pesticide *Aspergillus flavus* AF36 is in the public interest. Long-term and area-wide influences of applications become an emphasis of AF36 development. Many users of atoxigenic

strains seek long-term modifications to the fungal communities in order to reduce risks of aflatoxin contamination in both treated and rotation crops.

The application rate for AF36 is 10 lb/acre. The end use product consists of steam sterilized wheat seed colonized by the atoxigenic *A. flavus* strain AF36. The product is axenic with only the intended atoxigenic strain present. After colonization, the product is dried and stored for up to 9 months prior to delivery to farms. The product is produced in a manufacturing facility operated by a farmer run organization, the Arizona Cotton Research and Protection Council (ACRPC, Figure 3). The process and facility were developed by a partnership between ARS and ACRPC. Quality controls agreed to for the Experimental Registration are maintained as useful insurance of quality product to farmers. For AF36, the registration process was approached as a scientific one. EPA was open to many non-traditional types of information that were ecological in nature. Ecological approaches lead to investigation of the populations of aflatoxin-producing and closely related fungi in natural habitats of the Sonoran desert and in the air above agricultural regions. Several discoveries from these studies have significance well beyond the registration process. *Aspergillus flavus* strain AF36 was found to be endemic not only to agricultural fields, but also to natural habitats. These studies showed that agricultural practices inevitably alter *A. flavus* communities both qualitatively and quantitatively and this can result in average aflatoxin-producing potentials being greater for fungal communities in agricultural fields than for fungal communities in natural habitats. Later, detailed studies showed that soil type, location, and crop rotation all alter the composition and thus the average aflatoxin-producing potential of *A. flavus* communities resident in a given region. Highly toxic *A. flavus* was found in natural habitats along with incidences of deadly aflatoxin levels in samples of native leguminous seeds.

In selecting AF36 as the atoxigenic strain to develop as a commercial biocontrol agent, its efficacy was contrasted with those of other atoxigenic strains in greenhouse and field plot tests. The natural distribution of AF36 on the target crop (cotton) across target regions was also considered. Potential adverse environmental impacts were avoided by not applying *A. flavus* strains to fields in regions where they did not naturally occur. Relative incidence of strains (defined here as VCGs) on the target crop in target regions was taken as a measure of relative adaptation to the crop. It was thought that the most common strains on the crop in these regions would have the greatest adaptation to both the crops and the environments in which the biocontrol needed to work. Atoxigenic strains used for biocontrol need to be highly competitive during crop colonization and infection and throughout the crop environment. AF36 occurred in all the target regions and was the most common atoxigenic strain on the cotton crop throughout these regions. AF36 is an excellent choice for biocontrol strategies based on single atoxigenic strains. However, many other atoxigenic strains also have efficacy. Certain less common strains may be active under specific conditions or in minor components of fields. Natural communities of *A. flavus* endemic to agricultural fields and associated with crops are highly diverse and are composed of many VCGs. After fields are treated with AF36, the resident communities become dominated by AF36. Increased displacement and greater long-term stability of modified communities might be achieved by utilizing mixtures of atoxigenic strains that more closely reflect the complexity of natural *A. flavus* communities. Furthermore, strain mixtures could be customized for various regions, crop rotations, or soil types. The nature of the product may allow the cost of generating such mixtures to be minor. However, the registration process may impede utilizing strain mixtures. Strategies such as atoxigenic strain technology might be particularly limited by pesticide regulations because a continually growing list of strains (reaching perhaps hundreds) may need approval. Instigation of a low cost path to registration of additional strains (similar but not identical to those originally registered) might make further development of atoxigenic strain technology faster and more economically feasible, particularly for the public sector. This would facilitate development and optimization of such technologies for multiple crops, locations, and environments.

