Study Title

Evaluation of the Potential for Interactions among Cry Proteins Produced by MON 89034 × TC1507 × MON 88017 × DAS-59122-7 by Insect Bioassay

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Statement of Compliance

This study does not meet the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160. Measures taken to ensure study quality have been included in the Quality Measures section of the report.

Study Director: Steven Levine Date: 2/28/08
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Submitter: _____ Date: _____

Quality Control Statement

This report was reviewed to assure that it accurately reflects the raw data of this study. The raw data were audited by the Quality Assurance Unit for compliance with Monsanto Company Guidelines for Keeping Research Records (GRR 10/1/99), the protocol, and where applicable, Monsanto SOPs.

Reviewed by:

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2/28/08

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Study Certification

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

Signatures of Approval:

Study Director: Steven Levine Date: 2/28/2008
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Statistician: Changjian Jiang Date: 02/28/08
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Study Information Page

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1.0 Summary

Monsanto Company and Agrigenetics (Dow AgroSciences) have used conventional breeding techniques to develop the combined-trait corn product MON 89034 × TC1507 × MON 88017 × DAS-59122-7 that confers insect resistance and herbicide tolerance.

The purpose of this study was to evaluate the potential for interactions among the proteins produced by MON 89034 × TC1507 × MON 88017 × DAS-59122-7. This was evaluated by concurrently assessing the potential for interactions with the lepidopteran active proteins (combination of Cry1A.105 and Cry2Ab2 proteins with the Cry1F protein) and assessing the potential for interactions between the lepidopteran-active and coleopteran-active proteins Cry3Bb1 and Cry34Ab1/Cry35Ab1 (hereafter Cry34/35Ab1).

To assess the potential for interaction between the combination of the Cry1A.105 and Cry2Ab2 proteins and the Cry1F protein in MON 89034 × TC1507 × MON 88017 × DAS-59122-7, MON 89034 × TC1507 × NK603 was used as the test substance because it expresses the Cry1A.105, Cry2Ab2 and Cry1F proteins but not the Cry3Bb1 and Cry34/35Ab1 proteins. This allowed the potential interactions between the combination of the Cry1A.105 and Cry2Ab2 proteins and the Cry1F protein to be directly evaluated. To perform this assessment, European corn borer larvae (ECB, *Ostrinia nubilalis* (Hübner)) were exposed to a series of lyophilized leaf tissue concentrations of either MON 89034, TC1507 or MON 89034 × TC1507 × NK603 along with the appropriate control tissues in 7-day diet-incorporation bioassays run in parallel. The hypothesis of no interaction was tested under a concentration addition model by statistically comparing the estimated (observed) GI_{50} value (50% growth inhibition) for MON 89034 × TC1507 × NK603 with the predicted GI_{50} value for MON 89034 × TC1507 × NK603. The predicted GI_{50} value for MON 89034 × TC1507 × NK603 was based on calculations using the GI_{50} values from MON 89034 and TC1507. This analysis indicated no significant difference between the estimated and predicted GI_{50} values for MON 89034 × TC1507 × NK603 ($p = 0.38$), which indicates no interaction between the combination of the Cry1A.105 and Cry2Ab2 proteins and the Cry1F protein.

To assess whether combined Cry1A.105, Cry2Ab2 and Cry1F activity is altered by the presence of the Cry3Bb1 and Cry34/35Ab1 proteins, the biological activity of MON 89034 × TC1507 × MON 88017 × DAS-59122-7 and MON 89034 × TC1507 × NK603 were compared. To perform this comparison, ECB were exposed to a series of lyophilized leaf tissue concentrations of either MON 89034 × TC1507 × NK603 or MON 89034 × TC1507 × MON 88017 × DAS-59122-7 along with the appropriate control tissues in 7-day diet-incorporation bioassays run in parallel. The hypothesis of no interaction was tested by statistically comparing the estimated GI_{50} values from MON 89034 × TC1507 × NK603 and MON 89034 × TC1507 × MON 88017 x DAS-59122-7. The GI_{50} values for MON 89034 × TC1507 × NK603 and MON 89034 × TC1507 ×

MON 88017 × DAS-59122-7 were not significantly different ($p = 0.53$) and were estimated to be 0.048 and 0.046 mg tissue/ml diet, respectively. Observing nearly identical GI_{50} values for MON 89034 × TC1507 × NK603 and MON 89034 × TC1507 × MON 88017 × DAS-59122-7, indicates that the Cry3Bb1 and Cry34/35Ab1 proteins do not interact with the combined activity of the Cry1A.105, Cry2Ab2 and Cry1F proteins.

The results from this study demonstrate no interaction among the *Bt* Cry proteins expressed in MON 89034 × TC1507 × MON 88017 × DAS-59122-7. First, the results indicate no interaction between the combination of the Cry1A.105 and Cry2Ab2 proteins and the Cry1F protein. Additionally, the results indicate no interaction between combined Cry1A.105, Cry2Ab2 and Cry1F activity and the Cry3Bb1 and Cry 34/35Ab1 proteins.

2.0 Background and Purpose

Monsanto Company and Agrigenetics (Dow AgroSciences) have used conventional breeding techniques to develop the combined trait corn products MON 89034 × TC1507 × NK603 and MON 89034 × TC1507 × MON 88017 × DAS-59122-7 that confers insect resistance and herbicide tolerance. Each biotechnology-derived trait contributes specific benefits to the final combined product as follows:

MON 89034 produces two insecticidal proteins that protect against feeding damage caused by European corn borer (ECB, *Ostrinia nubilalis*) and other lepidopteran insect pests. MON 89034 produces two *Bacillus thuringiensis* (*Bt*) proteins, Cry2Ab2 (subsp. *kurstaki*) protein and Cry1A.105, a modified Cry1A *Bt* protein. The combination of the two insecticidal proteins provides enhanced insect control and offers an additional insect-resistance management tool.

