July 10, 2002

MEMORANDUM

Subject: Review of aerobic soil degradation study submitted by Monsanto Co. in support of registering *Bacillus thuringiensis* Cry3Bb1 protein expressed in field corn. EPA Reg. No. 524-LRA; Barcode No. D262045; Case No. 066221; Submission No. S572997. MRID No 451568-04.

From: Robyn Rose, Entomologist
Biopesticides and Pollution Prevention Division, 7511C

Through: Chris Wozniak, Biologist
Biopesticides and Pollution Prevention Division, 7511C

To: Mike Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division, 7511C

Classification: Supplemental to conducting the study in the field with all plant tissue incorporated into the soil in fields that have had MON 863 corn grown for one to three consecutive years. Studies should also be conducted in a variety of soil types particularly soil high in clay and humic acids.

Background

Monsanto Company has requested a registration for *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material (ZMIR13L) necessary for its production in all corn lines and varieties. The Cry3Bb1 protein is intended to control corn rootworms (CRW, *Diabrotica* spp.), a coleopteran pest of corn. Studies were submitted to support registration of the transformation event MON 863. CRW, a primary pest of corn in the U.S., feeds on corn roots as larvae leading to a reduction in the plant's ability to absorb water and nutrients from soil and lodging. In areas where CRW is a pest (e.g., Corn Belt), significant financial losses are realized from a decrease in production and chemical insecticide usage. Significant acres of corn are treated annually to control CRW with organophosphate, carbamate and pyrethroid insecticides.

This soil degradation study was submitted as part of the ecological risk assessment in support of registering CRW protected Bt corn event MON 863. However, Cry3Bb1.11231 (event MON 859) rather than Cry3Bb1.11098 (event MON 863) was utilized in this study. According to Monsanto, Cry3Bb1.11231 "demonstrates an eight-fold increase in activity against the SCRW as a result of four strategically placed amino acid substitutions in the wild type protein sequence."

Cry3Bb1.11098 "demonstrates an eight-fold increase in activity against the SCRW as a result of five strategically placed amino acid substitutions in the wild type protein sequence." Cry3Bb1.11098 has the same amino acid sequence as Cry3Bb1.11231 with an additional substitution. It is acceptable that this test was conducted with event MON 859 rather than MON 863 because they produce nearly identical Cry3Bb1 protein variants.

Conclusions

Results from this study show the DT_{50} and DT_{90} for Cry3Bb1 protein in sandy loam soil based on ELISA test are 2.76 and 9.16 days. However, the value of these results are not necessarily correlated with activity in insect guts because it is unknown if the extractable protein in the ELISA test was functional or non-functional. The DT_{50} and DT_{90} determined by insect bioassays with CPB were 2.37 and 7.87 days respectively.

Based on these results it is likely that Cry3Bb1 protein degrades rapidly in sandy loam soil. However, corn is not necessarily grown in sandy loam soil in all regions. Corn is grown in other soil types such as clay loam and silty loam soils in various regions of the U.S. In addition, this test does not account for all plant tissue such as roots. It is possible that root tissue is degrading slower then plant tissue in the soil which may result in a longer duration of degradation time of the Cry3Bb1 protein. Therefore, field tests should be conducted in a variety of soil types including clay and humic acid soils over a three year period of time to determine the long-term degradation rate of Cry3Bb1 in soil. It is important to include tests using clay soils since it has been determined that Bt Cry proteins may readily bind to clay and humic acid soil particles potentially leading to increased accumulation and persistence of the protein (Crecchio and Stotzky 1998, Koskella and Stotzky 1997, Tapp and Stotzky 1995, Tapp and Stotzky 1998) . Testing clay soils would, therefore, be considered a "worst case scenario."

