STUDY TITLE

Toxicity of the Cry1F Protein to Neonate Larvae of the Monarch Butterfly (*Danaus plexippus* (Linnaeus))

DATA REQUIREMENTS

None

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STUDY COMPLETED ON

05-04-00

PERFORMING LABORATORY

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LABORATORY STUDY ID

GH-C 5073

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Cry1F Maize

Title: Toxicity of the Cry1F Protein to Neonate Larvae of the Monarch Butterfly (*Danaus plexippus* (Linnaeus)

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: Diane Shanahan

Title: Regulatory Manager

Signature: Diane Shanahan

Date: 4/20/2000

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

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

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Toxicity of the Cry1F Protein to Neonate Larvae of the Monarch Butterfly (*Danaus plexippus* (Linnaeus))

Study Initiation Date:	N/A	Study Completion Date:	05-04-00
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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
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This study does not meet requirements of 40 CFR Part 160.

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QUALITY ASSURANCE STATEMENT

Compound: Cry1F Maize

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Study Initiation Date: N/A Study Completion Date: 05-04-00

NON-GLP STUDY

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Toxicity of the Cry1F Protein to Neonate Larvae of the Monarch Butterfly (*Danaus plexippus* (Linnaeus))

ABSTRACT

Microbially produced, chromatographically pure truncated Cry1F delta endotoxin from *Bacillus thuringiensis* variety *aizawai* was incorporated into a modified lepidopteran diet. Neonate larvae of the monarch butterfly (*Danaus plexippus* (Linnaeus)) were placed on this diet for seven days, after which mortality and growth inhibition were assessed. The LC_{50} for Cry1F to monarch butterfly neonates could not be determined because there was no mortality at the highest dose tested (10,000 ng/ml diet), indicating that Cry1F is essentially non-toxic to the monarch butterfly.

INTRODUCTION

Statement of Problem

There has been substantial concern recently over the potential risk to the monarch butterfly *Danaus plexippus* (Linnaeus) posed by the pollen of transgenic maize. The purpose of this document is to report on the current state of knowledge of the toxicity of the Cry1F protein from *Bacillus thuringiensis* var *aizawai*, in the form expressed in transgenic plant tissue, to the neonate larval stage of the monarch butterfly.

Background

Although the concern about the risk to monarch butterflies involves the maize pollen, the issue arose too quickly to conduct the necessary studies to validate the procedures for collecting, handling, storing, and diet incorporating pollen. Historically, studies like these have not been conducted with pollen, and there were no pre-existing protocols for such work. In order to have results in a timely fashion, and to minimize the need to use incompletely validated procedures, it was decided to use pre-existing supplies of proteins and duplicate procedures used for other lepidopterous larvae as closely as possible. All work was performed at the laboratory of Dr. Blair Siegfried, Department of Entomology, University of Nebraska in Lincoln, Nebraska.

CRY1F PROTEIN SOURCE

The Cry1F protein used in this study was produced in the fall of 1998 at the San Diego, CA facilities of Dow AgroSciences. It was produced through fermentation in *Pseudomonas flourescens*. At the completion of fermentation, the *Pseudomonas* cell walls are enzymatically digested and the Cry1F crystals are separated and purified. Following digestion to the trypsin resistant core they are purified across an ion exchange column, eluted with a salt gradient, and stabilized in 20mM Tris-Cl pH 7.4/ 1mM EDTA buffer. The Cry1F content is quantified using

SDS Page. Material for the current study was provided to the University of Nebraska, where it has been stored at –80°C.

The protein identifier number for this lot is 1497-52 and it was created for the European corn borer susceptibility monitoring program. Its activity on European corn borers was confirmed just prior to, and immediately following its use for this study.

METHODS AND MATERIALS

The methods and materials used are explained in Appendix I.

RESULTS

Cry1 F

Cry1F was found to cause no mortality to neonate larvae at the highest dose tested, which was 10,000 ng/ml diet. Higher doses were not tested because this dose was high enough to cause consistency problems with the diet, and because the Cry1F protein was considered too valuable to waste on further testing. Some growth inhibition was seen at the highest rate.

Other Bt delta endotoxins

Toxicity to monarch neonates was observed with Cry1Ab, Cry1Ac, and Cry9C. The LC_{50} for these proteins was 1.37, 13.8, and 316 ng/ml diet respectively. Growth inhibition was seen at rates substantially lower than the LC_{50}.

