Study Title

Evaluation of Potential for Interaction Between the *Bacillus thuringiensis* Proteins Cry3Bb1, Cry34Ab1, and Cry35Ab1

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Sponsor and Performing Laboratory

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Study 07-RA-52-02
MSL0020554

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Signature Date

Statement of Compliance

This study does not meet the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160. Insect handling and details of diet preparation were not documented in the raw data. Measures taken to ensure study quality have been included in the Quality Measures section of this report, and an independent review of the data and final reports was conducted by the Monsanto Quality Assurance Unit.

Submitter Date

Sponsor Representative 1-18-08
Date

Study Director 1/18/08
Date

Study Title: Evaluation of Potential for Interaction Between the *Bacillus thuringiensis* Proteins Cry3Bb1, Cry34Ab1, and Cry35Ab1

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Quality Control Review

This report was reviewed to ensure that it accurately reflects the raw data of the study. The raw data was audited for compliance to the Monsanto Company guidelines for Keeping Research Records (GRR 10/1/99).

Reviewed by:

Mary Stephenson 1/18/08

Mary Stephenson — Date
Quality Assurance Specialist
Monsanto Regulatory, Monsanto Company

Study Certification

This report is an accurate and complete representation of the study/project activities.

Signatures of Approval:

Ted C. Mackee 1/18/08

Study Director Date

LeAnna G. Stout 1/18/08

Contributor Date

Hudson Head 1-18-08

Sponsor Representative Date

Study Information

Study Number:	07-RA-52-02
Report ID:	MSL0020554
Title:	Evaluation of Potential for Interaction Between the *Bacillus thuringiensis* Proteins Cry3Bb1, Cry34Ab1, and Cry35Ab1
Facility:	Monsanto Company Agronomic Traits Program 700 Chesterfield Parkway Chesterfield, MO 63017
Study Director:	Ted C. MacRae
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Quality Measures

All raw data were documented in accordance with Monsanto's Guidelines for Research Records (GRRs), and all study data will be retained in Monsanto's Regulatory archives. An independent review of the data and final reports was conducted by the Monsanto Quality Assurance Unit, statistical analysis was independently conducted by the Monsanto Regulatory Statistics Technology Center, and the study was conducted in compliance with the Monsanto Company guidelines for Keeping Research Records GRR 10/1/99. All of these measures were taken to ensure the integrity of the study.

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Abbreviations and Definitions

CI – Confidence interval
GI_{50} – Median growth inhibitory concentration, the concentration of a toxin that results in 50% biomass reduction.
HEPA - High-Efficiency Particulate Air (filter)
LC_{50} – Median lethal concentration, the concentration of a toxin that results in 50% mortality.
L:D – Light:dark
R.H. – Relative humidity
SCRW – Southern corn rootworm, *Diabrotica undecimpunctata howardi* (Coleoptera: Chrysomelidae)
UTC – Untreated control

1.0 Summary

Monsanto and Dow AgroSciences have independently developed unique corn products for protection from damage by corn rootworms, *Diabrotica* spp. MON 88017, produces the *Bacillus thuringiensis* (*Bt*) δ-endotoxin Cry3Bb1, and DAS-59122-7, produces the binary *Bt* δ-endotoxins Cry34Ab1 and Cry35Ab1 (Cry34/35Ab1). Combining these traits in a single corn product could provide greater efficacy and enhance insect resistance management. The purpose of this study was to characterize the potential for interactive effects between the Cry3Bb1 protein and the Cry34/35Ab1 binary protein. Southern corn rootworm (SCRW), *Diabrotica undecimpunctata howardi* Barber, was exposed to purified Cry3Bb1 protein, a mixture of the Cry34Ab1 and Cry35Ab1 proteins, and a combination of all three proteins in 6-day diet-overlay bioassays. Predicted LC_{50} values and GI_{50} values for the combination of all three proteins were based on the model of multiple-toxicity presented by Finney (1971). This is an established model for examining joint action of substances having a similar mode of action[1] (simple similar action), such as Bt proteins (Tabashnik 1992). The results from the 6-day bioassay demonstrated that the predicted LC_{50} values and GI_{50} values of the combination were not significantly different from the observed LC_{50} values and GI_{50} values. Based on these results, it can be concluded that there is no evidence of interaction (i.e., synergism or antagonism) between the Cry3Bb1 and the Cry34Ab1 and Cry35Ab1 proteins.

