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

OFFICE OF
PREVENTION, PESTICIDES, AND
TOXIC SUBSTANCES

AUG 18 2009

MEMORANDUM

Decision: 385454
DP Barcode: 345961

SUBJECT: Review of a three-year soil fate study to assess persistence and accumulation of plant expressed Cry1Ac and Cry1F required as a condition of registration for Dow AgroSciences' WideStrike cotton (MRID 472145-01), EPA Reg. No. 68467-3.

FROM: Jeannette Martinez, Ecologist
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PEER REVIEW: Zigfridas Vaituzis, Senior Scientist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P) 8/19/09

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

WideStrike Insect Resistant Cotton Seed was conditionally registered in 2004. Among the conditions of registration was the requirement that the registrant, Mycogen Seeds/Dow Agrosciences, submit a soil fate study (nonguideline) to assess persistence and accumulation of plant expressed Cry1F and Cry1Ac proteins from fields planted with WideStrike cotton for three consecutive years. The study has been submitted to meet the condition of registration, and is reviewed in this memorandum. A summary of the study is presented below, and a Data Evaluation Record is attached.

Study Summary

Study: Persistence and accumulation of plant expressed Cry1F and Cry1Ac proteins in the soil after three years of cropping with WideStrike cotton
MRID: 472145-01

Classification: ACCEPTABLE

Summary: Field studies were conducted in California, Mississippi, and Texas to assess the persistence and accumulation of the Cry1F and Cry1Ac proteins in soils where WideStrike cotton was grown in the same plots for three consecutive years. Each site included a control plot of PSC355 (non-transgenic) cotton for comparison. Cry1F and Cry1Ac test strips were used in the field to confirm that WideStrike was expressing the Cry1F and Cry1Ac proteins during the first two seasons. During and after the third season, bulk soil, rhizosphere soil, and root samples were collected and analyzed for Cry1F and Cry1Ac using enzyme-linked immunosorbent assay (ELISA). A tobacco budworm (*Heliothis virescens*, TBW) feeding assay was also conducted to detect Cry1F/Cry1Ac in the rhizosphere soil samples.

ELISA Results:

No Cry1F and Cry1Ac protein residues were detected in the bulk soil and rhizosphere soil samples above the LOD (0.0045 ng of protein/mg soil) after three years of continuous cropping with WideStrike cotton. As expected, root samples of WideStrike cotton contained detectable levels of Cry1F and Cry1Ac at mid-bloom and post harvest (LOD 0.10 ng protein/mg soil).

Insect Bioassay Results:

Bioassay results with TBW did not reveal significant differences in weight of larvae from different soil treatments (WideStrike and control), and mortality did not differ and exceed 13%. As expected, diet treated with WideStrike roots resulted in significant growth reduction for surviving TBW larvae compared to control treatments. Interestingly, all treatments impeded TBW growth compared to soil, suggesting the presence of potent natural plant protectants in the roots of cotton plants that are active against cotton pests. This observation has been previously made with leaf tissue from cotton (Greenplate, 1999).

Conclusions:

No detectable Cry1F or Cry1Ac was detectable in the soil after three years of continuous cropping with WideStrike Cotton. This three year soil fate study is acceptable and satisfies the condition of registration for WideStrike Cotton.

DATA EVALUATION RECORD

Contractor Primary Reviewers: E. Lewis, A. Armstrong, Oak Ridge National Laboratory
EPA Secondary Reviewer: Jeannette Martinez JCM
EPA Peer Reviewer: Zigfridas Vaituzis ZV

STUDY TYPE:	Soil Accumulation (Nonguideline)
MRID NO:	47214501
DP BARCODE:	DP346371
DECISION NO:	370934
SUBMISSION NO:	799398
TEST MATERIAL:	WideStrike Insect Resistant Cotton Seed (a.i., *Bacillus thuringiensis* var. aizawai Cry1F (Synpro) and the genetic material (from the insert of plasmid pGMA281) necessary for its production in cotton and *Bacillus thuringiensis* var. kurstaki Cry1Ac (Synpro) and the genetic material (from the insert of plasmid pMYC3006) necessary for its production in cotton)
STUDY NO:	030036
SPONSOR:	Dow AgroSciences, LLC, Indianapolis, IN 46268
TESTING FACILITY:	Dow AgroSciences, LLC, Regulatory Laboratories – Indianapolis Lab, 9330 Zionsville, Road, Indianapolis, IN 46268
TITLE OF REPORT:	Soil Accumulation of Cry1F and Cry1Ac Proteins After Three Years of Cropping with WideStrike Cotton
AUTHORS:	Shan, G., S.K. Embrey, R.A. Herman, et al.
STUDY COMPLETED:	August 6, 2007
CONFIDENTIALITY CLAIMS:	None
GOOD LABORATORY PRACTICE:	A signed and dated compliance statement was provided. The study was conducted in accordance with 40 CFR Part 160, with the following exceptions: 1) at some sites, documentation is incomplete by GLP standards for climatological data, field history, pesticide maintenance, sample weights, and crop information, and 2) characterization of seed test substances was not performed.

