

CRY 1F *BACILLUS THURINGIENSIS* VAR. *AIZAWAI* DELTA ENDOTOXIN:
A DIETARY TOXICITY STUDY WITH THE LADYBIRD BEETLE

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 354-113B

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

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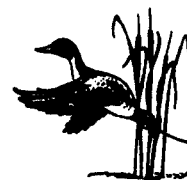
SUBMITTED TO:

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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).

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

Company Agent: Diane Shanahan (Typed Name)

Title: Registration Manager

Signature: Diane Shanahan Date: 12/10/99

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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 the Ladybird Beetle

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 354-113B


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:



John R. Porch
Senior Biologist

DATE 8 Dec 99**SPONSOR:**

Mycogen c/o
Dow AgroSciences

Sponsor


Applicant/Submitter


DATE 12/10/99DATE 12/10/99

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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:

ACTIVITY	DATE CONDUCTED	DATE REPORTED TO:	
		STUDY DIRECTOR	MANAGEMENT
Protocol	March 22, 1999	March 23, 1999	March 24, 1999
Initial Trial 354-113			
Test Substance Preparation and Test Initiation	March 24, 1999	March 25, 1999	March 29, 1999
Second Trial 354-113A			
Test Substance Preparation	April 9, 1999	April 9, 1999	April 9, 1999
Definitive Trial 354-113B			
Data and Draft Report	August 24, 1999	August 24, 1999	September 3, 1999
Final Report	December 8, 1999	December 8, 1999	December 8, 1999



 Timothy A. Springer
 Manager, Technical and Regulatory Support

DATE 12/8/99

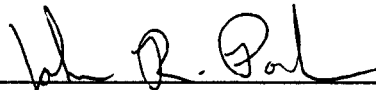
REPORT APPROVAL

SPONSOR: Dow AgroSciences LLC/Mycogen Corporation

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

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 354-113B

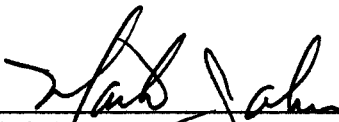
STUDY DIRECTOR:



John R. Porch
Senior Biologist

DATE 8 Dec 99

MANAGEMENT:



Mark Jaber
Wildlife Toxicologist

DATE 12/8/99

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SUMMARY

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

WILDLIFE INTERNATIONAL LTD. PROJECT NO.:	354-113B
TEST SUBSTANCE:	Cry 1F <i>Bacillus thuringiensis</i> var. <i>aizawai</i> delta endotoxin
STUDY:	Cry 1F <i>Bacillus thuringiensis</i> var. <i>aizawai</i> delta endotoxin: A Dietary Toxicity Study with the Lady- bird Beetle
NOMINAL TEST CONCENTRATIONS:	Negative Control and 480 ppm a.i.
TEST DATES:	Experimental Start (OECD)- March 24, 1999 Experimental Start (EPA)- June 21, 1999 Experimental Termination - July 20, 1999
LENGTH OF EXPOSURE:	29 Days

TEST ORGANISM:	Ladybird Beetle (<i>Hippodamia convergens</i>)
SOURCE OF TEST ORGANISMS:	A-1 Unique Insect Control 5504 Sperry Drive Citrus Heights, California 95621
LIFE STAGE OF TEST ORGANISMS:	Adult

DIETARY LC50:	> 480 ppm a.i.
NO OBSERVED EFFECT CONCENTRATION:	480 ppm a.i.

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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 twice due to high control mortality. The definitive test was conducted from June 21, 1999 to July 20, 1999. Raw data generated at Wildlife International Ltd. and a copy of the final report are filed under Project Number 354-113B in archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of the study was to evaluate the dietary toxicity of Cry 1F *Bacillus thuringiensis* var. *aizawai* delta endotoxin administered to the ladybird beetle (*Hippodamia convergens*) in the diet.

EXPERIMENTAL DESIGN

Ladybird beetles 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 30X 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 beetles 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 29 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. The nominal test concentration to which the ladybird beetles were exposed was 480 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 the Ladybird Beetle". 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 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 ladybird beetle (*Hippodamia convergens*) is useful in evaluating the potential hazards of agricultural chemicals and insecticidal proteins to nontarget insects and is an important predator of a great variety of agricultural pests. Beetles used in the test were obtained from A-1 Unique Insect Control, Citrus Heights, California, and appeared healthy upon receipt. Upon receipt, the beetles were placed in cold storage, then were transferred to an incubator prior to the test. The incubator was set to maintain a temperature range of approximately 26°C to 28°C, with relative humidity above approximately 40%. During the three-day holding period prior to testing, the beetles were provided *ad libitum* access to food (commercial honey) and water.

