Study Sponsor

Mycogen
c/o Dow AgroSciences LLC
5501 Oberlin Drive
San Diego, CA 92121

Laboratory Report for the Study Titled:

Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed *Bt* Cry 1F Delta-Endotoxin and Pollen from Maize Expressing *Bt* Cry1F Delta Endotoxin

Data Requirements:

EPA Assessment Guidelines, Series 885 - Microbial Pesticide Test Guidelines
Group D - Nontarget Organism and Environmental Expression
Test Guidelines - OPPTS 885.4380
Honeybee Testing, Tier I

Author

Victor L. Maggi, M.S.

Study Report Date

December 2, 1999

Laboratory Study Number

CAR 172-99

Performing Laboratory

California Agricultural Research, Inc.
4141 N. Vineland
Kerman, CA 93630
(559) 843-2997

CERTIFICATION

This report provides a true and accurate representation of the raw data.

Study Director: Victor L. Maggi **Date:** 12/2/99

Victor L. Maggi, M.S.
Laboratory Research Manager
California Agricultural Research, Inc.

Acknowledged By: Diane Shanahan **Date:** 12/9/99

Diane Shanahan, Product Registration Manager
Mycogen
c/o Dow AgroSciences LLC

STATEMENT OF NO DATA CONFIDENTIALITY

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

We submitted this material to the United States Environmental Protection Agency (EPA) specifically under provisions contained in FIFRA as amended, and thereby consent to use and disclosure of this material by EPA according to FIFRA. Some pages of this report may be stamped with the following: **CONTAINS TRADE SECRET OR OTHERWISE CONFIDENTIAL INFORMATION OF MYCOGEN, c/o DOW AGROSCIENCES LLC.** This claim of confidentiality is not meant to convey supplemental claims of confidentiality regarding data subject to disclosure under section 10(d) and 10(e) of FIFRA. In submitting this material to the EPA according to method and format requirements contained in PR Notice 86-5, we do not waive any protection of rights involving this material that would not have been claimed by the company if this material had not been submitted to the EPA.

Sponsor: **Mycogen; c/o Dow AgroSciences LLC**

Signature: Diane Shanahan 12/9/99

Diane Shanahan, Product Registration Manager **Date**
Mycogen; c/o Dow AgroSciences LLC

GLP COMPLIANCE STATEMENT

This study was completed in accordance with the Good Laboratory Practice Standards, as published by the Environmental Protection Agency in 40 CFR 160, dated August 17, 1989, with the following exceptions:

- *The sponsor was responsible for the characterization of the test material and all calculations and dilutions of the purified Bacillus thuringiensis Cry 1F Delta-Endotoxin.*
- *Offsite beekeeping practices regarding maintenance and pesticide history were not performed or recorded under Good Laboratory Practice Standards.*

Victor L. Maggi 12/2/99

Victor L. Maggi, M.S., Study Director **Date**
California Agricultural Research, Inc.

STATEMENT OF THE QUALITY ASSURANCE CONSULTANT

The Quality Assurance Consultant of California Agricultural Research, Inc., conducted inspections and reported to the Study Director and Management as follows:

Type of Inspection	Date of Inspection	Date Reported To Management*	Date Reported To Study Director
Preparation & Dosing of Larvae	07-10-99	07-12-99	07-12-99
Laboratory Logbook	10-20-99	10-20-99	10-20-99
Final Report	10-20-99	10-20-99	10-20-99

*The term management refers to Study Director Management

Duke C. Wiley 12/2/99

Duke C. Wiley, Quality Assurance Consultant
GLP Research and Consulting
1447 Lofty Lane
Paradise, CA 95967
(530) 872-8006

TABLE OF CONTENTS

TABLE OF CONTENTS
(Continued)

LIST OF TABLES

LIST OF APPENDICES

SIGNATURES OF STUDY PERSONNEL

STUDY TITLE: Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed *Bt* Cry 1F Delta-Endotoxin and Pollen from Maize Expressing *Bt* Cry 1F Delta Endotoxin

CAR STUDY NUMBER: CAR 172-99

NAME **FUNCTION**	**SIGNATURE**	
Victor L. Maggi, M.S. Study Director	Victor L. Maggi	12/2/99 Date
Michael Beevers, Ph.D. Research Director		12/2/99 Date
Kathy B. Richards Office Manager/Archivist	Kathy B. Richards	12/2/99 Date
Roberto Moreno Field Technician	Roberto Moreno	12/2/99 Date
David Richards Field Technician		12/2/99 Date
Seressa Valdez Field Technician	Seressa Valdez	12-2-99 Date

STUDY IDENTIFICATION

Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed *Bt* Cry 1F Delta-Endotoxin and Pollen from Maize Expressing *Bt* Cry 1F Delta Endotoxin

Study Director:
Victor L. Maggi, M.S.
California Agricultural Research, Inc.
4141 N. Vineland
Kerman, California 93630
(559) 843-2997

Study Sponsor:
Diane Shanahan, Product Registration Manager
Mycogen
c/o Dow AgroSciences LLC
5501 Oberlin Drive
San Diego, CA 92121
(619) 453-8030 ext. 435

Performing Laboratory:
California Agricultural Research, Inc.
4141 N. Vineland
Kerman, California 93630
(559) 843-2997

CAR Study Number: CAR 172-99

Study Initiation Date: July 1, 1999

Experimental Start Date: July 10, 1999

Experimental Termination Date: July 26, 1999

Study Completion Date: December 2, 1999

STUDY IDENTIFICATION
(Continued)

Abstract

The purpose of this study was to evaluate the effect of using bacterially expressed *Bt* Cry1F protein added to the diet of larval honey bees (*Apis mellifera* L.), maturing within honeycomb brood cells. The ingestion of protein along with other natural pollen and nectar is anticipated to be the primary route of honey bee exposure.

This study was conducted in which 3 to 5 day old larval honeybees were administered a single dose of 1) pollen from non-genetically modified maize, 2) pollen expressing *Bacillus thuringiensis* var. *aiz*awai (*Bt*) Cry 1F delta-endotoxin, 3) pollen form non-genetically modified maize + potassium arsenate (positive control), or 4) *Bacillus thuringiensis* var. *aizawai* (*Bt*) Cry 1F delta-endotoxin, produced by a recombinant strain of *Pseudomonas fluorescens* (strain MR872) and suspended in a 30% sucrose solution. For Treatments 1 and 2 each respective pollen type was moistened with 30% sucrose to form a paste and applied to larval honeybees at the bottom of their brood cells. Immediately following the pollen application, a 10 µl drop of 30% sucrose was pipetted into the same cell.