STUDY SUMMARY:	Field studies were conducted in California, Mississippi, and Texas to assess the persistence and accumulation of the Cry1F and Cry1Ac proteins in soils where WideStrike cotton was grown in the same plots for three consecutive years. Each site included a control plot of PSC355 (non-transgenic) cotton for comparison. Cry1F and Cry1Ac test strips were used in the field to confirm that WideStrike was expressing the Cry1F and Cry1Ac proteins during the first two seasons. During and after the third season, bulk soil, rhizosphere soil, and root samples were collected and analyzed for Cry1F and Cry1Ac using enzyme-linked immunosorbent assay (ELISA). A tobacco budworm (*Heliothis virescens*) feeding assay was also conducted to detect Cry1F/Cry1Ac in the rhizosphere soil samples. Cry1F and Cry1Ac were below the ELISA limit of detection in all soil samples. Both proteins were detected in the root samples at both sampling times. There was no statistically significant difference in the mortality of tobacco budworm larvae fed diet containing soil from WideStrike or control plots.
CLASSIFICATION:	**ACCEPTABLE**

I. BACKGROUND

The Cry1F (synpro) and Cry1Ac (synpro) Bt plant-incorporated protectants as expressed in WideStrike™ cotton (EPA Reg. No. 68467-3) were registered on September 30, 2004 and first deployed during the 2005 cotton growing season. In the southeastern U.S., the two major target pests of *Bt* (*Bacillus thuringiensis*) cotton are tobacco budworm (TBW, *Heliothis virescens*) and cotton bollworm (CBW, *Helicoverpa zea*). WideStrike cotton is a pyramided event that expresses both the Cry1Ac and Cry1F Bt toxins at high levels to control tobacco budworm and cotton bollworm. As a condition of the registration, EPA required that Dow AgroSciences conduct a three-year soil fate study to determine the potential for Cry1Ac and Cry1F to accumulate and persist in the soil.

II. MATERIALS AND METHODS

WideStrike cotton expresses two proteins: Cry1F and Cry1Ac. The product also contains the PAT gene (phosphinothricin acetyltransferate), which confers resistance to the herbicide glufosinate ammonium. The analytical standards used as reference materials were Cry1F, TSN 104301, at a concentration of 0.164 mg/mL; and Cry1Ac, TSN 102337, at a concentration of 0.26 mg/mL.

In this study, WideStrike cotton was grown in the same plots for three consecutive years to assess the persistence and accumulation of Cry1F and Cry1Ac in soils. The tests were conducted in three states from May, 2003 to November, 2005 at sites in California, Mississippi, and Texas.

The soil characteristics and management practices are given in Table 1. Temperature and moisture conditions during the three-year study were typical of weather encountered in cotton producing areas. The registrant provided climatological data for each state over the course of the growing seasons (pp.24-25 of MRID 47214501). Supplemental irrigation was used at all the sites at some time during the three-year study. Information regarding use of maintenance chemicals over the course of the field trials was provided on pp.26-27 of MRID 47214501.

The test site plot design consisted of control plots of PSC355 (non-transgenic) cotton and plots with WideStrike cotton. The plot size was 12 rows by 100 feet with a minimum of 26 feet between control and WideStrike plots. Each plot was divided into three subplots for sampling, and *bulk soil*, *rhizosphere soil*, and *root samples* were collected. At the end of the three year experiment, five subsamples from each sample type were taken per subplot and combined into one composite sample, hence, making six samples for each subplot at each sampling interval: three (composite) control samples and three (composite) WideStrike samples. The bulk soil samples were collected approximately four inches away from the plant toward the row center. To obtain the rhizosphere samples, the plant had to be dug in such a fashion that the soil/root material was approximately eight inches in diameter and six inches deep. Then the roots were gently tapped against the side of a bucket to collect the soil. Rhizosphere soil samples were sieved through a 10-mesh sieve to remove any unwanted root material. The remaining plant root was washed to remove any additional soil, and the belowground tissue was cut off to obtain the root sample.