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 480 ppm a.i. (Appendix II). A calculated amount of the test substance was weighed and a sufficient amount of commercial honey was added until the final volume of 50 mL was achieved. 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 beetles were immobilized by refrigeration and impartially distributed to the test chambers. Twenty-five beetles were placed in each test chamber with the appropriate test or control diet. Fresh diets were presented to the beetles at least twice weekly 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 beetles were allowed *ad libitum* access to the test diets throughout the test period. Fresh water was supplied to the beetles as needed throughout the test period.

Environmental Conditions

During the test, the beetles 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 $26.7 \pm 1.0^\circ\text{C}$ (SD) with a range of 26.4 to 26.9°C, while average relative humidity was $79 \pm 8\%$ (SD) with a range of 52 to 92%. 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 beetles 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 $\frac{3}{4}$ hour and $1\frac{3}{4}$ hours after test initiation on Day 0, and then continued daily throughout the remainder of the test period, with the exception of Day 20. The test was terminated after mortality exceeded 20% in the negative control on Day 29 of the test.

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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. The no-observed-effect-concentration (NOEC) was determined by examination of the mortality and clinical observation data.

RESULTS AND DISCUSSION

Observations and Measurements

The data from observations of the beetles for mortality and other clinical signs are shown in Table 1. At test termination on Day 29, mortality in the negative control group was 21% (16 of 75). A small number of lethargic and immobile beetles were observed during the test. All other surviving beetles in the control group were normal in appearance and behavior throughout the test period.

Mortality in the 480 ppm a.i. treatment group was 16% (12 of 75) at test termination. A small number of lethargic and immobile beetles were observed during the test. All other surviving beetles in the 480 ppm a.i. treatment group were normal in appearance and behavior throughout the test period. The mortalities that occurred in the treatment group were less than those in the negative control group. Therefore, the mortalities were not considered to be related to treatment with the test substance.

CONCLUSION

Ladybird beetles exposed to a single test concentration of Cry 1F *Bacillus thuringiensis* var. *aizawai* delta endotoxin in the diet (480 ppm a.i.) showed no treatment-related mortality and no signs of toxicity over the 29-day test period. The test substance concentration, which represented up to 30X 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 480 ppm a.i..

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REFERENCES

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

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TABLE 1

Cumulative Mortality and Observations of Ladybird Beetles
Exposed to Cry 1F *B.t.* var. *aizawai* Delta Endotoxin

Day	Replicates	Negative Control		480 ppm a.i.	
		Mortality ²	Observations ³	Mortality	Observations
Day 0 ¹ - ¾ 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 - 1¾ 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 1	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	1L; 24 AN
Day 2	A	0/25	25 AN	0/25	25 AN
	B	0/25	25 AN	0/25	25 AN
	C	1/25	24 AN	0/25	1L; 24 AN
Day 3	A	0/25	25 AN	0/25	25 AN
	B	0/25	25 AN	1/25	24 AN
	C	1/25	24 AN	1/25	24 AN
Day 4	A	0/25	25 AN	0/25	25 AN
	B	0/25	25 AN	1/25	24 AN
	C	3/25	22 AN	1/25	24 AN
Day 5	A	0/25	25 AN	0/25	25 AN
	B	0/25	25 AN	1/25	24 AN
	C	3/25	22 AN	1/25	24 AN
Day 6	A	0/25	25 AN	0/25	25 AN
	B	0/25	25 AN	1/25	24 AN
	C	3/25	1 L; 21 AN	1/25	24 AN

¹Day 0 observation times represent the approximate number of hours after completion of diet presentation.

²Mortality data are presented as the cumulative number dead per number exposed.

³Observations: Number of beetles exhibiting clinical signs: AN = appear normal; I = immobile; L = lethargic.

⁴Observations on Day 20 were inadvertently not conducted.

⁵Percent mortality includes number of immobile beetles at test termination.

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TABLE 1

Cumulative Mortality and Observations of Ladybird Beetles
Exposed to Cry 1F *B.t.* var. *aizawai* Delta Endotoxin
Page 2