REVIEWED BY:	Robyn Rose, Entomologist Biopesticides and Pollution Prevention Division
PEER REVIEWER:	Chris Wozniak, Ph.D., Biologist Biopesticides and Pollution Prevention Division
STUDY TYPE:	Aerobic soil degradation study
MRID NO.:	451568-04
DP BARCODE NO.:	D262045
STUDY NO.:	XX-99-015
SUBMISSION NO.:	S572997
SPONSOR REPORT NO.:	MSL-16440
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	PTRL East, Inc., 3945 Simpson Lane, Richmond, KY 40475 & Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198.
TEST MATERIAL:	*Bacillus thuringiensis* Cry3Bb1 protein
AUTHORS:	John W. Martin, Michael J. McKee, Samuel Dubelman and Yelena A. Dudin
STUDY DATED:	April 26, 2000
GLP:	Good Laboratory Practice statement signed
CLASSIFICATION:	

Title: "Aerobic Soil Degradation of the *B.t.* Protein 11098 as a Component of Insect Protection Protected Corn."

Objective: This study was conducted to evaluate the rate of Cry3Bb1 degradation in a soil common to corn growing areas.

Study Summary

Materials and Methods:

Materials

Test Substance: Finely ground lyophilized field corn leaf tissue from event MON 859 plants grown in the greenhouse expressing Cry3Bb1 insecticidal protein. Tissue from the youngest whorl leaf of two to four week old corn plants (e-mail communication from Michael McKee to Robyn Rose on 7-10-02) was lyophilized in plastic bags sealed with a rubber stopper until sample weights were invariate. Leaf tissue from individual bags was combined and mixed before removing 200 g of each corn event/line to be ground in a blender with liquid nitrogen. The lyophilized plant material was stored at -80°C until test initiation.

Enzyme-linked immunosorbent assay (ELISA) was used to determine the Cry3B1 protein concentration as 1745 µg/g dry weight in lyophilized leaf tissue. The amount of lyophilized corn tissue added to the soil in this study was based on the amount of plant tissue that could potentially be incorporated into the top six inches of soil under field conditions. According to Monsanto's submission there is 616 m^3 of soil in one acre six inches deep. The calculation provided by Monsanto to determine the amount of plant tissue incorporated in the soil was:

$$\frac{(650 \text{ g dry wt/plant}) \times (40{,}000 \text{ plants/acre})}{(616 \text{ m}^3/\text{acre}) \times (10^6 \text{ cm}^3/\text{m}^3) \times (1.5 \text{ g soil/cm}^3)} = 0.028 \text{ g dry wt plant tissue/g soil}$$

Based on this calculation 0.03 g (rounded up from 0.028) dry weight plant tissue was added to each gram of dry soil; therefore, 3% of the dry weight of soil was dry weight of lyophilized plant tissue. An additional test was conducted with 10% of the soil containing lyophilized dry weight plant tissue (0.10 g leaf tissue/g soil).

Control Substance: Finely ground lyophilized field corn leaf tissue from event MON 846 that does not express the Cry3Bb1 insecticidal protein. ELISA was used to determine that no detectable levels (limit of detection = 0.10 µg/g dry weight) of Cry3Bb1 protein were present in the leaf tissue.

Based on this calculation 0.03 g (rounded up from 0.028) dry weight plant tissue was added to each gram of dry soil; therefore, 3% of the dry weight of soil was dry weight of lyophilized plant tissue. An additional test was conducted with 10% of the soil containing lyophilized dry weight plant tissue (0.10 g leaf tissue/g soil).

Test System: The top 6 in (horizon A) of sandy loam soil which is commonly found in corn growing areas was collected by PTRL East, Inc. near the testing facility in Fayette County of Lexington, KY. Soil properties were determined by A & L Great Lakes and are listed in Table 1. In addition, a soil microbial analysis was conducted by PTRL East, Inc. (Table 2.).

Soil was prepared for testing by PTRL East, Inc. by passing moist soil through a 2 mm screen and incubating it at 25 ± 1°C. Soil remained moist until testing when it was air-dried. After drying, soil was remoistened to 75% field capacity (0.33 bar) with deionized water.

