UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF PREVENTION,
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: BPPD Review of Soil and Air Monitoring Studies and Product Performance Testing (Efficacy) Submitted by USDA Southern Regional Research Center/IR-4 as a Condition of Registration and EUP Extension (Texas) for *Aspergillus flavus* AF36 (Submission #: S630862; DP Barcode D288777; ID# 071693-R, Chemical No. 006456) [MRID #s 453072-01; 453072-02; 1 volume *sans* MRID]

FROM: Gail S. Tomimatsu, Ph.D.
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division, 7511C

PEER REVIEW: John Kough, Ph. D., Senior Scientist 5/15/2003
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division, 7511C

TO: Shanaz Bacchus, Regulatory Action Leader
Biopesticides and Pollution Prevention Division 7511C

ACTION REQUESTED

BPPD has summarized the information and data contained in MRID#s 453072-01 and 453072-02). A complete Data Evaluation Record (DER) for these studies is included with this memorandum. It is noted that MRID 453072-02 appears to be an exact replicate of #453072-01. Another submission (*sans* MRID), with a cover letter from Dr. Michael Braverman, USDA/IR-4, dated March 7, 2003 is summarized also (see **SUMMARY AND RECOMMENDATIONS-Texas Studies**).

SUMMARY AND RECOMMENDATIONS

Reported results of several field and laboratory studies (MRID#s 453072-01 and 453072-02) address guideline requirements of **OPPTS 885.4000** *Nontarget Organism and Environmental*

Expression Test Guidelines (Group D, Background for Nontarget Organism Testing of Microbial Pest Control Agents, OPP 154A-1,2,3,4,5); **OPPTS 885.5000** *Environmental Expression Test Guidelines* (Group E, Background for microbial pesticides testing, OPP155A-1,2) and **OPPTS 810.1000** *Product Performance Guidelines* (Group A, Overview, definitions and general considerations, OPP 90-1, 90-3, 90-30)

These studies were necessary to characterize potential hazards and the efficacy associated with the MPCA, *Aspergillus flavus* AF36 to "displace" (or suppress) populations of aflatoxin-producing *A. flavus*, on cotton in Arizona and Texas [Review of Request for Waiver of Non-Target Organism Safety Effects Tests for EUP Renewal and Expansion for Biopesticide Containing *Aspergillus flavus* AF36 (AF36), from D. Gurian-Sherman, June 23, 1999], and were conducted during the EUP (69224-EUP-1).

Texas Studies

A document with cover letter dated March 7, 2003 (no MRID) included "Efficacy data for AF-36 Texas as a public interest data" was submitted for review, as well. The data (summarized in graphs) are results of 2 year field trials, conducted on 5 fields in 2000, and 4 fields in 2001 in southern Texas; each field was less than 1.0 acre of cotton. It is noted that 2 other atoxigenic strains were tested in the trial; there were no data which could directly support their claim that aflatoxin contamination in harvested cotton was reduced as a result of treatment with these atoxigenic strains, including AF36. No further review will be conducted for this submission.

A complete set of efficacy data conducted on larger acreage is necessary for appropriate and complete science review. This should be a condition of registration. In addition to measuring incidence of atoxigenic and toxigenic strains, aflatoxin levels from harvested cotton from treated and untreated fields should be submitted to support efficacy claims of aflatoxin reduction. Appropriate procedures and protocols should be discussed with Agency scientists prior to submitting studies.

BACKGROUND

In 1999, EPA approved expansion of the treated cotton acreage with AF36 to 20,000 acres in different locations of Arizona. The requested increase in acreage was to evaluate efficacy of AF36 in reducing aflatoxin-producing strains of *A. flavus* on cotton seed. Efficacy data generally are required for pesticides which claim control of pests known to endanger public health (PRN 2002-1).

BPPD therefore recommended continual monitoring of AF36 and "total" *Aspergillus flavus* population levels in treated and non-treated cotton fields. Air monitoring of *A. flavus* spore levels were required, especially when levels are expected to be highest, from July to October (crop maturity and harvest) [Review, June 23, 1999].