Treatment 3 included moistened, unmodified pollen and a 10 µl drop of 30% sucrose mixed with Potassium arsenate (@ 500 ppm) to act as a known stomach poison. Treatment 5 was mapped only and received no additional treatments. All treated bees were rated as survived to the capping stage and later observed once emerged as adult bees. Each test group included four replicates of 20 larval bees (total of 80 bees per test group).

Six days after treatment administration the bees were evaluated for survival. Survival in the pollen- and protein-treated bees ranged from an average of 93% (*Bacillus* endotoxin) to 100% in the mapped only bees. The arsenic treated bees yielded an average survival rate of only 75%. The arsenic treated bees exhibited the only statistically significant group separate from the other four treatments. No behavioral nor morphological abnormalities were observed in bees exposed to the test or non-arsenic control substances.

No effects on larval bee survival nor adult behavior were observed in the bees given genetically modified pollen or endotoxin protein.

INTRODUCTION

Purpose

The objective of this study is to evaluate the effect of *Bt* Cry1F Delta-Endotoxin protein administered into the diet of the larvae of the honey bee, (*Apis mellifera* L.)

Guideline Requirement

The study was carried out to satisfy the EPA Pesticide Assessment Guidelines, Series 885, Microbial Pesticide Test Guidelines, Group D, Nontarget Organism and Environmental Expression Test Guidelines, OPPTS 885.4380, Honeybee Testing, Tier I.

Protocol

This study was conducted according to the protocol titled *Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed Bt Cry1F Delta-Endotoxin and Pollen from Maize Expressing Bt Cry1F Delta Endotoxin.* The protocol is provided as Appendix A. Amendments and/or deviations to the study protocol are described in Appendix B.

EXPERIMENTAL SECTION

TEST SUBSTANCES

Name:	Pollen from maize expressing *Bacillus thuringiensis* var. *aizawai* (*Bt*) Cry1F delta-endotoxin
Lot Number:	TC1507
Storage Conditions:	Stored at near or below -80°C test material will be shipped and stored on dry ice (temperatures will be monitored and recorded daily)

Pollen samples were collected at the Mycogen Research Station in Santa Isabel, Puerto Rico. Pollen was collected at the R1 stage of growth from the Cry1F inbred line, 5XH751 x TC1507 [BC4] Source #PR980675. Pollen collection, as well as characteristics of the pollen sample expressing the *Bt* Cry1F delta-endotoxin, including bioactivity, will be the responsibility of the study sponsor (See Appendix D). Methods of synthesis, fabrication, or derivation of the test substance shall be documented by the Sponsor and the location of such documentation shall be specified. With the exception of Treatments 3 and 5, all treatments were used as received from the study sponsor except where 30% sucrose was added to form a workable pollen paste.

Preparation of Test and Control Substances - Calculations

Three to four drops of the 30% sucrose solution was added directly to the pollen in order to form a paste that could be applied to the cells. Treatment 3 (Potassium Arsenate) consisting of 500 ppm (0.5 mg/ml) arsenic in 30% sucrose solution was prepared, based on the following calculations:

By proportion, first find the amount of Potassium Arsenate (arsenic) to prepare a 500 ppm solution:

$$\frac{\text{500 gms (arsenic)}}{\text{1,000,000 mls deionized water}} = \text{500 ppm}$$

Therefore by proportion,

$$\frac{\text{500 gms (arsenic)}}{\text{1,000,000 mls deionized water}} = \frac{\text{X grams (arsenic)}}{\text{500 mls deionized water}}$$

$$X = 0.25 \text{ grams arsenic}$$

Therefore, 0.25 gms of arsenic was mixed into 500 mls of 30% sucrose solution, 500 mls of a 500 ppm arsenic solution. **Mathematical Check:**

$$\textbf{500 ppm} \quad \frac{\text{500 gms (arsenic)}}{\text{1,000,000 mls solvent}} = \frac{\text{0.25 gms (arsenic)}}{\text{500 mls solvent}}$$

EXPERIMENTAL SECTION

Preparation of Test and Control Substances - Calculations (continued)

The 30% sucrose solution was prepared by mixing 300 grams sucrose (table sugar) into 700 grams (=700 mls) of distilled water. For Treatment 4, as per study sponsor direction, 5.6 mg of test substance was blended into 10 mls of 30% sucrose to obtain a 10 μl dose volume equivalent to 5.6 μg/larvae.

MATERIALS AND METHODS

Test System

Honeybee, *Apis mellifera lingustica* Spin. - (Italian or Italian hybrid strains)

Bee Hives

All twenty-four beehives present at the California Agricultural Research, Inc. bee yard were delivered by Peter Kupina (Kerman, CA) by April 22, 1999, and remained at CAR for the duration of the study. Previously, all hives were rented off-location to various pollination sites in Fresno County, CA. This is a normal operating procedure for beehives maintained by professional beekeepers in this area.

In March and October of each year, all hives receive a dosage of approximately 50g of Terabee Mix 2X Terramycin (5.5% oxytetracycline hydrochloride). The March dosage was administered prior to placement at California Agricultural Research, Inc. These bactericidal applications are routinely applied to beehives in the beekeeping industry as a preventive measure against European and American foulbrood diseases. In addition to these treatments, two to three Apistan® acaricide (10.0% Fluvalinate) strips were placed into each hive in November of 1998 prior to their arrival at the CAR bee yard. The Apistan® strips were placed into hives as per label specifications to eliminate any possible infestations of the larval and adult honeybees by Varroa Mites. From our previous experiences, these mites have been known to seriously interfere with larval honeybee development and survival. These procedures are a part of normal beekeeping maintenance procedures carried out annually by professional beekeepers in California.

Hives number 12, 14,17, and 21 were used for this study and were acclimated in the CAR bee yard for a minimum of 30 days prior to actual treatment administration. A supplemental diet consisting of approximately 30% sucrose was administered to the hives. All hives were fed two to three times a week approximately 3/4 qt of a 30% sucrose solution until June 14, 1999. At that time evaluations showed healthy hives and feeding was discontinued.

On April 21st all hives were inventoried for their general health, vigor, and brood conditions. These inventories were repeated July 9th one day prior to treatment to identify those hives able to provide adequate larval brood frames for treatment purposes. Brood frames from hives 12, 14, 17,& 21 were chosen specifically for this study due to the presence of two or more frames with large, uniformly-sized populations of larval brood.

MATERIALS AND METHODS
(Continued)

Cage Description - Emergence Cages

The adult emergence cages were made of 3.2 mm wire mesh, metal hardware cloth measuring approximately 1.5 cm tall. Cages were cut to custom fit over each group of treated brood cells. The average size of such a cage was roughly 3 x 6 cm. The edge of each cage was bent down and the cages pressed into the wax of the honeycomb in order to contain any bees emerging from the treated area.

