4) Dudin, Y. A., B. P. Tonnu, L. D. Albee and R. P. Lirette (2001). Amended report for MSL-16559: B.t. Cry3Bb1.11098 and NPTII protein levels in tissue collected from corn event MON 863 grown in 1999 field trials. MSL-17181, an unpublished study conducted by Monsanto Company.

Study Title

Amended Report For MSL-16559: *B.t.* Cry3Bb1.11098 and NPTII Protein Levels in Samples Tissue Collected from Corn Event MON 863 Grown in 1999 Field Trials

Authors

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Study Completed on

Amendment 1
April 10, 2001

Performing Laboratory

Monsanto Company
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Laboratory Project ID

Study 99-01-39-22
MSL - 17181

Statement of No Data Confidentiality Claim

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

"We submit this material to the United States Environmental Protection Agency specifically under the requirements set forth in FIFRA as amended, and consent to the use and disclosure of this material by EPA strictly in accordance with FIFRA. By submitting this material to EPA in accordance with the method and format requirements contained in PR Notice 86-5, we reserve and do not waive any rights involving this material that are or can be claimed by the company notwithstanding this submission to EPA."

Monsanto Company — 7 May 2001
Company — Date

Dennis P. Ward — 7 May 2001
Company Agent — Date

Regulatory Affairs Manager
Title — Date

Signature — 7 May 2001
Date

Statement of Compliance

This study meets the requirements for 40 CFR Part 160.

Submitter — Date: 7 May 2001

Sponsor — Date: April 10, 2001

Study Director — Date: April 10, 2001

Quality Assurance Statement

Study Title: Amended Report For MSL-16559: *B.t.* Cry3Bb1.11098 and NPTII Protein Levels in Tissue Samples Collected from Corn Event MON 863 Grown in 1999 Field Trials

Study Number: 99-01-39-22

Reviews conducted by the Quality Assurance Unit (QAU) confirm that the final report reflects the raw data.

Following is a list of reviews conducted by the Monsanto Regulatory QAU on the study reported herein.

Dates Of Inspection / Audit	Phase	Date Reported To: Study Director	Date Reported To: Management
October 5, 1999	Extraction	October 5, 1999	October 5, 1999
October 26, 1999	ELISA	October 26, 1999	October 26, 1999
November 10, 1999	ELISA	November 11, 1999	November 11, 1999
May 19, 2000	Data Audit & Report Review	May 19, 2000	May 19, 2000
April 4, 2001	Amended Report Audit	April 9, 2001	April 9, 2001

Michelle R. Hessin
Quality Assurance Specialist
Monsanto Regulatory, Monsanto Company

04-10-01
Date

Signatures of Approval

Study Number:	99-01-39-22
Title:	Amended Report For MSL-16559: *B.t.* Cry3Bb1.11098 and NPTII Protein Levels in Tissue Samples Collected from Corn Event MON 863 Grown in 1999 Field Trials
Facility:	Monsanto Company 700 Chesterfield Parkway North St. Louis, Missouri 63198
Study Director:	Yelena A. Dudin
Contributors:	Monsanto (St. Louis): Bich-Phuong Tonnu, Lee D. Albee and Ronald P. Lirette
Sponsor:	Monsanto Company
Study Initiation Date:	September 29, 1999
Original Study Completion Date:	May 19, 2000
Amended Report Completion Date:	April 10, 2001
Records Retention:	All study specific raw data, protocols, final reports and facility records will be retained at Monsanto, St. Louis.
Sample Storage:	Any study samples that are to be retained will be stored at Monsanto, St. Louis.

Signatures of Approval (cont'd)

Amendments to Report:

This amendment modifies the final report to reflect the new Cry3Bb1 protein information. The following changes do not affect the quality or integrity of the data.

MSL-17181 Amended Report	MSL-16559 Original Report	Amendments
1. Title Page (Pg 1)	1. Title Page (Pg 1)	a. Added "Amended Report For MSL-16559" in front of the study title b. Added a new line: "Amendment 1" after "Report Completed on" c. Added new report completion date
2. QA Statement - (Pg 4)	2. QA Statement - (Pg 4)	Added a new line: "Amended Report Audit" to the list of phases
3. Signatures of Approval - (Pg 5)	3. Signatures of Approval - (Pg 5)	a. Added "Amended Report For MSL-16559" in front of the study title b. Added the word "Original" to the Study Completion Date and immediately following, inserted a new line "Amended Report Completion Date"
4. Signatures of Approval (cont'd) Pg 6	4. Not included	Added "Amendments to Report" section
5. Page 7	5. Page 6	Table of contents - changed pagination
6. Page 12	6. Page 11	Reworded the background paragraph to reflect new protein information
7. Page 14	7. Page 13	Paragraphs 2 and 3 - updated references
8. Pages 22-23	8. Page 21	a. Added a new line "Amended version of the report is in-progress as MSL-17180" to the Dudin et al., 1999. b. Inserted a new reference: Hileman, R. E. and Astwood, J. D, 2001. c. Updated the previous reference of Hileman et al., 1999 (MSL-15531) with a new title, publication year, and MSL#

