CRY 1F *BACILLUS THURINGIENSIS* VAR. *AIZAWAI* DELTA ENDOTOXIN: A DIETARY TOXICITY STUDY WITH PARASITIC HYMENOPTERA

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 354-114D

U.S. ENVIRONMENTAL PROTECTION AGENCY
SERIES 885 MICROBIAL PESTICIDE TEST GUIDELINES
OPPTS NUMBER 885.4340

AUTHORS:

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STUDY INITIATION DATE: March 16, 1999

STUDY COMPLETION DATE: December 8, 1999

SUBMITTED TO:

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA section 10(d) (1)(A), (B), or (C).

Company: Mycogen c/o Dow AgroSciences (Typed Name)

Company Agent: Diane Shanahan (Typed Name)

Title: Registration Manager

Signature: Diane Shanahan Date: 12/10/99

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Dow AgroSciences LLC/Mycogen Corporation

TITLE: Cry 1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin: A Dietary Toxicity Study with Parasitic Hymenoptera

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 354-114D

STUDY COMPLETION DATE: December 8, 1999

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice, ENV/MC/CHEM (98) 17, Paris 1998; and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984, with the following exceptions:

Verification of the test concentrations, stability and homogeneity of the test substance in the diet were not determined.

The stability of the test substance under the conditions of storage at the test site was not conducted in compliance with Good Laboratory Practice Standards.

STUDY DIRECTOR:

_____ DATE 8 Dec 99

John R. Porch
Senior Biologist

SPONSOR:

Mycogen
c/o Dow AgroSciences _____ DATE 12/10/99
Sponsor

_____ DATE 12/10/99
Applicant/Submitter

QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice, ENV/MC/CHEM (98) 17, Paris 1998; and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984. The dates of all inspections and audits, and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

		DATE REPORTED TO:	
ACTIVITY	DATE CONDUCTED	STUDY DIRECTOR	MANAGEMENT
Protocol	March 22, 1999	March 22, 1999	March 22, 1999
Initial Trial 354-114			
Test Substance Preparation and Test Initiation	March 24, 1999	March 25, 1999	March 29, 1999
2nd Trial 354-114A			
Test Substance Preparation	April 9, 1999	April 9, 1999	April 9, 1999
Definitive Trial 354-114D			
Test Substance Preparation	July 26, 1999	July 26, 1999	July 27, 1999
Raw Data and Draft Report	August 23 & 24, 1999	August 24, 1999	September 3, 1999
Final Report	December 8, 1999	December 8, 1999	December 8, 1999

Tim Springer

Timothy A. Springer
Manager, Technical and Regulatory Support

DATE 12/8/99

REPORT APPROVAL

SPONSOR: Dow AgroSciences LLC/Mycogen Corporation

TITLE: Cry 1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin: A Dietary Toxicity Study with the Parasitic Hymenoptera

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 354-114D

STUDY DIRECTOR:

John R. Porch DATE 8 Dec 99
John R. Porch
Senior Biologist

MANAGEMENT:

Mark Jaber DATE 12/8/99
Mark Jaber
Wildlife Toxicologist

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SUMMARY

SPONSOR:	Dow AgroSciences LLC/Mycogen Corporation
CONTACT:	Ms. Diane Shanahan
LOCATION OF STUDY, RAW DATA AND A COPY OF FINAL REPORT:	Wildlife International Ltd. Easton, Maryland 21601

WILDLIFE INTERNATIONAL LTD. PROJECT NO.:	354-114D
TEST SUBSTANCE:	Cry 1F *Bacillus thuringiensis* var. *aizawai* delta endotoxin
STUDY:	Cry 1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin: A Dietary Toxicity Study with Parasitic Hymenoptera
NOMINAL TEST CONCENTRATIONS:	Negative Control and 320 ppm a.i.
TEST DATES:	Experimental Start (OECD) - March 24, 1999 Experimental Start (EPA) - July 28, 1999 Biological Termination - August 9, 1999 Experimental Termination - August 9, 1999
LENGTH OF EXPOSURE:	12 Days

TEST ORGANISM:	Parasitic Hymenopteran (*Nasonia vitripennis*)
SOURCE OF TEST ORGANISMS:	Carolina Biological Supply Company 2700 York Road Burlington, North Carolina 27215-3398
LIFE STAGE OF TEST ORGANISMS:	Adult

DIETARY LC50:	>320 ppm a.i.
NO OBSERVED EFFECT CONCENTRATION:	320 ppm a.i.