Table 1. Physicochemical Characteristics of Soil

Parameter	Results/Units
PTRL East, Inc. Log. No.	Q-2
USDA Textural Class:	Sandy Loam

Soil Series:	Huntington Series
pH	7.7
Particle Size Distribution:	
Sand	61.6%
Silt	28.0%
Clay	10.4%
Water Holding Capacity (at 0.33 bar)	14.63%
Organic Matter	2.32%
Cation Exchange Capacity (meq/100g)	6.72
Bulk Density (disturbed) gm/cc	1.24

* Table 1 was copied from page 22 of MRID451568-04

Table 2. Microbial Characterization Evaluated by Enumerating the Total Colony Forming Units (CFU) Per Gram of Soil

Microbial Media	CFU/g soil
Pre-Study	
Aerobic Bacteria	1.3 E + 07
Actinomycetes	1.8 E + 07
Fungi	5.0 E + 04
Week 3	
Aerobic Bacteria	9.3 E + 06
Actinomycetes	9.8 E + 06
Fungi	3.1 E + 04
Week 6	
Aerobic Bacteria	8.7 E + 06
Actinomycetes	1.0 E + 07
Fungi	3.4 E + 04

Table 2. Continued

Microbial Media	CFU/g soil
Week 9	
Aerobic Bacteria	6.4 E + 06

Actinomycetes	8.6 E + 06
Fungi	3.2 E + 04
Post-Study	
Aerobic Bacteria	6.5 E + 06
Actinomycetes	8.4 E + 06
Fungi	7.7 E + 03

* Table 2 was copied from page 23 of MRID451568-04

Methods

Two levels of test and control substances including lyophilized transgenic and non-transgenic plant tissue were added to sandy loam soil at the testing facility, PTRL Inc. A high concentration and low concentration of test and control substances were added to 22 mL sterile, glass vials (Table 3). The 10% Cry3Bb1 vials contained 174.5 µg Cry3Bb1 protein/g of soil and the 10% control vials contained 0.30 g control substance/g of soil. The 3% Cry3Bb1 vials contained 0.09 g of the test substance per 2.91 g of dried sandy loam soil for a Cry3Bb1 protein concentration of 52.4 µg/g soil. The 3% concentration control vials contained 0.09 g of the control substance. There were also tests conducted on "control-only", "soil-only" and "microbial characterization" samples. All samples contained enough deionized water to achieve 75% field capacity at 0.33 bar and vials were covered by rubber caps that were vented with three holes to allow air flow. This was achieved by adding 0.33 mL or 0.36 mL deionized water to the 10% (2.7 g soil) or 3% (2.91 g soil) test concentrations.

Table 3. Application of Test and Control Substance to Soil

# of Samples	Soil (g)	Test Substance (g)	Test Substance (g)	Cry3Bb1 (µg/g)	Designation
			High Concentration (10%)		
40	2.7	0.3	0	174.5	"test-soil"
40	2.7	0	0.3	0	"control soil"
4	0	0.3	0	175.5	"test only"
4	0	0	0.3	0	"control only"

Table 3. Continued

# of Samples	Soil (g)	Test Substance (g)	Test Substance (g)	Cry3Bb1 (µg/g)	Designation
4	2.7	0	0	0	"microbial characterization"

4	2.7	0	0	0	"soil only"
Low Concentration (3%)					
20	2.91	0.09	0	52.4	"test-soil"
20	2.91	0	0.09	0	"control soil"
4	0	0.09	0	52.4	"test only"
4	0	0	0.09	0	"control only"
4	2.91	0	0	0	"microbial characterization"

* Table 3 was copied from page 24 of MRID451568-04

At test initiation, samples of test and control substances were sent to Monsanto to determine if any protein degradation occurred during handling and transport. Soil-only samples were adjusted for FMC twice a week and incubated until microbial activity was evaluated on weeks 3, 6, 9 and at test termination. On days 1, 3, 7, 14, 21, 28, 35, 42, 49, 56, 63, 71, 77, 84, 91, 99, 105, 112 and 119 high dose samples were removed from the incubator and on days 1, 3, 7, 14, 28, 42, 56, 71 and 80 low dose samples were removed from the incubator and shipped to Monsanto on dry ice.

