

CRY 1F *BACILLUS THURINGIENSIS* VAR. *AIZAWAI* DELTA ENDOTOXIN:
AN ACUTE TOXICITY STUDY WITH THE EARTHWORM
IN AN ARTIFICIAL SOIL SUBSTRATE

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 354-112

OECD GUIDELINE 207

AUTHORS:

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STUDY INITIATION DATE: March 16, 1999

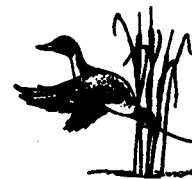
STUDY COMPLETION DATE: December 8, 1999

SUBMITTED TO:

Dow AgroSciences LLC/Mycogen Corporation
5501 Oberlin Drive
San Diego, California 92121



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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: Myrogen c/o DowAgroSciences (Typed Name)

Company Agent: Diane Shanahan (Typed Name)

Title: Registration Manager

Signature: Diane Shanahan Date: 12/9/99

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Dow AgroSciences LLC/Mycogen Corporation

TITLE: Cry 1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin: An Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 354-112


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This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Part 160, 17 August 1989; 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 soil were not determined.

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

STUDY DIRECTOR:



John R. Porch
Senior Biologist

DATE 8 Dec 99

SPONSOR:

Mycogen c/o
Dow AgroSciences

Sponsor

DATE 12/9/99



Applicant/Submitter

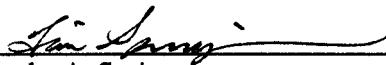
DATE 12/9/99

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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 in 40 CFR Part 160, 17 August 1989; 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 23, 1999	March 23, 1999	March 24, 1999
Test Substance Preparation	April 1, 1999	April 1, 1999	April 2, 1999
Light Measurements	April 5, 1999	April 6, 1999	April 7, 1999
pH Measurements	April 15, 1999	April 19, 1999	April 20, 1999
Data and Draft Report	June 18 & 21, 1999	June 21, 1999	June 22, 1999
Final Report	December 8, 1999	December 8, 1999	December 8, 1999



 Timothy A. Springer
 Manager, Technical and Regulatory Support

DATE 12/8/99

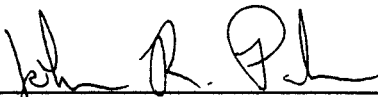
REPORT APPROVAL

SPONSOR: Dow AgroSciences LLC/Mycogen Corporation

TITLE: Cry 1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin: An Acute Toxicity Study
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WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 354-112

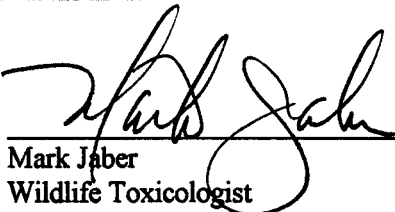
STUDY DIRECTOR:



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 A COPY OF THE FINAL REPORT:	Wildlife International Ltd. 8598 Commerce Drive Easton, Maryland 21601
WILDLIFE INTERNATIONAL LTD. PROJECT NO.:	354-112
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: An Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate
NOMINAL TEST CONCENTRATIONS:	Negative Control and 1.7 mg a.i./kg dry soil
TEST DATES:	Experimental Start - April 1, 1999 Biological Termination - April 15, 1999 Experimental Termination - April 16, 1999
LENGTH OF EXPOSURE:	14 Days
TEST ORGANISM:	Earthworm (<i>Eisenia fetida</i>)
SOURCE OF TEST ORGANISMS:	Willingham Worm Farm Rt. #1, Box 241 Butler, Georgia 31006
AGE OF TEST ORGANISMS:	Adult (with clitellum)
LC50 IN SOIL:	> 1.7 mg a.i./kg dry soil
NO OBSERVED EFFECT CONCENTRATION:	1.7 mg a.i./kg dry soil

INTRODUCTION

The study was conducted by Wildlife International Ltd. for Dow AgroSciences LLC/Mycogen Corporation at the Wildlife International Ltd. toxicology facility in Easton, Maryland. The in-life portion of the test was conducted from April 1, 1999 to April 15, 1999. Raw data generated at Wildlife International Ltd. and a copy of the final report are filed under Project Number 354-112 in the archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of this study was to determine the acute effects of Cry 1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin on earthworms during a 14-day exposure period in an artificial soil substrate.