Ravinder S Sidhu — April 10, 2001
Sponsor — Date

Yolena A Dudin — April 10, 2001
Study Director — Date

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Abbreviations

Abs	absorbance or optical density
B.t.	*Bacillus thuringiensis*
BSA	bovine serum albumin
CRW	corn rootworm
cry3Bb1	coding sequence for Class III (Coleoptera-specific) crystal protein
Cry3Bb1	Class III (Coleoptera-specific) crystal protein
CV	coefficient of variation
ELISA	Enzyme-Linked Immunosorbent Assay
fwt	fresh weight of tissue
HRP	horseradish peroxidase
x g	acceleration of gravity
LOD	limit of detection
N.A.	not applicable
N.D.	not done
NPTII	neomycin phosphotransferase II protein
nptII	coding sequence for neomycin phosphotransferase II protein
OSL	leaf tissue collected over corn growing season
OSWP	whole plant tissue (above ground portion) collected over corn growing season
OSR	root tissues collected over corn growing season
PBST	phosphate-buffered saline with Tween-20™
SD	standard deviation
SDS	sodium dodecyl sulfate
SOP	standard operating procedure
T:B	tissue weight to buffer volume ratio (g:mL)
TBA	Tris borate with ascorbic acid buffer
TMB	(3,3',5,5'-tetramethylbenzidine) or HRP substrate
Trizma Base®(Sigma)	tris(hydroxymethyl)-aminomethane
µg/g fwt	microgram per gram fresh weight tissue
wt	weight
w/v	weight to volume
v/v	volume to volume
YL	young leaf, usually collected at the V-4 corn growing stage or approximately three weeks post-planting

1.0 Summary

Corn plants (*Zea mays* L.) have been modified to produce a *Bacillus thuringiensis* (*B.t.*) Cry3Bb1 insecticidal protein that protects against corn rootworm (CRW, *Diabrotica*), a major North American corn pest. The DNA sequence used for corn transformation and the inserted DNA in the corn event MON 863 (Monsanto designation) encodes two expression cassettes: one containing the *cry3Bb1* coding region and the other containing the *nptII* (neomycin phosphotransferase II) coding region. The NPTII enzyme was used as a selectable marker during early development phases of the modified corn.

The purpose of this study was to evaluate the production of the proteins encoded by the integrated DNA in the corn event MON 863. Specifically, the objective was to estimate the levels of Cry3Bb1 and NPTII proteins in various tissues collected from MON 863 corn plants grown under varied conditions in the 1999 field trials. Tissue samples of young leaf, forage, mature root and grain were collected at four U.S. field sites. Also, various tissue samples during the corn growing season were collected at three U.S. field sites. In addition, pollen analyses was conducted using tissue samples from one site in U.S. and three sites in Argentina. Finally, silk was collected at one U.S. field site.

Cry3Bb1 protein levels were measured in forage and grain because these tissues are most relevant to food and animal feed product safety. Since Cry3Bb1 protein levels are relevant to the insect control performance of the corn plants, and are also necessary to assess the environmental exposure, the levels of this protein were analyzed in several additional corn tissue types: leaf, mature root, silk, pollen and overseason tissue samples of leaf, whole plant (aerial portion) and root. NPTII protein was measured in leaf, forage, and grain to evaluate the levels of introduced protein.

Direct double antibody sandwich ELISA (enzyme-linked immunosorbent assay) methods were developed and validated to estimate the levels of Cry3Bb1 and NPTII proteins in tissue extracts of CRW-protected corn plants. Protein values were reported as micrograms (µg) of protein per gram (g) on a fresh weight basis of collected tissues (fwt). Reported Cry3Bb1 and NPTII protein levels were corrected for method bias as established during validation.