Treatments and Rates

Treatment Name	Dosage Per Cell
1. Non-genetically modified pollen + 30% Sucrose Solution	~ 2 mg + 1droplet (10$\mu\ell$)
2. Genetically Modified Pollen + 30% Sucrose Solution	~ 2 mg + 1droplet (10$\mu\ell$)
3. Positive Control: Unmodified Pollen + Potassium arsenate (Arsenic @ 500 ppm) in a 30% Sucrose Solution	~ 2 mg + 1droplet (10$\mu\ell$)
4. *Bacillus thuringiensis* var. *aizawai* (*Bt*) Cry1F delta-endotoxin, produced by a recombinant strain of *Pseudomonas fluorescens* (Strain MR872) (560 μg/mL of a 30% sucrose solution	~ 10 μL
5. Untreated Control (mapped only)	

Replications

For each treatment, four replicates, each containing approximately 20 honeybee larvae were used.

Treatment Procedures

Prior to treatment administration, cells receiving treatments were mapped on an acetate sheet laid over the larval frame.

MATERIALS AND METHODS
(continued)

Treatment Procedures (contd.)

In order to verify the dosing technique and the ingestion of the supplied treatment substances, a positive control treatment (potassium arsenate (arsenic)), containing a substance known to have a detrimental effect to maturing larvae was included (Treatment 4). Each arsenic-treated cell received a 10 $\mu\ell$ droplet of approximately 0.5 mg/ml (=500 ppm) arsenic.

For treatment administration, the larval frames were transported to the laboratory, one replicate at a time, inside a clean, insulated container (ice chest) which was wiped clean with a mild (~10%) Clorox solution between each replicate (hive). Each one of the five selected hives represented a complete replicate of five treatments. In all cases, two frames were taken from each hive. One frame at a time was selected indiscriminately for dosing from the ice chest. It was first mapped using the acetate sheet. Then ~ 2 mg of pollen was placed into each cell. After placement of the pollen a 10 μl droplet of the 30% sucrose solution was pipetted into the cell allowing the pollen to liquify and flow down to the mouth of the larva.

Larval cells in Treatment 3 received a 10 μl droplet of 500 ppm arsenic in 30% sucrose. Treatment 4 larval cells received 10 μl of a dosing solution containing approximately 5.6 μg of *Bacillus thuringiensis* var. *aizawai* (*Bt*) Cry1F delta-endotoxin suspended in 30% sucrose solution. In order to achieve a 10X exposure via the use of the bacterially expressed Cry1F delta-endotoxin, (which is 11.4% pure) each larvae was exposed to 5.6 μg (640 ng/0.114 = 5.6 μg) of the Cry1F delta-endotoxin preparation. To facilitate this exposure, a 30% sucrose solution containing 560 μg of the Cry1F delta-endotoxin preparation/ml was prepared. Ten μL of this solution was administered to each cell resulting in an exposure of 5.6 μg/larvae. Larval cells in Treatment 5 were simply mapped and received no pollen or liquid additives.

Once all five treatments were applied to a replicate, the treated frames were covered by a moistened towel in the ice chest to help maintain humid conditions for the larval bees. After at least 30 minutes under the moistened towel, during which time the larvae were expected to consume the treatment material, the frames were returned to their original hive. The frames for the next replicate were then carried into the laboratory and the process repeated as before.

The ice chest was wiped clean with a mild solution of chlorine bleach between each replicated group. To minimize the possibility of cross-contamination from the arsenic-treated larvae, the arsenic treatments were placed onto separate frames. During dosing, the laboratory was maintained under controlled and monitored environmental conditions. The two monitored conditions; temperature and humidity, were approximated and temperature ranged between 22° to 28°C with relative humidity of 27 to 37%.

MATERIALS AND METHODS
(continued)

Handling Procedures

Treatment application was conducted on July 10, 1999. Six days later (July 16, 1999) all frames were evaluated one at a time for larval removal or capping in a nearby greenhouse using the acetate maps to locate the treated brood cells. At this time, it was apparent that all capping was not complete for all larvae surviving to this point. In those cases where a larva was killed or injured for any reason, it would have been removed by attendant nurse bees prior to cell capping. Surviving larvae would then be capped (larval cell sealed over with beeswax) by nurse bees to allow pupation to occur.

Twelve days after treatment application (July 22, 1999), all treated frames were removed from their source hives for placement of the adult emergence cages and relocation into a screened hive box into an environmentally controlled and monitored growth chamber. Prior to placement of adult emergence cages a capping evaluation was repeated again using the acetate maps as before. Then all untreated larvae and pre-pupae in capped cells near the treated cell area were removed. The custom-fitted adult emergence cages were then put into place over each treated area of capped brood allowing only the treated bees to emerge into the enclosed space.

From this time on, the frames remained darkened in the growth chamber until the study was concluded. Observations and daily counts for emergence took place twice daily once emergence began on July 22, 1999, and continued until the final bee emerged on July 26, 1999

Environmental Conditions

Throughout the interval between dosing through to one day prior to the emergence of the first adult bees (approximately 12 days), larvae were allowed exposure to their natural hive environment. Thereafter, the frames were incubated in a darkened larval growth chamber. Temperatures in the larval growth chamber ranged from 27° to 31°C, while the relative humidity ranged from 35 to 58 percent.

Data Collection - Survival and Emergence

The typical developmental cycle of the honeybee is as follows: Approximately three days after the queen bee has laid the egg, hatching occurs. Capping occurs at approximately six days later, followed by emergence as adult bees approximately 21 days after eggs were laid. These are average development times which may vary slightly due to brood temperatures or genetic variations within each hive.

MATERIALS AND METHODS
(Continued)

Data Collection - Survival and Emergence (continued)

At 6 and 12 days after treatment (DAT) (July 16, and July 22, 1999, respectively) data were collected regarding survival of the larvae to the capping stage. After bees began to emerge (July 22, 1999, or 12 DAT), all frames were checked and evaluated twice daily. Frames which had emerged bees present in the emergence cages were temporarily removed from their screened hive box one at a time for evaluation and counting. Emergence was noted and recorded between 12 and 16 DAT (July 16 through July 22, 1999). Once the emerged bees were counted, they were removed from each emergence cage and euthanized by freezing.

Statistical Methods

ANOVA were carried out using Agriculture Research Manager (ARM) software, version 6.0.1 (Gyllings Data Management, Inc., Brookings, SD).

Raw Data Storage

Upon completion of the final report, a copy of all data pertinent to the study along with a certified copy of the final report, supporting raw data, and all original facility data will be archived at California Agricultural Research, Inc. 4141 N. Vineland, Kerman, CA. The original final report including the original supporting raw data pertinent to the study will be submitted to the Study Sponsor for archiving.