TC1507 produces the *Bt* var *aizawai* Cry1F protein to selectively control larvae of the ECB and other lepidopteran insect pests. In addition, TC1507 produces the phosphinothricin acetyl transferase (PAT) from *Streptomyces viridochromogenes*, to confer tolerance to glufosinate-ammonium, the active ingredient in Liberty® herbicide.

MON 88017 produces a modified *Bt* (subsp. *kumamotoensis*) Cry3Bb1 protein to protect against corn rootworm (CRW) larval feeding. In addition, MON 88017 is a Roundup Ready® corn that produces 5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium sp.* strain CP4 (CP4 EPSPS), which confers tolerance to glyphosate, the active ingredient in Roundup® agricultural herbicides.

DAS-59122-7 produces the *Bt* strain PS149B1 Cry34Ab1 and Cry35Ab1 (hereafter Cry34/35Ab1) proteins to protect against coleopteran pests such as CRW. In addition, DAS-59122-7 also produces the PAT protein from *Streptomyces viridochromogenes* which confers tolerance to glufosinate-ammonium, the active ingredient in Liberty herbicide.

NK603 is a Roundup Ready® corn that expresses the CP4 EPSPS from *Agrobacterium sp.* strain CP4, which confers tolerance to glyphosate, the active ingredient in Roundup agricultural herbicides.

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The combination of these biotechnology-derived events provide protection against lepidopteran and/or coleopteran insect pests and tolerance to the glyphosate and glufosinate herbicide families in a single product generated through conventional breeding techniques.

Previously, the potential for interactions has been evaluated with combinations of the *Bt* proteins produced by MON 89034 × TC1507 × MON 88017 × DAS-59122-7. The Cry1A.105 and Cry2Ab2 proteins were shown to have additive activity against two lepidopteran pests of corn (MacRae et al., 2005), the European corn borer (ECB, *Ostrinia nubilalis* (Hübner)) and the corn earworm (CEW, *Helicoverpa zea* (Boddie)). Subsequently, it was demonstrated that combined Cry1A.105 and Cry2Ab2 activity is not altered in the presence of the Cry3Bb1 protein and vise-versa in sensitive insect diet-bioassays (MacRae et al. 2006) and that protection against root feeding damage is additive when combining MON 88017 and DAS-59122-7 by conventional breeding (Levine and Uffman, 2008). In addition, no interaction was observed between the Cry1F and Cry34/35Ab1 proteins in sensitive insect diet-bioassays (Herman and Storer, 2004). Recently, the Cry3Bb1 and Cry34/35Ab1 proteins were shown to have additive activity against southern corn rootworm species in diet bioassays (MacRae, 2008).

The purpose of this study was to evaluate the potential for interactions among the proteins produced by MON 89034 × TC1507 × MON 88017 × DAS-59122-7. This was evaluated by concurrently assessing the potential for interactions with the lepidopteran active proteins (combination of Cry1A.105 and Cry2Ab2 proteins with the Cry1F protein) and assessing the potential for interactions between the lepidopteran-active and coleopteran-active proteins Cry3Bb1 and Cry34/35Ab1.

3.0 Experimental Design

To assess the potential for interaction between the combination of the Cry1A.105 and Cry2Ab2 proteins and the Cry1F protein in lyophilized leaf tissue of MON 89034 × TC1507 × MON 88017 × DAS-59122-7, MON 89034 × TC1507 × NK603 was used as the test substance because it expresses the Cry1A.105, Cry2Ab2 and Cry1F proteins but not the Cry3Bb1 and Cry34/35Ab1 proteins. This allowed the potential for potential interactions between the combination of the Cry1A.105 and Cry2Ab2 proteins and the Cry1F protein to be directly evaluated. To perform this assessment, ECB were exposed to a series of lyophilized leaf tissue concentrations of either MON 89034, TC1507 or MON 89034 × TC1507 × NK603 along with the appropriate control tissues in 7-day diet-incorporation bioassays run in parallel. ECB was chosen as the test organism because of its relatively high sensitivity to Cry1 and Cry2 class proteins (De Maagd et al, 2001; Wolt et al., 2005). The hypothesis of no interaction was tested with a concentration addition model by statistically comparing the estimated (observed) GI_{50} value (50% growth inhibition) for MON 89034 × TC1507 × NK603 with the predicted GI_{50} value for MON

89034 × TC1507 × NK603 (Finney, 1971, Tabashnik, 1992). Under the concentration addition model, the predicted GI_{50} value for MON 89034 × TC1507 × NK603 was based on calculations using the GI_{50} values from MON 89034 and TC1507.

To assess whether combined Cry1A.105, Cry2Ab2 and Cry1F activity is altered by the presence of the Cry3Bb1 and Cry34/35Ab1 proteins, the biological activity of MON 89034 × TC1507 × MON 88017 × DAS-59122-7 and MON 89034 × TC1507 × NK603 lyophilized leaf tissues were compared. To perform this comparison, ECB were exposed to a series of lyophilized leaf tissue concentrations of either MON 89034 × TC1507 × NK603 or MON 89034 × TC1507 × MON 88017 × DAS-59122-7 along with the appropriate control tissues in 7-day diet-incorporation bioassays run in parallel. The hypothesis of no interaction was tested by statistically comparing the estimated GI_{50} values from MON 89034 × TC1507 × NK603 and MON 89034 × TC1507 × MON 88017 x DAS-59122-7.