All samples were placed in bags, frozen within four hours after sampling, and shipped to Dow AgroSciences' laboratory in Indianapolis. The soil samples were stored at approximately -20°C; the root samples were stored at -80°C prior to lyophilization. After lyophilization, the roots were frozen in liquid nitrogen, coarsely ground using an Agvise Model 201 hammermill with a 1/8-inch screen, then finely ground using a Geno grinder, and finally stored at -80°C until the time of analyses.

In 2003 and 2004, Cry1F and Cry1Ac test strips (Strategic Diagnositics, Inc.) were used in the field to confirm that WideStrike cotton plants were expressing the Cry1F and Cry1Ac proteins. The *rhizosphere* and *bulk soil* samples collected in 2005 were analyzed (based on fresh weight basis) for Cry1F and Cry1Ac using Dow AgroSciences' methods GRM 04.10 and GRM 06.14, respectively. If WideStrike cotton had exuded crystal proteins from the roots, then the rhizosphere soil could be expected to have higher concentrations of Cry1F and Cry1Ac. The Cry1F and Cry1Ac proteins were extracted from the soil samples with a solution designed to mimic invertebrate digestive juice (Shan et al., 2005). The extract was centrifuged, and the aqueous supernatant was collected, diluted, and assayed using specific Cry1F and Cry1Ac enzyme-linked immunosorbent assay (ELISA) kits (Strategic Diagnostics, Inc.). An aliquot of the diluted sample was incubated in the wells of an anti-Cry1F or anti-Cry1Ac antibody-coated plate. After washing, an aliquot of enzyme-conjugated anti-Cry1F or anti-Cry1Ac antibody was added and incubated in the plate to form an antibody-protein-antibody/enzyme conjugate sandwich. After incubation, the unbound reagents were removed by washing with phosphate buffered saline solution (PBST). The presence of both proteins was detected by incubating the antibody-bound conjugate with an enzyme substrate, generating a colored product. Absorbance was read at 450 nm minus 650 nm using a plate reader. The limit of detection and limit of quantitation were 4.5 ng/g and 18 ng/g, respectively, for all protein assays in soil. A calibration curve resulting from the standard concentrations used generated a quadratic regression equation with a correlation coefficient $\geq$0.990.

Approximately 15 g of each *root tissue* sample were analyzed (based on dry weight basis) for the presence of Cry1F and Cry1Ac using DowAgrosciences' methods GRM 02.12 and GRM 02.11, respectively. Cry1F and Cry1Ac were extracted with PBST containing 0.05% Tween 20 and 1% polyvinylpyrrolidone. The extract was centrifuged, and the supernatant was collected, diluted, and assayed using Cry1F and Cry1Ac ELISA kits. The LOD and LOQ were 0.025 and 0.10 ng/mg for Cry1F and 0.025 and 0.25 ng/mg, respectively, for Cry1Ac in roots.

A series of calibration standards were analyzed (three times) on each ELISA plate along with the study samples. Calibration curves were generated, and the Cry1F and Cry1Ac concentrations (ng/mL) were calculated from the quadratic regression equation using Soft-MAX Pro v. 4.0 (Molecular Devices Corp).

Statistical analysis of the ELISA data consisted of calculating means, standard deviations, and regression analysis.

Table 1. Trial Site Details

Location	**Fresno, CA**	**Greenville, MS**	**Claude, TX**
Soil name and texture	Pachappa loam	Commerce loam	Pullman loam
pH	6.4	6.1	6.2
Organic carbon (%)	0.6	0.6	1.2
CEC	27	11	22
Row spacing (inches)	40	40	36
Tillage	Stalks shredded in fall, undercut 4-8 inches, then disked 4-6 inches. In spring, beds formed with a lister.	No-till stalks were bush-hogged after harvest.	Disked to 3 inches in fall. Disk or rotary tiller in spring.
2003 Planting date - harvest date	5/16/03 – 11/8/03	5/22/03 – 10/3/03	5/29/03 – 11/8, 11/14/03
2004 Planting date - harvest date	5/27/04 – 12/1/04	6/4/04 – 10/28/04	5/31 and 7/8/04[a] – 11/13/04
2005 Planting date - harvest date	5/4/05 – 11/28/05	5/16/05 – 11/29/05	5/21/05 – 11/23/05
Flowering sample date	7/29/05	8/11/05	8/30/05
Post harvest sample date	12/7/05	10/13/05	11/29/05

Data from p. 23, MRID 47214501; this table has been modified from the original submission.

[a] Hail destroyed the crop in June 2004, and it was replanted in July 2004.