The data included summarized results from field studies conducted over a period of several years in Arizona cotton fields. Also, comparative analyses of aflatoxin production of the MPCA AF36 and other *Aspergillus flavus* strains (L-strain phenotype) from diverse geographical locations of the southern U.S., and from treated cotton fields were included to demonstrate the environmental stability of AF36, in retaining its atoxigenic (non-aflatoxigenic) status.

DATA EVALUATION RECORD

EPA Primary Reviewer: Gail S. Tomimatsu, Ph. D. May 15, 2003
EPA Secondary Reviewer: John Kough, Ph.D.

STUDY TYPES: **OPPTS 885.4000** *Nontarget Organism and Environmental Expression Test Guidelines* (Group D, Background for Nontarget Organism Testing of Microbial Pest Control Agents, OPP 154A-1,2,3,4,5); **OPPTS 885.5000** *Environmental Expression Test Guidelines* (Group E, Background for microbial pesticides testing, OPP155A-1,2) and **OPPTS 810.1000** *Product Performance Guidelines* (Group A, Overview, definitions and general considerations, OPP 90-1, 90-3, 90-30)

DP BARCODE: 288777
CASE NO: 062458
SUBMISSION NO: S630862
TEST MATERIAL: *Aspergillus flavus* AF36 colonized sterile wheat seed [end-use product]
PROJECT NO: IR-4-PR No. 52B
SPONSOR: Arizona Cotton Research and Protection Council, 3721 East Wier Avenue, Phoenix, AZ 85040
TESTING FACILITY: Southern Regional Research Center, USDA/ARS, P.O. Box 19687, New Orleans, LA 70179
TITLE OF REPORT: *Aspergillus flavus* isolate AF36 Safety Information (Soil and Air Monitoring of Populations of *A. flavus*)
AUTHOR: Peter J. Cotty, Ph. D.
STUDY COMPLETED: January 9, 2001
GOOD LABORATORY PRACTICE: The studies were not in compliance with GLP of 40 CFR Part 160. However, the data presented in tables, and graphs were collected and analyzed utilizing accepted scientific or commercial practices and are considered scientifically valid.

SUMMARY AND CONCLUSION: The overall objectives were to reduce aflatoxin-producing potential of the fungi associated with the treated cotton crop, and to cause long-term and area-wide changes in the aflatoxin producing potential of fungal communities resident in treated fields and areas. Air and soil populations of *Aspergillus flavus* and AF36 were monitored for at least two or three years in several cotton fields in Arizona. Results of these studies demonstrated significant changes in the incidence of toxigenic *Aspergillus flavus* strains, without increasing the overall quantity of *A. flavus* in the cotton agroecosystem. Also, the atoxigenicity of the AF36 strains was compared with several hundred *A. flavus* isolates from Arizona soils, and from cottonseed bolls from 5 different cotton gin operations in the Southern U.S. having morphological similarity to, and genetic complementation with AF36 (vegetative compatibility). These results suggest "stability" of the AF36 MPCA, (or, isolates similiar to AF36), in its capability to maintain atoxigenicity status under a wide range of environmental conditions.

CLASSIFICATION: ACCEPTABLE for OPPTS 885.4000 and OPPTS 885.5000 Environmental Expression Testing. The submitted data fulfill the requirements for Tier I - Nontarget Organism Testing, and Tier II Environmental Expression testing. Applications of *Aspergillus flavus* AF36 to cotton fields at a rate of 10 lbs/acre should not cause incremental hazards or exposures to nontarget wildlife or the environment. **SUPPLEMENTAL** for efficacy claims and product performance testing, pending validation with currently accepted FDA standard procedures for aflatoxin assays of commercial cottonseed (AOAC Official Method 980.20). There are currently no harmonized OPPTS guideline requirements for product performance testing of microbial pesticides.