Control treatments included a diet-only assay control, lyophilized conventional corn tissue, lyophilized MON 88017 tissue and lyophilized DAS-59122-7 tissue. Lyophilized tissue from MON 88017 and DAS-59122-7 were included to confirm the absence of an effect on ECB growth at the tested concentrations.

4.0 Materials and Methods

Test and Control Substances

The test and control materials were lyophilized leaf tissue from V6 plants derived from the test and control substances. Leaf tissue was produced under Monsanto production plan number 07-01-52-07 (Klug, 2008) and stored at -80°C from the time of collection until lyophilization. All leaf tissues used in this study were lyophilized in a VirTis 24X48 GPFD Freeze Dryer (VirTis Company, Gardiner, NY). Lyophilized leaf tissue was analyzed for moisture content using an IR-200 moisture analyzer (Denver Instrument Company, Arvada, CO) according to SOP AG-EQ-1023-01. Because there was a limited amount of leaf tissue available from production 07-01-52-07, the moisture content of a pooled tissue sample was measured and considered to be representative. Moisture content was determined to be approximately 5%.

Test Substances:

1) MON 89034 (starting seed lot number GLP-0604-17104-S)
2) TC1507 (starting seed lot number GLP-0604-17103-S)
3) MON 89034 × TC1507 × NK603 (starting seed lot number GLP-0604-17107-S)
4) MON 89034 × TC1507 × MON 88017 × DAS-59122-7 (starting seed lot number GLP-0604-17108-S)

All test substances were in the XE6001 hybrid genetic background.

Control Substances:

1) MON 88017 (starting seed lot number GLP-0604-17100-S)
2) DAS-59122-7 (starting seed lot number GLP-0604-17101-S)
3) XE6001 Conventional corn standard (starting seed lot number GLP-0604-17109-S)

All control substances were in the XE6001 hybrid genetic background.

Assay Control:

Untreated diet was included as an assay control.

The presence or absence of MON 89034, TC1507, NK603, MON 88017 and DAS-59122-7 in the test and control substances were verified by event-specific polymerase chain reaction (PCR) analyses. The verifications were performed and documented by the Monsanto Biotechnology Regulatory Sciences Product Characterization Technology Center, and the raw data are retained in the Monsanto Regulatory Archives. Copies of the Certificates of Analysis for the test and control substance starting seed are included in the study file.

Laboratory Bioassay Testing Procedures

Standard diet-incorporation insect bioassay methodology described in the current version of Monsanto SOP BR-ME-0044 was used to characterize the biological activity of the test and control substances in 7-day bioassays. A single tissue-diet concentration was tested for MON 88017, DAS-59122-7 and the XE6001 conventional control, which was equivalent to the amount of tissue in the highest tissue-diet concentration for MON 89034 × TC1507 × NK603 and MON 89034 × TC1507 × MON 88017 × DAS-59122-7. The test concentrations for MON 89034, MON 89034 × TC1507 × NK603, MON 89034 × TC1507 × MON 88017 × DAS-59122-7 ranged from 0.016 to 0.256 mg tissue/ml diet with a two-fold dilution factor between each concentration level. The test concentrations

for TC1507 ranged from 0.40 to 6.4 mg tissue/ml diet with a two-fold dilution factor between each concentration level. These dietary concentrations were based on the results of range-finding assays and were chosen to adequately characterize the concentration-effect relationship as well as to accurately estimate GI_{50} values. It was found during method development that a tissue level of 6.24 mg lyophilized conventional tissue/ml diet had no adverse effect on ECB growth in 7-day bioassays. Therefore, it was not necessary to control for a tissue effect by having the same amount of tissue in all treatment groups.

Treatments were prepared by mixing 5 ml of purified water with finely-ground lyophilized leaf tissue (dosing solution) and then adding 20 ml of warm (52 - 54°C) agar-based multi-species diet (Southland Products) to a final volume of 25 ml. The diet was vortex-mixed until visually homogeneous. Additionally, diet-only assay controls were included that contained 5 ml of purified water and 20 ml of diet. A repeat pipettor was used to aliquot 0.5 mL of diet into individual wells of 128-well bioassay trays and the diet was allowed to solidify.

ECB eggs were received from Monsanto's Waterman facility (Waterman, IL). ECB eggs were handled following the current version of Monsanto SOP BR-ME-0989 and were held at a target temperature of 10°C prior to incubation and at a target temperature of 27°C for hatching. One newly hatched ECB larva (≤24 hours after the first observation of hatching) was added to each bioassay well the day the diet was prepared. Each replicate contained a target number of 24 individually-housed ECB larvae and were covered with a ventilated adhesive cover. For each independent assay, one replicate was maintained for each test concentration and three replicates for each control treatment. Bioassay trays were marked with the appropriate treatment designation, concentration and a unique bioassay identification number. Bioassay trays were incubated at a target temperature of 27°C, ambient relative humidity and a 14:10 light:dark photoperiod for 7 days. At the end of the 7-day bioassay, the actual number of insects in each treatment level, the number of surviving insects in each treatment level and the combined insect weight of the surviving insects in each treatment level were recorded. In each bioassay, all of the test and control substances were evaluated in parallel and all of the substances were independently tested in three separate bioassays.

5.0 Control of Bias

Dosing solutions for each concentration level and each protein were prepared independently. Assays with the test and control substances were run concurrently using the same batch of insects. Additionally, assays were replicated three times with three separate batches of insects. No potential sources of bias are expected to affect the results of the study.