RESULTS AND DISCUSSION

Adult bee emergence began 12 days after treatment (DAT) in all treatment groups and continued until 16 DAT (July 22 through July 26, 1999).

Temperature differences and genetic variability between beehives influence honeybee emergence times; therefore, emergence times occurred over a range of days. Because of this, emergence data were not statistically analyzed on a day by day basis. The emergence times of the treatment groups fall within an expected time frame typical of honeybee larvae.

The data presented in Table 1 indicates that all of the larval mortality occurred prior to the capping of the larval cells by nurse bees. Larvae injured or killed in the cells for whatever reason are readily removed by nurse bees in the hive. In these cases, the cells would appear empty or vacated and subsequently not capped. The most significantly affected larvae were those exposed to the arsenic treatment (Treatment 1) which exhibited an average of 25.0% mortality by the 6 DAT evaluation.

Statistical analysis of the total percent mortality by ANOVA revealed that significant differences in mortality occurred between the arsenic-treated (Treatment 3) and non-arsenic-treated larvae (Treatments 1, 2, 4, and 5). These results indicate that the presence of Cry1F protein had no effect on honeybee larval survival nor emergence.

CONCLUSION

Based on survival and mortality data evaluation, the results of this study indicate that no significant detrimental effects occurred among honeybee larvae when exposed to Cry1F protein when compared with an untreated control group of honeybee larvae.

ANOVA were performed for the following data. Capped cells at 6 days after treatment (DAT), total number larvae emerged as adults at 16 DAT, and percent survival for the same parameters. When comparing potassium arsenate versus the untreated control for the same parameters, significant differences were found in all cases. Clearly significant mortality (25.0%) was caused by the potassium arsenate. Considering the untreated control versus Cry1F, no significant differences were seen in the same parameters. There was no significant mortality in the Cry1F.

Larvae in the Cry1F protein-treated and genetically modified pollen groups exhibited no significant mortality (prob. $F = 0.10$) when compared with the larvae provided with unmodified pollen, indicating that the presence of Cry1F protein did not adversely affect either honeybee larval survival nor emergence.

Table 1. Larval Survival from Dosing to Final Emergence at 16 Days After Treatment (DAT)

Treatment and Rate (Dosage Per Cell for 20 Larvae)	Rep. #	Number capped and Assumed Alive (6 DAT)	Total Number of Larvae Emerged (16 DAT)	Total Percent Survival[1]
1. Non-genetically modified Pollen + 30% Sucrose Solution @ ~ 2 mg + 1 droplet (10 µl)	1	20	20	100
	2	20	20	100
	3	19	19	95
	4	20	20	100
	Mean	**19.8**	**19.8**	**98.8**
2. Genetically modified Pollen + 30 % Sucrose Solution @ ~ 2 mg + 1 droplet (10 µl)	1	20	20	100
	2	20	20	100
	3	19	18	90
	4	20	20	100
	Mean	**19.8**	**19.5**	**97.5**
3. Positive Control: Unmodified Pollen + Potassium Arsenate (arsenic 500 ppm) in a 30% Sucrose Solution	1	20	20	100
	2	17	17	85
	3	7	7	35
	4	16	16	80
	Mean	**15.0**	**15.0**	**75.0**
4. *Bacillus thuringiensis* var. *aizawai* (*Bt*) Cry1F delta-endotoxin, produced by a recombinant strain of *Pseudomonas fluorescens* (560 µg/mL of a 30% sucrose solution) @ 10 µL	1	20	20	100
	2	15	15	75
	3	19	19	95
	4	20	20	100
	Mean	**18.5**	**18.5**	**92.5**
5. Untreated Control (Mapped Only)	1	20	20	100
	2	20	20	100
	3	20	20	100
	4	20	20	100
	Mean	**20.0**	**20.0**	**100.0**

[1] Calculation: (Total number bees capped and assumed alive/20 bees dosed per replicate) x 100. For an explanation regarding the unaccounted for bees in these totals, see the Results and Discussion section in text.

Appendix A: Study Protocol

STUDY PROTOCOL

Study Title

Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed *Bt* Cry 1F Delta-Endotoxin and Pollen from Maize Expressing *Bt* Cry1F Delta Endotoxin

Sponsor

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

Study Guidelines

EPA Pesticide Assessment Guidelines, Series 885 - Microbial Pesticide Test Guidelines
Group D - Nontarget Organism and Environmental Expression
Test Guidelines
OPPTS Number: 885.4380
Honeybee Testing, Tier I

Testing Facility

California Agricultural Research, Inc.
4141 N. Vineland
Kerman, CA 93630
(559) 843-2997

Testing Facility Study Number

CAR 172-99

Date

July 1, 1999

California Agricultural Research, Inc. **CAR Study Number: CAR 172-99**

TABLE OF CONTENTS

California Agricultural Research, Inc. **CAR Study Number: CAR 172-99**

I. STUDY OBJECTIVE

The objective of this study is to evaluate the effects using bacterially expressed *Bt* Cry1F Delta-Endotoxin and pollen from maize expressing *Bt* Cry1F Delta Endotoxin when administered to the larval honeybee (*Apis mellifera* L.) The pollen contains a Cry1F protein derived from *Bacillus thuringiensis* which is known to be toxic to certain lepidopterous insects.

II. SUMMARY

A honeybee larval toxicity study will be conducted in which 3 to 5 day old larval honeybees will be administered a single dose of 1) pollen from non-genetically modified maize, 2) pollen expressing *Bacillus thuringiensis* var. *aizawai* (Bt) Cry1F delta-endotoxin, 3) pollen from non-genetically modified maize + potassium arsenate (positive control), and 4) *Bacillus thuringiensis* var. *aizawai* (Bt) Cry1F delta-endotoxin, produced by a recombinant strain of *Pseudomonas fluorsescens* (strain MR872). Dry pollen will be applied to larval honeybees at the bottom of their brood cells followed by a small drop of a 30% sucrose solution. The bacterially expressed Cry1F delta-endotoxin (Treatment 4) will be administered in a 30% sucrose solution.

Larvae will then be incubated until adult emergence (approximately 10 to 14 days later) at which time the treated and control bees will be evaluated for emergence.