EXPERIMENTAL DESIGN

Earthworms were exposed to one limit test concentration of Cry 1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin in the soil, and were observed for mortality and signs of toxicity on Days 7 and 14 of the test. The test substance concentration represented up to 100X (1.7 mg Cry 1F/kg dry soil) the concentration of Cry 1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin present in the top six inches of an acre of soil following the incorporation of 25,000 senescent corn plants. A control group was maintained concurrently. Four replicate test chambers were maintained in the treatment and control group, with 10 worms in each test chamber. The cumulative mortality percentages in the treatment group were used to determine the LC50 value at test termination. The no observed effect concentration was determined by visual examination of the mortality, body weight 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 earthworms were exposed was 1.7 milligrams of Cry 1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin per kilogram of dry soil (mg a.i./kg).

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "Bt Cry 1F delta-endotoxin: An Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate." The protocol

was based upon procedures outlined in the Organization for Economic Cooperation and Development (OECD) Guideline No. 207, *Guideline for Testing of Chemicals, Earthworm, Acute Toxicity Tests* (1).

Test Substance

The test substance was received from Mycogen 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 Worms

Adult earthworms (*Eisenia fetida*) were supplied by Willingham Worm Farm, Butler, Georgia. In the laboratory, the worms were maintained in culture chambers containing a mixture of moist peat and manure. Approximately 24 hours prior to the test, 115 worms were selected and placed in a container of prepared artificial soil substrate adjusted to a moisture content of approximately 33%, for the acclimation period. On the day of test initiation, the worms were rinsed briefly with deionized water, and impartially distributed by pairs into groups of 10 worms each. Each group of worms was weighed and placed on the soil in the appropriate test chamber. The worms were not fed during testing.

Test Chambers

The test chambers were one liter glass beakers covered with plastic wrap which was perforated for air exchange. The test chambers were identified with the project number, concentration, and replicate.

Artificial Soil Preparation

The artificial soil was prepared by blending 70% sand, 20% Kaolin clay, and 10% sphagnum peat (Appendix II). The pH of the bulk soil was adjusted to 5.8 using calcium carbonate. The soil was stored in a sealed container under ambient conditions until used to prepare the test soils.

To prepare the test soil substrate, the test substance was dissolved in deionized water and mixed directly into moistened artificial soil. Sufficient water was added to the soil to achieve a moisture content of approximately 33%. The soil was mixed for a total of fifteen minutes. The control soil was mixed in the same manner but without the addition of any test substance. Seven hundred fifty grams of prepared soil were added to each of four test chambers per treatment or control group. At test initiation and termination, soil samples were collected from each treatment and control group and were used to measure the initial and final pH and moisture content of the soils. The moisture content was determined by measuring the initial weight of the soil sample, then weighing the soil sample after drying for at least 18 hours at 105°C. The percent moisture was calculated using the following formula:

$$\% \text{ moisture} = [(\text{net wet weight} - \text{net dry weight}) \div \text{net wet weight}] * 100$$

Soil pH and moisture content at test initiation and termination are presented in Table 1.

Environmental Conditions

During the test, the worms were maintained in an environmental chamber. Temperature in the environmental chamber was monitored continuously. Soil temperature was measured in each treatment and control group on Days 0 and 14 of the test. The photoperiod during the test was twenty-four hours of continuous overhead florescent light per day. Light intensity was measured once during the test. Air and soil temperature and light intensity measurements are presented in Table 1.

Observations

On Day 0, the worms were placed on the surface of the soil in each test chamber and were observed for burrowing behavior. On Day 7 and Day 14 of the test, the contents of each test chamber were emptied onto a flat surface covered with clean paper, and the number of surviving worms counted. The worms were also observed for behavioral abnormalities. A record of any mortality, signs of toxicity, or abnormal behavior was maintained. On Day 7, following observations, the artificial soil was returned to the test chambers and the worms were replaced on the soil surface in order to observe burrowing behavior. On Day 14, following observations and body weight determination, all worms were euthanized, and all carcasses and soil substrate disposed of by incineration.

Body Weights

On Day 0, all worms in each replicate were weighed by group prior to being placed in the test chambers. On Day 14, all surviving worms were removed from each replicate test chamber, rinsed with deionized water and weighed as a group. Average individual body weights were calculated.

Reference Toxicity Test

A reference toxicity test was conducted under a separate protocol to determine the LC50 value for earthworms exposed to a reference toxicant, chloroacetamide, in the soil (2). The test was conducted using worms from the same source and under conditions similar to those in the test with Cry 1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin in order to monitor the techniques used and the sensitivity of the test population. The worms were exposed to chloroacetamide in the soil at concentrations of 7.5, 15, 30, and 60 mg a.i./kg dry soil.

Data Analysis

Mortality and body weight data were evaluated by visual inspection to determine if the single concentration tested was a no-observed-effect-concentration. The LC50 value was estimated based on biological observations obtained in the treatment group used in the limit test.