The concentration of active Cry3Bb1 protein in the soil samples was determined by conducting insect bioassays. Insect bioassays included a mixture of test and control substances with an agar-based insect diet added to wells of bioassay trays. Standard diet concentrations were prepared from purified protein. Colorado potato beetle (CPB; *Leptinotarsa decemlineata*) larvae were added to each well (one larva/well) and each treatment bioassay was replicated twice for a total of 16 CPB/replicate. CPB are more sensitive to the Cry3Bb1 protein then CRW and were, therefore, expected to result in a more measurable response then CRW, the target species. According to Monsanto, CPB are also preferred for this test because an adequate insect diet is available, whereas, a dependable commercial diet is not available for CRW. During the test, bioassay trays were kept in an incubator set at approximately 27°C for seven days. Survivorship and weight of surviving CPB were evaluated and recorded.

Of the four insect bioassays conducted, the first two were low dose (3%) with a target concentration of 2.3 µg Cry3Bb1 protein/mL insect diet. This rate was calculated from: (1.745 µg Cry3Bb1 protein/g tissue) × (0.03 g lyophilized corn tissue/mL soil) × (0.5 g soil/10 mL diet) × 0.893 (adjustment for 10.7% soil moisture) = 2.3 µg Cry3Bb1 protein/mL diet. The third bioassay contained 2.6 µg Cry3Bb1 protein/mL insect diet and was calculated from: (1.745 µg Cry3Bb1 protein/g tissue) × (0.03 g lyophilized corn tissue/g soil) × (0.56 g soil/10 mL diet) × 0.893 (adjustment for 10.7% soil moisture) = 2.6 µg Cry3Bb1 protein/mL diet. The fourth bioassay contained 3.9 µg Cry3Bb1 protein/mL insect diet and was calculated from: (1.745 µg Cry3Bb1 protein/g tissue) × (0.01 g lyophilized corn tissue/g soil) × (0.25 g soil/10 mL diet) × 0.90 (adjustment for 10.7% soil moisture) = 3.9 µg Cry3Bb1 protein/mL diet.

A bioassay was not conducted for "shipping control" samples at the 3% leaf tissue level because a lack of sensitivity resulted in inconclusive results. While attempting to analyze the 3% dosing level, a large portion of the "shipping control" samples were depleted which prevented analysis of the 10% leaf in soil level.

In addition to an insect bioassay, an ELISA was conducted to measure the level of functional and non-functional Cry3Bb1 protein present in samples. However, the ELISA test will only show extractable protein and does not distinguish between functional and non-functional proteins. A soil extraction buffer adopted from Palm *et al.* 1994 was used in the ELISA which was based on a one-step, two-site sandwich. According to Monsanto, the "ELISA consisted of a simultaneous incubation of 50 µL per well of standards, samples or quality control solutions, and 50 µL per well of a 1:5,000 dilution of Protein-A purified rabbit anti-Cry3Bb1 conjugated to horseradish peroxidase (HRP; Sigma Chemical, St. Louis, MO)." A detailed description of the ELISA methodology can be found on page 17 of MRID No. 451568-04.

The DT_{50} (time to 50% degradation of the bioactivity or concentration of the protein) and DT_{90} (time to 90% loss of bioactivity or concentration) degradation rates for the Cry3Bb1 protein were determined from a DISSFIT Excel Computer Program Analysis. A calculation was done for the percent insect mortality from bioassay data and an additional calculation was conducted from the concentration of extractable Cry3Bb1 protein remaining in the soil.