INTRODUCTION

This study was conducted by Wildlife International Ltd. for Dow AgroSciences LLC/Mycogen Corporation at the Wildlife International Ltd. toxicology facility in Easton, Maryland. The test was repeated once to lower the limit test concentration at the request of the Sponsor, once because of escaped insects, and two times due to high control mortality. The definitive test was conducted from July 28, 1999 to August 9, 1999. Raw data generated at Wildlife International Ltd. and a copy of the final report are filed under Project Number 354-114D in archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of the study was to evaluate the toxicity of Cry 1F *Bacillus thuringiensis* var. *aizawai* delta endotoxin administered to parasitic hymenoptera (*Nasonia vitripennis*) in the diet.

EXPERIMENTAL DESIGN

Parasitic hymenoptera were exposed to one limit test concentration of Cry 1F *Bacillus thuringiensis* var. *aizawai* delta endotoxin in a honey diet. The test substance concentration represented up to 20X the expression of Cry 1F *Bacillus thuringiensis* var. *aizawai* delta endotoxin present in pollen. A negative control group was maintained concurrently. Three replicate test chambers were maintained in each treatment and control group, with 25 wasps in each test chamber. Observations of mortality and clinical signs were conducted twice within the first four hours of test initiation, and then continued daily until negative control mortality exceeded 20% on Day 12 of the test. The LC50 value and the no-observed-effect-concentration (NOEC) were determined by visual examination of the mortality and clinical observation data.

Selection of the limit test concentration was based upon information supplied by the Sponsor and the results of the initial trial of the test. The nominal test concentration to which the parasitic hymenoptera were exposed was 320 ppm of Cry 1F *Bacillus thuringiensis* var. *aizawai* delta endotoxin (ppm a.i.).

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "Bt Cry 1F delta-endotoxin: A Dietary Toxicity Study with Parasitic Hymenoptera". The protocol was based upon procedures outlined in Series 885 of The U.S. Environmental Protection Agency's Microbial Pesticide Test Guidelines, OPPTS Number 885.4340 (1).

Test Substance

The test substance was received from Mycogen Corporation on March 9, 1999 and was assigned the Wildlife International Ltd. identification number 4807. The test substance was an off white powder, identified as: Cry 1F microbial (truncated); Lot no. 1599-45. The reported purity of the test substance was 11.4% active ingredient. The test substance was stored refrigerated. A summary of the GLP characterization of the test substance is presented in Appendix I.

Test Organism

The parasitic hymenopteran (*Nasonia vitripennis*) is useful in evaluating the potential hazards of agricultural chemicals and microbiological pest control agents to nontarget insects and is an important parasite on pupae of several fly species. Wasps used in the test were obtained from Carolina Biological Supply Company, Burlington, North Carolina as larvae in blowfly (*Sarcophaga* sp.) pupae. The pupae were placed in paper cups in an incubator over an eight day period to allow the parasitic wasps to emerge. The incubator was set to maintain a temperature of approximately 26 to 28 °C, with relative humidity above approximately 50%. During the eight-day holding period prior to testing, the wasps were provided *ad libitum* access to food (commercial honey).

Test Chambers

The test chambers were disposable half-pint rolled paper containers measuring approximately 9 cm in diameter and 5 cm high. Each container was covered with a disposable plastic petri dish (approximately 10 cm in diameter) through which an inverted 20 ml glass vial containing deionized water was inserted. A cotton swab coated with the appropriate diet was inserted through the side of each test chamber and was held in place with a small stopper. The test chambers were identified by study number, dosage group, and replicate.

Preparation of Diets

The test diet was prepared weekly at a nominal concentration of 320 ppm a.i. (Appendix II). A calculated amount of the test substance was weighed and commercial honey was added to bring the final volume of the diet to 50 mL. The negative control diet was prepared in the same manner without the addition of any test substance. The diets were stored under refrigeration.

Diet Administration

At initiation of the test, the newly emerged wasps were immobilized with nitrogen gas and impartially distributed to the test chambers. Twenty-five wasps of uniform size were placed in each test chamber with the appropriate test or control diet. Fresh diets were presented to the wasps twice each week by carefully replacing the diet-coated cotton swabs in each test chamber. The swabs were coated with the diets after thoroughly hand-mixing the refrigerated diets. The wasps were allowed *ad libitum* access to the test diets throughout the test period. Fresh water was supplied to the wasps as needed throughout the test period.

