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

OFFICE OF PREVENTION, PESTICIDES
AND TOXIC SUBSTANCES

DEC 14 2007

MEMORANDUM

SUBJECT: Review of "Evaluation of Potential Interactions between the *Bacillus thuringiensis* Proteins Cry1A.105, Cry2Ab2, and Cry3Bb1" for Monsanto's MON 89034 X MON 88017 Maize
MRID 469513-05 & 469513-06
DP# 335188

FROM: (for) Mika J. Hunter, Biologist (Z. Vaituzis)
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

THROUGH: Zigfridas Vaituzis, Ph.D, Senior Scientist Zigfridas Vaituzis 12/14/07
Microbial Pesticides Branch,
Biopesticides and Pollution Prevention Division (7511P)

TO: Susanne Cerrelli
Regulatory Action Leader
Microbial Pesticides Branch
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Introduction

Monsanto Company has developed MON 89034, a new corn line that produces Cry1A.105 and Cry2Ab2 *Bacillus thuringiensis* proteins. The Cry1A.105 is comprised of portions of Cry1Ab, Cry1Ac, and Cry1F proteins. Together, Cry1A.105 and Cry2Ab2 protect MON 89034 corn from lepidopteran pests. Monsanto is planning on combining MON 89034 with MON 88017. MON 88017 corn produces the Cry3Bb1 protein for protection against rootworm (Coleoptera) and is tolerant to Roundup® agricultural herbicides. The purpose of the submitted study (MRID 469513-05) was to test potential interactions between Cry1A.105, Cry2Ab2, and Cry3Bb1 proteins against sensitive Lepidopteran and Coleopteran species. This study and memorandum accompany the Agency's review of "Evaluation of the Potential for Interactions Between the *Bacillus thuringiensis* Proteins Cru1A.105 and Cry2Ab2", dated July 6, 2006. Attached at the end of this document is the supporting Data Evaluation Record (DER).

MRID 469513-05
Evaluation of the Potential for Interactions Between *Bacillus thuringiensis* Proteins Cry1A.105, Cry2Ab2, and Cry3Bb1

The purpose of this study was to characterize the potential for interaction between the lepidopteran-active proteins Cry1A.105 and Cry2Ab2 and the coleopteran-active protein Cry3Bb1. The Cry1A.105 and Cry1A.105 and Cry2Ab2 proteins were tested alone and in combination with either the Cry3Bb1 protein against European corn borer (ECB, *Ostrinia nubilalis*) and corn ear worm (CEW, *Helicoverpa zea*) in diet incorporation studies. Likewise, the Cry3Bb1 protein was tested alone and with the Cry1A.105 and/or the Cry2Ab2 proteins, against the Colorado potato beetle (CPB, *Leptinotarsa decemlineata*). The test substances used were purified *E. coli*-produced Cry1A.105 (Lot # 20-100073), *E. coli*-produced Cry2Ab2 protein (Lot # 20-100071) and *E. coli*-produced Cry3Bb1.pvzmir39 (Lot # 20-100025). The test insects were neonate larvae (>24 hours post-hatch) of ECB, CEW, and CPB. Each protein or buffer control dosing solutions were incorporated at a 1:4 ratio into insect diet. Treated diet was dispensed (0.5 mL/well) into 128-well bioassay trays. A target number of 64 insects in the untreated control and 32 insects for each protein and buffer treatment level were used in each bioassay. Each well contained one insect. In total, three assay replicates were used for ECB and CEW and two replicates were used for CPB. Two CPB and two CEW replicates were rejected because they did not meet the acceptance criteria defined in the study plan. The PROC PROBIT procedure in SAS (version 9.1) was used to calculate LC_{50} and MIC_{50} (median molt inhibitory concentration) values for the combined data based on mortality and arrestment. The 95% confidence intervals (CIs) were used to make comparisons within ECB, CEW, and CPB treatments. Overlap of 95% CIs demonstrates comparable activity among treatments. Fisher's Exact tests were used to compare CPB, CEW, and ECB responses between the spiked protein groups and their respective buffer control groups.