Results:

Data from the 3% lyophilized corn plant tissue were not reported because the samples taken at test initiation in the first two bioassays did not show a response by the CPB. The third bioassay which included a 3% lyophilized plant tissue sampled resulted in high contamination and high mortality so these results were not reported. A Cry3Bb1 dissipation rate for the 10% lyophilized plant tissue samples was calculated based on insect survival. CPB mortality in the control treatments ranged from 3.1% to 12.5% (0 = 7.3%) during the 42 day testing period (Table 4). The DT_{50} was calculated from data collected on days 0, 1, 3 and 7 (Table 5). On day 7, percent mortality reached and remained at zero. The average background mortality of 7.3% observed in the control group was used for the day 7 time point in the DT_{50} calculation rather than zero. The **DT_{50}** based on linear plots from calculations in Table 4 (see Figure 1 on page 29 of MRID No. 451568-04) was determined to be **2.37 days** and **DT_{90}** was **7.87 days**.

Table 4. Percent Mortality of Colorado Potato Beetle Larvae as a Function of Time after exposure to samples containing Control and Test Substances added to soil at 10% (w:w).

Treatment	Cry3Bb1 starting concentration in diet mg/mL	Soil Incubation (days)	# of Insects at Start	# of Insects Dead	% Mortality
Test Substance (10%)	3.9	0	32	16	50
Test Substance (10%)	3.9	1	32	15	46.8

Test Substance (10%)	3.9	3	32	4	12.5
Test Substance (10%)	3.9	7	32	0	0
Test Substance (10%)	3.9	14	32	0	0
Test Substance (10%)	3.9	21	32	3	9.4
Test Substance (10%)	3.9	28	31	1	3.2
Test Substance (10%)	3.9	42	32	1	3.1
Control Substance (10%)	0	0	32	2	6.3
Control Substance (10%)	0	1	32	1	3.1
Control Substance (10%)	0	3	32	4	12.5
Control Substance (10%)	0	7	32	2	6.3
Control Substance (10%)	0	14	32	3	9.4
Control Substance (10%)	0	21	32	1	3.1
Control Substance (10%)	0	28	32	3	9.4
Control Substance (10%)	0	42	32	3	9.4

* Table 4 was copied from page 25 of MRID451568-04

Table 5. Soil Dissipation Analysis Based on Insect Bioassay Results

		Curve Fitting Parameters				
Days	% Morality	LN % Mortality	Trnsfmd Days	LN Pred Conc.	Pred Conc.	% Error
0	50	3.91202	0	4.03920	56.7807	13.56
1	46.8	3.84588	0.85811	3.52369	33.9093	-27.54
3	12.5	2.52573	2.057882	2.80394	16.5096	32.08
7	7.3	1.98787	3.555999	1.90469	6.7173	-7.98

* Table 5 was copied from page 26 of MRID451568-04

The extractable Cry3Bb1 protein of the 10% plant tissue soil samples were below the limit of quantification (LOQ) of an ELISA test. All values were <2 ng Cry3Bb1/mL which is the lowest detectable protein standard. Days 0, 1 and 3 sample values exceeded the range of the ELISA curve so the test was repeated at a high dilution rate (Table 6). On day 7 samples were analyzed at the same dilution rate to allow for data bridging. Data on day 7 showed values of 2578 and 2304 ng/mL (0 = 2441 ng/mL) which were considered equivalent and consistent by Monsanto. The final value used for day 7 was 2441 ng/mL and a quality control sample utilizing known concentrations of Cry3Bb1 protein resulted in 59.4 ng/mL and 52.6 ng/mL on the two respective sample dates (8/24/99 & 9/8/99) (Table 6). The extractable protein on days 28 and 42 were

<LOQ. The DT_{50} determined by the ELISA was 2.76 days and the DT_{90} was 9.16 days (Table 7).