2.0 Introduction

Corn rootworms (*Diabrotica* spp.) (Coleoptera: Chrysomelidae) are major pests of corn, *Zea mays* L., in the U.S. Larvae cause damage by feeding on the roots of the plant and may seriously impact corn yields. Management techniques have traditionally involved crop rotation and chemical insecticide use. Recently, several δ-endotoxins that exhibit insecticidal activity against rootworms were characterized from *Bacillus thuringiensis*, and *in planta* expression of these endotoxins (Cry proteins) provides effective control of these pests. Monsanto has developed a biotechnology-derived corn product producing the Cry3Bb1 protein, MON 88017, while Dow AgroSciences has similarly developed, DAS-59122-7 producing the Cry34/35Ab1 binary proteins. Combining these traits in a single corn product could provide greater efficacy against corn rootworms and enhance insect resistance management.

The purpose of this study was to characterize the potential for interactive effects between the Cry3Bb1 protein and the Cry34/35Ab1 binary proteins. Southern corn rootworm (SCRW), *Diabrotica undecimpunctata howardi* Barber, was exposed to purified Cry3Bb1 protein, a mixture of purified Cry34Ab1 and Cry35Ab1 proteins, and a combination of all three purified proteins in 6-day diet-overlay bioassays. A range of concentrations that characterized the dose-effect relationship were tested concurrently to determine 50% effect

[1] "Mode of action" in this context refers to the general mechanism of toxicity, which for *Bt* proteins involves pore formation in the insect midgut. The term is used differently in the context of insect resistance to *Bt* proteins, where "similar mode of action" implies that two *Bt* proteins bind to the same midgut receptors. The simple similar action model does not rely on *Bt* proteins sharing the same receptors.

values for Cry3Bb1, the Cry34Ab1 + Cry35Ab1 mixture, and the 3-protein mixture. The concentration-dependent responses for each of the treatments were used to evaluate potential interactions using the "simple similar action" model (Finney, 1971; Tabashnik, 1992). Predicted 50% effect values for the 3-protein mixture, assuming no interaction (i.e., activity is additive), were calculated based on the observed values obtained for Cry3Bb1 and the Cry34Ab1 plus Cry35Ab1 mixture. Predicted LC_{50} values and GI_{50} values for the mixture were compared to observed LC_{50} values and GI_{50} values and their 95% confidence intervals to determine whether there is a significant interaction (i.e., synergism or antagonism) for the combination of the Cry3Bb1 and Cry34/35Ab1 proteins.

3.0 Materials and Methods

3.1 Test and Control Substances

The test substances were: 1) purified *E. coli*-produced Cry3Bb1 stored in 50-mM sodium carbonate/bicarbonate, 1-mM EDTA buffer, pH ~10.1 (hereafter: Buff-1), with a purity of 87% and a purity corrected concentration of 4.1 mg/mL, which was received July 5, 2006 from the Monsanto Product Characterization Center (Monsanto Lot #20-100025); 2) lyophilized Cry34Ab1 protein powder with a purity of 69.9% (protein to powder mass ratio) which was received June 14, 2006 from Dow AgroSciences (Indianapolis, IN) (Dow Lot #TSN104463) and subsequently suspended in 10-mM potassium phosphate buffer, pH ~7.5 (hereafter: Buff-2) to a purity-corrected concentration of 2.0 mg/mL; and 3) lyophilized Cry35Ab1 protein powder with a purity of 40.3% (protein to powder mass ratio) which was received June 14, 2006 from Dow AgroSciences (Dow Lot #TSN104462) and subsequently suspended in Buff-2 to a purity-corrected concentration of 2.0 mg/mL. The Cry34Ab1 and Cry35Ab1 suspensions were combined in a ratio of 9:1 (w/w) (hereafter: Cry34/35Ab1) for use in bioassays. Buffers of the same composition and pH but without protein were used as control substances. Proteins and buffers were stored at approximately 4°C during the course of the study. Data supporting the stability or characterization of the test substances in 10mM potassium phosphate buffer and in the diet from the point of overlay to the end of the assay are not available.