Tissues from the non-transgenic corn line (MON 846) of comparable genetic background were also collected and analyzed. Results of analyses of MON 846 for Cry3Bb1 and NPTII protein levels were not reported since these control tissues were utilized to verify the limit of detection for each tissue type established during validation. DNA analyses confirmed the expected absence of the *cry3Bb1* and *nptII* expression cassettes in the control samples of MON 846 line. The limit of detection for each tissue type in the Cry3Bb1 protein ELISA varied from 0.08 µg/g in silk to 0.76 µg/g in root tissues. The

limit of detection for the NPTII protein ELISA varied from 0.075 µg/g in forage to 0.093 µg/g in leaf tissues.

Young leaf, forage, mature root and grain were analyzed using extracts prepared from tissue samples collected from four U.S. field sites. Young leaf samples of the MON 863 event were collected approximately at the V-4 growth stage or 21 days post-planting and the level of the Cry3Bb1 protein averaged 81 µg/g. The levels of the Cry3Bb1 protein in grain averaged 70 µg/g. The Cry3Bb1 protein levels in mature root and forage averaged 41 µg/g and 39 µg/g, respectively. The levels of this protein in pollen were estimated using tissue samples collected from four field sites (one U.S. field site and three sites in Argentina), and averaged 62 µg/g. Finally, Cry3Bb1 protein level was approximately 10 µg/g in silk collected as a single composite from one U.S. field site.

Site to site variation at four U.S. locations of the Cry3Bb1 protein levels in the MON 863 event was observed to be approximately two-fold as demonstrated by the following ranges: leaf (65 - 93 µg/g), forage (24 - 45 µg/g), root (25 - 56 µg/g) and grain (49 - 86 µg/g).

In addition, various tissues of MON 863 were collected during the corn growing season at three U.S. field sites. The level of the Cry3Bb1 protein in leaves decreased from 81 µg/g at the V-4 stage to about 43 µg/g prior to pollination (approximately 49 days post-planting). The level of the Cry3Bb1 protein in whole plants followed the same pattern: 46 µg/g (at two weeks prior to pollination) decreased to 25 µg/g at senescence or post-harvest. Similarly, root tissues collected at the same sampling times as whole plant tissues, indicated a decrease in the Cry3Bb1 protein levels from 58 µg/g to 24 µg/g.

The NPTII protein levels in young leaf across four U.S. field sites ranged from 0.74 to 1.4 µg/g and the levels in forage tissues ranged from 0.17 to 0.23 µg/g. The NPTII protein levels in grain tissue were below the LOD (<0.076 µg/g) in all of the samples analyzed from four U.S. field sites.

In summary, it was observed that the mean concentrations of the Cry3Bb1 protein in the MON 863 event were highest in leaf (81 µg/g), followed by grain (70 µg/g), pollen (62 µg/g), root (41 µg/g), forage (39 µg/g), and silk (10 µg/g).

Field trials were conducted using agronomic practices and field conditions typical of commercial corn cultivation and provided a variety of environmental situations representative of the various geographical regions where CRW-protected corn lines would be grown. Therefore, data generated should be representative of the expected protein levels in the commercial corn cultivation of the MON 863 event.

2.0 Introduction

2.1 Background

Bacillus thuringiensis (*B.t.*) is a gram-positive bacterium commonly present in soil. Many different strains of *B. thuringiensis* have been shown to produce crystal proteins or inclusion bodies that are specifically effective in controlling certain orders and species of insect pests. Microbially-based products, such as those derived from *B.t*, have been widely used as pesticides since 1961 (McClintock et al., 1995). Microbial pesticides have been commercially available and used as environmentally acceptable insecticides because they are specific for the targeted insect pests and are typically harmless to plants and other non-targeted organisms. *B.t.* proteins have been generally classified based on their insecticidal activity (*e.g.* Cry1, Cry2, Cry3 and Cry4 proteins are toxic to lepidopteran, lepidopteran/dipteran, coleopteran and dipteran pests, respectively) (Bravo, 1997; Hofte and Whiteley, 1989). The Cry3 class protein, Cry3Bb1, has natural insecticidal activity against the coleopteran pest, corn rootworm genus *Diabrotica* (Von Tersch et al., 1994), a major North American corn pest.