6.0 Quality Measures

This study does not meet the requirements of 40 CFR Part 160. As stated in the protocol, there was no intention to conduct this study according to Good Laboratory Practice (GLP) Standards. However, the following quality control measures were taken to ensure the integrity and validity of the study:

- This study was conducted according to a protocol that was peer-reviewed and reviewed by Monsanto QAU.
- Sample transfer forms were used to document chain-of-custody of the test and control substances.
- Customized worksheets were used to document experimental information and data which followed Monsanto's guidelines for keeping research records and Regulatory Science's Best Practices.
- Bioassays and data collection were monitored by the study director.
- The study director reviewed the data in detail.
- The Monsanto Regulatory Quality Assurance Unit audited the raw data and final report.
- The Monsanto Statistics Technology Center performed and reviewed the statistical analysis, statistics report and final report.
- The study file will be retained in the Monsanto Regulatory Archive.

7.0 Statistical Analysis

A brief summary of the statistical methods is presented below and a detailed summary of the statistical analyses and results is provided in Appendix I. Statistical significance was determined at the 95% confidence level.

Planned comparisons on mean body weights among the control treatments were performed with a mixed regression model in SAS. The planned comparisons included the following:

1) XE6001 (conventional control) versus DAS-59122-7
2) XE6001 (conventional control) versus MON 88017
3) XE6001 (conventional control) versus diet only assay control

A joint logistic model analysis was run under PROC NLMIXED in SAS to, (1) model the concentration responses and estimate GI_{50} values for MON 89034, TC1507, MON 89034 × TC1507 × NK603, and MON 89034 × TC1507 × MON 88017 × DAS-59122-7 and (2) to statistically compare the GI_{50} values from MON 89034 × TC1507 × NK603, and MON 89034 × TC1507 × MON 88017 × DAS-59122-7.

The potential for interaction between the Cry1A.105, Cry2Ab2 and the Cry1F proteins was performed by statistically comparing the estimated GI_{50} value from MON 89034 × TC1507 × NK603 with the predicted GI_{50} value for MON 89034 × TC1507 × NK603. The predicted GI_{50} value for MON 89034 × TC1507 × NK603 is a function of the GI_{50} values of MON 89034 and TC1507 and was based on the concentration addition model presented by Finney (1971) and Tabashnik (1992).

The model is represented by: $1/\text{predicted } GI_{50} = \pi a/GI_{50}a + \pi b/GI_{50}b$

where πa and πb are the proportions of the two single trait products, a = MON 89034 and b = TC1507 in the combined trait product MON 89034 × TC1507 × NK603.

Concentration addition is based on the premise that (1) activity of a mixture can be predicted directly from the concentrations of the single compounds in the mixture, (2) two non-interacting compounds will behave as dilutions of one another when combined and (3) that a compound cannot interact with itself. This is an established model for examining interactions between substances having a similar mode of action, such as *Bt* proteins (Tabashnik, 1992; Olmstead and LeBlanc, 2005; U.S. EPA, 2006). A mode of action can be viewed as a category of mechanisms that share particular key features or steps.

8.0 Results and Discussion

The purpose of this study was to evaluate the potential for interactions among the proteins produced by MON 89034 × TC1507 × MON 88017 × DAS-59122-7. This was evaluated by concurrently assessing the potential for interactions with the lepidopteran active proteins (combination of Cry1A.105 and Cry2Ab2 proteins with the Cry1F protein) and assessing the potential for interactions between the lepidopteran-active and coleopteran-active proteins Cry3Bb1 and Cry34/35Ab1. Importantly an expression analysis was performed to measure levels of the Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1 and Cry34Ab1 and Cry35Ab1 proteins in the single and combined trait products. This analysis indicated comparable Cry protein levels in the single and combined trait products (Stillwell and Silvanovich, 2007; Phillips, 2008).

To assess the potential for interaction between the combination of the Cry1A.105 and Cry2Ab2 proteins and the Cry1F protein in MON 89034 × TC1507 × MON 88017 × DAS-59122-7, MON 89034 × TC1507 × NK603 was used as the test substance because it expresses the Cry1A.105, Cry2Ab2 and Cry1F proteins but not the Cry3Bb1 and Cry34/35Ab1 proteins. This allowed the potential interactions between the combination of the Cry1A.105 and Cry2Ab2 proteins and the Cry1F protein to be directly evaluated.

To perform this assessment, ECB were exposed to a series of lyophilized leaf tissue concentrations of either MON 89034, TC1507 or MON 89034 × TC1507 × NK603 along with the appropriate control tissues in 7-day diet-incorporation bioassays run in parallel. ECB exhibited concentration-dependent responses to MON 89034, TC1507 and MON 89034 × TC1507 × NK603 (Figures 1) and GI_{50} values are summarized in Table 1. The concentration-response curve for TC1507 was to the right of the concentration-response curves for MON 89034 and MON 89034 × TC1507 × NK603, indicating less potency against ECB (Figure 2). Additionally, the slopes of these concentration response curves were visually comparable (Figure 2), which is consistent with similar modes of action (Oris and Bailer, 1997).

The hypothesis of no interaction (additivity) was tested under the concentration addition model by statistically comparing the estimated GI_{50} value for MON 89034 × TC1507 × NK603 with the predicted GI_{50} value for MON 89034 × TC1507 × NK603. The predicted GI_{50} value for MON 89034 × TC1507 × NK603 was based on calculations using the GI_{50} values from MON 89034 and TC1507. This analysis indicated no significant difference between the estimated (0.48 mg tissue ml/diet) and predicted (0.51 mg tissue ml/diet) GI_{50} values for MON 89034 × TC1507 × NK603 ($p = 0.38$), which indicates no interaction between the combination of the Cry1A.105 and Cry2Ab2 proteins and the Cry1F protein.