Environmental Conditions

During the test, the wasps were placed in an incubator set to maintain a temperature range of approximately 26 to 28 °C, with relative humidity above approximately 40%. Temperature and relative humidity were measured in the incubator at least once daily. During the test the temperature in the incubator averaged 26.7 ± 0.2 °C (SD) with a range of 26.2 to 27.0 °C, while average relative humidity was 76 ± 7% (SD) with a range of 59 to 86%. The photoperiod during the test was approximately 12 hours of light and was controlled with an automatic timer. Overhead fluorescent lighting was used during testing.

Observations

The wasps were observed periodically in order to evaluate the numbers of mortalities and the numbers of individuals exhibiting clinical signs of toxicity or abnormal behavior. Observations were made approximately ½ hour and 1½ hours after test initiation on Day 0, and then continued daily throughout the remainder of the test period. The test was terminated after mortality in the negative control exceeded 20% on Day 12 of the test.

Data Analysis

Because this was a limit test, the LC50 value could not be statistically defined. Therefore, an estimation of the value was made by a visual inspection of the mortality data. Comparisons of the test and control groups were conducted using a t-test (2) to determine if there were any significant differences in mortality at test termination. The no-observed-effect-concentration (NOEC) was determined by examination of the mortality and clinical observation data.

RESULTS AND DISCUSSION

Observations and Measurements

The test was run initially with the test concentration of 480 ppm a.i., which represents up to 30X the expression of Cry 1F *Bacillus thuringiensis* var. *aizawai* delta endotoxin present in pollen. Mortality in the control and treatment groups at test termination were 23 and 47%, respectively (Table 1). Since the test substance could not be precluded as the cause of increased mortality in the 480 ppm a.i. group, the test was repeated using a lower concentration. The definitive limit test was conducted with the test concentration of 320 ppm a.i., which represents up to 20X the expression of Cry 1F *Bacillus thuringiensis* var. *aizawai* delta endotoxin present in pollen.

The data from observations of the wasps for mortality and other clinical signs during the definitive test are shown in Table 2. The test terminated on day 12 after mortality in the negative control group exceeded 20%. At test termination, mortality in the negative control group was 39% (27 of 69). Six wasps were eliminated from final mortality results since they were found stuck in honey. Several wasps in the control group exhibited lethargy and immobility during the test. All other surviving wasps in the control group were normal in appearance and behavior throughout the test period.

Mortality at test termination in the 320 ppm a.i. treatment group was 47% (34 of 72). Three wasps were not included in the final mortality results since they were stuck in honey. Several wasps in the 320 ppm a.i. treatment group exhibited lethargy and immobility during the test, which was comparable to the negative control. All other surviving wasps in the 320 ppm a.i. treatment group were normal in appearance

and behavior throughout the test period. Mortality at test termination was compared to the negative control group using a t-test. Differences were not statistically significant ($p>0.05$). Therefore, the mortalities and clinical signs observed were not considered to be related to treatment with the test substance.

CONCLUSION

Parasitic hymenoptera exposed to a single test concentration of Cry 1F *Bacillus thuringiensis* var. *aizawai* delta endotoxin in the diet (320 ppm a.i.) showed no treatment-related mortality and no signs of toxicity over the 12-day test period. The test substance concentration, which represented up to 20X the expression of Cry 1F *Bacillus thuringiensis* var. *aizawai* delta endotoxin present in pollen, was therefore determined to be a no-observed-effect-concentration, and the LC50 value was estimated to be greater than the limit test concentration of 320 ppm a.i..

REFERENCES

1 **U.S. Environmental Protection Agency**. 1996. Series 885-Microbial Pesticide Test Guidelines, OPPTS Number 885.4340: Nontarget Insect Testing, Tier 1.

2 **Finney, D. J**. 1971. *Statistical Methods in Biological Assay*, 2nd Ed. Griffin Press, London.