Dosing solutions for the CEW bioassays included:

1. Cry1A.105 (1.56-100 µg/mL diet)
2. Cry1A.105 (1.56-100 µg/mL diet) + Cry3Bb1 (30 µg/mL diet)
3. Cry2Ab2 (1.25 – 80 µg/mL diet)
4. Cry2Ab2 (1.25 – 80 µg/mL diet) + Cry3Bb1 (30 µg/mL diet)
5. Cry1A.105 + Cry2Ab2 (dose range of 1:1 mixture at 1.56 – 100 µg/mL)
6. Cry1A.105 + Cry2Ab2 (dose range of 1:1 mixture at 1.56 – 100 µg/mL) + Cry3Bb1 (30 µg/mL diet)
7. Cry3Bb1 (30 µg/mL diet)

Dosing solutions for the ECB bioassays included:

1. Cry1A.105 (0.039-2.5 µg/mL diet)
2. Cry1A.105 (0.039-2.5 µg/mL diet) + Cry3Bb1 (30 µg/mL diet)
3. Cry2Ab2 (0.156 – 10 µg/mL diet)
4. Cry2Ab2 (0.156 – 10 µg/mL diet) + Cry3Bb1 (30 µg/mL diet)
5. Cry1A.105 + Cry2Ab2 (dose range of 1:1 mixture at 0.078 – 5.0 µg/mL)
6. Cry1A.105 + Cry2Ab2 (dose range of 1:1 mixture at 0.078 – 5.0 µg/mL) + Cry3Bb1 (30 µg/mL diet)
7. Cry3Bb1 (30 µg/mL diet)

Dosing solutions for the CPB bioassays included:

1. Cry3Bb1 (0.078- 5.0 µg/mL diet)
2. Cry3Bb1 (0.078- 5.0 µg/mL diet) + Cry1A.105 (30 µg/mL diet)
3. Cry3Bb1 (0.078- 5.0 µg/mL diet) + Cry2Ab2 (30 µg/mL diet)
4. Cry3Bb1 (0.078- 5.0 µg/mL diet) + Cry1A.105 + Cry1Ab2 (1:1 mixture with each protein at 30 µg/mL diet)
5. Cry1A.105 (30 µg/mL diet)
6. Cry2Ab2 (30 µg/mL diet)
7. Cry1A.105 + Cry2Ab2 (1:1 mixture with each protein at 30 µg/mL diet)

Results

Activity Against CEW

The LC_{50} and MIC_{50} values for the 1:1 mixture of the Cry1A.105 and Cry2Ab2 proteins with and without the addition of the Cry3Bb1 protein had overlapping 95% confidence intervals, indicating comparable activity. The LC_{50} and MIC_{50} values for Cry1A.105 protein and Cry2Ab2 protein spiked with and without 30 µg Cry3Bb1/mL diet had overlapping CIs also indicating comparable activity. Mortality and arrestment in the untreated control was low and did not exceed 1%. No significant effect of Cry3Bb1 protein on CEW survival was observed ($p>0.05$).

Activity Against ECB

The LC_{50} and MIC_{50} values for the 1:1 mixture of Cry1A.105 and Cry2Ab2 with and without the addition of Cry3Bb1 protein had overlapping 95% CIs. The LC_{50} and MIC_{50} values for larvae feeding on the diet containing Cry2Ab2 protein combined with Cry3Bb1 protein and Cry2Ab2 alone had overlapping 95% confidence intervals indicating comparable activity. The LC_{50} value for the Cry1A.105 protein spiked with Cry3Bb1 protein had an overlapping 95% CI with the LC_{50} values for the Cry1A.105 protein alone. However, in the Cry1A.105 protein treatments with and without the addition of the Cry1Bb1 protein there was a small separation of the 95% CIs for the MIC_{50}s due to very narrow CIs for each treatment. The difference in MIC_{50} values was minor, with values for the Cry1A.105 protein and Cry1A.105 protein with the Cry3Bb1 protein of 0.13 µg/mL diet and 0.10 µg/mL diet, respectively. Mortality and arrestment in the untreated control was low and did not exceed 1%. The Cry3Bb1 protein diet had no significant effect on ECB survival ($p>0.05$).