Similar mean ECB weights were observed for the diet only assay control treatment, the conventional control (XE6001) treatment, the MON 88017 treatment and the DAS-59122-7 treatment (Figure 3). There were no significant differences for the following planned comparisons: XE6001 versus DAS-59122-7 ($p = 0.47$), XE6001 versus MON 88017 ($p = 0.57$) and XE6001 versus diet only assay control ($p = 0.36$). This result justified using the diet only assay controls as the zero concentration level for all of the concentration response curves and indicated no insecticidal activity for MON 88017 and DAS-59122-7 at the tested concentration levels.

To assess whether combined Cry1A.105, Cry2Ab2 and Cry1F activity is altered by the presence of the Cry3Bb1 and Cry34/35Ab1 proteins, the biological activity of MON 89034 × TC1507 × MON 88017 × DAS-59122-7 and MON 89034 × TC1507 × NK603 were compared. To perform this comparison, ECB were exposed to a series of lyophilized leaf tissue concentrations of MON 89034 × TC1507 × NK603 or MON 89034 × TC1507 × MON 88017 × DAS-59122-7 along with the appropriate control tissues in 7-day diet-incorporation bioassays run in parallel. ECB exhibited nearly identical concentration-dependent responses to MON 89034 × TC1507 × NK603 and MON 89034 × TC1507 × MON 88017 × DAS-59122-7 (Figures 2 and 4). The hypothesis of no interaction was tested by statistically comparing the estimated GI_{50} values from MON 89034 × TC1507 × NK603 and MON 89034 × TC1507 × MON 88017 × DAS-59122-7. The GI_{50} values for MON 89034 × TC1507 × NK603 and MON 89034 × TC1507 × MON 88017 × DAS-59122-7 were not significantly different ($p = 0.53$) and

were estimated to be 0.048 and 0.046 mg tissue/ml diet, respectively. Observing nearly identical GI_{50} values for MON 89034 × TC1507 × NK603 and MON 89034 × TC1507 × MON 88017 × DAS-59122-7, indicates that the Cry3Bb1 and Cry34/35Ab1 proteins do not interact with the combined activity of the Cry1A.105, Cry2Ab2 and Cry1F proteins.

All acceptance criteria defined in the protocol were met for these assays. No mortality was observed in the diet-only assay controls. Additionally, all concentration responses MON 89034, TC1507, MON 89034 × TC1507 × NK603, MON 89034 × TC1507 × MON 88017 × DAS-59122-7 had at least one concentration level with between 10% and 50% growth inhibition relative to the mean control response and at least two treatments between 50% - 99% growth inhibition relative to the mean control response.

9.0 Conclusions

The results from this study indicate no interaction among the *Bt* Cry proteins expressed in MON 89034 × TC1507 × MON 88017 × DAS-59122-7. First, the results indicate no interaction between the combination of the Cry1A.105 and Cry2Ab2 proteins and the Cry1F protein. Additionally, the results indicate no interaction between combined Cry1A.105, Cry2Ab2 and Cry1F activity and the Cry3Bb1 and Cry 34/35Ab1 proteins.

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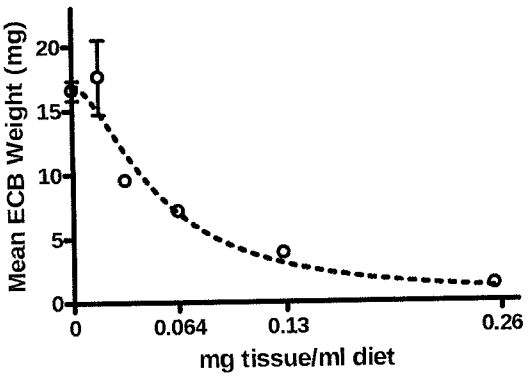
11.0 Tables and Figures

Table 1. Estimated 7-day GI_{50} values and standard errors for MON 89034, TC1507, and MON 89034 × TC1507 × NK603 against ECB

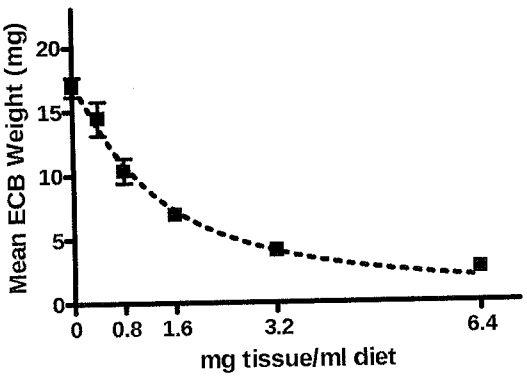
Treatment	**GI_{50} Value ± Standard Error (mg tissue/ml diet)**
MON 89034	0.052 ± 0.0061
TC1507	1.7 ± 0.22
MON 89034 × TC1507 × NK603	0.048 ± 0.0054

Figure 1. Concentration-response for A. MON 89034, B. TC1507 and C. MON 89034 × TC1507 × NK603 in 7-day diet incorporation bioassays with the European corn borer. Data points represent means with standard errors.

A.



B.



C.

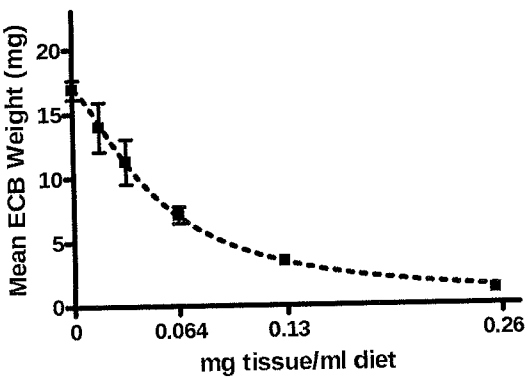


Figure 2. Concentration-responses for MON 89034 × TC1507 × MON 88017 × DAS-59122-7, MON 89034 × TC1507 × NK603, MON 89034 and TC1507 in 7-day diet incorporation bioassays with the European corn borer. Data points represent means with standard errors

Tissue concentrations were log transformed to illustrate the relative potency of MON 89034 × TC1507 × MON 88017 × DAS-59122-7, MON 89034 × TC1507 × NK603, MON 89034 and TC1507 as well as the overall similarity in slopes of the concentration response curves.