I. Materials and Methods

A. Population Monitoring Studies

1. Soil: Samples of soil were collected in May and early June from fields before treatment with AF36 and from untreated fields annually from 1996 through 1999. Samples were collected in a manner which represented a cross-section of the field, for dilution plating on a modified agar medium, optimized for *A. flavus* recovery, and isolation for phenotyping to either L or S strains. and for complementation testing with AF36 in vegetative compatibility group analyses (VCG) and for demonstrate of stability of AF36. Fields, ranging in size from 10 to 80 acres were treated a maximum of one time per year; 10 lbs of AF36-colonized wheat seed $acre^{-1}$ were applied either by ground or air. Composite samples of 100 to 150 g each were retrieved along a 10 m length and consisted of 20 to 40 subsamples from the top 2 cm of soil. Fields were sampled dry, soil clods were hammered, mixed and stored at room temperature in a sealed container. Fungal populations were quantified by dilution plating, and representative isolates of the *A. flavii* group were processed within 50 days of field collection.

Raw data were summarized in tabular and graphic formats in the submitted studies. Relevant citations of methodology and materials for sample collection and dilution plating onto semi-selective agar media [3] were noted as well. Results were analyzed statistically with the t-test for dependent samples (Statistica for Windows, Statsoft, Inc., Tulsa, OK), since a "repeated measures" experimental design for sampling was utilized in these studies. In this type of statistical analysis, values for that portion of the field were contrasted with values from subsequent years, within the same location.

2. Air: Populations of *Aspergillus flavus* in the air were monitored with Burkard cyclone samplers and modified rotorod samplers continuously between May 1997 to March 1999 at two AZ sites approximately 1 km apart, surrounded by commercial agriculture. Particle size sampling efficiency was characterized previously and reputedly is excellent for particles within the size range of *A. flavus* spores (3 to 10 μm). Fields around one site were initially planted with cotton and treated with an atoxigenic strain of *A. flavus* (strain isolate was not provided); whereas fields around the other site were not treated with the atoxigenic strain and initially planted to barley. Weekly analyses were performed on each sampler's catch from the prior week's continuous sampling, which involved dilution plating of the sample on 4 different agar media to estimate the total microflora per unit volume (includes total fungi, *A. flavus* and bacteria per m^{-3}) of the air sample. Every 4 weeks, subcultures of *A. flavus* were performed on 25 *A. flavus* isolates per 20-l

sample for characterization to either the S or L strains(on the basis of sclerotial morphology) and for estimating the proportion of *A. flavus* which could be accounted for by the applied atoxigenic strain (by vegetative compatibility group analysis).

Weather data were recorded; and all raw data were included in the submitted studies with appropriate references of methodology and materials for sample collection and dilution plating onto semi-selective agar media optimized for *A. flavus* recovery. Appropriate statistical tests were performed (Statistica V3.0 and SAS V8.0), using T-tests, correlation analyses, analysis of variance and Tukey's HSD means separation analysis to analyze the data. from treated and untreated sites. Results of statistical analyses were also reported in the submitted documents.

B. Testing to Vegetative Compatibility Groups and Separation into L or S phenotypes

Morphological criteria (based on sclerotial size, cultural characteristics and virulence to cotton) were used to assign *Aspergillus flavus* isolates to either the S or L strains [2]. Isolates which have large sclerotia (> 400 μm diameter) were designated as L strains; a significant percentage of L strains do not produce aflatoxins. Isolates which have smaller sclerotia (< 400 μm diameter) were designated S strains; and typically produce very large quantities of aflatoxins. The MPCA, AF36 is an L-strain and does not produce aflatoxins ("atoxigenic") [2, 6].

All "L" strain isolates were subjected to complementation testing and vegetative compatibility analyses, cited in another publication [1]. Thousands of isolates were characterized from soil samples (10 to 15 isolates from 2 independent soil dilutions from each soil sample); and several of these isolates were analysed for aflatoxin production.