III. TIMELINES

Proposed Experimental Start Date:	July, 1999
Proposed Experimental Termination Date:	September, 1999
Proposed Final Report Date:	October, 1999

IV. TEST SUBSTANCES

A

Name:	Pollen from maize expressing *Bacillus thuringiensis* var. *aizawai* (Bt) Cry1F delta-endotoxin
Lot Number:	TC1507
Storage Conditions:	Stored at near or below -80°C test material will be shipped and stored on dry ice (temperatures will be monitored and recorded daily)

Pollen samples were collected at the Mycogen Research Station in Santa Isabel, Puerto Rico. Pollen was collected at the R1 stage of growth from the Cry1F inbred line, 5XH751 x TC1507 [BC4] Source #PR980675. Pollen collection, as well as characteristics of the pollen sample expressing the Bt Cry1F delta-endotoxin, including bioactivity, will be the responsibility of the sponsor. Methods of synthesis, fabrication, or derivation of the test substance shall be documented by the Sponsor and the location of such documentation shall be specified.

IV. TEST SUBSTANCES (continued)

B

Name:	Pollen from non-genetically modified maize
Lot Number:	5XH751
Storage Conditions:	Stored at near or below -80°C test material will be shipped and stored on dry ice (temperatures will be monitored and recorded daily)

Pollen samples were collected at the Mycogen Research Station in Santa Isabel, Puerto Rico. Pollen was collected at the R1 stage of growth from the isogenic inbred line, 5XH751, which will serve as the control or non-genetically modified pollen. Pollen collection will be the responsibility of the Sponsor. Methods of synthesis, fabrication, or derivation of the test substance shall be documented by the Sponsor and the location of such documentation shall be specified.

C

Name:	*Bacillus thuringiensis* var. *aizawai* (Bt) Cry1F delta-endotoxin, produced by a recombinant strain of *Pseudomonas fluorsescens* (strain MR872)
Lot Number:	TSN 101788
Storage Conditions:	Stored at near or below 4°C test material will be shipped and stored on dry ice (temperatures will be monitored and recorded daily)

Purified *Bacillus thuringiensis* (Bt) Cry1F delta-endotoxin, produced by a recombinant strain of *Pseudomonas fluorscescens* strain (MR872) containing the Cry1F gene, will be provided by the Sponsor. The *Pseudomonas fluorscescens* batch culture was concentrated, truncated, and then purified for the truncated Cry1F delta-endotoxin. The sample was identified ad TSN 101788 and analyzed to contain 11.4% Bt Cry1F delta-endotoxin. Methods of synthesis, fabrication, or derivation of the test substance shall be documented by the Sponsor and the location of such documentation shall be specified.

V. TEST SYSTEM

Justification of Test System

A dietary study was chosen because ingestion of genetically modified pollen along with other natural pollen and nectar is anticipated to be the primary route of honeybee exposure. Small amounts of pollen are known to be fed directly to larval honeybees by nurse bees in the hives.[1]

---

[1] Winston, Mark. L., 1987 *The Biology of the Honey Bee*. pg. 59.

V. TEST SYSTEM (continued)

Species and Life Stage

The larval honeybee, *Apis mellifera* (Italian or Italian-hybrid Strain) from 3 to 5 days old.

Physical Description of the Test Apparatus

Bee larvae will be tested in an in-hive study. Colonies used for the study will be vigorous, healthy, and maintained at California Agricultural Research, Inc. (CAR), Kerman, California. Young bee larvae of the appropriate age range will be selected from at least four hives.

Route of Administration and Reason for Its Choice

Larvae are in an appropriate life stage for exposure to the test substance because the larval stage appears to be the most sensitive developmental stage.

VI. MATERIALS AND METHODS

Treatments	Dosage Rate Per Cell
1. Non-genetically modified pollen+30% Sucrose Solution	~ 2 mg + 1 droplet (10μl)
2. Genetically Modified Pollen+30% Sucrose Solution	~ 2 mg + 1 droplet (10μl)
3. Positive Control: Unmodified Pollen + Potassium arsenate (arsenic 500 ppm) in a 30% Sucrose Solution	~ 2 mg + 1 droplet (10μl)
4. *Bacillus thuringiensis* var. *aizawai* (Bt) CryIF delta-endotoxin, produced by a recombinant strain of *Pseudomonas fluorsescens* (Strain MR872). (560 μg/mL of a 30% sucrose solution)	- 10μL
5. Untreated Control (mapped only)	

Replicates

Four replicates of each treatment will be included. If possible, one entire replicate block of treatments will be included in one hive. Each hive will therefore constitute a randomized complete block. For each replicate, treatments will be administered to each cell when larvae are approximately 3 to 5 days old, using approximately 20 larvae per treatment in each replicate. To facilitate the caging of the emerging adult bees in each treatment group, the 20 treated larvae will be located in relatively close proximity on the brood frame. Frames and mapped cells will be identified on acetate overlay maps to indicate the study, hive, replicate, and treatment numbers.

VI. MATERIALS AND METHODS (continued)

Bias Control

An untreated control, replication, and randomization of treatments will provide adequate control of bias.

Dosing Procedure

Prior to treatment administration, cells receiving treatments will be mapped on an acetate sheet laid over the larval frame. Cells assigned to Treatments 1, 2, and 3 will each receive 10μl of 30% sucrose solution. Each cell assigned to Treatment 4 will receive 10 μL of the sucrose/bacterially expressed Cry1F delta-endotoxin preparation. This is intended to allow the dry pollen to reach the mouth parts of the treated larva. As a check for larval removal by nurse bees due to handling activities, an untreated control will be included (Treatment 5) which will receive neither the sucrose solution nor the pollen and will simply be mapped. The frames of the untreated control bees (Treatment 5) will otherwise be handled in a manner similar to the frames with the treated bees. This will include removal from the hive, mapping, temporary incubation, and timing out of the hive. They will remain with the treated frames by replicate for all handling activities.

In order to verify the dosing technique and the ingestion of the supplied treatment substances, a positive control treatment, containing a substance known to have a detrimental effect to maturing larvae is included (Treatment 3). The targeted dosage volume of approximately 2 mg of pollen will be administered to each pollen-treated larval cell with the use of a toothpick. The dose volume size will be verified by weighing several practice volumes gravimetrically until each one approximates 2 mg.

An additional treatment (Treatment 4) will be included to expose the larvae to a 10X dosage of the amount of Cry1F delta-endotoxin found in pollen. In Treatment 2, each cell will receive 2 mg of pollen from maize expressing *Bacillus thuringiensis* var. *aizawai* (Bt) Cry1F delta-endotoxin. The expression of the Cry1F delta-endotoxin in pollen is approximately 32 ng/mg (ppm). Consequently if all of the pollen is consumed, the larve will be exposed to 64 ng of Cry1F delta-endotoxin.

