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

OFFICE OF PREVENTION,
PESTICIDES AND TOXIC
SUBSTANCES

SEP 13 2007

MEMORANDUM

SUBJECT: Review of Product Chemistry and Efficacy Data to satisfy Conditions of Registration for *Aspergillus flavus* AF36 [EPA Reg. No. 71693-1]

TO: Shanaz Bacchus, Regulatory Action Leader
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511P)

FROM: Annabel Waggoner, Environmental Protection Specialist
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511P)

Annabel Waggoner

THROUGH: John L. Kough, Ph.D., Biologist
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511P)

John L. Kough

ACTION REQUESTED: To review product chemistry and efficacy data, submitted by the Arizona Cotton Research and Protection Council, to satisfy conditions of registration for *Aspergillus flavus* AF36 [EPA Reg. No. 71693-1].

CONCLUSION: The methods and data submitted by the registrant demonstrate complete analyses of the five batches required at production. The efficacy data from a large-scale field trial in TX demonstrate an increase of the non-aflatoxin producing strain and a decrease of the aflatoxin producing S- strain after one year post-treatment. However, these tests are not a true measure of the mycotoxin reduction, because these tests are comparing the percentages of displacement, rather than quantitatively measuring the amount of aflatoxins in ppb. However, cotton is not a direct dietary commodity and additional screening methods are utilized to confirm the atoxigenic strain of *A. flavus* AF36 strain before production. Moreover, cotton and its by-products are screened for aflatoxin prior to introduction into the channels of commerce. Therefore, the data submitted are classified as **ACCECTABLE** and the registrant has submitted sufficient data in support of satisfying conditions of registration for *A. flavus* AF36. If more extensive use patterns are sought for treatment of other agricultural terrestrial sites or crops that are a direct food commodity, quantification of aflatoxin levels should be required to demonstrate efficacy.

THIS REVIEW DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

DATA REVIEW RECORD

Active Ingredient:	*Aspergillus flavus* AF36 [EPA Reg. No. 71693-1]
Company Name:	Arizona Cotton Research and Protection Council
ID No:	71693
Chemical Number:	006456
Decision No:	368607
DP Barcode:	330590

MRID No:

468595-01 **Product Analysis, Certification of Limits, Determination of Unintentional Ingredients, Quality Control, Storage Stability and Viability**

468595-02 **Product Performance Data**

BACKGROUND:

Aspergillus flavus AF36 (also referred to as AF36) is a non-aflatoxin-producing and naturally occurring strain that was isolated in Arizona from cottonseed, and is also indigenous to Texas. Some toxigenic straings of *A. flavus* produce mycotoxins, such as aflatoxin, a potent carcinogen produced. AF36 is a specific strain of *A. flavus* and is identified by its lack of aflatoxin production and its unique vegetative compatibility group, which does not allow exchange of genetic material with the aflatoxin-producing strains. Pre-bloom applications of AF36, as sterilized wheat seeds colonized with the AF36 fungus, are expected to displace the aflatoxin-producing strains of *A. flavus* from the cotton crop and fields.

The Arizona Cotton Research and Protection Council submitted data in support for registration of *A. flavus* AF36, as an active ingredient for use in microbial pesticides. In June 2003, the Agency issued a Section 3(c)(7)(C) conditional registration [EPA Reg. No. 71693-1] for use on cotton in Arizona and Texas. Concurrent with the conditional registration, an exemption from the requirement of a tolerance was established for residues of the microbial pesticide *Aspergillus flavus* AF36 in or on cotton and its food/feed commodities in 40 CFR § 180.1206 [68 FR 41541, July 14, 2003]. The Agency previously determined that this registration was in the public interest, and AF36 is not likely to pose any unreasonable risk to health or the environment (EPA, 2003). However, Section 3(c)(7)(C) of FIFRA stipulated that certain conditions apply to *A. flavus* biopesticide, which are outlined below:

Analyses of five batches are required at production and must include data relevant to detection, identification, enumeration and rejection limits of metabolites (including aflatoxin) and potential human pathogens (bacterial and fungal), using quality control and assurance methods for large scale production. Batch analysis must also include:

(i) certifications of limits;
(ii) identification of *A. flavus* AF36 by either DNA analysis or some other method different from the vegetative compatibility method now in use;

(iii) analysis and quantification of metabolites and other unintentional ingredients;
(iv) identification and enumeration of potential human pathogens;
(v) storage stability; and
(vi) viability data.

Efficacy data are also required from a large-scale field trial in TX to demonstrate that *Aspergillus flavus* AF36 reduces aflatoxin-producing strains of *Aspergillus flavus*.

The registrant has submitted the required data and these data are reviewed in this report.

RECOMMENDATION:

The methods and data submitted by the registrant demonstrate complete analyses of the five batches required at production. These data include: detection, identification, enumeration and rejection limits of metabolites (including aflatoxin) and potential human pathogens (bacterial and fungal), as well as using quality control and assurance methods to be used during large scale production. The efficacy data from a large-scale field trial in TX demonstrate an increase of the non-aflatoxin producing strain and a decrease of the aflatoxin producing S- strain after one year post-treatment. These tests are not a true measure of the mycotoxin reduction, because these tests are comparing the percentages of displacement, rather than quantitatively measuring the amount of aflatoxins in ppb. However, the Agency previously determined that the proposed food use pattern for cotton is not likely to result in dietary exposure or residues on food and feed because cotton is not a direct dietary commodity (EPA, 2003). Additional screening methods are utilized to confirm the atoxigenic strain of *A. flavus* AF36 strain before production. Moreover, cotton and its by-products are screened for aflatoxin prior to introduction into the channels of commerce. Therefore, these factors were taken into consideration in the review and negate the need for additional efficacy testing based on the quantification level. If more extensive use patterns are sought for treatment of other agricultural terrestrial sites or crops that are a direct food commodity, quantification of aflatoxin levels should be required to demonstrate efficacy.

Therefore, the data submitted are classified as **ACCECTABLE** and the registrant has submitted sufficient data in support of satisfying conditions of registration for *A. flavus* AF36. The Agency previously determined that this registration was in the public interest, and AF36 is not likely to pose any unreasonable risk to health or the environment (EPA, 2003). This includes all anticipated dietary exposures and all other exposures for which there is reliable information.