RESULTS AND DISCUSSION

Observations

The data from weekly observations of the worms for mortality and other signs of toxicity are shown in Table 2. There were no apparent treatment-related effects on mortality or body weight of worms in the test. One mortality was observed in each of three control replicates during the 14-day test. All other control worms were normal in appearance and behavior throughout the test. One mortality was observed in the 1.7 mg a.i./kg group. Surviving worms showed no signs of behavioral abnormalities on Days 0, 7, or 14. No aversion to the test soil was observed on Day 0 or 7.

Body Weights

Average individual body weights and the change in body weight were calculated from the Day 0 and Day 14 replicate measurements (Table 3). When compared to the control group, there were no

apparent treatment-related effects upon body weight in the 1.7 mg a.i./kg treatment group. A slight loss in body weight from Day 0 to Day 14 is typical, since the test organisms are not fed during the test. Both the control and the treatment group lost an average of 0.04 g per worm over the course of the 14-day test.

Reference Toxicity Test

The 14-day LC50 value for earthworms exposed to chloroacetamide in an artificial soil substrate was determined to be approximately 15.7 mg a.i./kg dry soil, with a 95% confidence interval of 7.5 to 30 mg a.i./kg dry soil (2). These results are consistent with those observed in previous studies, and verify the adequacy and consistency of the methods used in the test with Cry 1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin.

CONCLUSION

Earthworms (*Eisenia fetida*) exposed to a single test concentration of Cry 1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin in the soil showed no mortality and no signs of toxicity over the 14 day test period. The test substance concentration, which represented up to 100X (1.7 mg Cry 1F/kg dry soil) the concentration of Cry 1F *Bacillus thuringiensis* var. *aizawai* Delta Endotoxin present in the top six inches of an acre of soil following the incorporation of 25,000 senescent corn plants, was therefore determined to be a no-observed-effect-concentration. The LC50 value was therefore determined to be greater than the test concentration of 1.7 mg a.i./kg dry soil.

REFERENCES

- 1 OECD Guideline 207. 1984. *Guideline for Testing of Chemicals, Earthworm, Acute Toxicity Tests*. Organization for Economic Cooperation and Development.
- 2 Wildlife International Ltd. 1999. Chloroacetamide: An Acute Toxicity Study With the Earthworm in an Artificial Soil Substrate. Project No. 100R-104.

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TABLE 1
ENVIRONMENTAL CONDITIONS

Moisture Content, pH and Temperature of the Artificial Soil

Nominal Concentration (mg a.i./kg)	Day 0			Day 14 ²		
	Moisture ¹ Content (%)	pH ¹	Temperature ² (°C)	Moisture Content (%)	pH	Temperature (°C)
0 (Control)	32.2	7.85	22	31.1	7.75	21
1.7	31.7	7.87	21	31.9	7.83	21

¹ Measurements taken from prepared soil immediately after preparation.
² Measurements taken from one replicate test chamber at each concentration.

Light Intensity and Air Temperature

Light Intensity (lux)		Temperature (°C)
Mean (± SD)	Range (Min - Max)	
645 (± 120)	480-771	20.0-20.5

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TABLE 2

**CUMULATIVE MORTALITY AND OBSERVATIONS OF EARTHWORMS
EXPOSED TO *Bacillus thuringiensis* var *aizawai* Cry 1F Delta Endotoxin
IN AN ARTIFICIAL SOIL SUBSTRATE**

Nominal Concentration (mg a.i./kg)	Rep.	Day 7		Day 14		Replicate Percent Mortality	Group Percent Mortality
		No. Dead ¹	Effects ²	No. Dead	Effects		
0 (Control)	A	1/10	9 AN	1/10	9 AN	10	7.5
	B	0/10	10 AN	0/10	10 AN	0	
	C	1/10	9 AN	1/10	9 AN	10	
	D	0/10	10 AN	1/10	9 AN	10	
1.7	A	0/10	10 AN	0/10	10 AN	0	2.5
	B	0/10	10 AN	1/10	9 AN	10	
	C	0/10	10 AN	0/10	10 AN	0	
	D	0/10	10 AN	0/10	10 AN	0	

¹ Mortality data are presented as the number dead per number exposed.