TABLE 1

Cumulative Mortality and Observations of Parasitic Hymenoptera
Exposed to Cry 1F *B.t.* var. *aizawai* Delta Endotoxin in the Initial Test (354-114A)

		Negative Control		480 ppm a.i.	
Day	Replicate	Mortality[2]	Observations[3]	Mortality	Observations
Day 0[1] - ½ Hour	A	0/25	25 AN	0/25	25 AN
	B	0/25	25 AN	0/25	25 AN
	C	0/25	25 AN	0/25	25 AN
Day 0 - 2 Hours	A	0/25	25 AN	0/25	25 AN
	B	0/25	25 AN	0/25	25 AN
	C	0/25	25 AN	0/25	25 AN
Day 1	A	2/25	23 AN	0/25	25 AN
	B	1/25	24 AN	4/25	21 AN
	C	0/25	25 AN	0/25	25 AN
Day 2	A	3/25	22 AN	1/25	24 AN
	B	2/25	23 AN	6/25	19 AN
	C	0/25	25 AN	1/25	24 AN
Day 3	A	6/25	19 AN	1/25	24 AN
	B	2/25	23 AN	7/25	18 AN
	C	0/25	24 AN;1 L	4/25	21 AN
Day 4	A	7/25	18 AN	3/25	22 AN
	B	2/25	23 AN	8/25	17 AN
	C	0/25	25 AN	5/25	20 AN
Day 5	A	7/25	18 AN	3/25	22 AN
	B	2/25	23 AN	8/25	17 AN
	C	0/25	25 AN	6/25	19 AN
Day 6	A	7/25	18 AN	4/25	21 AN
	B	2/25	23 AN	8/25	17 AN
	C	0/25	25 AN	8/25	17 AN

[1]Day 0 observation times represent the approximate number of hours after completion of diet presentation.
[2]Mortality data are presented as the cumulative number dead per number exposed.
[3]Observations: Number of wasps exhibiting clinical signs: AN = appear normal; I = immobile; L = lethargic.

TABLE 1

Cumulative Mortality and Observations of Parasitic Hymenoptera
Exposed to Cry 1F *B.t.* var. *aizawai* Delta Endotoxin in the Initial Test (354-114A)
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		Negative Control		480 ppm a.i.	
Day	Replicate	Mortality[2]	Observations[3]	Mortality	Observations
Day 7	A	8/25	17 AN	5/25	20 AN
	B	2/25	23 AN	8/25	17 AN
	C	0/25	25 AN	8/25	17 AN
Day 8	A	8/25	17 AN	5/25	20 AN
	B	2/25	23 AN	8/25	17 AN
	C	0/25	25 AN	8/25	17 AN
Day 9	A	9/25	16 AN	8/25	17 AN
	B	3/25	22 AN	8/25	17 AN
	C	0/25	25 AN	10/25	15 AN
Day 10	A	10/25	15 AN	9/25	16 AN
	B	4/25	21 AN	9/25	16 AN
	C	0/25	25 AN	10/25	15 AN
Day 11	A	13/25	12 AN	14/25	11 AN
	B	4/25	21 AN	11/25	14 AN
	C	0/25	25 AN	10/25	15 AN

Percent Mortality:		Replicate	Group	Replicate	Group
	A	52		56	
	B	16		44	
	C	0	23	40	47

[1]Day 0 observation times represent the approximate number of hours after completion of diet presentation.
[2]Mortality data are presented as the cumulative number dead per number exposed.
[3]Observations: Number of wasps exhibiting clinical signs: AN = appear normal; I = immobile; L = lethargic.

TABLE 2

Cumulative Mortality and Observations of Parasitic Hymenoptera
Exposed to Cry 1F *B.t.* var. *aizawai* Delta Endotoxin in the Definitive Test (354-114D)

		Negative Control		320 ppm a.i.	
Day	Replicate	Mortality[2]	Observations[3]	Mortality	Observations
Day 0[1] - ½ Hour	A	0/25	25 AN	0/25	25 AN
	B	0/25	25 AN	0/25	25 AN
	C	0/25	25 AN	0/25	25 AN
Day 0 - 1½ Hours	A	0/25	25 AN	0/25	25 AN
	B	0/25	25 AN	0/25	25 AN
	C	0/25	25 AN	0/25	25 AN
Day 1	A	0/25	25 AN	2/25	23 AN
	B	0/25	25 AN	0/25	25 AN
	C	0/25	25 AN	0/25	25 AN
Day 2	A	0/25	25 AN	2/25	23 AN
	B	0/25	25 AN	0/25	25 AN
	C	0/25	25 AN	1/25	24 AN
Day 3	A	0/25	25 AN	2/25	23 AN
	B	0/25	25 AN	0/25	25 AN
	C	0/25	25 AN	1/25	24 AN
Day 4	A	0/25	25 AN	2/25	23 AN
	B	1/25	24 AN	0/25	25 AN
	C	0/25	25 AN	1/25	24 AN
Day 5	A	0/25	25 AN	2/25	23 AN
	B	1/25	24 AN	0/25	25 AN
	C	0/25	25 AN	1/25	24 AN
Day 6	A	0/25	25 AN	2/25	23 AN
	B	1/25	24 AN	2/25	23 AN
	C	0/25	25 AN	1/25	24 AN