SUMMARY OF DATA SUBMITTED:

MRID No. 468595-01: Review of Product Analysis, Certification of Limits, Determination of Unintentional Ingredients, Quality Control, Storage Stability and Viability

Several procedures were performed on samples from five production batches of *Aspergillus flavus* AF36. The average spore counts in samples from the batches ranged from approximately 3.6×10^9 to 7.4×10^9 spores/gram product. Two pyrosequencing assays were used to differentiate AF36 from other *A. flavus* strains. The pksA assay is based on the quantification of a single nucleotide polymorphism (SNP) that is responsible for the failure of AF36 to produce a functional polyketide synthase The aflR assay is based on identifying a SNP in the gene coding for the aflatoxin

pathway-specific transcription factor, aflR. Two additional sequencing methods that can be used to identify AF36 involve the identification of SNPs in gene segments that are unique to AF36. AF36 is also distinguishable from other atoxigenic *A. flavus* strains by its ability to convert aflatoxin precursors to aflatoxin. No aflatoxins were detected in production batch samples analyzed by HPLC. One percent of one batch had bacterial contamination; however, enterobacteria were not detected in any samples. The analytical results of three storage/viability tests in which product batches were stored under warehouse conditions for greater than 12 months indicated spore counts of greater than 2×10^9 spores/gram.

As stated in the Biopesticides Registration Action Document for *A. flavus* AF36 in the Product Identity and Manufacturing Data sections, the identification of AF36 (non-aflatoxin-producing strain) is verified on the basis of vegetative compatibility. Starter cultures are monitored for aflatoxin production by standard thin layer chromatography (tlc) procedures and visualization via scanning fluorescence densitometry [MRID 44626101; BPPD Review, March 29, 1999]. One of the conditions of registration for *Aspergillus flavus* AF36 stipulates that *A. flavus* AF36 should be identified by either DNA analysis or some other method (different from the vegetative compatibility method now in use). Since these methods and data were found acceptable, future identification methods should utilize the methods presented in this report, which include: pksA and aflR pyrosequencing assays, in addition to vegetative compatibility as described in the initial production methods.

CLASSIFICATION: ACCEPTABLE

MRID No. 468595-02: Review of Product Performance Data

Product efficacy tests were conducted at five sites in Texas by evaluating the ability of AF36 to displace other *A. flavus* strains. One year after application, the percent incidence of AF36 on treated cottonseed crops ranged from 80% to 100%; the percent incidence of the high aflatoxin-producing S strain ranged from 0% to 11%; the S strain was detected in 0/4, 2/5, 2/2, 3/30 and 0/9 cottonseed samples at the five sites, respectively. For the five sites, the average percent incidence of AF36 in soil prior to treatment compared with one year post-treatment was 4%/55%, 1%/73%, 1%/56%, 0%/67%, and 1%/60%, respectively. Data available for four of the sites indicate the average percent incidence of the S strain in soil prior to treatment compared with one year post-treatment as 58%/11%, 43%/6%, 43%/14%, and 0%/7%, respectively. These data demonstrate an increase of the non-aflatoxin producing strain and a decrease of the aflatoxin producing S- strain after one year post-treatment. These tests are not a true measure of the mycotoxin reduction, because these tests are comparing the percentages of displacement, rather than quantitatively measuring the amount of aflatoxins in ppb.

However, the Agency previously determined that the proposed food use pattern for cotton is not likely to result in dietary exposure or residues on food and feed (EPA, 2003). This is because cotton is not a direct dietary commodity. If AF36 can be found on cotton seed, residues are not likely to survive the heating and pressure associated with the processing of cottonseed into cottonseed meal. Moreover, AF36 will not separate into the edible fraction, cottonseed oil. Thus, potential transfer of residues of AF36 to edible cotton food/feed commodities is not expected.

Consequently, human dietary exposure to AF36 via cottonseed oil, or by secondary transfer of AF36 residues to meat and milk via cottonseed meal, is unlikely to be above naturally occurring background levels.

It should also be noted that additional precautions have been implemented in the manufacturing process of AF36 because aflatoxin is considered a public health hazard. These include: starter cultures of AF36 are screened by thin layer chromatography and scanning fluorescence densitometry for lack of aflatoxin, as well as pksA and aflR pyrosequencing assays to confirm the culture as the atoxigenic AF36 strain. Even if AF36 does not control aflatoxin levels in the treated cotton food/feed commodities, cotton and its by-products are screened for aflatoxin prior to their introduction into the channels of commerce and are regulated by the FDA. Therefore, these factors were taken into consideration in the review of the data submitted and negate the need for additional efficacy testing based on the quantification level. However, if more extensive use patterns are sought for treatment of other agricultural terrestrial sites or crops that are a direct food commodity, quantification of aflatoxin levels should be required to demonstrate efficacy.

CLASSIFICATION: ACCEPTABLE

REFERENCE:

EPA, 2003. Biopesticide Registration Action Document (BRAD) for *Aspergillus flavus*, strain AF36, PC Code: 006456, dated July 3, 2003.

DATA EVALUATION RECORD

Primary Reviewer: Isabel Mandelbaum, Ph.D., DABT, Tetrahedron
EPA Secondary Reviewer: Annabel Waggoner, Environmental Protection Specialist AW

STUDY TYPES:	Product Identification (OPPTS 885.1100), Discussion of Formation of Unintentional Ingredients (OPPTS 885.1300), Analysis of Samples (OPPTS 885.1400), Certification of Limits (OPPTS 885.1500), Storage Stability and Viability (OPPTS 885.2400)
MRID NO:	468595-01
DECISION NO:	368607
DP BARCODE:	330590
TEST MATERIAL:	*Aspergillus flavus* AF36 [EPA Reg. No. 71693-1]
PROJECT NO.:	IRA PR No. 0052B
SPONSOR:	Arizona Cotton Research and Protection Council 3721 E. Wier Avenue Phoenix, AZ 85040
TESTING FACILITY:	USDA-ARS, Forbes 204, 1140 East Campus Drive Tucson, AZ 85721
AUTHOR:	Dr. Peter Cotty
STUDY COMPLETED:	June 9, 2006
GOOD LABORATORY PRACTICE:	Not GLP Compliant- but based on accepted scientific and/or commercial practice and are considered by the applicant to be valid.