C. Aflatoxin Assays & Methodology to Demonstrate Field/Environmental Stability of AF36.

Three independent studies were initiated to determine the stability of atoxigenecity of AF36. Aflatoxin assay methodology was described in other publications [4, 5].

1. Comparison with L-strain isolates from other cotton production areas. Aflatoxin analyses were performed on several "L" strains collected from commercially produced cottonseed supplied by oil mills from different states: Texas (Sweetwater, Harlingen), Arkansas (Little Rock), Alabama (Montgomery), or Mississippi (Greenville).

2. Comparison with L-strain isolates from non-agricultural natural habitat, Sonoran desert, AZ. Fifty seven L-strain isolates of *A. flavus* were examined independently for aflatoxin-producing ability and by vegetative compatibility analysis for membership in the VCG of AF36.

3. Long-term field stability of AF36 in treated AZ cotton fields. Three to four years following treatment of fields in 1996, *A. flavus* was isolated and subjected to both strain classification (by VCG grouping to identify AF36 and by morphological criteria to separate the S and L strains) and assessment of aflatoxin producing potential. Isolates from five fields were examined, characterized and strains were assayed for aflatoxin levels.

II. RESULTS

A. Population Monitoring

1. Soil:

Complete sets of results were provided for five to eight fields in 3 distinct geographical areas of Arizona. There were other fields that were similarly treated and processed, however, samples may not have been collected every year, and sometimes grower practices and environment sometimes precluded sampling.

In summary, data from many of these samples showed statistically significant changes in the aflatoxin-producing potential of fungi resident in agricultural fields following applications of atoxigenic AF36, relative to fields not receiving AF36 treatment. Treatments were frequently associated with decreases in incidences of highly toxigenic S strains. Furthermore, treatments did not appear to change the overall quantity of *A. flavus* (atoxigenic + toxigenic strains), possibly because of the extreme variability in the plating results, typical for this type of environmental data.

2. Air: Results of these studies showed no significant differences between the two sites in either the mean number of *A. flavus* propagules m^{-3}, nor in total fungal propagules m^{-3}. Population counts of *A. flavus* ranged from <1 to 406 m^{-3} (no atoxigenic application) to <1 to 416 m^{-3} (receiving atoxigenic application). Peaks in *A. flavus* (and total fungal) propagules coincided with boll maturation and cotton harvest within the area. Both S and L strain isolates of *A. flavus* were detected at the treated and untreated sites. Overall, a significantly greater proportion (p= 0.006) of the L strains were observed at the treated site (80.2%) compared to the untreated site (68.5%); although it appeared that the L strain was most abundant from September through December . Greatest quantities of S strain isolates occurred between May and August of both years at the untreated site, when compared to the treated site. Furthermore the proportion of the L strains did not appear to change significantly between 1997 and 1998, which suggests that the effect of treatment did not disappear after a single season.

The applied atoxigenic strain accounted for an average of 38% (range = 5 to 75%) of the L strains recovered from the treated site; whereas it only accounted for an average of 19% (range = 0 to 47%) of the L strains recovered from the untreated site during 1997 through 1998. The high incidence of the applied atoxigenic VCG isolate (47%) recovered from the untreated site suggests dispersal from the site treated in 1996 and 1997. This is a significant finding, since quantities of *A. flavus* propagules sampled at both the treated ($\bar{x}$ = 28.6 m^{-3}) and untreated sites ($\bar{x}$ = 29.7 m^{-3}) were similar throughout the 1997 season, thereby suggesting that application of the atoxigenic isolate may have altered the composition of *A. flavus* in the air, without altering the total quantity (toxigenic + atoxigenic) *A. flavus* recovered. It is commonly accepted that *A. flavus* is aerially dispersed, and can travel several hundred meters, depending on air currents, speed or other biological dispersal agents, e.g., insects.

B . Results of Phenotyping and VCG Analyses , Aflatoxin Assays & Methodology to Demonstrate Field/Environmental Stability of AF36.

Results of the following three studies provide further evidence of the atoxigenic "stability" of AF36, outside of controlled conditions.