[1]Day 0 observation times represent the approximate number of hours after completion of diet presentation.
[2]Mortality data are presented as the cumulative number dead per number exposed.
[3]Observations: Number of wasps exhibiting clinical signs: AN = appear normal; I = immobile; L = lethargic.
[4]Six wasps were stuck in honey and were omitted from mortality results.
[5]Three wasps were stuck in honey and were omitted from mortality results.
[6]Percent mortality includes nimber of immobile beetles at test termination.

TABLE 2

Cumulative Mortality and Observations of Parasitic Hymenoptera
Exposed to Cry 1F *B.t.* var. *aizawai* Delta Endotoxin in the Definitive Test (354-114D)
Page 2

		Negative Control		320 ppm a.i.	
Day	Replicate	Mortality[2]	Observations[3]	Mortality	Observations
Day 7	A	0/25	1L; 24 AN	2/25	23 AN
	B	1/25	24 AN	2/25	23 AN
	C	0/25	1L; 24 AN	2/25	2L; 21 AN
Day 8	A	1/25	24 AN	2/25	23 AN
	B	1/25	24 AN	3/25	1I; 21AN
	C	2/25	1I; 22 AN	3/25	3I; 19 AN
Day 9	A	4/25	1L; 20 AN	2/25	23 AN
	B	2/25	23 AN	8/25	1I; 16 AN
	C	2/25	1I; 3L; 19 AN	5/25	5L; 15 AN
Day 10	A	4/25	1L; 20 AN	2/25	23 AN
	B	3/25	22 AN	9/25	16 AN
	C	3/25	3L; 19 AN	6/25	8L; 11 AN
Day 11	A	7/25	5L; 13 AN	2/25	23 AN
	B	4/25	4L; 17 AN	13/25	2L; 10 AN
	C	3/25	3L; 19 AN	8/25	2I; 6L; 9 AN
Day 12	A	8/25	1I; 3L; 13 AN	2/22[5]	3L; 17 AN
	B	9/25	2I; 3L; 11 AN	15/25	2I; 2L; 6 AN
	C	6/19[4]	1I; 3L; 9 AN	14/25	1I; 5L; 5 AN

Percent Mortality[6]:		Replicate	Group	Replicate	Group
	A	36		9	
	B	44		68	
	C	37	39	60	47

[1]Day 0 observation times represent the approximate number of hours after completion of diet presentation.
[2]Mortality data are presented as the cumulative number dead per number exposed.
[3]Observations: Number of wasps exhibiting clinical signs: AN = appear normal; I = immobile; L = lethargic.
[4]Six wasps were stuck in honey and were omitted from mortality results.
[5]Three wasps were stuck in honey and were omitted from mortality results.
[6]Percent mortality includes number of immobile wasps at test termination.

APPENDIX I

Test Substance Characterization

SUMMARY

(In accordance with 40 CFR part 152, this summary is available for public release after registration)

STUDY TITLE

Characterization of Expressed Cry1F Protein in Maize Tissues (Pollen, Grain, Grain-Containing Feed, and Purified Maize-Expressed Cry1F Protein) and Microbial Expressed Cry1F Delta Endotoxin by Biological and Biochemical Procedures

DATA REQUIREMENTS

Not Applicable

AUTHORS

D. L. Young, R. A. Herman

STUDY COMPLETED ON

November 18, 1999

PERFORMING LABORATORIES

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Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

Pioneer Hi-Bred International
7300 NW 62nd Ave.
Johnston, Iowa 50131

LABORATORY STUDY ID

990027

Characterization of Expressed Cry1F Protein in Maize Tissues (Pollen, Grain, Grain-Containing Feed, and Purified Maize-Expressed Cry1F Protein) and Microbial Expressed Cry1F Delta Endotoxin by Biological and Biochemical Procedures

SUMMARY

This report contains characterization information of maize lines that have been modified to express the Cry1F protein to support regulatory submissions including equivalency and toxicological studies. Maize tissues expressing Cry1F protein (pollen, grain, grain-containing feed and purified maize-expressed Cry1F protein) and microbial expressed Cry1F protein were evaluated and characterized by biological and biochemical analysis. The biological analysis results confirmed the biological activity of the pollen, grain, purified maize-expressed Cry1F protein and bacterially derived Cry1F protein when tested with susceptible insect species, either European corn borer or tobacco budworm. The biochemical analysis was performed to quantify and characterize the extractable Cry1F protein of the pollen, grain, purified maize-expressed Cry1F protein and bacterially derived Cry1F protein. The biochemical analysis of the tissues included ELISA and SDS-PAGE followed by Western Blotting. Biochemical analysis data demonstrated the test materials contained immunoreactive Cry1F protein at the expected molecular weight.