CONCLUSION: Several procedures were performed on samples from five production batches of *Aspergillus flavus* AF36. The average spore counts in samples from the batches ranged from approximately 3.6×10^9 to 7.4×10^9 spores/gram product. Two pyrosequencing assays were used to differentiate AF36 from other *A. flavus* strains. The pksA assay is based on the quantification of a single nucleotide polymorphism (SNP) that is responsible for the failure of AF36 to produce a functional polyketide synthase The aflR assay is based on identifying a SNP in the gene coding for the aflatoxin pathway-specific transcription factor, aflR. Two additional sequencing methods that can be used to identify AF36 involve the identification of SNPs in gene segments that are

unique to AF36. AF36 is also distinguishable from other atoxigenic *A. flavus* strains by its ability to convert aflatoxin precursors to aflatoxin. No aflatoxins were detected in production batch samples analyzed by HPLC. One percent of one batch had bacterial contamination; however, enteric bacteria were not detected in any samples. The analytical results of three storage/viability tests in which product batches were stored under warehouse conditions for greater than 12 months indicated spore counts of greater than 2×10^9 spores/gram.

CLASSIFICATION: ACCEPTABLE

I. INTRODUCTION:

The information/data in this report was based on five consecutive product batches produced at the USDA-ACRPC manufacturing facility in Phoenix, AZ on December 6, 7, 9, 13, and 15, 2004, respectively. The data submitted in support of each data requirement in fulfillment of the conditions of registration are summarized under each heading.

II. CERTIFICATION OF LIMITS (OPPTS 885.1500):

A. Methods:

Five product batches were analyzed for spore counts according to the "Quality Control Plan" (not provided). Four replicates were analyzed for each batch, and the range and average spores/gram were calculated (see Table 1).

B. Results:

Table 1. Batch Identification of *Aspergillus flavus* AF36 with Range and Average Spore Count

Batch Number	Range of Spore Counts (spores/gram)	Average Spore Count (spores/gram)
041206B	$3.3 \times 10^9 - 4.1 \times 10^9$	3.6×10^9
041207B	$5.2 \times 10^9 - 6.1 \times 10^9$	5.7×10^9
041209A	$6.1 \times 10^9 - 7.2 \times 10^9$	6.7×10^9
041213A	$7.2 \times 10^9 - 7.7 \times 10^9$	7.4×10^9
041215B	$6.7 \times 10^9 - 8.0 \times 10^9$	7.2×10^9

C. Reviewer's Comment:

The spore count data were submitted to satisfy the requirement of "certification of limits"; however the accompanying text does not identify the certified limits. The only identified certified limits available to the reviewer were those listed on a Confidential Statement of Formula (CSF) dated August 8, 2002 and which was not part of the current submission. On the CSF, the upper and lower certified limits of the active ingredient (*Aspergillus flavus* AF36) are listed as 0.0009% and 0.0007% by weight, respectively. The CSF indicates that the active ingredient will produce at least 2.5×10^9 spores/gram. The spore counts from the batch analyses are much higher than the minimum of 2.5×10^9 gram listed on the CSF. Therefore, the upper and lower limits for the active ingredient are beyond the acceptable certified limits. However, the Agency recognizes it is not uncommon for the certification limits of the a.i. to exceed the EPA

recommended certified limit ranges. This is due to the variability in the production of biological products active components.

Moreover, the Agency has previously accepted wider ranges of certified limits for other products and recognizes the variability in the production of active components in biological products, so as to promote better product performance and application. Therefore, the registrant's results of the five batch analysis are adequate.

III. DISCUSSION OF FORMATION OF UNINTENTIONAL INGREDIENTS (OPPTS 885.1300)

1. Analysis and Quantification of Unintentional Ingredients and Aflatoxin analysis by High Performance Liquid Chromotography (HPLC):

A. Methods:

An independent laboratory (Romer™ Labs, Inc., Union, MO) analyzed the five batches for aflatoxins (B1, B2, G1, G2) as well as two control samples by HPLC (see Table 2). Although the methods were not submitted in this report, the registrant later submitted the HPLC protocol [Kobra Cell Method for Aflatoxin by HPLC, code: afl-lc-01-00.1] *via* email on June 11, 2007 from M. Braverman of IR-4 to S. Bacchus of EPA/BPPD, which are summarized below:

The solid samples were extracted by filtering 25 g of the sample from each batch with 100 ml of 84/16 acetonitrile/water that was previously mixed for two minutes. The standard was prepared by mixing 100 µl of the aflatoxin working standard (5 µg/ml total aflatoxins: 2 µg/ml each of B1 and G1 and 0.5 µg/ml each of B2 and G2) to 50 ml of 84/16 acetonitrile/water, followed by transfer to an autosampler vial with 440 µl of deionized water, and a 100 µl injection into the HPLC. These standards were used to calibrate the HPLC and spikes were prepared to measure the percent recovery and to check the accuracy of the run. Spike recovery of 100% resulted in a total concentration of 40 ppb of aflatoxin: 16 ppb each of B1/G1 and 4 ppb each of B2/G2 injected into the HPLC. The percent recovery for B1, B2, G1, and G2 aflatoxin were 98.0%, 94.3%, 109.1%, and 104.4%, respectively. The samples were also purified by pushing 500 µL of each sample through a MycoSep® clean-up column. The standards, spikes, samples and quality controls (100µl) were injected into the HPLC in a specific sequence. The detection limit for all aflatoxins is 1 ppb.

B. Results:

Table 2. Five batch Analysis for Types of Aflatoxin Content by HPLC conducted by Romer™ Labs, Inc.

Test Description	LOD (ppb)	Batch Identification							
		041206B	C	041207B	041213A	NI	041209A	C	041215B
Aflatoxin B1	1.0	ND	ND	ND	ND	ND	ND	ND	ND
Aflatoxin B2	1.0	ND	ND	ND	ND	ND	ND	ND	ND
Aflatoxin G1	1.0	ND	ND	ND	ND	ND	ND	ND	ND
Aflatoxin G2	1.0	ND	ND	ND	ND	ND	ND	ND	ND

LOD= Detection Limit
C= control
ND= None Detected
NI= Not Identified

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C. Reviewer's Comment:
The results of the independent laboratory testing for aflatoxin content by HPLC indicated no aflatoxin in the product and no aflatoxin in two control samples of raw wheat which was the starting material from which *Aspergillus flavus* AF36 product is manufactured. However, only seven of the samples were identified by the registrant, whereas, the independent laboratory tested eight samples. Although there was no detection of aflatoxin in the unidentified sample, this sample should be identified. It is also noted that the DNA sequencing techniques used to identify AF36 (see Section IV) also demonstrated the lack of aflatoxin detection in the five batches submitted for analyses.