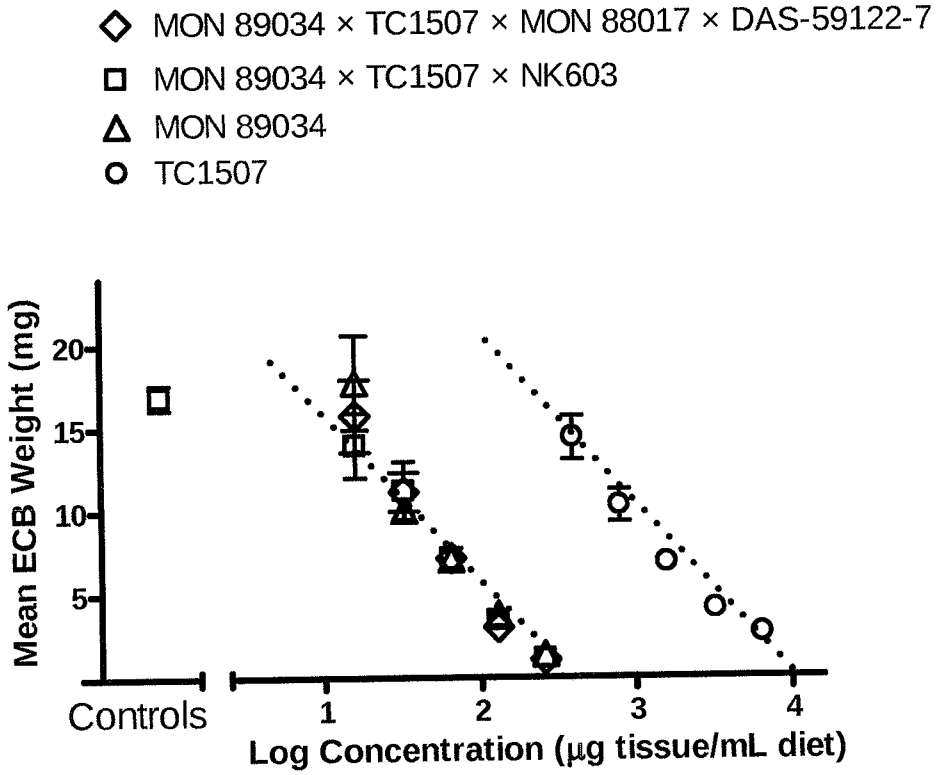


Figure 3. Mean European corn borer weights with standard errors after 7-days of feeding for the diet only assay control, conventional corn tissue (XE6001) incorporated into diet, MON 88017 tissue incorporated into diet and DAS-59122-7 tissue incorporated into diet. Bars represent means with standard errors.

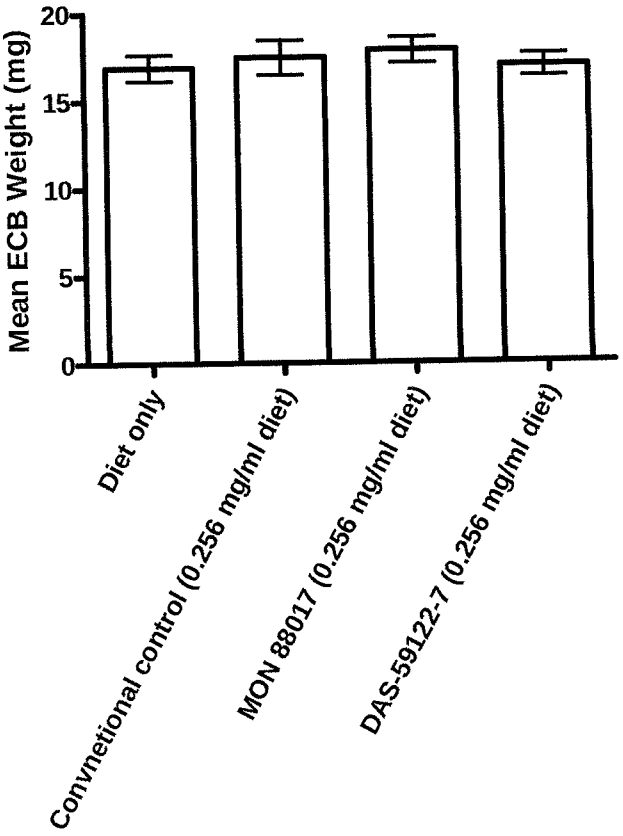
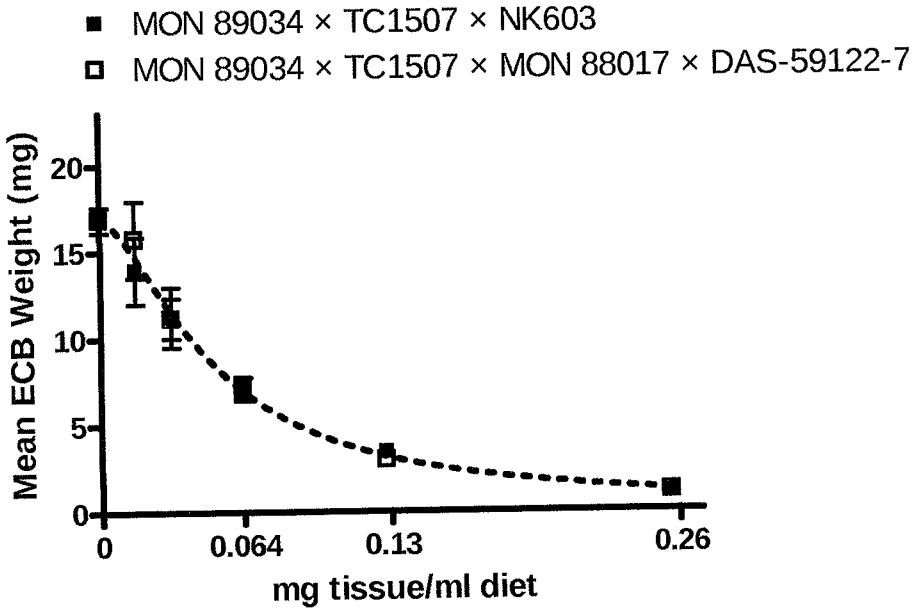