1. Comparison with L-strain isolates from other cotton production areas. None of the isolates which belong to the same VCG as AF36 and originating from locations in cotton areas outside of Arizona produced aflatoxins in six tests, using liquid fermentation tests of Bayman and Cotty [5]. Other L-strain isolates belonging to 5 VCGs from diverse states produced varying quantities of aflatoxin. Isolates of one VCG (AF13) consistently produced very high levels of aflatoxins (>300,000 ng per 70 ml fermentation). Isolates which belonged to 2 other VCGs (P19 and G) produced moderate quantities of aflatoxins (28,915 to 207,216 ng per 70 ml fermentation). Isolates of the 5th VCG group in this study (VCG V) had the most variation in aflatoxin levels, 0 to 1,066,991 ng per 70 ml fermentation). In general, members of a VCG tend to produce similar quantities of aflatoxin.

2. Comparison with L-strain isolates from Arizona natural habitat,. Five of 57 isolates from the Sonoran desert were identified as belonging to the AF36 VCG; none produced aflatoxins. Sixteen of the 57 isolates did not produce aflatoxins. Aflatoxin production-producing isolates ranged from 20 to 1,025,529 ng aflatoxin B_1 per 70 ml fermentation.

3. Long-term field stability of AF36 in treated AZ cotton fields. Isolates from five fields were examined in this study. In one field, isolates were collected three years after the last treatment. In no case did an isolate belonging to the AF36 VCG group produce aflatoxin. S strain isolates produced very high levels of aflatoxins; and L strain isolates ranged from non-producers to strains that produced significant quantities of aflatoxins.

III. Study Authors Conclusions: Several conclusive statements were summarized for the submitted studies (paraphrased from Summary) :

a) "[A]pplication of *Aspergillus flavus* AF36 replaces aflatoxin producers with a non-producer without increasing the overall quantity of *A. flavus* in the soil.

b) ...Applications of atoxigenic AF36 induce clear, significant changes in the aflatoxin-producing potential of fungi resident in agricultural fields. The process of increasing the incidence of AF36 greatly decreases the incidences of aflatoxin producing strains of *A. flavus*.

c) ...Results demonstrate that AF36 can be used in commercial agriculture to decrease the burden of aflatoxins within the environment without increasing the overall quantity of *A. flavus* present.

d) ....Results demonstrate the stability of the atoxigenic phenotype among members of the genetic (VCG) group to which AF36 belongs; this holds even among members of that group from diverse geographical locations.

e) ....it is demonstrated that AF36 isolated from treated fields, even fields treated several years earlier, does not produce aflatoxins in tests where S strain isolates from the same fields produce very large quantities of aflatoxins."

IV. EPA Reviewer's Comments:

For the most part, EPA agrees with the scientific conclusions noted by the study author. However, there were no data to support concomitant reduction of the aflatoxin levels in cottonseed from treated fields. The submitted study contains data showing aflatoxin production in field isolates which may, or may not be present in the raw agricultural commodity. The evaluation of soil and air monitoring data, are however, critical for the overall conclusion, that no incremental hazards to nontarget organisms and the environment are anticipated from the intended

use on cotton fields, and that continued seasonal application of AF36 likely induces significant changes in incidence of toxigenic *A. flavus*.

More specifically,

1) There was no information relating aflatoxin analyses as coordinated in the present study to the FDA (AOAC) method of aflatoxin measurement in cottonseed products (AOAC Official Method 980.20). Consequently, EPA agrees that the incidence of toxigenic populations may be reduced (or changed), such that the amount of aflatoxins in cottonseed products (e.g., meal or oil) may be reduced.