Day	Replicates	Negative Control		480 ppm a.i.	
		Mortality ²	Observations ³	Mortality	Observations
Day 7	A	0/25	25 AN	0/25	25 AN
	B	0/25	25 AN	1/25	24 AN
	C	3/25	1L; 21 AN	1/25	24 AN
Day 8	A	0/25	25 AN	0/25	25 AN
	B	0/25	25 AN	1/25	24 AN
	C	3/25	22 AN	2/25	23 AN
Day 9	A	0/25	25 AN	0/25	25 AN
	B	0/25	25 AN	1/25	24 AN
	C	3/25	22 AN	2/25	23 AN
Day 10	A	0/25	25 AN	0/25	25 AN
	B	0/25	25 AN	1/25	24 AN
	C	4/25	21 AN	2/25	23 AN
Day 11	A	0/25	25 AN	0/25	25 AN
	B	0/25	25 AN	2/25	23 AN
	C	4/25	21 AN	2/25	23 AN
Day 12	A	0/25	25 AN	1/25	24 AN
	B	0/25	1L; 24 AN	2/25	23 AN
	C	4/25	21 AN	2/25	23 AN
Day 13	A	0/25	25 AN	1/25	24 AN
	B	1/25	24 AN	2/25	23 AN
	C	4/25	21 AN	3/25	22 AN
Day 14	A	0/25	25 AN	1/25	24 AN
	B	1/25	24 AN	2/25	23 AN
	C	4/25	21 AN	3/25	22 AN

¹Day 0 observation times represent the approximate number of hours after completion of diet presentation.

²Mortality data are presented as the cumulative number dead per number exposed.

³Observations: Number of beetles exhibiting clinical signs: AN = appear normal; I = immobile; L = lethargic.

⁴Observations on Day 20 were inadvertently not conducted.

⁵Percent mortality includes number of immobile beetles at test termination.

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TABLE 1

Cumulative Mortality and Observations of Ladybird Beetles
Exposed to Cry IF *B.t.* var. *aizawai* Delta Endotoxin
Page 3

Day	Replicates	Negative Control		480 ppm a.i.	
		Mortality ²	Observations ³	Mortality	Observations
Day 15	A	0/25	1I; 24 AN	1/25	24 AN
	B	1/25	24 AN	2/25	23 AN
	C	6/25	19 AN	4/25	1I; 20 AN
Day 16	A	1/25	24 AN	2/25	23 AN
	B	1/25	24 AN	2/25	1I; 22 AN
	C	7/25	18 AN	5/25	20 AN
Day 17	A	1/25	24 AN	2/25	23 AN
	B	1/25	24 AN	3/25	22 AN
	C	7/25	18 AN	5/25	20 AN
Day 18	A	1/25	24 AN	2/25	23 AN
	B	1/25	24 AN	3/25	22 AN
	C	7/25	1I; 17 AN	5/25	20 AN
Day 19	A	1/25	24 AN	2/25	23 AN
	B	1/25	24 AN	3/25	22 AN
	C	8/25	17 AN	5/25	20 AN
Day 21 ⁴	A	2/25	23 AN	2/25	23 AN
	B	1/25	24 AN	3/25	22 AN
	C	8/25	1I; 16 AN	5/25	20 AN
Day 22	A	2/25	23 AN	2/25	23 AN
	B	1/25	24 AN	3/25	22 AN
	C	11/25	14 AN	5/25	20 AN
Day 23	A	2/25	23 AN	2/25	23 AN
	B	1/25	24 AN	3/25	22 AN
	C	11/25	14 AN	5/25	20 AN

¹Day 0 observation times represent the approximate number of hours after completion of diet presentation.

²Mortality data are presented as the cumulative number dead per number exposed.

³Observations: Number of beetles exhibiting clinical signs: AN = appear normal; I = immobile; L = lethargic.

⁴Observations on Day 20 were inadvertently not conducted.

⁵Percent mortality includes number of immobile beetles at test termination.

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TABLE 1

Cumulative Mortality and Observations of Ladybird Beetles
Exposed to Cry 1F *B.t.* var. *aizawai* Delta Endotoxin

Page 4

Day	Replicates	Negative Control		480 ppm a.i.	
		Mortality ²	Observations ³	Mortality	Observations
Day 24	A	2/25	23 AN	2/25	23 AN
	B	1/25	24 AN	3/25	22 AN
	C	11/25	14 AN	5/25	20 AN
Day 25	A	2/25	23 AN	2/25	23 AN
	B	1/25	1I; 23 AN	3/25	22 AN
	C	11/25	14 AN	5/25	20 AN
Day 26	A	2/25	23 AN	2/25	23 AN
	B	1/25	24 AN	3/25	22 AN
	C	11/25	14 AN	5/25	20 AN
Day 27	A	3/25	22 AN	2/25	23 AN
	B	1/25	24 AN	3/25	22 AN
	C	11/25	14 AN	5/25	20 AN
Day 28	A	3/25	22 AN	2/25	23 AN
	B	1/25	24 AN	3/25	22 AN
	C	11/25	14 AN	5/25	20 AN
Day 29	A	3/25	22 AN	3/25	22 AN
	B	1/25	24 AN	3/25	22 AN
	C	12/25	13 AN	6/25	19 AN
<hr/>					
Percent Mortality ⁵	Replicate	Group	Replicate	Group	
A	12		12		
B	4		12		
C	48	21	24	16	
<hr/>					
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 beetles exhibiting clinical signs: AN = appear normal; I = immobile; L = lethargic.					
⁴ Observations on Day 20 were inadvertently not conducted.					
⁵ Percent mortality includes number of immobile beetles at test termination.					