In order to achieve a 10X greater exposure via the use of the bacterially expressed Cry1F delta-endotoxin, (which is 11.4% pure) each larvae will be exposed to 5.6 μg (640 ng/0.114 = 5.6 μg) of the Cry1F delta-endotoxin preparation. To facilitate this exposure, a 30% sucrose solution containing 560 μg of the Cry1F delta-endotoxin preparation/ml will be prepared. Ten μL of this solution will be administered to each cell resulting in an exposure of 5.6 μg/larvae.

VI. MATERIALS AND METHODS (continued)

Dosing Procedure (continued)

To administer the small amount of protein, the full 20 dosages each of the four (replicate sets of) treatments will be measured out and divided into the twenty dosages needed per each of the four replicates. For treatment administration, larval frames will be transported to the laboratory, one replicate at a time, inside an insulated container (i.e., ice chest, without ice). During dosing, the laboratory will be maintained under controlled and monitored environmental conditions (temperature and relative humidity targeted at 20° to 35°C and 20 to 60%, respectively). To avoid possibly lethal cross-contamination from the positive control treatment, Treatment 3 will be placed on a frame surface separate from the other four treatments, if an adequate number of brood frames are available..

Incubation of Dosed Larvae in the Hive

Upon completion of dosing, frames will be held in the insulated container for a minimum of 30 to 40 minutes to allow the larvae to ingest the test substance before being returned to their original hive location. Prior studies using dye-colored corn pollen have demonstrated, upon dissection, that the larvae consume the pollen within this time period.

Prior to adult emergence of the treated larvae, a cage will be pressed into each mapped area of the combs to contain the adult worker bees as they emerge. Once emergence is anticipated (approximately 14 days after treatment), all treated frames will be moved into a screened hive box which will be placed into a growth chamber under controlled and monitored conditions (temperature and relative humidity will be targeted at 20° to 40°C and 20% to 100%, respectively).

Data Evaluations

Five to ten days after dosing, cells in each mapped area will be scored as either capped or not capped. This process will be repeated again when the emergence cages are put into place. The percent cumulative survival to capping will then be calculated.

After first emergence, daily counts will be made of emerged adult bees. Once recorded, adults will be removed from the emergence cages. Data will be recorded until 48 hours after the last bee emerges from the untreated control treatment (Treatment 5).

VI. MATERIALS AND METHODS (Continued)

Data Evaluations (Continued)

The study will be terminated if, at any time prior to capping, the average mortality or absence of larvae in the untreated control treatment exceeds 20%. Once the study is concluded, areas receiving Treatments 2, 3, and 4 will be scraped from the frames and disposed of before the frames are returned to their source hive. All worker bees which have emerged from the treatments will be frozen and disposed.

VII. FINAL REPORT AND LOCATION OF ARCHIVES

A final report for the study will be prepared in compliance with the EPA PR Notice 86-5 and shall include, but not necessarily be limited to, those items enumerated in 40 CFR 160.185, "Reporting of Study Results" and current applicable FIFRA Data Reporting Guidelines. Upon completion of the final report, a copy of all data pertinent to the study along with a certified copy of the final report, original supporting raw data, and all original facility data will be archived at California Agricultural Research, Inc. 4141 N. Vineland, Kerman, CA. The original final report will be submitted to the Study Sponsor for archiving.

VII. STUDY RECORDS TO BE MAINTAINED

Records to be maintained for the study include all raw data and observations recorded during the conduct of the study, documentation, and study related correspondence. This includes, but is not limited to, the following items.

- A description of equipment (with serial or identification number) used in the study.
- Completed laboratory logbook.
- Chemical storage facility temperature records.
- List of SOPs used in the study.
- Chain of custody for the test substance, including air bills and shipping receipts.
- Copies of current Curricula Vitae (CVs) from all personnel involved in the study.

IX. GLP COMPLIANCE

This study will be conducted and reported in accordance with the EPA FIFRA Good Laboratory Practices Standards (40 CFR 160). A statement of compliance will be signed by the study director and included in the final report.

X. SOP AND GLP DEVIATIONS

California Agricultural Research, Inc., will assume responsibility for SOPs used to conduct this study. SOPs will be in place for all phases and activities of this study and will be adhered to unless superseded by specific provisions of this protocol. Certification of SOP adherence will be documented at the signing of the Good Laboratory Practice Statement by the study director in the final report.

Any deviations from Standard Operating Procedures or Good Laboratory Practices will be authorized by the Study Director and documented in the study records, noting the nature of the deviation, potential effect on the study, and corrective action taken.

XI. DATA ANALYSIS

Comparison of the treated and control groups with respect to mortality will be made using analysis of variance with a suitable mean comparison test, such as Duncan's Multiple Range Test (DMRT), to separate differences among means. Mean emergence times between treatment groups will also be compared to determine if treatment related growth delays occurred. Any negative behavioral effects noted will be tabulated by treatment group, but will not be subjected to statistical analyzes.

XII. PROTOCOL AMENDMENTS AND DEVIATIONS

Planned changes to the approved protocol shall be documented by amendments that clearly describe the change and justification for the change. Amendments will be signed and dated by the study director and sponsor representative and will be maintained with the study protocol.

Protocol deviations, which are one time and unplanned deviations from the protocol, shall be documented in the study records, noting the nature of the deviation, potential effects on the study, and corrective action.

XIII. QUALITY ASSURANCE

This study will be monitored by an independent Quality Assurance Consultant for California Agricultural Research, Inc. Selected critical phases of the study, techniques, documentation, and the final report will be audited. A statement will be made by the independent QA Consultant within five working days listing the dates that the study inspections were made and reported to the Study Director and Sponsor Management.

XIV. TEST SUBSTANCE STORAGE AND DISPOSAL

Following the last evaluation, unless specifically directed otherwise by the Study Sponsor, all remaining test substance will be held and then returned to the Sponsor in the same manner in which it was sent. Disposal of the remaining test substance in any manner other than its return will be done only at the written request of the Study Sponsor.