Activity Against CPB

LC_{50} values for the Cry3Bb1 protein against CPB, and Cry3Bb1 protein treatments with the addition of Cry1A.105, or Cry1A.105 and Cry2Ab2 combined were similar and had overlapping 95% CIs. The 95% CI for the LC_{50} value for the Cry3Bb1 diet that also contained Cry2Ab2 protein was narrowly separated from the 95% CI for the LC_{50} for the Cry3Bb1 protein alone. However, LC_{50} values for each CPB treatment with the Cry3Bb1 protein were similar to historical values (as reported by Monsanto). Mortality in the untreated control was low and did not exceed 7%. There was no significant effect on CPB survival in treatments that received the Cry1A.105 protein, the Cry2Ab2 protein or a combination of both proteins ($p>0.05$).

Conclusions/Recommendations

This study is acceptable. The activity of Cry1A.105 and Cry2Ab2 proteins was not significantly altered by the presence of Cry3Bb1, and the activity of Cry3Bb1 was not significantly altered by the presence of Cry1A.105 and/or Cry2Ab2. This study, along with the previously reviewed interaction study between Cry1A.105 and Cry2Ab2 indicate that MON 89034 x MON 88017 maize will not result in any unexpected interaction with regards to target and non-target insects.

Reference:

Hunter, Mika. Review of "Evaluation of the Potential for Interactions Between *Bacillus thuringiensis* Proteins Cry1A.105 and Cry2Ab2" for Monsanto's MON 89034 X MON 88017 Maize Experimental Use Permit 524-EUP-OT. July 6, 2006.

DATA EVALUATION RECORD

Primary Reviewer: Eric B. Lewis, M.S., Oak Ridge National Laboratory, Contract Review
EPA Secondary Reviewer: Mika J. Hunter, Biologist

STUDY TYPE:	Product Performance (810.3300)
MRID NO:	46951305
DP BARCODE:	DP335188
DECISION NO:	371190
SUBMISSION NO:	Not provided
TEST MATERIAL:	Cyr1A.105, Cry2Ab2, and Cry3Bb1 proteins
STUDY NO:	05-01-39-19
SPONSOR:	Monsanto Company 800 N. Lindbergh Blvd. St. Louis, MO 63167
TESTING FACILITY:	Monsanto Company Agronomic Traits 700 Chesterfield Parkway West St. Louis, MO 63017
TITLE OF REPORT:	Evaluation of Potential for Interactions Between the *Bacillus thuringiensis* Proteins Cry1A.105, Cry2Ab2, and Cry3Bb1
AUTHORS:	MacRae, T.C., C.R. Brown, and S.L. Levine
STUDY COMPLETED:	May 30, 2006
CONFIDENTIALITY CLAIMS:	None
GOOD LABORATORY PRACTICE:	A signed and dated GLP statement was provided. The study does not meet the GLP requirements specified in 40 CFR Part 1060.
STUDY SUMMARY:	Laboratory bioassays using artificial diet were conducted to characterize the potential for interaction between the lepidopteran-active proteins Cry1A.105 and Cry2Ab2 and the coleopteran-active protein Cry3Bb1, which are present in MON 89034 x MON 88017 corn. The Cry1A.105 and Cry2Ab2 proteins were fed alone and in combination with each other or Cry3Bb1 to European corn borer (*Ostrinia nubilalis*) and corn earworm (*Helicoverpa zea*) larvae. The Cry3Bb1 protein was fed alone and in combination with Cry1A.105 and/or Cry2Ab2 to Colorado potato beetle (*Leptinotarsa decemlineata*) larvae. At study end median lethal concentration and median molt inhibitory concentration values were calculated. The activity of the Cry1A.105 and Cry2Ab2 proteins was not significantly altered by the presence of Cry3Bb1, and the activity of Cry3Bb1 was not altered by the presence of Cry1A.105 and/or Cry2Ab2.
CLASSIFICATION:	**Acceptable**.