The Cry3Bb1 protein was previously referred to as CryIIIB2 (or Cry3B2) as well as Cry3Bb or CryIIIC. This protein should be referred to as Cry3Bb1 protein according to the most recent and accepted nomenclature (Crickmore et al., 1998). The Cry3Bb1 protein nomenclature will be used in this report. Two variants of the wild type *cry3Bb1* coding sequence (GenBank Accession No. M89794) have been designed to encode for proteins with enhanced insecticidal activity against corn rootworm. These Cry3Bb1 protein variants were initially produced for investigation with a *B.t.* cloning vector and expression system. Protein variants, Cry3Bb1.11231 and Cry3Bb1.11098, were produced by the recombinant *B.t.* strains EG11231 and EG11098, respectively. Cry3Bb1.11231 and Cry3Bb1.11098 proteins differ in amino acid sequence from the wild type protein by a total of four and five amino acids, respectively. To facilitate optimum expression in plants, these coding sequences were further manipulated prior to their use in transforming corn (*Zea mays*). Corn events expressing variants of the *cry3Bb1.11231*and *cry3Bb1.11098* coding sequences produce proteins that differ in amino acid sequence from the wild type protein by five and seven amino acids, respectively (Hileman and Astwood, 2001). The DNA insert sequence used for corn transformation and the inserted DNA in the corn event MON 863 (Monsanto designation) encodes two expression cassettes: one containing the *cry3Bb1.11098* coding region, and the other containing *nptII* (neomycin phosphotransferase II) coding region. The NPTII enzyme was used as a selectable marker (Fraley et al., 1983) during early production phases enabling the selection of plants in tissue culture that contain transgenic DNA.

2.2 Purpose

The purpose of this study was to estimate the levels of Cry3Bb1 and NPTII proteins in tissues samples collected from MON 863 corn plants grown under varied conditions in 1999 U.S. field trials (Production Plan #99-01-39-08). In addition, pollen tissues collected in Argentina (Production Plan #99-04-39-01) were added to this study by a protocol amendment for analysis of the Cry3Bb1 protein levels. As a control for bias, tissues of comparable genetic background from non-transgenic control corn plants (MON 846) were collected and analyzed in the same manner as the CRW-protected corn event. Validated ELISA (enzyme-linked immunosorbent assay) methods were used to determine the Cry3Bb1 and NPTII protein levels.

3.0 Materials

3.1 Test, Control and Reference Substances

3.1.1 Test substances. The test substance for this study was the CRW event MON 863. The originally transformed plant (R0) was crossed to corn line MTCY10412 to produce F1 plants that were next self-pollinated to produce F2 generation plants. The progeny of F2 crossed to corn line A1 provided the plant material for this study. MON 863 is the Monsanto designation assigned to this event. An additional event was discontinued as a commercial candidate during the conduct of this study and was deleted from the study by a protocol amendment.

3.1.2 Control Substance. The control substance for this study was corn line MON 846 which has not been genetically modified, but has the background genetics representative of the test substance. The control plant material was harvested from the progeny of non-transgenic corn line MTCY10412 that was crossed to A1. MON 846 is the Monsanto designation assigned to this control material. An additional control line for the dropped test event was also deleted from the study by a protocol amendment.

3.1.3 Characterization of Test and Control Substances. The identity of each test and control substance was confirmed by the Study Director prior to their use in the study by reviewing the chain-of-custody documentation. The test and control substances were characterized at the DNA level to distinguish between test events during this study.

3.1.4 Reference Substances. The reference substances were the Cry3Bb1 and NPTII purified protein standards.

Cry3Bb1 protein standard. DNA encoding two modified Cry3Bb1 proteins were engineered into *B.t.* strains EG11098 and EG11231. Proteins derived from these strains, Cry3Bb1.11098 and Cry3Bb1.11231, contain a total of 5 and 4 amino acid changes, respectively, compared to the Cry3Bb1 wild-type protein sequence; and as a result, these modified proteins differ from each other by only one amino acid.

The Cry3Bb1.11098 protein standard was shown to be comparable to the Cry3Bb1.11231 protein standard with respect to the immunoreactivity and other physiochemical properties (Dudin et al, 1999; Hileman et al., 2001; Hileman, R. E. and Astwood, J. D. 2001). The ELISA assay is based on polyclonal antibodies which were generated against wild-type Cry3Bb1 protein. Since the ELISA method is based on the polyclonal antibodies, one amino acid difference between these proteins is indistinguishable when immunological methods such as ELISA or immunoblotting are applied. Cry3Bb1.11231 protein was chosen as the standard for the ELISA method.