2. Identification and Enumeration of Potential Human Pathogens:

A. Methods:
The five batches were also analyzed for other quality parameters (such as moisture content, % seed with bacteria, % seed from which AF36 grew, % seed with growth of enterobacteria). The method(s) used to determine microbial contamination or to identify enterobacteria were not provided in this study.

B. Results:

Table 3. Other Quality Parameters of *Aspergillus flavus* AF36 Batches

Batch Number	Moisture Content	% Seed with Bacteria	% Seed from which AF36 grew	% Seed with growth of Enterobacteria
041206B	5.6%	0	100%	0
041207B	4.0%	0	100%	0
041209A	4.5%	0	100%	0
041213A	3.8%	0	100%	0
041215B	4.6%	1%	100%	0

One percent (1%) of the seed of one batch (Batch 041215B) was reported to have bacterial contamination; however, this level is lower than the stated limit to indicate contamination, which are <6 of 50 seeds of bacteria (12%) and <2 of 50 seeds (2%) with coliform. Moreover, enterobacteria were not detected and no microbial contamination was found in the other four batch samples.

C. Reviewer's Comment:
The method(s) used to determine microbial contamination or to identify enterobacteria should be provided in this report, however, quality control procedures for microbial contaminates in the end-use product are described in another report (MRID No. 446261-01). These methods were found acceptable (see memorandum- from M. Watson, Ph.D., through J. Kough, Ph.D., to S. Bacchus, dated March 29, 1999). Therefore, the data and the previously accepted study are sufficient to demonstrate the lack of microbial contamination or coliforms for this study.

IV. PRODUCT IDENTIFICATION and ANALYSIS OF SAMPLES (OPPTS 885.1100 and 1400):

1. Identification of *A. flavus* AF36 by DNA analysis

A. Methods:

Two pyrosequencing assays were performed to confirm that the wheat seed samples from the five batch analyses were colonized by the appropriate *A. flavus* AF36 strain. The first pyrosequencing assay (pksA assay) was based on the quantification of a single nucleotide polymorphism (SNP) near the beginning of the coding sequence for the polyketide synthase gene. This gene is required for aflatoxin synthesis. The coding change introduces a premature stop codon into the coding sequence, preventing enzyme production and aflatoxin accumulation. It has previously been demonstrated that the isolate of *Aspergillus flavus* (AF36) used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase gene (Ehrlich and Cotty, 2004). All members of the vegetative compatibility group to which AF36 belongs, do not produce aflatoxin and have been examined to contain this polymorphism.

The second pyrosequencing assay (aflR assay) was based on identifying a SNP in the gene coding for the aflatoxin pathway specific transcription factor, aflR. Aflatoxin production requires the expression of this biosynthetic pathway regulatory gene. It has previously been demonstrated that sequence comparisons from different *Aspergillus* species via the aflR assay provides evidence for variability in regulation of aflatoxin production. (Ehrlich, Montalbano, and Cotty, 2003).

To isolate DNA, approx. 10 grams of wheat seeds that had been inoculated with strain AF36 were ground to a powder. DNA was isolated from a 20 mg powder sample using DNeasy® Plant Mini kit (Qiagen, Valencia CA), with a minor modification. The DNA was measured using a Bio-Rad SmartSpec™ 3000 spectrophotometer.

Nested polymerase chain reaction (PCR) was conducted for both assays, by first amplifying a 400 to 450 base pair gene segment and then amplifying a smaller 95 to 150 base pair segment from the first amplicon. A HotMaster PCR kit from Eppendorf AG (Hamburg, Germany) was used to amplify the outer segments. To amplify the inner segments, the PCR products were diluted 10x, then amplified as for the outer segments.

In pyrosequencing, a sequencing primer is first hybridized to a single stranded, PCR-amplified DNA template. A deoxynucleotide triphosphate (dNTP) is then added. The dNTP is incorporated into the DNA strand if it is complementary to the base in the template, releasing equimolar pyrophosphate (PPi). The PPi is converted to ATP, which drives the conversion of luciferin to oxyluciferin, generating proportional amounts of visible light. The light is detected by a charge coupled device (CCD) camera, and appears as a peak in a pyrogram™. Unincorporated dNTPs and excess ATP are degraded by apyrase. dNTPs are added one at a time; as the complementary DNA strand is built up, the nucleotide sequence is determined from the signal peak in the pyrogram.

B. Results:
The results of the pksA assay and the aflR assay demonstrated that 100% of the DNA isolated from the five batch samples was from AF36 (0% was from other *A. flavus*) (see Table 4). For the control (raw wheat), it was determined that 0% of the DNA was from AF36 (100% was from other *A. flavus*).

Table 4. The percent of *A. flavus* DNA extracted from the Five Batch Analysis as Identified by the aflR P and the pks Pyrosequencing Assays.

Batch Number	Sample Type	Code for Batch	pksA Assay		aflR Assay	
			% of DNA from AF36	% of DNA from other *A. flavus*	% of DNA from AF36	% of DNA from other *A. flavus*
041206B	AF36 product	A-121	100	0	100	0
041207B	AF36 product	C-341	100	0	100	0
041209A	AF36 product	H-891	100	0	100	0
041213A	AF36 product	F-671	100	0	100	0
041215B	AF36 product	D-451	100	0	100	0
N/A	Raw wheat-Control	B-231	0	100	0	100

Using both the pksA assay and the aflR assay, it was determined that 100% of the DNA isolated from the five batch samples was from AF36 (0% was from other *A. flavus*). For the control (raw wheat), it was determined that 0% of the DNA was from AF36 (100% was from other *A. flavus*).