Test Material

Purified Cry1A.105 protein, Lot No. 20-100073, suspended in 25 mM CAPS, 1 mM benzamidine HCl, 0.1 mM EDTA, 0.2 mM DTT buffer, pH ~11, received from the sponsor and stored at -80°C.

Purified Cry2Ab2 protein, Lot No. 20-100071, suspended in 50 mM CAPS, 2 mM DTT buffer, pH ~11, received from the sponsor and stored at -80°C.

Purified Cry3Bb1.pvzmir39 (hereafter: Cry3Bb1) protein, Lot No. 20-100025, suspended in 50 mM sodium carbonate/bicarbonate, 1 mM EDTA buffer, pH ~10.1, received from the sponsor and stored at -80°C.

The buffer control substances were the buffers used for the test materials, received from the sponsor and stored at 4°C.

The assay control was sterile water.

Test Methods

The study was conducted to characterize the potential for interaction between the lepidopteran-active proteins Cry1A.105 and Cry2Ab2 and the coleopteran-active protein Cry3Bb1, which are present in MON 89034 x MON 88017 corn. The test insects were neonate larvae (<24 hrs post-hatch) of the European corn borer (ECB, *Ostrinia nubilalis*) and corn earworm (CEW, *Helicoverpa zea*), which are lepidopterans, and the Colorado potato beetle (CPB, *Leptinotarsa decemlineata*), a coleopteran. ECB eggs were obtained from laboratory colonies maintained at Iowa State University (Ames, IA), CEW eggs from Benzon, Inc. (Carlisle, PA), and CPB eggs from the New Jersey Dept. of Agriculture (Trenton, NJ). Upon receipt at the test facility, the eggs were incubated in environmental chambers and maintained at appropriate temperatures to manipulate the timing of egg hatch.

Treatments used for the study were as follows:

Summary of CEW bioassay treatments
Cry1A.105 (dose range 1.56 – 100 µg/mL of diet)
Cry1A.105 (dose range 1.56 – 100 µg/mL of diet) + Cry3Bb1 (30 µg/mL of diet)
Cry2Ab2 (dose range 1.25 – 90 µg/mL of diet)
Cry2Ab2 (dose range 1.25 – 90 µg/mL of diet) + Cry3Bb1 (30 µg/mL of diet)
Cry1A.105 + Cry2Ab2 (dose range of 1:1 w/w mixture 1.56-100 µg/mL of diet)
Cry1A.105 + Cry2Ab2 (dose range of 1:1 w/w mixture 1.56-100 µg/mL of diet) + Cry3Bb1 (30 µg/mL of diet)
Cry3Bb1 spike (30 µg/mL of diet)

Summary of ECB bioassay treatments
Cry1A.105 (dose range 0.039 – 2.5 µg/mL of diet)
Cry1A.105 (dose range 0.039 – 2.5 µg/mL of diet) + Cry3Bb1 (30 µg/mL of diet)
Cry2Ab2 (dose range 0.156 – 10 µg/mL of diet)
Cry2Ab2 (dose range 0.156 – 10 µg/mL of diet) + Cry3Bb1 (30 µg/mL of diet)
Cry1A.105 + Cry2Ab2 (dose range of 1:1 w/w mixture 0.078 – 5.0 µg/mL of diet)
Cry1A.105 + Cry2Ab2 (dose range of 1:1 w/w mixture 0.078 – 5.0 µg/mL of diet) + Cry3Bb1 (30 µg/mL of diet)
Cry3Bb1 spike (30 µg/mL of diet)