3.2 Test Insects

Test insects were neonate larvae (<24 hours post-hatch) of southern corn rootworm (SCRW), *Diabrotica undecimpunctata howardi*, hatched from eggs supplied in soil by Crop Characteristics, Inc. (Farmington, MN). Eggs were separated from soil using a 60-mesh sieve covered with a 30-mesh sieve and rinsing gently with tepid tap water until soil was removed. Recovered eggs were rinsed into a beaker, transferred into a separatory funnel and allowed to settle, and floating debris was decanted. Eggs were transferred back into the separatory funnel, and excess water was withdrawn using a transfer pipette. A $MgSO_4$ solution (350 mL $MgSO_4$ + 250 mL water) warmed to room temperature was added in excess to the separatory funnel and agitated by inversion to float the eggs from the soil. The $MgSO_4$ solution and soil were drained, and a second $MgSO_4$ solution (50 mL $MgSO_4$ + 950 mL water) was added in excess to the separatory funnel and agitated by inversion to float lighter debris from the eggs. The eggs were drained to separate them from the floating debris, transferred to a glass beaker, and triple-rinsed in deionized water, decanting excess water and

floating debris after each rinse. Eggs were surface sterilized by adding excess 1% Clorox® bleach (0.0615% NaOCl) solution (1 mL Clorox® bleach (6.15% NaOCl v/v) + 99 mL autoclaved Millipore-filtered deionized water, hereafter: sterile water) to the beaker and decanting after 90 seconds, triple rinsing/decanting with sterile water, adding 100 ml of 25% peracetic acid/sodium alkylaryl sulfonate (0.714 mL 32% peracetic acid [Aldrich Chemical Company] + 2 mL 2% wt/vol sodium alkylaryl sulfonate solution (Fisher Scientific) raised to 100 mL with sterile water) and decanting after 90 seconds, and triple rinsing/decanting again with sterile water. A plastic transfer pipette (tip cut off) was used to distribute the eggs evenly inside double-stacked coffee filters (Mr. Coffee, 8–12 cup size). Coffee filters were evenly moistened with sterile water and placed in a 473-mL deli cup (Solo #DM-16 cup) with ~10–15 holes poked in the lid (Solo #LG-8) with a #2 insect pin. Deli cups were incubated in Percival environmental chambers at 10±1° C, 60±10% R.H., and a 0:24 L:D photoperiod for a maximum of seven days before transferring to 25±1° C, 60±10% R.H., and a 0:24 L:D photoperiod until hatch.

3.3 Bioassay Procedure

Each bioassay contained seven concentrations each of Cry3Bb1 and Cry34/35Ab1 prepared using standard serial dilution techniques and a 3-fold dilution factor with sterile water as the diluent in Nalgene® 5-mL cryogenic vials (#5000-0050) to achieve nominal concentrations ranging from 0.137–100 µg/cm^2 when applied to the diet. This range of concentrations was selected based on results of range-finding bioassays conducted prior to initiation of the study. The Cry3Bb1 and Cry34/35Ab1 dilutions series were used to prepare a third dilution series consisting of Cry3Bb1 + Cry34/35Ab1 and in which equal volumes of each Cry3Bb1 dilution and its corresponding Cry34/35Ab1 dilution were combined to achieve a 1:1 (w/w) mixture of the two individual protein treatments. Each bioassay also contained one treatment each of Buff-1 and Buff-2 diluted to the same level as the highest concentration of its corresponding protein, as well as a third buffer treatment consisting of a mixture of the Buff-1 and Buff-2 treatments prepared in the same manner as the Cry3Bb1 + Cry34/35Ab1 mixture. Buffer treatments served as negative controls to assess bioassay quality. Lastly, each bioassay included two treatments of sterile water to serve as untreated controls (UTC).