2) The study author infers conclusions not necessarily supported by the data. For example, in conclusion "e", the reviewer is left with the impression that the MPCA was isolated..."[A]F36 was isolated from treated fields, even fields treated several years earlier". The conclusion is an overstatement of the current state of the science. At best, it is an inferential statement, deduced from results of thousands of gross morphological comparison and genetic complementation studies. These studies are sufficient to characterize isolates from natural populations as "similar to" AF36, however, they should not be misconstrued to indicate that such isolates are "AF36". More definitive molecular methods should be employed to confirm the authenticity and field stability of "AF36". Atoxigenic *A. flavus* isolates which are vegetatively compatible with AF36 may not necessarily originate from the proliferation, or survival of the applied microbial active ingredient, AF36.

Relevant References Cited (not included in MRID)

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

2. Cotty, 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79:808-814.

3. Cotty, P. 1994a. Comparison of Four Media for the Isolation of Aspergillus flavus group Fungi. Mycopathologia 125:157-162.

4. Cotty, P. 1994b. Influence of field application of atoxigenic strain of Aspergillus flavus on the populations of A. flavus infecting cotton bolls and on aflatoxin content of cottonseed. Phytopathology 84:1270-1277.

5. Cotty, P. J. 1997. Aflatoxin producing potential of communities of *Aspergillus* section Flavi from cotton producing areas in the United States. Mycological Research 101:698-704.

6. Garber, R.K. and Cotty, P.J. 1997. Formation of sclerotia and aflatoxins in developing cotton bolls infected by the S strain of *Aspergillus flavus* and potential for biocontrol with an atoxigenic strain. Phytopathology 87:940-945.

DP BARCODE: D288777

CASE: 062458 DATA PACKAGE RECORD DATE: 03/11/03
SUBMISSION: S600862 BEAN SHEET Page 1 of 1

* * * CASE/SUBMISSION INFORMATION * * *

CASE TYPE: REGISTRATION ACTION: 194 ACTN INI BY AGCY-ADDL REQ
CHEMICALS: 006456 Aspergillus flavus 36 colonized wheat seed 0.0000%

ID#: 071693-R Aspergillus Flavus AF36
COMPANY: 071693 ARIZONA COTTON RESEARCH AND PROTECTION COUNCIL
PRODUCT MANAGER: 90 JANET ANDERSEN 703-308-8128 ROOM: CS1 5TH FL
PM TEAM REVIEWER: SHANAZ BACCHUS 703-308-8097 ROOM: CS1 5TH FL
RECEIVED DATE: 03/11/03 DUE OUT DATE: 09/07/03

* * * DATA PACKAGE INFORMATION * * *

DP BARCODE: 288777 EXPEDITE: N DATE SENT: 03/11/03 DATE RET.: / /
CHEMICAL: 006456 Aspergillus flavus 36 colonized wheat seed
DP TYPE: 001
CSF: Y LABEL: Y

ASSIGNED TO	DATE IN	DATE OUT	ADMIN DUE DATE: 07/29/03
DIV : BPPD	03/11/03	/ /	NEGOT DATE: / /
BRAN: BPPD-IO	03/11/03	/ /	PROJ DATE: / /
SECT: IO	03/11/03	/ /	
REVR : GTOMIMAT	03/11/03	/ /	
CONTR:	/ /	/ /	

* * * DATA REVIEW INSTRUCTIONS * * *

Please review the data submitted to demonstrate the efficacy of AF36 on cotton in AZ and TX The registrant is considering efficacy as displacement of the toxigenic strain of A. flavus by the atoxigenic strain A.flavus AF36. If any deficiencies are noted, please describe what information is required to satisfy the deficiency.
Thanks.
shawn

* * * DATA PACKAGE EVALUATION * * *

No evaluation is written for this data package

* * * ADDITIONAL DATA PACKAGES FOR THIS SUBMISSION * * *

DP BC	BRANCH/SECTION	DATE OUT	DUE BACK	INS	CSF	LABEL

13544

R141754

Chemical: Aspergillus flavus 36 colonized wheat seed

PC Code:
000456
HED File Code: 41300 BPPD Eco Effects
Memo Date: 5/15/2003
File ID: DPD288777
Accession #: *000-00-9002*

HED Records Reference Center
4/13/2007