Summary of CPB bioassay treatments
Cry3Bb1 (dose range 0.078 – 5.0 µg/mL of diet)
Cry3Bb1 (dose range 0.078 – 5.0 µg/mL of diet) + Cry1A.105 (30 µg/mL of diet)
Cry3Bb1 (dose range 0.078 – 5.0 µg/mL of diet) + Cry2Ab2 (30 µg/mL of diet)
Cry3Bb1 (dose range 0.078 – 5.0 µg/mL of diet) + Cry1A.105 + Cry2Ab2 (spiked with a 1:1 w/w mixture with each protein at 30 µg/mL of diet)
Cry1A.105 (30 µg/mL of diet)
Cry2Ab1 (spiked with 30 µg/mL of diet)
Cry1A.105 + Cry2Ab2 (spiked with a 1:1 w/w mixture with each protein at 30 µg/mL of diet)

At least six concentrations (only a range was provided in MRID 46951305) of each protein treatment were prepared by serial dilution with water. The range of concentrations was based on results of range-finding bioassays conducted prior to the study. Control treatments included a) the appropriate buffer diluted with water to the same level as in the highest concentration of the corresponding protein treatment, b) the Cry3Bb1 protein at a concentration of 30 µg/mL of diet, and c) two treatments of sterile water alone.

Diet for the CEW and ECB bioassays was Multiple Species Lepidoptera Diet with mold inhibitor (Southland Products, Lake Village AR), which was modified by replacing the standard agar with analytical grade agar. Diet for the CPB bioassays was Colorado Potato Beetle Diet (BioServe #F9380, Frenchtown, NJ), modified by replacing the standard agar with analytical grade agar and adding 1.25 mL Formalin per 500 mL batch of diet. The liquid diets were prepared in a blender, transferred to a 500-mL squirt bottle, and maintained in a water bath until they were mixed with the dosing solutions.

Each treatment and control dosing solution was incorporated 1:4 into the appropriate insect diet by adding diet to a final volume of 1.75 mL and agitating until visually homogeneous. The treated diet was then dispensed (0.5 mL/well) into 128-well bioassay trays and allowed to cool and solidify. A single larva was then randomly placed into each well. Each test material and buffer treatment included 32 insects, and the untreated control included 32 insect. The trays were then sealed with ventilated covers and incubated in darkness in an environmental chamber targeted at 27°C and 60% relative humidity. At test end (7 days for CEW and CPB; 12 days for ECB) the number of dead, first, second, and third or greater instars in each treatment was recorded.

The PROC PROBIT procedure (SAS v.9.1) was used to calculate LC_{50} and MIC_{50} (median molt inhibitory concentration) values based on mortality and arrestment (not developing past first instar), respectively. Fisher's Exact Test was used to compare responses between the spiked protein groups and their respective buffer control groups.

Results Summary

Results for the CEW test are provided in Table 1. The LC_{50} and MIC_{50} values for the Cry1A.105 + Cry2Ab2 protein mixture with and without the addition of Cry3Bb1 had overlapping 95% confidence intervals, indicating comparable activity. The LC_{50} and MIC_{50} values for Cry1A.105 and Cry2Ab2 with and without Cry3Bb1 also had overlapping 95% confidence intervals, indicating comparable activity. Mortality and arrestment in the untreated control were stated to be <1%, and the Cry3Bb1 protein control was stated to have no statistically significant effect on CEW survival (data not provided in MRID 46951305).

TABLE 1. LC_{50} and MIC_{50} for corn earworm fed diet containing Cry1A.105 and/or Cry2Ab2 protein with or without Cry3Bb1

Treatment	Cry3Bb1 spike (µg/mL)	LC_{50} (95% C.I.)	MIC_{50} (95% C.I.)
Cry1A.105	--	25.58 (21.07–31.62)	8.00 (6.85-9.30)
	30	28.09 (23.52-34.10)	9.08 (7.80-10.55)
Cry2Ab2	--	9.91 (8.63-11.37)	6.6 (5.77-7.53)
	30	11.37 (9.90-13.07)	6.28 (4.98-7.87)
Cry1A.105 + Cry2Ab2	--	20.69 (13.98-31.86)	7.79 (6.83-8.86)
	30	18.73 (14.09-25.28)	6.65 (5.73-7.67)