SCRW insect diet (Marrone et al. 1985, modified as follows: 18 g/L Serva agar (#11393) added in place of Phytagar, wheat germ increased to 50 g/L; and 2.5 mL/L mold inhibitor solution (10 ml phosphoric acid + 99.37 ml proprionic acid + 128 ml sterile water) added in place of streptomycin, chlorotetracycline, and KOH) was dispensed (200 µL per well) into 96-well U-bottom assay plates (Falcon® #353910) under a HEPA-filtered laminar flow hood, and allowed to solidify. Insect-diet plates were "pre-dried" for 3 min under a Packard aspirator (Turbo Driver®) to facilitate absorption of applied test/control substances. An Eppendorf pipettor (Repeater® #4780) fitted with a 1.25-mL Combitip® (#22 26 110-0) was used to dispense 25 µL per well of test/control substance onto the surface of the insect diet, after which the treated diet plates were placed under a HEPA-filtered biological hood until the surface of the insect diet was dry. Bioassay trays for each replicate were infested with a target number of 48 insects in each test/control treatment by placing a single, randomly chosen neonate larva (0–24 hours post-hatch) on the surface of the diet in each well using a fine camel hair brush. A tacking iron was used to seal a mylar sheet containing a heat-activated adhesive on one side over the top of the plate, and wells were ventilated by piercing the

mylar over each well with a #000 insect pin. Infested plates were incubated in an environmental chamber for 6 days at 27°C, 60% RH, and 0:24 L:D photoperiod, after which the number of dead and live larvae in each treatment were recorded. Live larvae were removed from assay plates, pooled by treatment, and weighed using a microbalance.

3.4 Data Analysis and Interpretation

Response data were used to calculate percent mortality and growth inhibition. Growth inhibition (GI) was based on reduction in total live weight, which incorporates mortality and yields a measure of efficacy that is proportional to the biomass of live insects (Herman et al. 2002). Bioassay data sets were accepted as valid if UTC mortality did not exceed 20% and at least three concentrations produced mortality and growth inhibitory responses bracketing 50%. Response data from valid replicates were pooled for further analysis. LC_{50} values with associated slopes and 95% confidence intervals (CIs) were estimated from mortality response data using PROC PROBIT with a logistic distribution in SAS® version 9.1.3 (SAS Institute, Inc., Cary, NC). GI_{50} values and associated 95% CIs were estimated from growth inhibition response data with the following 3-parameter logistic model under PROC NLIN in SAS® version 9.1.3:

$$\text{Weight} = W_0/(1+(\textit{concentration}/\text{GI}_{50})^B)$$

where W_0 is the expected control weight, *concentration* is the protein concentration in units of $\mu g/cm^2$, GI_{50} is the protein concentration expected to reduce total live weight by 50%, and B is the logistic function slope parameter (Sims & Berberich, 1996).

The "simple similar action" model (also known as the "dose addition model") was used to test a null hypothesis of no interaction between Cry3Bb1 and Cry34/35Ab1. This model is useful for examining potential interactions in *Bt* proteins (Tabashnik, 1992). Predicted LC_{50} and GI_{50} values for the mixture were calculated as follows:

$$\text{Prob}_{50(m)} = [(r_a/\text{Prob}_{50(a)})+(r_b/\text{Prob}_{50(b)})]^{-1}$$

where r_a and r_b are the relative proportions of Cry3Bb1 and Cry34/35, respectively, $\text{Prob}_{50(a)}$ and $\text{Prob}_{50(b)}$ are the observed LC_{50} or GI_{50} values of Cry3Bb1 and Cry34/35Ab1, respectively, and $\text{Prob}_{50(m)}$ is the predicted LC_{50} or GI_{50} of the mixture. No significant interaction (i.e., synergism or antagonism) is shown if the observed 95% CI of the mixture brackets the predicted LC/GI_{50} value – i.e., activity by the mixture is not inconsistent with additivity. On the other hand, synergism (antagonism) is claimed if the observed 95% CI of the mixture lies significantly below (above) the predicted LC/GI_{50} value.