² Number of worms exhibiting clinical signs: AN = normal in appearance and behavior

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TABLE 3

**AVERAGE BODY WEIGHTS OF EARTHWORMS EXPOSED
TO *Bacillus thuringiensis* var *aizawai* Cry 1F Delta Endotoxin
IN AN ARTIFICIAL SOIL SUBSTRATE**

Nominal Concentration (mg a.i./kg)	Rep.	Average Individual Body Weights (g)		
		Day 0	Day 14	Total Change
0 (Control)	A	0.38	0.33	-0.05
	B	0.42	0.39	-0.03
	C	0.39	0.33	-0.06
	D	0.36	0.34	-0.02
	$\bar{x} \pm \text{Std. Dev.}$	0.39 ± 0.03	0.35 ± 0.03	-0.04 ± 0.02
1.7	A	0.39	0.38	-0.01
	B	0.39	0.34	-0.05
	C	0.42	0.36	-0.06
	D	0.38	0.35	-0.03
	$\bar{x} \pm \text{Std. Dev.}$	0.40 ± 0.02	0.36 ± 0.02	-0.04 ± 0.02

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APPENDIX I

Test Substance Characterization

Dow AgroSciences LLC
Study ID: 990027
Page 1 of 2

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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9330 Zionsville Road
Indianapolis, Indiana 46268-1054

Pioneer Hi-Bred International
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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

Global Environmental Chemistry Laboratory—Indianapolis Lab
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

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.

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

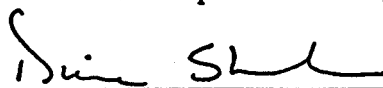
This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

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.



D. M. Shanahan, Sponsor
Dow AgroSciences LLC

Date

11/17/99



D. M. Shanahan, Submitter
Dow AgroSciences LLC

Date

11/17/99



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

Date

11/18/99

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



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Author
Dow AgroSciences LLC

11/18/99

Date



R. A. Herman
Co-Author
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10/21/99

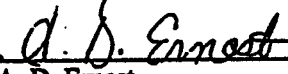
Date



G. A. Borrett
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Dow AgroSciences LLC

10/21/99


Date



A. D. Ernest
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Dow AgroSciences LLC

10/21/99


Date



C. K. Robb
Reviewer
Dow AgroSciences LLC

10/21/99

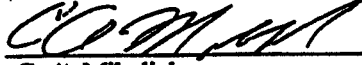
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V. A. Korjagin
Reviewer
Dow AgroSciences LLC

10/21/99

Date



C. A. Mihaliak
Global ECL Group Leader
Dow AgroSciences LLC

10/21/99

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.

APPENDIX II

ARTIFICIAL SOIL SUBSTRATE PREPARATION

The artificial soil was prepared in bulk as follows:

Quartz Sand:	31,500 g
Kaolin Clay:	9,000 g
Sphagnum Peat:	4,500 g
Calcium Carbonate:	1,125 g

The above constituents were weighed, placed in a Patterson Kelly Twinshell mixer and mixed for approximately 20 minutes.

A total of 3.25 kg (wet weight) of soil was prepared for each control and treatment group. Based on the desired test soil moisture of 33%, the calculated water content for 3.25 kg of test soil was $3250 \text{ g} * 0.33 = 1072.5 \text{ g}$ (or mL) H_2O . The remaining 67% (2177.5 g) constitutes the dry weight of the soil. Therefore, to prepare test soils, a constant ratio of 2177.5 g dry soil to 1072.5 g H_2O is maintained.

Bulk soil is used to obtain the 2177.5 g of dry soil. Since bulk soil contains only 96% dry soil by weight, the amount required to yield 2177.5 g dry soil equals $2177.5 \div 0.96 = 2268.2 \text{ g}$ bulk soil. The difference in the gross weight and the dry weight of the bulk soil ($2268.2 \text{ g} - 2177.5 \text{ g} = 90.7 \text{ g}$) equals the amount of water contained in the soil prior to test soil preparation, and must be subtracted from the 1072.5 g (or mL) total water component of the test soils. The remaining water ($1072.5 - 90.7 = 981.8 \approx 982 \text{ mL}$) must be added during test soil preparation.

The artificial soil was weighed in a mixing bowl. Five hundred milliliters of deionized water was added to the soil and mixed on a Hobart mixer for approximately five minutes. The test substance was weighed (0.0483 g) into a tared 100 mL glass beaker and dissolved in 50 mL of deionized water. This solution was added to the artificial soil. The beaker was rinsed with 50 mL of water, and the rinsate was added to the soil. The remaining 382 mL of deionized water was added to the soil in the mixing bowl and the soil was then mixed for an additional 15 minutes. 750 g of the soil mixture was placed in each test chamber. The control soil was prepared in the same manner, without the addition of test substance.

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APPENDIX III
CHANGES TO THE PROTOCOL

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

1. The protocol was amended to correct three minor typographical errors.
2. The protocol was amended to increase the amount of test soil prepared for each treatment group to provide adequate soil for pH and moisture content measurements to be made.