Data from p. 19, MRID 46951305

Results for the ECB test are provided in Table 2. The LC_{50} and MIC_{50} values for the Cry1A.105 + Cry2Ab2 protein mixture with and without the addition of Cry3Bb1 had overlapping 95% confidence intervals, indicating comparable activity. The LC_{50} and MIC_{50} values for Cry2Ab2 with and without Cry3Bb1 also had overlapping 95% confidence intervals, indicating comparable activity. The LC_{50} values for Cry1A.105 with and without Cry3Bb1 had overlapping 95% confidence intervals, indicating comparable activity. There was a small separation in the MIC_{50} values for Cry1A.105 with and without Cry3Bb1, but the differences were slight. Mortality and arrestment in the untreated control were stated to be <1%, and the Cry3Bb1 protein control was stated to have no statistically significant effect on CEW survival (data not provided in MRID 46951305).

TABLE 2. LC_{50} and MIC_{50} for European corn borer fed diet containing Cry1A.105 and/or Cry2Ab2 protein with or without Cry3Bb1

Treatment	Cry3Bb1 spike (µg/mL)	LC_{50} (95% C.I.)	MIC_{50} (95% C.I.)
Cry1A.105	--	0.43 (0.35-0.53)	0.13 (0.12-0.14)
	30	0.53 (0.47-0.61)	0.10 (0.09-0.11)
Cry2Ab2	--	1.48 (1.33-1.64)	0.97 (0.74-1.27)
	30	1.64 (1.47-1.83)	0.85 (0.77-0.95)
Cry1A.105 + Cry2Ab2	--	0.62 (0.55-0.71)	0.20 (0.18-0.23)
	30	0.74 (0.54-1.01)	0.18 (0.16-0.20)

Data from p. 20, MRID 46951305

Results for the CPB test are provided in Table 3. The LC_{50} values for Cry3Bb1 alone or combined with either Cry1A.105 or Cry1A.105 + Cry2Ab2 had overlapping 95% confidence intervals, indicating comparable activity. There was a separation in the confidence intervals for the LC_{50} values for Cry3Bb1 alone and Cry3Bb1 combined with Cry2Ab2; however, the LC_{50} values for each CPB treatment with Cry3Bb1 alone were stated to be similar to historical LC_{50} values. Mortality in the untreated control was stated to be <7%, and there was no statistically significant effect on survival in treatments that received Cry1A.105, Cry2Ab2, or Cry1A.105 + Cry2Ab2.

TABLE 3. LC_{50} and MIC_{50} for Colorado potato beetle fed diet containing Cry1A.105 and/or Cry2Ab2 protein with or without Cry3Bb1

Treatment	Cry1A.105 spike (µg/mL)	Cry2Ab2 spike (µg/mL)	LC_{50} (95% C.I.)
Cry3Bb1	--		0.41 (0.29-0.58)
	30		0.28 (0.23-0.33)
Cry3Bb1	--	30	0.23 (0.20-0.27)
	30	30	0.25 (0.21-0.30)

Data from p. 21, MRID 46951305

Conclusions

The study authors concluded that the combined activity of Cry1A.105 and Cry2Ab2 in artificial diet against CEW and ECB were not significantly altered by the presence of Cry3Bb1. Also, Cry3Bb1 activity in artificial diet against CPB was not significantly altered by the presence of Cry1A.105 and Cry2Ab2. Based on these results, there is no interaction between functional activity of the Cry1A.105/Cry2Ab2 and Cry3Bb1 proteins.

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R155632

Chemical: **Bacillus thuringiensis Cry1A.105 protein and genetic material necessary (vector PV-ZMIR245) for its production in corn**
Bacillus thuringiensis Cry2Ab2 protein and the genetic material necessary (vector PV-ZMIR245) for its production in corn
Bacillus thuringiensis Cry3Bb1 protein and the genetic material necessary (vector ZMIR39) for its production in corn

PC Code:
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006498

HED File Code: **41600 BPPD Other**
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