The Cry3Bb1 protein standard (lot #6812812) utilized for ELISA was purified from *Bacillus thuringiensis* (Hileman et al., 2001; Hileman, R. E. and Astwood, J. D. 2001). Lyophilized protein powder (lot #6307780) was dissolved in 50 mM sodium bicarbonate containing 0.05% (v/v) Tween 20™, pH 9.6. The protein standard solution was stored in aliquots in a −80°C freezer. Total protein concentration of the purified standard was estimated to be 0.291 mg/mL as determined by amino acid compositional analysis. The purity was 93.2% as determined using densitometry of electrophoresed protein by Colloidal Brilliant Blue G in SDS (sodium dodecyl sulfate) polyacrylamide gel. The final protein standard concentration used in ELISA was corrected for purity and calculated to be 0.271 mg/mL (Dudin et al., 1999). The performance of this standard during the study was consistent based on the positive and negative quality control samples, and was monitored using ELISA assay evaluation criteria cited in the SOP (standard operating procedure) as previously established during method validation.

NPTII protein standard. The neomycin phosphotransferase II coding sequence (*nptII*) was cloned and transferred to *Escherichia coli* for fermentation and purification (Berberich et al., 1993). Lyophilized standard protein powder (lot #4821020) was dissolved in 50 mM sodium bicarbonate. The protein standard solution was stored as aliquots in a −80°C freezer. Total protein concentration by amino acid compositional analysis was estimated to be 1.15 mg/mL and the purity was 99.5% as determined using densitometry of electrophoresed protein stained by Colloidal Brilliant Blue G in SDS polyacrylamide gel. The final protein standard concentration used in the ELISA

was 1.15 mg/mL (Bhakta et al, 1999). The performance of this standard during the study was consistent based on the positive and negative quality control samples, and was monitored using ELISA assay evaluation criteria cited in the SOP as previously established during method validation.

3.2 Test system

There was no test system. This study used analytical methods to characterize the protein levels in the MON 863 corn event.

3.3 Generation of Plant Tissue Samples for Analysis

3.3.1 Field Summary. The test and control substances were produced at four varied corn-growing U.S. locations during the 1999 field season. The location of each field site was as follows: Richland (Jefferson county) and Van Horne (Benton County) in the State of Iowa; York (York county) in the State of Nebraska; and Monmouth (Warren county) in Illinois State. These field sites are located within the major U.S. corn-growing region. These locations provided a variety of environmental conditions representative of the various geographical regions where commercial CRW-protected corn lines could be grown.

Young leaf, forage, mature root (at forage stage) and grain samples were collected from plants at four U.S. locations. Additional samples collected during the growing season included leaf, whole plants and roots from three U.S. field sites. Silk and pollen tissues were collected as a composite sample from one field site in the U.S. In addition, pollen was also analyzed from three field sites in Argentina (Production Plan #99-04-39-01). The locations of the field sites were as follows: Fontezuela in Pergamino county, Salto in Salto county, and Rojas in Rojas county. Corresponding control line tissues of MON 846 were collected at all field sites in U.S and Argentina.

During collection, sample identity was maintained by appropriate measures known to protect sample integrity (e.g. self-pollination by hand; bagging of tassels and ears) and by using unique sample identifiers. These were shipped using dry-ice, except for grain (ambient) and immediately transferred to the −80°C freezers except for grain (ambient) at the Sponsor facility (Monsanto). The YL-2 (young leaf), OSWP-2 (overseason whole plant), and OSR-2 (overseason root) samples from one of three overseason field sites arrived thawed and were destroyed immediately after receipt (Bhatti, 2000). These tissue samples were deleted from the study by a protocol amendment. This did not affect the assessment of the overseason Cry3Bb1 protein levels since one replicate was collected from two other U.S. field sites.

3.3.2 Young Leaf. The first young leaf (YL-1) sampling was collected ≈21 days post-planting or at the V-4 growth stage at four field sites and as four replicated plots per site. The number of MON 863 composited leaves per plot across all sites ranged from 37 to 50. Due to a lower number of planted seed, the number of leaves per plot across all field sites for MON 846 ranged from 20 to 32. The leaves of each event/line were pooled per replicate plot, frozen on dry ice and shipped to the Sponsor facility. Only one replicated plot from each field site was analyzed by ELISA.

3.3.3 Overseason Leaf. The young leaf sampling (YL) was repeated at approximately two week intervals until pollination at three field sites. Two additional young leaf samplings (YL-2 and YL-3) were collected at three field sites at about 35 and 49 days post-planting, respectively. Each sampling time point consisted of 15 non-systematically selected plants. Since only one replicate was collected, the leaves of each event/line were pooled at each site, frozen on dry ice and shipped to the Sponsor. All tissue samples collected in the field were analyzed by ELISA.