DISCUSSION AND CONCLUSIONS

All four Bt proteins tested were found to have some effect on monarch butterfly neonates, but there were dramatic differences in the magnitude of these effects. Cry1Ab was found to be relatively toxic to the monarch, while Cry1F was found to be nontoxic at the highest dose tested, which was the highest dose that physically could be mixed with the diet. It is improbable that doses higher than this will occur in nature, so Cry1F poses essentially no hazard to the monarch butterfly.

APPENDIX 1: DOSE-RESPONSE OF *DANAUS PLEXIPPUS* NEONATE LARVAE TO PURIFIED BT ENDOTOXINS

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INTRODUCTION: The following investigation was initiated to contribute to the overall risk assessment of transgenic Bt corn and its potential effects on monarch butterfly populations. Preliminary results are presented on the dose-response of neonate monarch butterfly larvae to purified endotoxins from *Bacillus thuringiensis* that are currently employed or are being developed for expression in transgenic corn hybrids.

OBJECTIVES

1. Develop reliable bioassay methods for assessment of toxicity to neonate larvae.
2. Provide comparative assessment of neonate monarch larval susceptibility to purified Bt toxins.
3. Assess sublethal effects of Bt exposure.
4. Provide data that can be incorporated into risk assessment models.

METHODS:

Insects: Monarch eggs were purchased from Monarch Butterfly Farm, (St. Petersburg, FL) or from Monarch Watch (Lawrence KS) and shipped by express mail to the University of Nebraska. Eggs were held in environmental chambers at 20° C until hatching

Bioassays: Bioassay of neonate monarch larvae involved exposure to Bt toxins incorporated into artificial diet. A multi-species Lepidopteran diet (Southland Products, Lake Village, AK) containing 2% powdered leaf tissue from air-dried common millkweed plants was used in all bioassays. This diet provide consistent levels of growth of monarch larvae for at least 7 days and has been used successfully to rear individuals to the adult stage.

The trypsin resistant core of four Bt toxins (Cry1Ab, Cry1Ac, Cry1F, Cry9C) were uniformly incorporated into the larval diet prior to solidifying. Depending on availability of larvae, each bioassay was replicated twice for each toxin with 16 larvae at 5 different Bt concentrations per replication. Bioassays were performed in 128 well trays (each well 16 mm diam. x 16 mm high; CD International, Pitman, NJ). Approximately 1 ml of diet was dispensed into each well and allowed to solidify. Controls consisted of artificial diet in the absence of Bt toxin. Neonate larvae (<24 hr after hatching) were transferred to individual wells which were covered with vented lids (CD International), and trays were held at 27° C, 24 h scotophase, and 80% RH.

Both larval weight and mortality were recorded after 7-days. Mortality data were analyzed by probit analysis (Finney 1971, LeOra Software. 1987) to determine lethal concentrations. Observed mortality was corrected for mortality in control treatments, and lethal concentrations with 95% fiducial limits were calculated. Larval weights were transformed to % growth inhibition relative to the controls.

RESULTS AND DISCUSSION

The bioassay diet provided consistent growth and development throughout the 7-day exposure period, and control mortality was less than 10%. Concentration–response curves for the four Bt toxins are shown in Figure 1. In all cases, growth of monarch larvae was significantly inhibited at concentrations below those which caused mortality suggesting that there was a sublethal effect of the toxins.

Calculated LC_{50}'s and LC_{90}'s for the four toxins are shown in Table 1. Monarch larvae were not equally sensitive to all toxins tested. The order of sensitivity was Cry1Ab > Cry1Ac > Cry9C > Cry1F. Significant concentration mortality regressions were obtained for all toxins except Cry1F which did not produce significant mortality at any of the concentration tested, although an increase in growth inhibition was observed at the highest concentration (10,000 ng/ml).

Table 1. Comparative toxicity of the four Bt endotoxins tested against neonate monarch larvae.

TOXIN	N	LC_{50}* (95% Confidence Interval)	LC_{90}* (95% Confidence Interval)	χ^2
Cry1Ab	187	1.37 (0.64 - 3.1)	6.47 (2.92 -48.3)	9.3
Cry1Ac	96	13.8 (3.04 - 26.2)	70.8 (36.3 - 546)	0.6
Cry9c	164	316 (203 - 428)	753 (539 - 1560)	1.5
Cry1F	198	>10,000†	>10,000†	–

* ng toxin/ml diet

† highest concentration tested

Preliminary results from this investigation indicate that monarch larvae are sensitive to the purified Bt toxins expressed by transgenic corn plants although there are differences among the four toxins examined. These data should provide a mechanism to calculate the necessary exposure to transgenic corn pollen that is required to produce a toxic response assuming that the expression levels have been previously identified. It is not appropriate at this time to compare these susceptibility levels with other species until direct comparisons using similar exposure methods and similar sources of toxins are available. Incorporation of these data with measurement of exposure to Bt pollen under field conditions should provide information relative to the formulation of risk assessment models for this species.