Table 6. ELISA Results for Concentration of Cry3Bb1 Protein in Soil Samples at Various Incubation Times

Day #	ELISA 8/24 Total µg/mL[1]	ELISA 9/8 Total ng/mL[2]	Results (ng/mL)	Results (µg/g soil)
0	–[3]	22,744 / 23,062	22,903	115[5]
1	–[3]	20,448 / 19,682	20,065	100
3	–[3]	7,226 / 7,469	7,348	36.7
7	2578	2304	2,441	12.2
14	997	N/A	997	4.99
21	87.2	N/A	87.2	0.44
28	<LOQ[4]	N/A	<LOQ[4]	<LOQ[4]
42	<LOQ[4]	N/A	<LOQ[4]	<LOQ[4]

* Table 6 was copied from page 27 of MRID451568-04
(1) 1:16 dilution rate used for all samples
(2) Days 0 and 1 dilution rates were 1:100 and 1:200; Day 3 dilution rates were 1;50 and 1:100; Day 7 dilution rate was 1:16 which was repeated as a bridging control.
(3) Could not be quantified because samples exceeded the highest standard concentration
(4) LOQ = 0.1 µg/g dry weight
(5) Initial protein concentration on day zero was 115 µg/g which represents 66% of 174.5 µg/g (theoretical initial concentration)

Table 7. Soil Dissipation Analysis Based on ELISA Results

		Curve Fitting Parameters				
Days	Conc. (µg/g)	LN Conc.	Trnsfmd Days	LN Pred Conc.	Pred Conc.	% Error
0	115	4.74493	0	4.63445	102.9715	-10.46
1	100	4.60517	1	4.38302	80.0794	-19.92
3	36.7	3.60278	3	3.88015	48.4316	31.97
7	12.2	2.50144	7	2.87442	17.7151	45.21

Table 7. Continued

Days	Conc. (µg/g)	LN Conc.	Trnsfmd Days	LN Pred Conc.	Pred Conc.	% Error
14	4.99	1.60744	14	1.11438	3.0477	-38.92
21	0.44	-0.82098	21	-0.64565	0.5243	19.16

* Table 7 was copied from page 28 of MRID451568-04

Monsanto's Conclusions:

According to the data and results presented by Monsanto the DT_{50} based on insect bioassays and ELISA were 2.37 and 2.76 days respectively. The DT_{90} estimates for the insect bioassays and ELISA were 7.87 and 9.16 days respectively. These results verify that the Cry3Bb1 protein degrades rapidly in the soil.

Reviewer's Comments:

This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with two exceptions that did not affect the integrity of the test. According to the signed GLP statement, "1. Equipment SOPs were not available for the incubators used for the insect bioassay. However, appropriate maintenance and calibration was conducted and documented in the study data or facility records. There was no impact on the results of the bioassay. 2. Due to a machine malfunction at PTRL East, Inc., temperature data are not available for a portion of the incubation period. This had no impact on the study."

Results from this study show the DT_{50} and DT_{90} for Cry3Bb1 protein in sandy loam soil based on ELISA test are 2.76 and 9.16 days. However, the value of these results need to be considered with regard to biological activity because it is unknown if the extractable protein in the ELISA test was functional or non-functional. The DT_{50} and DT_{90} determined by insect bioassays with CPB were 2.37 and 7.87 days respectively.

Based on these results it is likely that Cry3Bb1 protein degrades rapidly in sandy loam soil. However, corn is not necessarily grown in sandy loam soil in all regions. Corn is grown in other soil types such as clay loam and silt loam soils in various regions of the U.S. In addition, this test does not account for all plant tissue such as roots. It is possible that root tissue is degrading slower then leaf tissue in the soil which may result in a longer duration of degradation time of the Cry3Bb1 protein. Therefore, field tests should be conducted in a variety of soil types including clay and humic acid soils over a three year period of time to determine the long-term degradation rate of Cry3Bb1 in soil. It is important to include tests using clay soils since it has been determined that Bt Cry proteins may readily bind to clay and humic acid soil particles potentially leading to increased accumulation and persistence of the protein (Crecchio and Stotzky 1998, Koskella and Stotzky 1997, Tapp and Stotzky 1995, Tapp and Stotzky 1998) . Testing clay soils would, therefore, be considered a "worst case scenario."

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