STUDY TITLE

Characterization of Expressed Cry1F Protein in Maize Tissues (Pollen, Grain, Grain-Containing Feed, and Purified Maize-Expressed Cry1F Protein) and Microbial Expressed Cry1F Delta Endotoxin by Biological and Biochemical Procedures

DATA REQUIREMENTS

Not Applicable

AUTHORS

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R. A. Herman

STUDY COMPLETED ON

November 18, 1999

PERFORMING LABORATORY

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Indianapolis, Indiana 46268-1054

Pioneer Hi-Bred International
7300 NW 62nd Ave.
Johnston, Iowa 50131

LABORATORY STUDY ID

990027

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Cry1F Delta Endotoxin Protein

Title: Characterization of Expressed Cry1F Protein in Maize Tissues (Pollen, Grain, Grain-Containing Feed, and Purified Maize-Expressed Cry1F Protein) *and Microbial* Expressed *Cry1F* Delta Endotoxin by Biological and Biochemical Procedures

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10 (d)(1)(A)(B), or (C).*

Company: Dow AgroSciences LLC

Company Agent: D. M. Shanahan

Title: Regulatory Manager

Signature: [signature]

Date: 11/17/99

*In the United States, the above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Characterization of Expressed Cry1F Protein in Maize Tissues (Pollen, Grain, Grain-Containing Feed, and Purified Maize-Expressed Cry1F Protein) *and Microbial* Expressed *Cry1F* Delta Endotoxin by Biological and Biochemical Procedures

Study Initiation Date: August 4, 1998 Study Completion Date: November 18, 1999

Experimental Start Date: August 4, 1998 Experiment Termination Date: September 24, 1999

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organisation for Economic Co-Operation and Development
ISBN 92-64-12367-9, Paris 1982

At Pioneer Hi-Bred, during the first three biological experiments (8/98, 9/98, and 2/99) the laboratory was working towards being GLP compliant; therefore, several GLP-required elements were not yet in place. GLP training and personnel record information was instituted for scientists performing bioassay tests during the course of this study. Protocols and SOPs had been approved, and Quality Assurance conducted in-phase inspections but in some instances SOPs were not present or available during the conduct of the study. On several occasions data were not recorded or corrected exactly as required by GLPs. Maintenance logs were not in place for some equipment used in the study, some reagents were not properly labeled and calibrations were not always performed. The GLP required documentation of the two reference substances used in the biochemical study was not performed (the bacterially derived Cry1F protein and the BioRad BSA protein).

At Dow AgroSciences, management-approved SOPs specific to the insect bioassay were not in place. The GLP required documentation for reference standards were not met.

Diane Shanahan 11/17/99

D. M. Shanahan, Sponsor
Dow AgroSciences LLC
Date

Diane Shanahan 11/17/99

D. M. Shanahan, Submitter
Dow AgroSciences LLC
Date

D R Young 11/18/99

D. L. Young, Study Director/Author
Dow AgroSciences LLC
Date

Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page

Compound: Cry 1F Protein **Study ID:** 990027

Title: Characterization of Expressed Cry1F Protein in Maize Tissues (Pollen, Grain, Grain Containing Feed, and Purified Maize-Expressed Cry1F Protein) and Microbial Expressed Cry1F Delta Endotoxin by Biological and Biochemical Procedures

Study Initiation Date: 8/4/98 **Study Completion Date:** 11/18/99

GLP Quality Assurance Inspections

Date of GLP Inspection(s)	Date Reported to the Study Director and to Management	Phases of the Study which received a GLP Inspection by the Quality Assurance Unit
8/4/98	8/12/98	Elisa, extraction, Bradford assay, Bioassay of pollen (PHI)
2/23/99	3/1/99	Bioassay of microbial tox lot
6/17/99	6/18/99	Bioassays of pollen, microbial protein (PHI)
8/11/99	8/12/99	Bioassay for Amendment 8 – Test/Control substance preparation, dilution, application, test system placement
8/19/99	8/25/99	Sample prep for Elisa assay of corn grain, quail and fish feed
9/22/99	9/23/99	Raw data and draft report (PHI)
9/22-24/99	9/24/99	Raw data and draft report (PHI)
11/1-4/99	11/16/99	Raw data and draft report

QUALITY ASSURANCE STATEMENT:

The Quality Assurance Unit has reviewed the final study report and has determined that the report reflects the raw data generated during the conduct of this study.