4.0 Results

Five experimental replicates were conducted. The first two were rejected because %GI did not bracket a 50% response in one of the three treatments. Acceptability criteria were satisfied in all subsequent replicates. Mortality of the UTC averaged 11% in the accepted experimental replicates, with UTC-corrected mortality and growth inhibition of 1% or less in the three buffer controls. LC_{50} values and associated 95% CIs for Cry3Bb1, Cry34/35Ab1, and a 1:1 (w/w) mixture of Cry3Bb1 + Cry34/35Ab1 are presented in Table 1 and illustrated in Figure 1. Also indicated is a predicted LC_{50} value for the Cry3Bb1 + Cry34/35Ab1

mixture, based on observed values obtained for Cry3Bb1 and Cry34/35Ab1 independently using the "simple similar action" model. Under this model, the LC_{50} predicted for the mixture fell within the observed 95% CI obtained for the mixture, showing no evidence of significant interaction (i.e., synergism or antagonism) as measured by mortality. GI_{50} values and associated 95% CIs for Cry3Bb1, Cry34/35Ab1, and the Cry3Bb1 + Cry34/35Ab1 mixture are presented in Table 1 and illustrated in Figure 2. Again, under this model, the GI_{50} predicted for the mixture fell within the observed 95% CI obtained for the mixture, showing no evidence of a significant interaction (i.e., synergism or antagonism) as measured by growth inhibition. Therefore, the results for mortality and growth inhibition are not inconsistent with additivity. A second statistical model, the "independent joint action" model (assumes responses rather than doses are additive), was also considered. However, Tabashnik (1992) has argued that this model is less appropriate than the simple similar action model for assessing interactions between *Bt* proteins. A preponderance of subsequent published works have adopted Tabashnik's concepts, thus, results from the independent joint action model analysis are not included in this report.

5.0 Conclusions

The simple similar action model is the most appropriate model for examining the potential for interactions for substances having similar modes of action, such as Bt proteins (Tabashnik 1992). LC_{50} and GI_{50} values obtained with the 6-day SCRW bioassay using the combination of the Cry3Bb1 protein and the Cry34/35Ab1 binary proteins did not show evidence of a significant interaction. Therefore, the results for mortality and growth inhibition are not inconsistent with additivity.

6.0 Acknowledgment

We thank O. M. Ilagan (Monsanto Agronomic Traits) for providing SCRW neonates and prepared insect bioassay plates and G. P. Head, S. L. Levine, and M. A. Nemeth (Monsanto Regulatory Sciences) for guidance on experimental design and statistical analyses.

7.0 References

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8.0 Tables

Table 1. Observed LC_{50} and GI_{50} values and associated 95% CIs for Cry3Bb1 Cry34/35Ab1, and a 1:1 (w/w) mixture of the two protein treatments against SCRW versus expected values for the mixture, interaction type, and percent difference in observed from expected activity.

	LC_{50} (µg of toxin / cm^2 diet)					
	Observed					
Toxin(s)	Mean	Lower[a]	Upper[a]	Expected[b]	Interaction Type	% Diff. from Expected
Cry3Bb1	68.74	52.46	95.17	-	-	-
Cry34/35	58.55	36.20	98.16	-	-	-
Cry3Bb1 + Cry34/35Ab1	65.37	53.51	79.13	63.24	additive	-3%
	GI_{50} (µg of toxin / cm^2 diet)					
	Observed					
	Mean	Lower[a]	Upper[a]	Expected[b]	Interaction Type	% Diff. from Expected
Cry3Bb1	31.23	18.19	44.28	-	-	-
Cry34/35	4.01	2.48	5.53	-	-	-
Cry3Bb1 + Cry34/35Ab1	10.90	6.95	14.85	7.10	additive	-35%

[a] 95% confidence interval.

[b] Calculated by the following formula: $[(0.5 \div \text{mean Cry3Bb1}) + (0.5 \div \text{mean Cry34/35Ab1})]^{-1}$