Figure 4. Concentration-responses for MON 89034 × TC1507 × NK603 and MON 89034 × TC1507 × MON 88017 × DAS-59122-7 in 7-day diet incorporation bioassays with the European corn borer. Data points represent means with standard errors.



12.0 **Appendix I.** Statistical Report for REG-07-275

Purpose: The purpose of this analysis was to: (1) Assess the potential for interaction between the combination of the Cry1A.105 and Cry2Ab2 proteins with the Cry1F protein. (2) Assess the potential for combined Cry1A.105, Cry2Ab2 and Cry1F activity to be altered in the presence of the combination of the Cry3Bb1 and Cry34/35Ab1 proteins.

Experimental Design: The test organism used in the bioassays was the European cornborer (ECB). The study included eight treatments: 4 test materials (lyophilized leaf tissues from MON 89034, TC1507, MON 89034 × TC1507 × NK603, and MON 89034 × TC1507 × MON 88017 × DAS-59122-7), 4 control materials (lyophilized leaf tissues from MON 88017, DAS-59122-7 and XE6001 designated as the Conventional Corn in raw data, and a diet only treatment designated as Assay Control in the raw data). ECB larvae were exposed to diet containing a series of tissue concentration levels for each test material, and only one level for each control treatment. Three replicates (assays) were conducted with all treatments evaluated concurrently in each replicate. Each concentration level of each treatment contained approximately 24 insects and very little mortalities occurred in the experiment. The response variable measured was total weight of the surviving insects. The average insect weight by treatment, concentration, and replicate was calculated and used for the analysis.

Data file: The data were supplied in an Excel file ("REG_07_275_ECB.xls"). The worksheet was read and the data were analyzed with Release 9.1.3 of the SAS statistical program (SAS Institute Inc., 2002-2003) running under Windows XP.

Statistical Analysis, Results and Statistical Conclusions

(1) Summary of mean insect weight by treatment by concentration

Mean insect weight and the standard deviation over replicates were calculated and listed in Table 1 by treatment and concentration level. A large dependence of the standard deviation on the mean insect weight could be observed. The standard deviation and the mean decreased almost proportionally as the tissue concentration in diet increased.

(2) Comparisons among 4 control treatments

A mixed model analysis with a randomized complete block design was applied on 4 control treatments. Results of the 3 designated comparisons are listed in Table 2. No statistical significance was found for any of the three comparisons.

(3) Joint logistic model analysis of four test materials and significance testing of designated comparisons among treatments

In this analysis, data from all 4 treatments MON 89034, TC1507, MON 89034 × TC1507 × NK603, and MON 89034 × TC1507 × MON 88017 × DAS-59122-7 were included in a joint analysis in order to perform the designated comparisons. In addition, the diet only treatment was used for zero concentration level. The analysis used the following form of the logistic model:

$$y_{ij} = \frac{w_0}{1+\left(Conc_i / GI_{50t}\right)^B} + r_j + e_{ij}$$

$$GI_{50t} = \begin{cases} GI_{501} & \text{if treatment} = \text{MON 89034} \\ GI_{502} & \text{if treatment} = \text{TC1507} \\ GI_{503} & \text{if treatment} = \text{MON 89034} \times \text{TC1507} \times \text{NK603} \\ GI_{504} & \text{if treatment} = \text{MON 89034} \times \text{TC1507} \times \text{MON 88017} \times \text{DAS - 59122 - 7} \end{cases}$$

y_{ij}: Average insect weight (mg) under concentration level i in the j^{th} replicate;

w_0: Expected weight at zero concentration;

$Conc_i$: Tissue concentration at the level i;

GI_{50t}: Concentration for 50% growth inhibition for treatment t;

B: Rate of the weight change as concentration increases;

r_j: Random replicate (assay) effect;

e_{ij}: Residual effect.

The model also assumes the replicate effect and the residual effect being proportional to the expected mean insect weight for a particular concentration level based on the observation from the results in Table 1. The analysis was performed by using SAS procedure PROC NLMIXED.