2. Identification of *A. flavus* AF36 by additional sequencing methods

A. Methods:
Two additional sequencing methods that can also be used to identify AF36 were presented. AF36 can be identified by SNPs in two sequences of gene segments. The first segment (ver1-Segment1) is a portion of the intergenic space between genes ver1 and norA. This region contains two SNPs that are unique to AF36. This gene region was sequenced for 9 field isolates of AF36 and 55 isolates of other *A. flavus* (determined by vegetative compatibility analysis). The DNA sequence of the entire region amplified by the PCR protocol is indicated in Figure 1. The second segment (ordA-Segment 1) extends from the promotor region of ordA into the coding region. This gene region contains one SNP that is unique to AF36. This gene region was sequenced for10 field isolates of AF36 and 51 isolates of other *A. flavus* (determined by vegetative compatibility analysis). The DNA sequence of the entire region amplified by the PCR protocol is indicated in Figure 2. In both procedures the DNA is extracted, PCR performed, and the amplicon submitted to a commercial laboratory for clean-up and sequencing.

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Figure 1. The DNA sequence of the region containing ver1-Segment1, amplified by PCR.

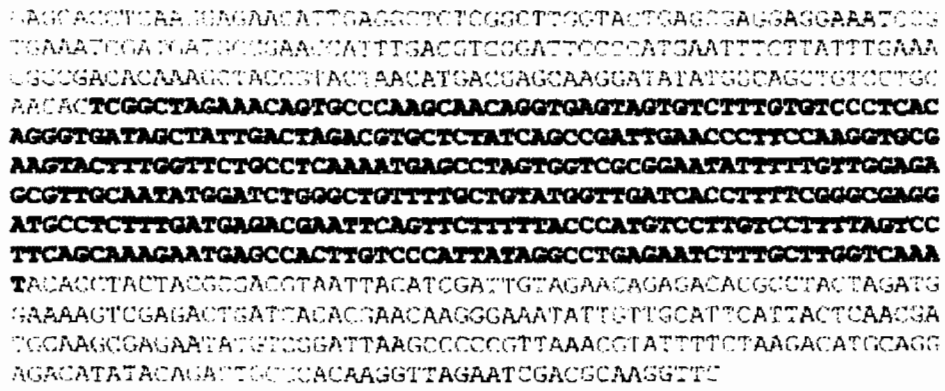
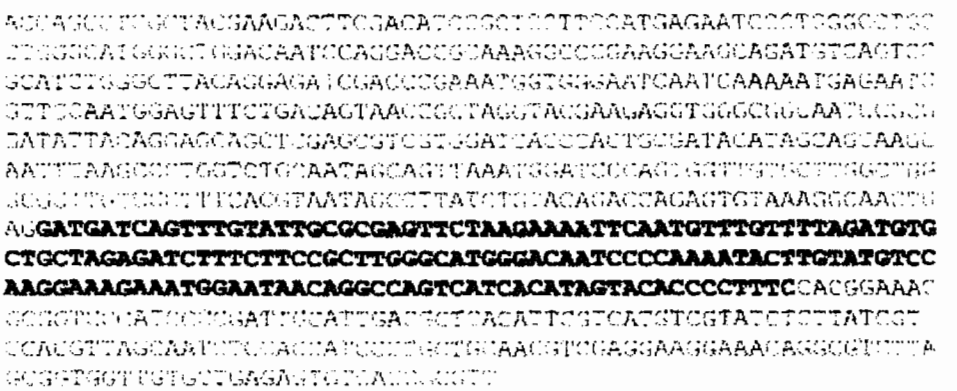


Figure 2. The DNA sequence of region containing ordA-Segment 1, amplified by PCR.



B. Results:

The portions of the sequences contained in the appendices are also indicated in Figures 1 and 2 by shading and bold letters. The multiple sequence alignments of ver1 and ordA containing the polymorphisms for differentiating AF36 are presented in Appendices A and B, respectively. In the ver1 sequence, the SNPs unique to AF36 are thymine instead of cytosine (located at base number 95) and adenine instead of thymine (located at base number 267). In the ordA sequence, the SNP unique to AF36 is thymine instead of guanine or adenine (located at base number 112).

It has also been previously demonstrated that another characteristic of AF36 is that it can convert early aflatoxin precursors to aflatoxin, although it cannot produce aflatoxins (Cotty and Bhatnagar, 1994). This distinguishes AF36 from other atoxigenic strains.

C. Reviewer's Comment:

The DNA sequencing methods presented in this report (for verifying that wheat seed samples from the lots of AF36 product used for the five batch analyses was colonized by the intended *A. flavus*, strain AF36) are accurate and precise methods to screen for the genetic lesions in AF36

and complement the vegetative compatibility analysis. It is recommended that the DNA sequencing assays be utilized in future identification of *A. flavus* AF36 and perhaps to screen cultures being stored as seed. It should also be noted that pages 19 and 20 of the report are duplications of pages 17 and 18.

V. STORAGE STABILITY AND VIABILITY DATA (OPPTS 885.2400):

A. Methods:

Three tests were conducted for storage stability using the primary storage container, Buckhorn Flow Bulk Containers. The products were stored in a warehouse adjacent to the manufacturing facility.

For all tests, spore yield was determined for each sample by placing one seed into each well of a 24-well cell culture plate. Approximately 10 mL of water was added to the spaces between the plates, and the plates covered and placed inside sealed plastic containers. The plates were incubated at 31° C for 7 days. Spore yield was measured for three sets of two seeds each, one set from the center four wells, two sets from wells along the edges of the plate. Seeds were rinsed 3x with 100% ethanol. The washes from two similarly located seeds were combined for a volume of 11 mL. The suspension was diluted 50% with distilled de-ionized water and poured into a turbidimeter vial. The turbidity of the suspension was measured in NTU (Nephelometric Turbidity Unit) and the concentration of spores in the suspension was extrapolated from an NTU versus CFU (colony forming unit) standard curve.

Test 1

Test 1 was conducted on samples from 2,114 lb of Batch 041209C, stored on December 9, 2004. The product was sampled periodically over a period of close to 500 days. The results were graphed as spore counts (spores/gram x 10^9) versus day after manufacture. The graph shows one data point per sampling event prior to 300 days (10 events); thereafter 2 data points were graphed per sampling event (6 events). Two samples (200g each) were taken from the center of the storage container; five samples were taken from 6 inches below the surface with a trowel.