9.0 Figures

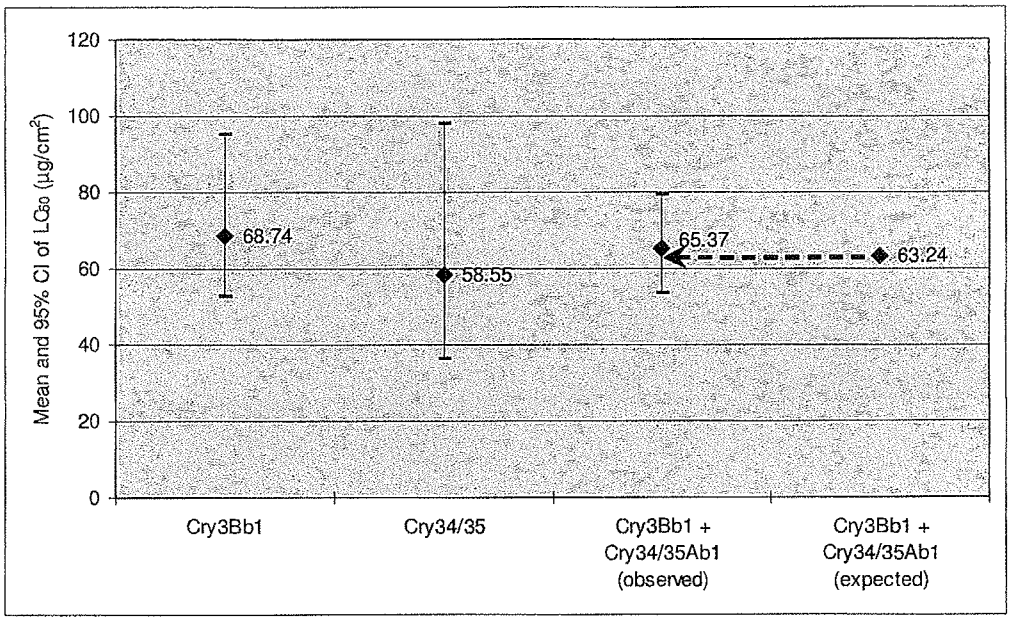

Figure. 1. Observed LC_{50} ($\mu g/cm^2$) and associated 95% CI for Cry3Bb1, Cry34/35Ab1, and a 1:1 (w/w) mixture of the two proteins in combination against SCRW compared to a predicted LC_{50} of the mixture under simple similar action. The arrow indicates the predicted activity falls within the 95% CI of the observed activity of the mixture.

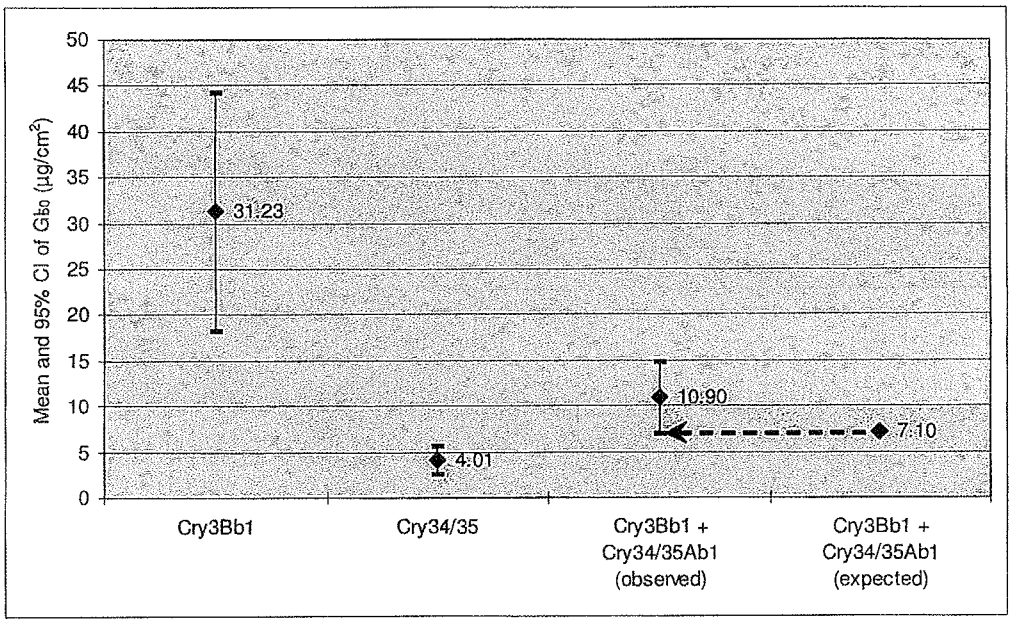

Figure. 2. Observed GI_{50} ($\mu g/cm^2$) and associated 95% CI for Cry3Bb1, Cry34/35Ab1, and a 1:1 (w/w) mixture of the two against SCRW compared to a predicted GI_{50} of the mixture under simple similar action. The arrow indicates the predicted activity falls within the 95% CI of the observed activity of the mixture.