California Agricultural Research, Inc. **CAR Study Number: CAR 172-99**

STUDY TITLE

Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed *Bt* Cry 1F Delta-Endotoxin and Pollen from Maize Expressing *Bt* Cry1F Delta Endotoxin

I CERTIFY THAT I HAVE READ AND APPROVED THIS PROTOCOL

Study Director: Victor L. Maggi 7/1/99

Victor L. Maggi, M.S. Date
California Agricultural Research, Inc.
4141 N. Vineland
Kerman, CA 93630
(559) 843-2997

Study Sponsor Representative: Diane Shanahan

Diane Shanahan, Product Registration Manager Date
Mycogen Corporation
5501 Oberlin Drive
San Diego, CA 92121
(619) 453-8030 ext. 435

CAR Management: 7-1-99

Michael Beevers, Ph.D. Date
California Agricultural Research, Inc.
4141 N. Vineland
Kerman, CA 93630
(559) 843-2997

I CERTIFY THAT I HAVE READ AND REVIEWED THIS PROTOCOL

Quality Assurance Consultant: Duke C Wiley 7/1/99

Duke C. Wiley Date
California Agricultural Research, Inc.
4141 N. Vineland
Kerman, CA 93630
(559) 843-2997

California Agricultural Research, Inc. CAR Study Number: CAR 172-99

STUDY TITLE

Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed *Bt* Cry 1F Delta-Endotoxin and Pollen from Maize Expressing *Bt* Cry1F Delta Endotoxin

I CERTIFY THAT I HAVE READ AND APPROVED THIS PROTOCOL

Study Director: Victor L. Maggi 7/1/99

Victor L. Maggi, M.S. Date
California Agricultural Research, Inc.
4141 N. Vineland
Kerman, CA 93630
(559) 843-2997

Study Sponsor Representative: Diane Shanahan 7/6/99

Diane Shanahan, Product Registration Manager Date
Mycogen Corporation
5501 Oberlin Drive
San Diego, CA 92121
(619) 453-8030 ext. 435

CAR Management:

Michael Beevers, Ph.D. Date
California Agricultural Research, Inc.
4141 N. Vineland
Kerman, CA 93630
(559) 843-2997

I CERTIFY THAT I HAVE READ AND REVIEWED THIS PROTOCOL

Quality Assurance Consultant:

Duke C. Wiley Date
California Agricultural Research, Inc.
4141 N. Vineland
Kerman, CA 93630
(559) 843-2997

Appendix B: Amendments and/or Deviations to the Study Protocol

CALIFORNIA AGRICULTURAL RESEARCH, INC.

Protocol Amendment Documentation and Change Form

Sponsor Study Number: None	**CAR Study Number:** CAR 172-99
Study Title: Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed *Bt* Cry 1F Delta-Endotoxin and Pollen from Maize Expressing *Bt* Cry1F Delta Endotoxin	
Amendment Number: 1	
Study Director: Victor L. Maggi, M.S.	
Protocol Section(s) affected: Section II. Summary	

Description of change(s):

Section II. Summary

The second line reads, "Dry pollen will be applied....of a 30%sucrose solution."

This is changed to read as follows:

"Dry pollen moistened with a small amount of 30% sucrose to form a paste, will be applied to larval honeybees at the bottom of their brood cells followed by a small drop (10μl) of a 30% sucrose solution.

Section VI. Material and Methods; Subsection: Dosing Procedure

1st paragraph second line reads: "Cells assigned to ....sucrose solution." Changed to read as follows: Cells assigned to Treatments 1, 2, and 3 will each receive 10μL of 30% sucrose solution in addition to their respective pollen dosages.

Addition to paragraph three: To allow for proper dosing, a small amount of 30% sucrose will be mixed with pollen in order to form a compact paste. This will enable the dosing technicians to pick up a single dosage with the toothpick.

Justification for change(s):

To clarify dosing procedures.

Date Change(s) Took Affect: 07-10-99

Study Director/Principal Investigator Signature/Date:	Victor L. Maggi	7/10/99
Quality Assurance Consultant Signature/Date:	Debbie C. Wiley	7/10/99
Sponsor Representative Signature/Date:	Blaine Shaw	7/26/99

Appendix C: Summary of Statistical Analysis

CALIFORNIA AG. RESEARCH

Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed Bt Cry1F Delta-Endotoxin and Pollen from Maize Expressing Bt Cry1F....

Trial ID: CAR 172-99 Investigator: Victor L. Maggi, M.S.
Location: CAR Bee Yard Entered By: Kathy Richards

Weed Code						Honeybee	Honeybee	Honeybee
Crop Code						Survival	Total	Percent
Part Rated						to	Number	of
Rating Data Type						Capping	Emerged	Emergenc
Rating Unit						Capped		
Rating Date						07-16-99	07-26-99	07-26-99
Trt-Eval Interval						6 DAT	16 DAT	16 DAT
ARM Action Codes							T1	T2
# Subsamples, Dec.							1	1
Trt No.	**Treatment Name**	**Form Conc**	**Form Type**	**Rate**	**Rate Unit / Plot**			
1	Non-genetically modified				101	20.0	20.0	100.0
1	pollen + 30% Sucrose				201	20.0	20.0	100.0
1	Solution @ ~ 2 mg +				301	19.0	19.0	95.0
1	1 droplet (10 ul)				401	20.0	20.0	100.0
					Mean =	19.8	19.8	98.8
2	Genetically modified				102	20.0	20.0	100.0
2	pollen + 30 % Sucrose				202	20.0	20.0	100.0
2	Solution @ ~ 2 mg +				302	19.0	18.0	90.0
2	1 droplet (10 ul)				402	20.0	20.0	100.0
					Mean =	19.8	19.5	97.5
3	Positive Control:				103	20.0	20.0	100.0
3	Unmodified Pollen +				203	17.0	17.0	85.0
3	Potassium Arsenate				303	7.0	7.0	35.0
3	(arsenic 500 ppm) in a				403	16.0	16.0	80.0
3	30% Sucrose Solution							
3	@ ~ 2 mg +							
3	1 droplet (10 ul)							
					Mean =	15.0	15.0	75.0
4	Bacillus thuringiensis				104	20.0	20.0	100.0
4	var. aizawai (Bt) Cry1F				204	15.0	15.0	75.0
4	delta-endotoxin, produced				304	19.0	19.0	95.0
4	by a recombinant strain				404	20.0	20.0	100.0
4	of Pseudomonas							
4	fluorsescens							
4	(Strain MR872)							
4	(560 ug/mL of a 30%							
4	sucrose solution)							
4	@ 10 uL							
					Mean =	18.5	18.5	92.5
5	Untreated Control				105	20.0	20.0	100.0
5	(Mapped Only)				205	20.0	20.0	100.0
					305	20.0	20.0	100.0
					405	20.0	20.0	100.0
					Mean =	20.0	20.0	100.0

AOV Means Table

CALIFORNIA AG. RESEARCH

Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed Bt Cry1F Delta-Endotoxin and Pollen from Maize Expressing Bt Cry1F....