Test 2

Test 2 was conducted on samples from all five batches: 041206B (2,134 lb), 041207B (1,866 lb), 041209A (1,885 lbs), 041213A (1,907 lbs), 041215B (2,085 lb). The batches were sampled five times over a period of 449 days. The results were graphed as spore counts versus day of the year. Two samples (200g each) were taken from the center of the storage container; one sample was taken from the surface with a trowel, and one sample was taken from 2 ft beneath the surface with a grain sampling probe.

Test 3

The third test involved a retrospective analysis of previously sampled batches that were stored for varying periods of time: 1-3 months (5 batches), 4-6 months (12 batches), 10-12 months (4 batches), 13-15 months (6 batches), and 16-18 months (7 batches). The samples from a total of 34 batches were collected as per Test 2.

B. Results:

Test 1
During the course of the test, the spore counts ranged from approximately 5 x 10^9 to slightly greater than 8 x 10^9 spores/gram (actual counts not provided in text). Yields were stated to have exceeded minimum spore yield requirements throughout the test (the CSF indicates that the active ingredient will produce at least 2.5 x 10^9spores/gram).

Test 2
During the first 235 days of storage, the average spore yields (2.99 x 10^9 to 3.56 x 10^9 spores/gram) from all five batches were stated to be similar to those determined immediately after manufacture (at least 2.5 x 10^9spores/gram). At 449 days, the range of average counts decreased to 1.98 x 10^9 to 2.91 x 10^9 spores/gram. These spore yields were still considered to be sufficient to be effective.

Test 3
The results were presented as a bar graph (spore counts versus age of product at time of assay). The average counts in spores/gram, estimated from the graph by the reviewer, were approximately 3.1 x 10^9 after 1-3 months, 2.8 x 10^9 after 4-6 months, 2.7 x 10^9 after 10-12 months, 2.4 x 10^9 after 13-15 months, and 2.9 x 10^9 after 16-18 months.

C. Reviewer's Comment:
The method of calculation of the graphed data, assumed by the reviewer to be an average, was not provided.

The results presented above in Section V were also submitted to support the viability requirement. In addition, a copy of an article was included to further support the viability and stability requirements. The article presents data indicating that the dry biopesticide had multi-year stability without refrigeration (Bock and Cotty, 1999).

VI. CONCLUSION:

The methods and data submitted by the registrant demonstrate complete analyses of the five batches required at production. These data include: detection, identification, enumeration and rejection limits of metabolites (including aflatoxin) and potential human pathogens (bacterial and fungal), as well as using quality control and assurance methods to be used during large scale production.

VII. CLASSIFICATION:

This study packet is classified as **ACCEPTABLE.** The registrant has satisfied the remaining data gaps identified in the following study guidelines: Product Identification (OPPTS 885.1100), Discussion of Formation of Unintentional Ingredients (OPPTS 885.1300), Analysis of Samples (OPPTS 885.1400), Certification of Limits (OPPTS 885.1500), and Storage Stability and Viability (OPPTS 885.2400).

VIII. REFERENCES:

Bock, C.H. and P.J. Cotty. 1999. Wheat seed colonized with atoxigenic *Aspergillus flavus*: characterization and production of a biopesticide for aflatoxin control. Biocontrol Science and Technology 9:529-543.

Cotty, P.C. and D. Bhatnagar. 1994. Variability among atoxigenic *Aspergillus flavus* strains in ability to prevent aflatoxin contamination and production of aflatoxin biosynthetic pathway enzymes. Applied and Environmental Microbiology, 60(7): 2248-2251

Ehrlich, K.C. and P.J. Cotty, 2004. Appl. Microbiol. Biotechnol. 65:473-478

Ehrlich, K.C., Montalbano, B.G., and P.J. Cotty, 2003. Fungal Genetics and Biology 38:63-74.

DATA EVALUATION RECORD

Primary Reviewer: Isabel Mandelbaum, Ph.D., DABT, Tetrahedron
EPA Secondary Reviewer: Annabel Waggoner, Environmental Protection Specialist AW

STUDY TYPE:	Product Performance (OPPTS 810.1000)
MRID NO:	468595-02
DECISION NO:	368607
DP BARCODE:	330590
TEST MATERIAL:	*Aspergillus flavus* AF36 [EPA Reg. No. 71693-1]
PROJECT NO.:	IRA PR No. 0052B
SPONSOR:	Arizona Cotton Research and Protection Council 3721 E. Wier Avenue Phoenix, AZ 85040
TESTING FACILITY:	USDA-ARS, Forbes 204, 1140 East Campus Drive Tucson, AZ 85721
AUTHOR:	Dr. Peter Cotty
STUDY COMPLETED:	June 9, 2006
GOOD LABORATORY PRACTICE:	Not GLP Compliant- but based on accepted scientific and/or commercial practice and are considered by the study author to be valid.

CONCLUSION: Product efficacy tests were conducted at five sites in Texas by evaluating the ability of AF36 to displace other *A. flavus* strains. One year after application, the percent incidence of AF36 on treated cottonseed crops ranged from 80% to 100%; the percent incidence of the high aflatoxin-producing S strain ranged from 0% to 11%; the S strain was detected in 0/4, 2/5, 2/2, 3/30 and 0/9 cottonseed samples at the five sites, respectively. For the five sites, the average percent incidence of AF36 in soil prior to treatment compared with one year post-treatment was 4%/55%, 1%/73%, 1%/56%, 0%/67%, and 1%/60%, respectively. Data available for four of the sites indicate the average percent incidence of the S strain in soil prior to treatment compared with one year post-treatment as 58%/11%, 43%/6%, 43%/14%, and 0%/7%, respectively.

CLASSIFICATION: ACCEPTABLE

I. STUDY DESIGN:

Efficacy tests were performed from 2003 to 2005 in commercial cotton fields in south and southeast Texas at the following locations: Edroy, Port Lavaca, Gregory, Ganado, and Hidalgo. AF36 was applied according to label directions. Efficacy was evaluated as the ability of *Aspergillus flavus* AF36 to displace aflatoxin producers.

II. METHODS:

Crop samples were collected at all five sites one year post-treatment. Samples were collected soon after ginning by the commercial gins or by the VALCO oil mill in Harlington upon receipt of the seed from the gin. Gins were selected by the farmers. Analyses were performed on 12-15 fungal isolates from each cottonseed sample. Samples for all sites were analyzed for AF36 and the high aflatoxin-producing S strain.