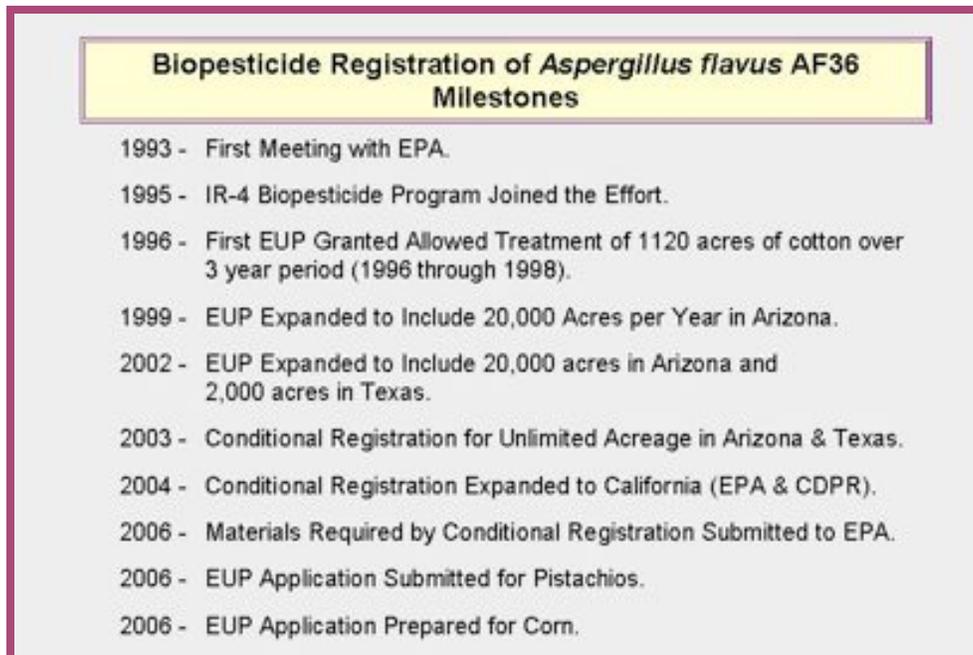


Figure 1. Timeline for the registration of *Aspergillus flavus* AF36. The registration process was undertaken by USDA-ARS with assistance from the IR-4 project. Input from the National Cotton Council and other industry source was important throughout the process. The registrant for the full registration is the Arizona Cotton Research and Protection Council.

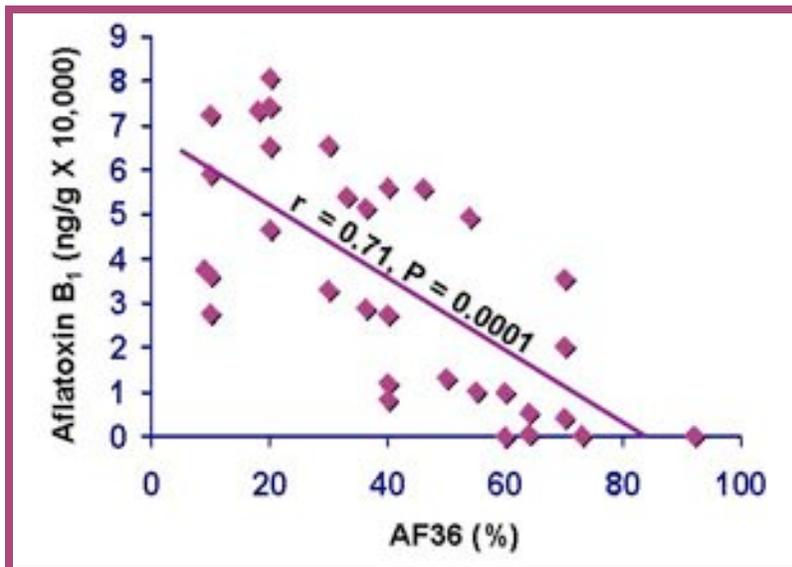


Figure 2. The quantity of aflatoxins in the cottonseed decreases as the percent of the *A. flavus* on the crop that is AF36 increases. Individual points are values from replicate plots either treated with AF36 in various ways or untreated controls. Values are for infected seed. From P.J. Cotty, 1994, *Phytopathology* 84:1270-1277.



Figure 3. Facility for manufacturing atoxigenic strain material run by the Arizona Cotton Research and Protection Council in Phoenix, Arizona. The process and facility were developed by a partnership between ARS and ACRPC. Photographs: P.J. Cotty.

**Composition of *Aspergillus flavus* Communities in Soil of Treated and Nearby Fields in May 1996 Prior to Application of AF36 and in May 1997 One Year After Application**

Field type	Fields (#)	AF36		S strain		<i>A. flavus</i>	
		(% <i>A. flavus</i> )		(% <i>A. flavus</i> )		(CFU/gram)	
		1996	1997	1996	1997	1996	1997
Treated	3	4% ab	85% a	52% a	4% d	582 a	365 a
Adjacent	4	2% b	48% b	41% a	18% c	411 a	157 a
Diagonal	4	2% b	16% c	52% a	33% b	61 a	100 a
Other	4	9% a	9% c	43% a	50% a	109 a	98 a

Other	Adjacent	Treated	Adjacent	Other	Other
Other	Diagonal	Adjacent	Diagonal	Other	Other

Figure 4. Proportion of *A. flavus* communities in soil composed of the biocontrol agent atoxigenic strain AF36 prior to treatment (1996) and one year after treatment (1997). Entire commercial cotton fields (approximately 40 acres each) were treated under an experimental use registration. Data on the incidence of the highly toxigenic strain and on the overall quantity of *A. flavus* in the soil is also included. Note that in treated fields, one year after treatment, incidence of the atoxigenic strain is increased and incidence of the high aflatoxin-producing S strain is decreased without increasing the overall quantity of *Aspergillus flavus* in the soil. Unpublished results, P.J. Cotty.

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References are available at: <http://cals.arizona.edu/research/cottylab/CottyPub.htm>