Trial ID: CAR 172-99 Investigator: Victor L. Maggi, M.S.
Location: CAR Bee Yard Entered By: Kathy Richards

Trt No.	Treatment Name	Form Conc	Form Type	Rate	Rate Unit	Honeybee Survival to Capping Capped 07-16-99 6 DAT	Honeybee Total Number Emerged 07-26-99 16 DAT T1 1	Honeybee Percent of Emergenc 07-26-99 16 DAT T2 1
1	Non-genetically modified pollen + 30% Sucrose Solution @ ~ 2 mg + 1 droplet (10 ul)					19.8 a	19.8 a	98.8 a
2	Genetically modified pollen + 30 % Sucrose Solution @ ~ 2 mg + 1 droplet (10 ul)					19.8 a	19.5 a	97.5 a
3	Positive Control: Unmodified Pollen + Potassium Arsenate (arsenic 500 ppm) in a 30% Sucrose Solution @ ~ 2 mg + 1 droplet (10 ul)					15.0 b	15.0 b	75.0 b
4	Bacillus thuringiensis var. aizawai (Bt) Cry1F delta-endotoxin, produced by a recombinant strain of Pseudomonas fluorsescens (Strain MR872) (560 ug/mL of a 30% sucrose solution) @ 10 uL					18.5 ab	18.5 ab	92.5 ab
5	Untreated Control (Mapped Only)					20.0 a	20.0 a	100.0 a
LSD (P=.05)						4.13	4.17	20.84
Standard Deviation						2.74	2.77	13.83
CV						14.72	14.91	14.91

Means followed by same letter do not significantly differ (P=.05, Duncan's New MRT)

CALIFORNIA AG. RESEARCH

Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed Bt Cry1F Delta-Endotoxin and Pollen from Maize Expressing Bt Cry1F....

Trial ID: CAR 172-99 Investigator: Victor L. Maggi, M.S.
Location: CAR Bee Yard Entered By: Kathy Richards

AOV For Honeybee Survival to Capping Capped 6 DAT

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F	Prob(F)
Total	19	182.800000			
Treatment	4	70.300000	17.575000	2.343	0.1019
Error	15	112.500000	7.500000		

AOV For Honeybee Total Number Emerged 16 DAT

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F	Prob(F)
Total	19	182.950000			
Treatment	4	68.200000	17.050000	2.229	0.1148
Error	15	114.750000	7.650000		

AOV For Honeybee Percent of Emergenc 16 DAT

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F	Prob(F)
Total	19	4573.750000			
Treatment	4	1705.000000	426.250000	2.229	0.1148
Error	15	2868.750000	191.250000		

Appendix D: Characterization of the Test Material

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

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

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

D. L. Young (317) 337-3649
[dlyoung@dowagro.com]
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: D. M. Shanahan

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 SL — 11/17/99

D. M. Shanahan, Sponsor
Dow AgroSciences LLC — Date

Diane SL — 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 — Date: 11/18/99

D. Keyes
Dow AgroSciences, Quality Assurance

SIGNATURE PAGE

D. L. Young — Date: 11/18/99
Author
Dow AgroSciences LLC

R. A. Herman — Date: 10/21/99
Co-Author
Dow AgroSciences LLC

G. A. Bormett — Date: 10/21/99
Reviewer
Dow AgroSciences LLC

A. D. Ernest — Date: 10/21/99
Reviewer
Dow AgroSciences LLC

C. K. Robb — Date: 10/21/99
Reviewer
Dow AgroSciences LLC

V. A. Korjagin — Date: 10/21/99
Reviewer
Dow AgroSciences LLC

C. A. Mihaliak — Date: 10/21/99
Global ECL Group Leader
Dow AgroSciences LLC

TABLE OF CONTENTS

TABLE OF CONTENTS (CONT.)

Page

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.

STUDY TITLE

Supplement to MRID 45041503: Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed *Bt* Cry 1F Delta-Endotoxin and Pollen from Maize Expressing *Bt* Cry 1F Delta-Endotoxin

DATA REQUIREMENTS

None

AUTHOR

M. A. Mayes 317-337-3200
[mamayes@dowagro.com]

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 5172

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Cry 1F

Title: Supplement to MRID 45041503: Evaluation of the Dietary Effect(s) *on* Honeybee Development Using Bacterially Expressed *Bt* Cry 1F Delta-Endotoxin and Pollen from Maize Expressing *Bt* Cry 1F Delta-Endotoxin

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: 1/16/01

*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 45041503: Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed *Bt* Cry 1F Delta-Endotoxin and Pollen from Maize Expressing *Bt* Cry 1F Delta-Endotoxin

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.

Penny K. Hunst — 1/16/01
P. L. Hunst — Date
Sponsor
Dow AgroSciences LLC

Penny K. Hunst — 1/16/01
P. L. Hunst — Date
Submitter
Dow AgroSciences LLC

Mark A Mayes — 1/16/01
M. A. Mayes — Date
Study Monitor/Author
Dow AgroSciences LLC

QUALITY ASSURANCE STATEMENT

Compound: Cry 1F

Title: Supplement to MRID 45041503: Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed *Bt* Cry 1F Delta-Endotoxin and Pollen from Maize Expressing *Bt* Cry 1F Delta-Endotoxin

Study Initiation Date: 1/12/01 Study Completion Date: 1/16/01

NON-GLP STUDY

SIGNATURE PAGE

Monte A Mayes — 1/16/01

M. A. Mayes
Author
Dow AgroSciences LLC

Date

for DLE — 1/16/01

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

Date

A reviewer noted that there was some ambiguity related to the actual concentration of Cry 1F delta-endotoxin to which the honeybee larvae were exposed. The report stated on page 16 that "in order to achieve a 10-X exposure via the use of the bacterially expressed Cry1F delta-endotoxin (which is 11.4% pure) each larvae was exposed to 5.6 µg (640 ng/0.114 = 5.6 µg) of the Cry1F delta-endotoxin preparation."

The value of 640 ng is derived from exposure of the honeybees to 2 mg of pollen which expresses Cry1F delta-endotoxin at 32 ng/mg (2 mg pollen X 32 ng Cry1F delta-endotoxin/mg = 64 ng Cry1F delta-endotoxin X 10 = 640 ng Cry1F delta-endotoxin). Because the Cry1F delta-endotoxin preparation is 11.4% active, 5.6 µg of the preparation/bee is necessary to achieve the desired dosing.

Another point of ambiguity may be that in Table 1 (page 21). Treatment 4 is defined as "*Bacillus thuringiensis* var *aizawai* (*Bt*) Cry 1F delta-endotoxin, produced by a recombinant strain of *Pseudomonas fluorescens* (560 µg/ml of a 30% sucrose solution) @ 10 µL". Treatment 4 may be better defined by: *Bacillus thuringiensis* var *aizawai* (*Bt*) Cry 1F delta-endotoxin, produced by a recombinant strain of *Pseudomonas fluorescens*: 640 ng/bee. (Dose achieved by administering 10 µl of a 560-µg/mL Cry1F delta-endotoxin preparation (64 µg.mL a.i.) in 30% sucrose solution).