Soil samples also were collected for analyses. At all five sites, soil samples were collected before application and one year post-treatment. At some sites, soil samples also were collected two years post-treatment. The presence of AF36 was determined by vegetative compatibility analyses. For four of the sites, soil samples were analyzed for the atoxigenic AF36 strain and the S strain. For one site, only AF36 was analyzed. Each sample consisted of 100 to 150 grams of soil and was composed of 40 to 50 subsamples collected from the top 2 cm of soil along a 30-40 meter transect. Sample replicates were collected from different locations within the same field. For each replicate, the same region was sampled each year.

Although only one toxigenic strain of *Aspergillus flavus* was analyzed (i.e., S strain), the majority of other *A. flavus* strains are toxigenic. Therefore, an increase in the incidence of AF36 makes crops less vulnerable to contamination by other toxigenic *A. flavus* strains.

III. RESULTS:

1) Edroy Treatment Area

Ten fields were used in the study. All treated fields and non-treated Fields 3 and 10 were irrigated. Fields 8 and 9 were not irrigated. The total area treated in 2003 was 482 acres. The total area treated in 2004 and the total untreated area was not provided. Six crop samples were analyzed in 2004, one year following treatment in 2003. The samples were from different fields or trucks of cottonseed.

The results of the soil and crop analyses are summarized in Table 1. The results of the soil analyses, based on the average of 4 replicates, are summarized in Table 2. The average incidence of AF36 in the Edroy soils prior to treatment in 2003 was 4%. The average soil incidence of AF36 one year after treatment (2004) was 55%. The incidence of the S strain decreased from an average of 58% prior to treatment in 2003 to 11% one year after treatment (2004).

Table 1. Sample Test Results for Edroy Treatment Area		
Soil Samples	**% AF36**	**% S Strain**
Prior to treatment 2003	4%	55%
After treatment 2004	58%	11%
Cottonseed Samples	**% AF36 in 2004**	**% S Strain in 2004**
Treated	100%	0%
Treated	100%	0%
Treated	93%	0%
Treated	93%	0%
Untreated	50%	0%
Untreated	38%	15%

Table 2. Soil Test Results for Edroy Treatment Area				
Field Number/ Treatment	**Average % AF36 in 2003**	**Average % AF36 in 2004 and 2005**	**Average % S Strain in 2003**	**Average % S Strain in 2004 and 2005**
Field 1 (treated in 2003 and 2004)	9%	31% (2004) 59% (2005)	3%	3% (2004) 3% (2005)
Field 2 (treated in 2003)	22%	50% (2004) No data (2005)	5%	2% (2004) No data (2005)
Field 3 (untreated)	0%	12% (2004) No data (2005)	21%	9% (2004) No data (2005)
Field 4 (treated in 2003)	2%	82% (2004) 78% (2005)	26%	2% (2004) 7% (2005)
Field 5 (treated in 2003)	5%	69% (2004) 78% (2005)	15%	2% (2004) 7% (2005)
Field 6 (treated in 2004)	No data	18% (2004) 96% (2005)	No data	30% (2004) 0% (2005)
Field 7 (treated in 2004)	0%	3% (2004) 76% (2005)	32%	12% (2004) 5% (2005)
Field 8 (untreated)	2%	13% (2004) 14% (2005)	5%	19% (2004) 13% (2005)
Field 9 (untreated)	No data	2% (2004) 31% (2005)	No data	15% (2004) 22% (2005)
Field 10 (untreated)	0%	10% (2004) No data (2005)	21%	10% (2004) No data (2005)

2) Port Lavaca Treatment Area

Seven fields were used in the study. The total treated area was 295 acres. The total untreated area was not provided. Crops were not irrigated during the test period. Five crop samples were analyzed in 2004, one year following treatment in 2003. The samples were from different fields or truckloads of seed.

The results of the crop analyses are summarized in Table 3. The results of the soil analyses, based on the average of 4 replicates, are summarized in Table 4. Prior to treatment, the average incidence of AF36 in Port Lavaca soil was 1%. One year following treatment, the average incidence was 73%. The average incidence of the S strain in soil decreased from 43% prior to treatment to 6% one year post-treatment.

Table 3. Cottonseed Sample Test Results for Port Lavaca Treatment Area

Treatment	% AF36 in 2004	% S Strain in 2004
Treated in 2003	80%	7%
Treated in 2003	87%	7%
Treated in 2003	100%	0%
Treated in 2003	100%	0%

Table 4. Soil Test Results for Port Lavaca Treatment Area

Field Number	Average % AF36 in 2003	Average % AF36 in 2004	Average % S Strain in 2003	Average % S Strain in 2004
PL1 (treated)	2%	45%	20%	9%
PL2 (treated)	0%	78%	52%	8%
PL3 (untreated)	0%	3%	20%	19%
PL4 (treated)	0%	95%	58%	0%
PL5 (untreated)	0%	24%	28%	36%
PL6 (untreated)	9%	9%	48%	38%
PL7 (untreated)	0%	35%	43%	27%

3) Gregory Treatment Area

Three fields were used in the study. The total treated area was 418 acres. The total untreated area was not provided. Crops were not irrigated during the test period. Four crop samples were analyzed in 2004, one year following treatment in 2003. The two treated crop samples were from different sides of the same field.

The results of the crop analyses are summarized in Table 5. The results of the soil analyses, based on the average of 4 replicates, are summarized in Table 6.

Table 5. Cottonseed Sample Test Results for Gregory Treatment Area

Sample Treatment	% AF36 in 2004	% S Strain in 2004
Treated in 2003	89%	11%
Treated in 2003	87%	4%
Untreated (from field adjacent to treated crop)	71%	11%
Untreated (from field adjacent to treated crop)	62%	9%

Table 6. Soil Test Results for Gregory Treatment Area

Field Number	Average % AF36 in 2003	Average % AF36 in 2004 and 2005	Average % S Strain in 2003	Average % S Strain in 2004 and 2005
Greg1 (treated)	1%	56% (2004) 76% (2005)	43%	14% (2004) 13% (2005)
Greg2 (untreated)	5%	26% (2004) 12% (2005)	33%	20% (2004) 43% (2005)
Greg3 (untreated)	2%	52% (2004) 44% (2005)	60%	9% (2004) 34% (2005)

4) <u>Ganado Treatment Area</u>

Sixteen fields appear to have been used in the study. The total treated area was 818 acres. The total untreated area was not provided. Crops were not irrigated during the test period. Thirty crop samples from fields treated in 2004 were analyzed one year post-treatment (2005). Each sample was taken at the gin from a separate module from one of the fields.