D. Keyes 11/18/99

D. Keyes
Dow AgroSciences, Quality Assurance

Date

SIGNATURE PAGE

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Characterization of Expressed Cry1F Protein in Maize Tissues (Pollen, Grain, Grain-Containing Feed, and Purified Maize-Expressed Cry1F Protein) and Microbial Expressed Cry1F Delta Endotoxin by Biological and Biochemical Procedures

ABSTRACT

This report contains characterization information used in support of regulatory submissions for maize lines that have been modified to express the Cry1F protein. The activity of maize tissues expressing Cry1F protein (pollen, grain, grain-containing feed and purified maize-expressed Cry1F protein) and microbial derived Cry1F protein were evaluated and characterized by biological and biochemical analysis.

Biological analysis of the purified maize-expressed Cry1F protein, the bacterially derived Cry1F protein, and maize pollen test substances demonstrates that the Cry1F protein present in all test substances was active against European corn borer (ECB) at all time points tested. Activity of each test substance analyzed is summarized in the following table:

ECB Potency

Test Substance	Activity
1507 – Maize pollen	100% mortality at high dose of 0.2 mg Cry1F/µL buffer diet overlay
5XH751 – Control pollen	No activity
1568-022A – Purified Maize-expressed Protein Control	0-36% Mortality
1568-022B – Purified Maize-expressed Cry1F Protein	LC_{50} = <0.03 µg Cry1F/mL diet
101788 – Microbial Cry1F Powder	LC_{50} = <0.02 µg - 0.06 µg Cry1F/mL diet

The potency of the test substance against tobacco budworm (TBW) was measured by determining the GI_{50} (concentration that inhibits growth by 50%). LC_{50}s (concentration that kills 50% of the insects) were not useful for indexing the potency of the test substances due to insufficient mortality at the highest concentrations tested. Biological analysis of the maize grain and feeds containing maize grain with TBW are summarized in the following tables:

TBW Potency Estimates with Cry1F Maize Grain, Quail Feed, and Fish Feed

Test Substance	GI_{50} (95% confidence limits) in % Cry1F Maize Grain[a]
maize grain expressing Cry1F	0.15 (0.07-0.32)
0-day quail feed containing Cry1F expressing maize	0.15 (0.06-0.41)
5-day quail feed containing Cry1F expressing maize	0.20 (0.05-0.77)
fish feed containing Cry1F expressing maize	>7.7

[a]Expressed as a percent of maize grain expressing Cry1F applied in the treatment suspensions.

TBW Weights with Fish Feed at 7.7% Maize

Test Substance	Insect Weight (mg)
Cry1F fish feed	875.7[a]
control fish feed	1032.3[a]
agar control	1214.9[a]
2:1 acetone:water	1253.7[a]

[a]The means were not significantly different ($\alpha = 0.05$) based on analysis of variance (1).

TBW results demonstrate comparable activity between the maize grain and the maize grain component of the quail feed. No statistically significant difference in activity was observed between fish feed containing Cry1F and the three controls.

Biochemical analysis by ELISA of the purified maize-expressed Cry1F protein, microbial derived Cry1F protein, maize grain, feeds containing maize grain and maize pollen test substances demonstrate that the Cry1F protein was present in all Cry1F expressed test substances. The range of quantitation of extractable Cry1F protein is summarized in the following table:

Test and Control Substances (sample number and identification)	Cry1F Concentration (ng Cry1F/mg)[a]
1507 – Maize pollen	30.7 – 32.8
5XH751 – Control pollen	ND[b]
1568-022A – Purified Maize-expressed Protein Control	ND
1568-022B – Purified Maize-expressed Cry1F Protein	1511.33 ± 268.9
101788 – Microbial Cry1F Powder	114,000
TSN101791 – maize grain containing Cry1F	2.2 - 3.5
TSN101792 – Control maize	ND
TSN101834 – fish feed containing control maize	ND
TSN101835 – fish feed containing Cry1F expressing maize	ND
TSN101862 – quail feed, Day 0 containing Cry1F expressing maize	0.2 - 1.1
TSN101863 – quail feed, Day 0 containing control maize	ND
TSN101864 – quail feed, Day 5 containing Cry1F expressing maize	0.2 - 0.6