References:

Finney, D.J. 1971. Probit analysis. Cambridge University Press, England, 333 pp.

LeOra Software. 1987. POLO-PC. A user's guide to probit and logist analysis. Berkeley. CA.

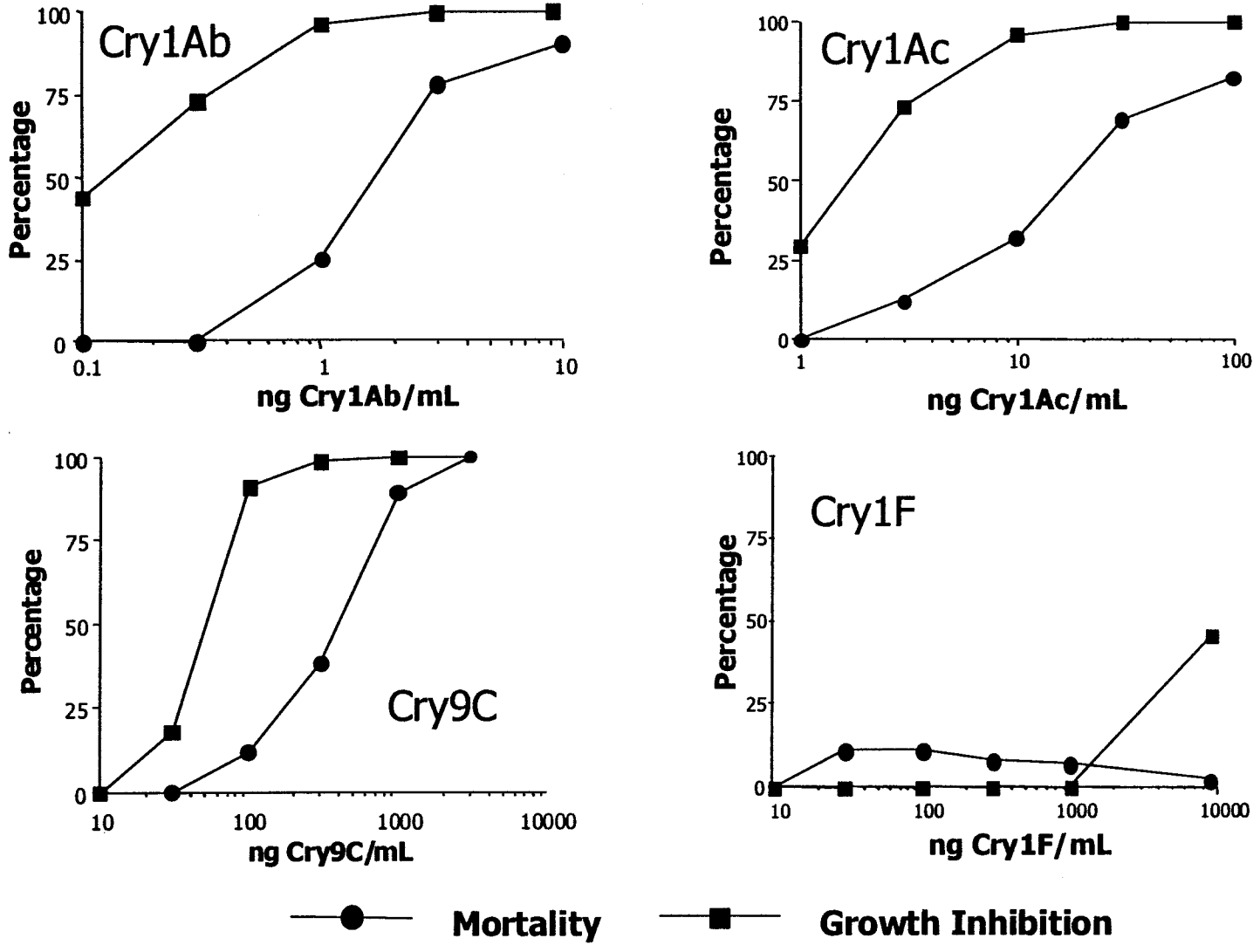

Figure 1. Concentration response curves for neonate Monarch larvae exposed to for different purified Bt endotoxins.