The incidence of AF36 was 100% for 15 of the samples and greater than 90% for 15% of the samples. The S strain was detected in 3 of the treated crop samples at less than 10%. (Note: results were presented as a bar graph only; percentages could only be estimated from the graph by the reviewer). The results of the soil analyses are summarized in Table 7.

Table 7. Soil Test Results for Ganado Treatment Area				
Field Number/ Treatment	**% AF36 in 2004**	**% AF36 in 2005**	**% S Strain in 2004**	**% S Strain in 2005**
Field G-1 (treated)	0%	67%	0%	7%
Field G-2 (untreated)	0%	40%	20%	0%
Field G-3 (untreated)	0%	15%	13%	0%
Field G-4 (untreated)	0%	7%	8%	0%
Field G-5 (untreated)	0%	0%	6%	7%
Field G-6 (untreated)	0%	0%	0%	13%
Field G-7 (untreated)	0%	0%	20%	13%
Field G- 8 (untreated)	0%	0%	0%	0%

5) Hidalgo Treatment Area

Twelve fields were used in the study. The total treated area was 388 acres. The total untreated area was not provided. Crops were irrigated according to need. Ten crop samples were analyzed in 2004, one year following treatment in 2003. The samples were from different fields.

The results of the crop analyses are summarized in Table 8. The results of the soil analyses are summarized in Table 9. The average incidence of AF36 in Hidalgo area soil was 1% prior to treatment, and 60% one year post-treatment. The average incidence of the S strain in soil was 26% prior to treatment and 5% one year post-treatment.

Table 8. Cottonseed Sample Test Results for Hidalgo Treatment Area		
Sample Treatment	**% AF36 in 2004**	**% S Strain in 2004**
Treated in 2003	100%	0%
Treated in 2003	80%	0%
Treated in 2003	80%	0%
Treated in 2003	87%	0%
Treated in 2003	93%	0%
Treated in 2003	100%	0%
Treated in 2003	100%	0%
Treated in 2003	100%	0%
Treated in 2003	100%	0%
Untreated	73%	0%

Table 9. Soil Test Results for Hidalgo Treatment Area		
Field Number/ Treatment	**% AF36 in 2004**	**% AF36 in 2005**
Field H-1 (treated in 2004)	0%	100%
Field H-2 (treated in 2004)	0%	93%
Field H-3 (treated in 2004)	0%	85%
Field H-4 (treated in 2004)	0%	73%
Field H-5 (treated in 2004)	0%	47%
Field H-6 (treated in 2004)	0%	70%
Field H-7 (untreated)	0%	38%
Field H-8 (untreated)	0%	36%
Field H-9 (untreated)	6%	33%
Field H-10 (untreated)	0%	27%
Field H-11 (untreated)	0%	13%
Field H-12 (untreated)	0%	6%

IV. REVIEWER'S COMMENT:

The identification of which specific batches or lots of AF36 used in the field efficacy trials were not provided.

Efficacy was evaluated as the ability of *Aspergillus flavus* AF36 to displace aflatoxin producers, but the efficacy tests are not a true measure of this mycotoxin. This is because these tests are comparing the percentages of displacement, rather than quantitatively measuring the amount of *A. flavus* in the ppb range. However, the Agency previously determined that the proposed food use pattern for cotton is not likely to result in dietary exposure or residues on food and feed (EPA, 2003). This is because cotton is not a direct dietary commodity. If AF36 can be found on cotton seed, residues are not likely to survive the heating and pressure associated with the processing of cottonseed into cottonseed meal. Moreover, AF36 will not separate into the edible fraction, cottonseed oil. Thus, potential transfer of residues of AF36 to edible cotton food/feed commodities is not expected. Consequently, human dietary exposure to AF36 via cottonseed oil, or by secondary transfer

of AF36 residues to meat and milk via cottonseed meal, is unlikely to be above naturally occurring background levels.

It should also be noted that additional precautions are made in the manufacturing process of AF36 because aflatoxin is considered a public health hazard. These include: starter cultures of AF36 are screened by thin layer chromatography and scanning fluorescence densitometry for lack of aflatoxin, as well as for the genetic lesions in pksA and aflR by pyrosequencing assays. Even if AF36 does not control aflatoxin levels in the treated cotton food/feed commodities, cotton and its by-products are screened for aflatoxin prior to their introduction into the channels of commerce and are regulated by the FDA. These factors were taken into consideration in the review of the data submitted and thereby, negate the need for additional efficacy testing based on the quantification level. However, if more extensive use patterns are sought for treatment of other agricultural terrestrial sites or crops that are a direct food commodity, quantification of aflatoxin levels should be required to demonstrate efficacy.

IV. CONCLUSION:

Product efficacy tests were conducted at five sites in Texas by evaluating the ability of AF36 to displace other *A. flavus* strains. One year after application, the percent incidence of AF36 on treated cottonseed crops ranged from 80% to 100%; the percent incidence of the high aflatoxin-producing S strain ranged from 0% to 11%; the S strain was detected in 0/4, 2/5, 2/2, 3/30 and 0/9 cottonseed samples at the five sites, respectively. For the five sites, the average percent incidence of AF36 in soil prior to treatment compared with one year post-treatment was 4%/55%, 1%/73%, 1%/56%, 0%/67%, and 1%/60%, respectively. Data available for four of the sites indicate the average percent incidence of the S strain in soil prior to treatment compared with one year post-treatment as 58%/11%, 43%/6%, 43%/14%, and 0%/7%, respectively.

V. CLASSIFICATION: This study packet is classified as **ACCEPTABLE.**

VI. REFERENCES:

EPA, 2003. Biopesticide Registration Action Document (BRAD) for *Aspergillus flavus*, strain AF36, PC Code: 006456, dated July 3, 2003.

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R153305

Chemical: Aspergillus flavus 36 colonized wheat seed

PC Code:
006456
HED File Code: 41500 BPPD Tox/Chem
Memo Date: 9/13/2007
File ID: DPD330590
Accession #: 000-00-9003

HED Records Reference Center
11/1/2007