[a] ng Cry1F/mg of tissue or powder weighed.
[b] ND = not detectable, below the limit of detection of the ELISA (0.04 ng/mg), 5 mg sample extracted.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting results indicated an expected immunoreactive molecular weight band of ~64kDa as previously reported (2) in both the microbial expressed Cry1F protein and the maize grain expressed Cry1F protein.

APPENDIX II

Diet Preparation

Nominal weights and volumes of constituents used to prepare test diets during the definitive test:

Nominal Concentration (ppm a.i.)	Test Substance Weight (g)	Final Volume in Honey (mL)
320	0.1404	50

The diet was prepared by weighing the appropriate amount of test substance into a tared 100 mL beaker (precalibrated to 50 mL). Small portions of honey were added and the mixture was hand stirred until the final volume was reached. The diet was stirred by hand until the test substance was in suspension, then was covered with parafilm. Cotton swabs were soaked in the diet prior to administering the diet to the wasps. The control diet was prepared in the same manner except no test substance was used.

APPENDIX III

Changes to Protocol

This study was conducted in accordance with the approved Protocol with the following changes:

1. The test concentration was changed by amendment to 20X the expression of the test substance in pollen, or 320 ppm.
2. The proposed test dates were changed twice during repeats of the test.
3. Half-pint rolled paper containers were used for test chambers.

APPENDIX IV

Personnel Involved In Study

The following key Wildlife International Ltd. personnel were involved in the conduct or management of this study:

(1) Henry O. Krueger, Ph.D., Director, Aquatic Toxicology & Non-Target Plants

(2) John R. Porch, Senior Biologist

(3) Kimberly A. Hoxter, Senior Biologist

(4) Mark Jaber, Wildlife Toxicologist

STUDY TITLE

Supplement to MRID 45020111: Cry 1F *Bacillus Thuringiensis* Var. *Aizawai* Delta Endotoxin: A Dietary Toxicity Study with Parasitic Hymenoptera

DATA REQUIREMENTS

None

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STUDY COMPLETED ON

January 16, 2001

SUBMITTED BY

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LABORATORY STUDY ID

GH-C 5170

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Cry 1F

Title: Supplement to MRID 45020111: Cry 1F *Bacillus Thuringiensis* Var. *Aizawai* Delta Endotoxin: A Dietary Toxicity Study with Parasitic Hymenoptera

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10 (d)(1)(A)(B), or (C).*

Company: Dow AgroSciences LLC

Company Agent: P. L. Hunst

Title: Regulatory Manager

Signature: Penny L Hunst

Date: 1/16/04

*In the United States, the above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Supplement to MRID 45020111: Cry 1F *Bacillus Thuringiensis* Var. *Aizawai* Delta Endotoxin: A Dietary Toxicity Study with Parasitic Hymenoptera

Study Initiation Date:	1/12/01	Study Completion Date:	1/16/01
Experimental Start Date:	1/12/01	Experiment Termination Date:	1/12/01

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organisation for Economic Co-Operation and Development
ISBN 92-64-12367-9, Paris 1982

No aspect of this study is subject to Good Laboratory Practice Standards.

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QUALITY ASSURANCE STATEMENT

Compound: Cry 1F

Title: Supplement to MRID 45020111: Cry 1F *Bacillus Thuringiensis* Var. *Aizawai* Delta Endotoxin: A Dietary Toxicity Study with Parasitic Hymenoptera

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NON-GLP STUDY

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A reviewer noted that based on current pollen expression data that the Margin of Exposure (MOE) as reported in this study is in error. The original protocol and subsequent report based the MOE on preliminary information that indicated that the expression of Cry1F delta-endotoxin in pollen was 16 μg/g. A definitive study appended to the original report indicated that the expression of Cry1F delta-endotoxin in pollen is 32 μg/g. Therefore, the MOE should be 10 rather than 20 as indicated in the original report. This change should be noted on pages 9, 12 and 13 of the original report. Additionally, the MOE for the initial test should be 15 rather than 30 as reported on page 12.