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 and 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 45020106: Cry 1F *Bacillus Thuringiensis* Var. *Aizawai* Delta Endotoxin:
An Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate

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
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

GH-C 5171

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Cry 1F

Title: Supplement to MRID 45020106: Cry 1F *Bacillus Thuringiensis* Var. *Aizawai*
Delta Endotoxin: An Acute Toxicity Study with the Earthworm in an Artificial
Soil Substrate

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 A Hunst*

Date: 1/16/01

*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.

QUALITY ASSURANCE STATEMENT

Compound: Cry 1F

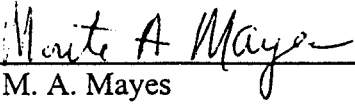
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Delta Endotoxin: An Acute Toxicity Study with the Earthworm in an Artificial
Soil Substrate

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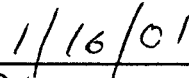
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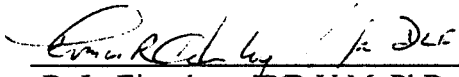
NON-GLP STUDY

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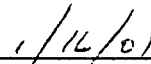


M. A. Mayes
Author
Dow AgroSciences LLC


Date



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


Date

A reviewer commented that our calculations for exposure of earthworms are incorrect. Dow AgroSciences have evaluated the calculations as provided on page 30 of the report and the attached protocol amendment. The total weight of the soil prepared was 3.5 kg wet weight. A total of 48.3 mg of the Cry1F microbial endotoxin preparation was added to the soil. This provides 14.9 mg of the preparation per kilogram moist soil. The preparation is 11.4% pure therefore exposure was 1.7 mg/kg of the active endotoxin per kilogram of moist soil. Based on wet weight of the soil, this provides a margin of safety (MOE) of 100 (estimated environmental concentration of 0.017 mg/kg). However, the report erroneously expresses exposure in dry weight. Therefore, on pages 8, 9, and 13, exposure should be expressed as 1.7 mg Cry 1F/kg moist soil. If, however, one wishes to make the assessment based on dry weight of the soil (2177.5 g), exposure is approximately 2.5 mg Cry 1F/kg dry soil and the MOE is 148.

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AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Bt Cry 1F delta-endotoxin: An Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate

PROTOCOL NO.: 354/022599/EWSDT/SUB354

AMENDMENT NO.: 1

SPONSOR: Mycogen Corporation

PROJECT NO.: 354-112

EFFECTIVE DATE: March 17, 1999

AMENDMENT:

Page 1:

CHANGE: Testing Guidelines: U.S. Environmental Protection Agency
Series 850 - Ecological Effects Test Guidelines
OPPTS Number 885-4340

TO: Testing Guidelines: Organization for Economic Cooperation and
Development (OECD) Guideline 207

REASON:

To correct a typographical error.

AMENDMENT:

Page 3, SUMMARY:

CHANGE: A single concentration, 1.6 mg/kg, will be tested

TO: A single concentration, 1.7 mg/kg, will be tested.

REASON:

To correct typographical errors.

AMENDMENT:

Page 5:

CHANGE: 20 ± 2°C and pH 6.0 ± 0.5

TO: 20 ± 2°C and pH 6.0 ± 0.5, respectively

REASON:

To correct typographical errors.

AMENDMENT:

Page 6: Change the calculations as follows:

- [4] Approximately 3.25 kilograms of soil (wet weight) are required to prepare dosing for the four replicates, and provide material for pH and soil moisture confirmation. The test substance concentration is based upon the wet weight of soil, therefore approximately 0.055 mg Cry 1F

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Project No. 354-112
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delta-endotoxin is required to establish a 1X test concentration.

- [5] The Cry 1F delta-endotoxin preparation is 11.4% pure, therefore 0.055 mg divided by 0.114 indicates that approximately 0.48 mg of this preparation is required to approximate the worst case 1X exposure.
- [6] For the limit test to be set at 100X the worst case exposure (0.017 mg/kg), 48 mg of the Cry 1F delta-endotoxin will be required in 3.25 kg (wet weight) of soil to achieve an exposure concentration of 1.7 mg/kg.

Therefore, 48 mg of the Cry 1F delta-endotoxin preparation will be used to prepare the test soil.

REASON:

Provision is made to have adequate test soil for measurement of pH and soil moisture.


STUDY DIRECTOR


17 Mar 99
DATE


LABORATORY MANAGEMENT

3/17/99
DATE


SPONSOR'S REPRESENTATIVE

3/19/99
DATE

CPA Reviewed by  3-17-99