The first purpose of this analysis was to assess the potential for interaction between the combination of the Cry1A.105 and Cry2Ab2 proteins with the Cry1F protein. This assessment was performed by statistically comparing the estimated GI_{50} from MON 89034 × TC1507 × NK603 with the prediction from MON 89034 and TC1507. The predicted GI_{50} value for MON 89034 × TC1507 × NK603 (GI_{503}) is a function of the combined GI_{50} values of MON 89034 (GI_{501}) and TC1507 (GI_{502}) and the prediction is

based on the concentration addition model presented by Finney (1971) and Tabashnik (1992).

$$\frac{1}{GI_{503}} = \frac{1}{GI_{501}} + \frac{1}{GI_{502}}$$

Therefore, the estimated GI_{50} value for MON 89034 × TC1507 × NK603 can be compared with the predicted GI_{50} value based on above equation. The absence of a statistically significant difference between the estimated and the predicted GI_{50} values implies no significant interaction between the combination of Cry1A.105 and Cry2Ab2 proteins (produced in MON 89034) with the Cry1F protein (produced in TC1507). For this analysis, the Cry1A.105, Cry2Ab2 and Cry1F protein expression in MON 89034 × TC1507 × NK603 were assumed to be equivalent with Cry1A.105 and Cry2Ab2 protein expression levels in MON 89034 and Cry1F protein expression levels in TC1507 (see main report for supporting citations).

Table 3 lists the parameter estimates from the logistic model used to jointly model the concentration-response for MON 89034, TC1507, MON 89034 × TC1507 × NK603 and MON 89034 × TC1507 × MON 88017 × DAS-59122-7, and the predicted GI_{50} value for MON 89034 × TC1507 × NK603 based on the GI_{50} values for MON 89034 and TC1507.

Table 4 lists the result of the significance testing of the difference between the predicted and the estimated GI_{50} values. No statistically significant difference was detected between the predicted and the estimated GI_{50} values. Note that SAS procedure PROC NLMIXED used delta method to obtain the standard error of the parameter function and the t-statistic was used for the significance testing of the hypothesis.

The second purpose of this analysis was to assess the potential for combined Cry1A.105, Cry2Ab2 and Cry1F activity to be altered in the presence of the combination of the Cry3Bb1 and Cry34/35Ab1 proteins by comparing the estimated GI50s between MON 89034 × TC1507 × NK603 and MON 89034 × TC1507 × MON 88017 × DAS-59122-7. The result of the significance testing of the difference in GI_{50} values between two stacked materials in Table 4 showed no statistical significance. Therefore, the results indicate no interaction between the combination of Cry1A.105, Cry2Ab2 (produced in MON 89034) and Cry1F proteins (expressed in TC1507) with the combination of Cry3Bb1 (produced in MON 88017) and Cry34/35Ab1 proteins (expressed in DAS-59122-7).

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Table 1. Mean insect weight and standard deviation (SD) among replicates (assays) by treatment and tissue concentration level (Conc) in diet

Treatment	Conc (mg tissue/ml diet)	N	Mean Wt(mg)	SD (mg)
Diet Only	0.256	9	16.88	2.23
XE6001	0.256	9	17.48	2.94
MON 88017	0.256	9	17.85	2.17
DAS-59122-7	0.256	9	17.00	1.87
MON 89034	0.016	3	17.75	4.93
	0.032	3	10.05	0.81
	0.064	3	7.11	0.73
	0.128	3	3.81	0.08
	0.256	3	1.38	0.24
TC1507	0.400	3	14.42	2.28
	0.800	3	10.33	1.70
	1.600	3	6.86	0.67
	3.200	3	4.07	0.27
	6.400	3	2.59	0.21
MON 89034 × TC1507 × NK603	0.016	3	13.97	3.35
	0.032	3	11.25	3.01
	0.064	3	7.08	1.16
	0.128	3	3.43	0.31
	0.256	3	1.10	0.16
MON 89034×TC1507×MON 88017× DAS-59122-7	0.016	3	15.75	3.82
	0.032	3	11.17	2.00
	0.064	3	7.18	1.02
	0.128	3	2.98	0.52
	0.256	3	1.06	0.08

Table 2. Least square mean (LSMean) insect weight and pair-wise comparisons of 4 control treatments

Treatment	LSMean (mg)	Standard Error
Diet Only	16.88	1.34
XE6001	17.48	1.34
MON 88017	17.85	1.34
DAS-59122-7	17.00	1.34

Comparison	Difference	Standard Error	DF	t Value	p Value
XE6001 vs DAS-59122-7	0.48	0.65	30	0.74	0.4661
XE6001 vs MON 88017	-0.38	0.65	30	-0.58	0.5670
XE6001 vs Diet Only	0.60	0.65	30	0.93	0.3623

Table 3. Parameter estimation (B: rate parameter of the weight change with concentration, GI_{50}: concentration (mg/ml diet) for 50% growth inhibition) in the joint analysis for MON 89034 (with GI_{501}) and TC1507 (with parameter GI_{502}), and MON 89034 × TC1507 × NK603 (with parameter GI_{503}) and MON 89034 × TC1507 × MON 88017 × DAS-59122-7 (with parameter GI_{504}), and Diet only as the common control for zero concentration.

Parameter	Estimate	Standard Error
GI_{501} (mg tissue/ml diet)	0.0523	0.0061
GI_{502} (mg tissue/ml diet)	1.7294	0.2176
GI_{503} (mg tissue/ml diet)	0.0478	0.0054
GI_{504} (mg tissue/ml diet)	0.0458	0.0051
B	1.5180	0.0863

Table 4. Significance testing of the difference between the estimated GI_{50} value for MON 89034 × TC1507 × NK603 (in Table 3) and the prediction from MON 89034 and TC1507, and significance testing of $GI_{503} = GI_{504}$ for MON 89034 × TC1507 × NK603 and MON 89034 × TC1507 × MON 88017 × DAS-59122-7

Parameter / Hypothesis	Estimate	Standard Error	DF	t Value	p Value
Predicted GI_{503}	0.0508	0.0059			
H_0: Est.GI_{503} - Pred.GI_{503} = 0	-0.0029	0.0034	62	-0.88	0.3841
H_0: GI_{503} - GI_{504} = 0	0.0020	0.0032	62	0.63	0.5286