Cry1F and Cry1Ac accumulation and persistence in rhizosphere soil at all locations were further assessed with the tobacco budworm bioassay. Root samples served as a positive control. Soil samples were collected post-harvest, while root samples from the California site were collected during the growing and post-harvest season. Aliquots of root and soil suspensions (diluted 10-fold) were applied to the surface of artificial insect diet into wells of the bioassay tray. After the wells had dried, one neonate TBW per well was introduced into the system. The diet bioassay was conducted at approximately 24°C for the duration of six days, after which mortality and insect weights were recorded.

ANOVA and Tukey's multiple range test were used to analyze the 'average body weight' data for surviving larvae and determine differences for each treatment (across subplots within a location). Soil and root analyses were kept separate.

III. RESULTS

- Field immunoassay tests showed that WideStrike cotton plants expressed Cry1F and Cry1Ac proteins during the study.

- Results of protein concentrations in the bulk and rhizosphere soil showed that residues of Cry1F and Cry1Ac were not detectable in soil at the LOD of 4.5ng/g (Table 2).

- Cry1F and Cry1Ac were both detected in the roots of WideStrike cotton from samples collected during the growing season as well as post harvest season (LOD 0.10 ng/mg) (Table 3).

- Bioassay mortality was less than 13% in all treatments from soil samples; there were no statistically significant weight differences between surviving insects fed on bulk and rhizosphere soil treated diets. However, diet treated with WideStrike roots (growing season and post harvest samples) significantly reduced survival of TBW compared to roots from control plants.

Table 2. Concentrations of Cry1F and Cry1Ac in Bulk and Rhizosphere Soil

Site	Event[a]	Treatment	Cry1F (ng/mg fresh wt)		Cry1Ac (ng/mg fresh wt)	
			Soil	Rhizosphere soil	Soil	Rhizosphere soil
CA	1	Control	ND[b]	ND	ND	ND
CA	1	Control	ND	ND	ND	ND
CA	1	Control	ND	ND	ND	ND
CA	1	WideStrike	ND	ND	ND	ND
CA	1	WideStrike	ND	ND	ND	ND
CA	1	WideStrike	ND	ND	ND	ND
CA	2	Control	ND	ND	ND	ND
CA	2	Control	ND	ND	ND	ND
CA	2	Control	ND	ND	ND	ND
CA	2	WideStrike	ND	ND	ND	ND
CA	2	WideStrike	ND	ND	ND	ND
CA	2	WideStrike	ND	ND	ND	ND
MS	1	Control	ND	ND	ND	ND
MS	1	Control	ND	ND	ND	ND
MS	1	Control	ND	ND	ND	ND
MS	1	WideStrike	0.021, ND	ND	ND	ND
MS	1	WideStrike	ND	ND	ND	ND
MS	1	WideStrike	ND	ND	ND	ND
MS	2	Control	ND	ND	ND	ND
MS	2	Control	ND	ND	ND	ND
MS	2	Control	ND	ND	ND	ND
MS	2	WideStrike	ND	ND	ND	ND
MS	2	WideStrike	ND	ND	ND	ND
MS	2	WideStrike	ND	ND	ND	ND
TX	1	Control	ND	ND	ND	ND
TX	1	Control	ND	ND	ND	ND
TX	1	Control	ND	ND	ND	ND
TX	1	WideStrike	ND	ND	ND	ND
TX	1	WideStrike	ND	ND	ND	ND
TX	1	WideStrike	ND	ND	ND	ND
TX	2	Control	ND	ND	ND	ND
TX	2	Control	ND	ND	ND	ND
TX	2	Control	ND	ND	ND	ND
TX	2	WideStrike	ND	ND	ND	ND
TX	2	WideStrike	ND	ND	ND	ND
TX	2	WideStrike	ND	ND	ND	ND

Data from p. 30, MRID 47214501; this table has been modified from the original submission.

[a] Event 1 = mid-bloom sampling, Event 2 = post harvest sampling

[b] ND = not detected, value less than the LOD (4.5 ng/g).

Two values for the same sample indicate a re-analysis of the sample.

Table 3. Concentrations of Cry1F and Cry1Ac in Roots of Control and WideStrike Cotton Plants