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APPENDIX I

Test Substance Characterization

Dow AgroSciences LLC
Study ID: 990027
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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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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.

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Dow AgroScience LLC
Study ID: 990027
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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

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R. A. Herman

STUDY COMPLETED ON

November 18, 1999

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Pioneer Hi-Bred International
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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: 

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.

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Dow AgroSciences LLC
 Study ID: 990027
 Page 3

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

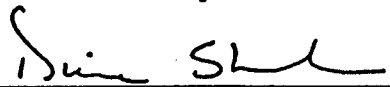
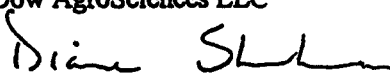
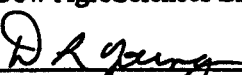
This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

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.

	<u>11/17/99</u>
D. M. Shanahan, Sponsor Dow AgroSciences LLC	Date
	<u>11/17/99</u>
D. M. Shanahan, Submitter Dow AgroSciences LLC	Date
	<u>11/18/99</u>
D. L. Young, Study Director/Author Dow AgroSciences LLC	Date

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Dow AgroSciences LLC
 Study ID: 990027
 Page 4

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
 D. Keyes
 Dow AgroSciences, Quality Assurance

11/18/99
 Date

SIGNATURE PAGE

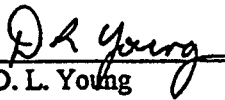


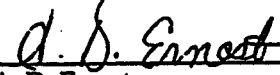

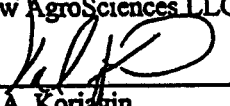

 D. L. Young Author Dow AgroSciences LLC	<u>11/18/99</u> Date
 R. A. Herman Co-Author Dow AgroSciences LLC	<u>10/21/99</u> Date
 G. A. Bornett Reviewer Dow AgroSciences LLC	<u>10/21/99</u> Date
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 C. A. Mihaliak Global ECL Group Leader Dow AgroSciences LLC	<u>10/21/99</u> Date

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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/ μ L 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 μ g Cry1F/mL diet
101788 - Microbial Cry1F Powder	LC ₅₀ = <0.02 μ g - 0.06 μ g 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.

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APPENDIX II

Diet Preparation

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

Nominal Concentration (ppm a.i.)	Test Substance Weight (g)	Final Volume in Honey (mL)
480	0.2110	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 diets 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 beetles. The control diet was prepared in the same manner except no test substance was used.

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APPENDIX III

Changes to Protocol

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

1. The proposed test dates were changed by amendment.
2. Observations on day 20 were inadvertently not performed.
3. Half-pint rolled paper containers were used for test chambers.
4. Dead beetles were removed from the test chambers daily during the test.

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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 45020110: Cry 1F *Bacillus Thuringiensis* Var. *Aizawai* Delta Endotoxin:
A Dietary Toxicity Study with the Ladybird Beetle

DATA REQUIREMENTS

None

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STUDY COMPLETED ON

January 16, 2001

SUBMITTED BY

Mycogen Seeds c/o
Dow AgroSciences LLC
9330 Zionsville Road
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LABORATORY STUDY ID

GH-C 5169

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Cry 1F

Title: Supplement to MRID 45020110: Cry 1F *Bacillus Thuringiensis* Var. *Aizawai*
Delta Endotoxin: A Dietary Toxicity Study with the Ladybird Beetle

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: *Perry J. Hunst*

Date: 11/2/01

*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 45020110: Cry 1F *Bacillus Thuringiensis* Var. *Aizawai* Delta
Endotoxin: A Dietary Toxicity Study with the Ladybird Beetle

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 45020110: Cry 1F *Bacillus Thuringiensis* Var. *Aizawai*
Delta Endotoxin: A Dietary Toxicity Study with the Ladybird Beetle

Study Initiation Date: 1/12/01

Study Completion Date: 1/16/01

NON-GLP STUDY

SIGNATURE PAGE

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D. L. Eisenbrandt, D. V. M., Ph.D. Date
Global Leader Toxicology
Dow AgroSciences LLC

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 $\mu\text{g/g}$. A definitive study appended to the original report indicated that the expression of Cry1F delta-endotoxin in pollen is 32 $\mu\text{g/g}$. Therefore, the MOE should be 15 rather than 30 as indicated in the original report. This change should be noted on pages 9 and 12 of the original report.