Site	Event[a]	Treatment	Cry1F (ng/mg dry wt)	Cry1Ac (ng/mg dry wt)
CA	1	Control	ND[b]	ND
CA	1	Control	ND	ND
CA	1	Control	ND	ND
CA	1	WideStrike	ND, 0.164	ND, ND
CA	1	WideStrike	0.458	(0.143)
CA	1	WideStrike	0.559	(0.205)
CA	2	Control	ND	ND
CA	2	Control	ND	ND
CA	2	Control	ND	ND
CA	2	WideStrike	0.349	0.545
CA	2	WideStrike	0.408	0.601
CA	2	WideStrike	0.412	0.515
MS	1	Control	ND	ND
MS	1	Control	ND	ND
MS	1	Control	ND	ND
MS	1	WideStrike	0.660	(0.136)
MS	1	WideStrike	0.947	(0.223)
MS	1	WideStrike	0.863	(0.242)
MS	2	Control	ND	ND
MS	2	Control	ND	ND
MS	2	Control	ND	ND
MS	2	WideStrike	0.462	ND
MS	2	WideStrike	0.409	ND
MS	2	WideStrike	0.273	ND
TX	1	Control	ND	ND
TX	1	Control	ND	ND, 0.329
TX	1	Control	ND	ND, 0.308
TX	1	WideStrike	0.635	ND
TX	1	WideStrike	0.702	0.258
TX	1	WideStrike	0.598	0.299
TX	2	Control	0.113, ND	ND
TX	2	Control	0.119, ND	ND
TX	2	Control	0.153, ND	ND
TX	2	WideStrike	1.237	0.291
TX	2	WideStrike	1.876	0.383
TX	2	WideStrike	1.164	0.436

Data from p. 31, MRID 47214501; this table has been modified from the original submission.

[a]Event 1 = mid-bloom sampling, Event 2 = post harvest sampling

[b]ND = not detected, value less than the LOD (0.025 ng/mg).

Values in parentheses are >LOD but <LOQ (0.10 ng/mg for Cry1F, 0.25 ng/mg for Cry1Ac)

Two values for the same sample indicate a re-analysis of the sample.

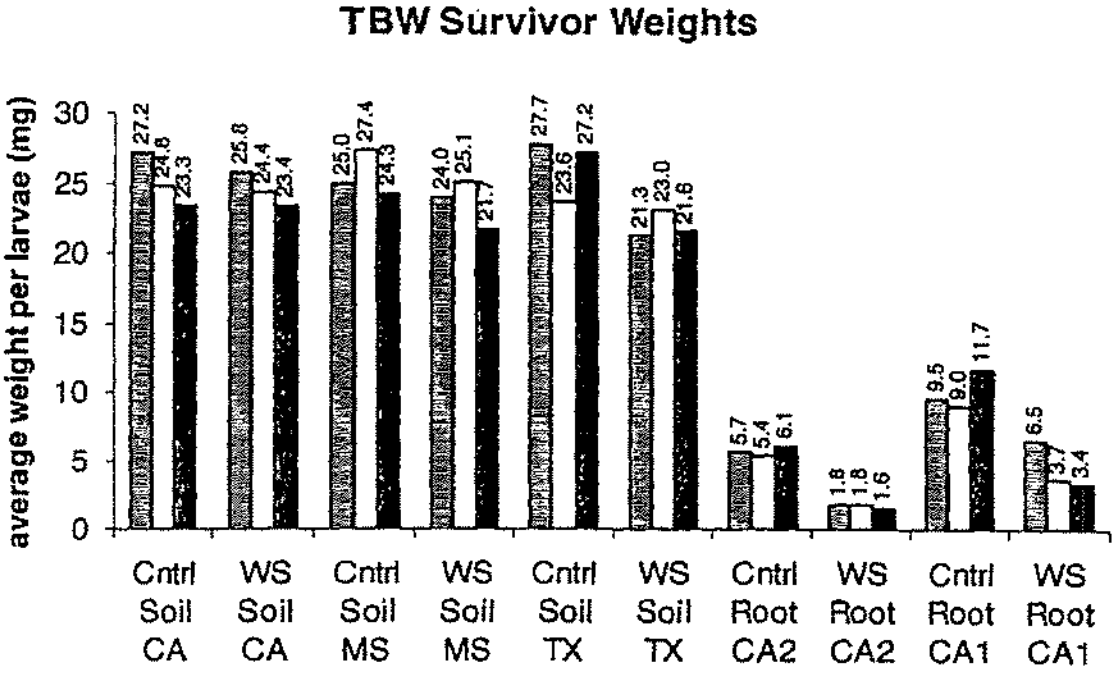

Figure 1. Results of diet bioassays with tobacco budworm exposed to soil and root from WideStrike cotton fields and plants (three subsamples/subplot). WS = WideStrike; Cntrl = control. CA1 indicates root samples collected during the mid-bloom growth stage; CA2 indicates root samples collected post harvest. (Figure extracted from p.43 MRID 472145-01)

IV. CONCLUSIONS

Study Authors:

The study authors concluded that there was no detectable Cry1F or Cry1Ac in the soil after three years of continuous cropping with WideStrike Cotton.

Reviewer's Conclusion

The reviewers agree with the study authors' conclusion.