3.3.4 Overseason Whole Plant and Forage. At each collection time point, two whole plants (entire aerial or above ground portions) were harvested for each test event and control line at multiple locations. The plants were cut into 4 to 6 inch segments, composited by line, frozen on dry ice and shipped to the Sponsor. Overseason whole plant samples, OSWP-1 and OSWP-2, were collected at ≈35 and ≈49 days post-planting, respectively. The first two collections of the OSWP-1 and OSWP-2 correspond to the YL-2 and YL-3 sampling time-points, respectively. The third and fourth overseason whole plant (OSWP-3 and OSWP-4) samples are respectively defined as the forage sample collected at the early dent stage (≈90 days post-planting) and a senescent plant sample collected at a stover stage (≈126 days post-planting). OSWP-1, OSWP-2, and OSWP-4 were collected at three U.S. field sites as one replicate with the exception of OSWP-3 (forage) which was collected from four field sites as four replicates per site. Only one replicate (i.e. sample) of forage tissues (OSWP-3) was analyzed from each of these locations.

3.3.5 Overseason Root and Mature Root. At each collection time point, the root mass from the two overseason whole plants was collected, thoroughly rinsed free of soil with water and placed in a uniquely labeled sample bag and frozen on dry ice for shipment to the Sponsor. The overseason root samples (OSR) were collected at three locations as one replicate per site with the exception of mature root samples at the forage stage (OSR-3; ≈90 days post-planting), which were collected at four field sites as four replicates per site.

Only one replicate of OSR-3 was analyzed from each field site. All of the root sample collection time points (OSR-1, OSR-2, OSR-3, and OSR-4) correspond to the respective tissue collection of the whole plants (OSWP-1, OSWP-2, OSWP-3, and OSWP-4, respectively).

3.3.6 Silk. This tissue type was collected approximately 58 days post-planting or at about 50% pollen shed. Each test and control sample was a composite of five plants collected from one plot in Monmouth (IL).

3.3.7 Pollen. A composite sample from one field site (Monmouth, IL, U.S.) was collected over a period of 7 days starting at about 60 days post-planting for the test event and the corresponding control line. Furthermore, additional pollen samples were collected in Argentina (Production Plan # 99-04-39-01) and were added to the study by amending the study protocol. A total of twelve additional tissue samples from the test event representing three field sites at Fontezuela, Salto, and Rojas (four replicates/site) were used to analyze the Cry3Bb1 protein levels in the pollen extracts of the MON 863 event. Corresponding control tissues of pollen were also collected and analyzed from the Argentina field sites.

3.3.8 Grain. All self-pollinated ears of each test event and control line were hand harvested at ≈35% moisture content and composited by plot for each event/line. Grain was air-dried to target storage moisture below 15% and was subsequently shipped to Monsanto at ambient temperature and stored in the same manner until processing. On the average across all sites, there were 126 days to corn maturity. For the MON 863 event, 28 to 41 corn ears were composited to produce each sample. Whereas, due to a lower number of planted seed of the MON 846 line, 11 to 29 corn ears were composited to produce each sample. Each field site contained four replicated plots and only one plot per site was analyzed.

4.0 Methods

4.1 Extraction of Protein from Corn Tissues

Corn tissues were processed as documented in the files of the Production Plan #99-01-39-08 (Bhatti, 2000). Pollen tissues are not processed before extraction. Extracts were prepared according to the SOPs (Appendix 1) or as documented in the study files. Tissue was processed by grounding to a fine powder on dry ice in a blender or a vertical cutter/mixer. Tissues were then stored in a -80°C freezer. During extract preparation, all tissue powders were kept on dry ice. In order to minimize contamination, control plant samples were extracted before test plant samples. The same buffer was used for extraction of the Cry3Bb1 and NPTII

proteins. PBST (phosphate buffered saline plus Tween-20™) buffer was formulated as follows: 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, and 0.05% (v/v) Tween-20™. Leaf and root samples were extracted using PBST containing 0.1% (w/v) BSA (bovine serum albumin) at a 1:100 tissue weight to buffer volume (T:B; g:mL) ratio. Silk and pollen tissue samples were extracted in the same buffer as leaf except at a 1:40 T:B ratio. Grain samples were extracted in PBST at a 1:100 T:B ratio. Forage and other whole plant samples were extracted in TBA buffer (100 mM Trizma® base, 10 mM sodium borate, 0.05% (v/v) Tween-20™, 5 mM $MgCl_2$ and 0.2% (w/v) L-ascorbic acid, pH 7.5) at a 1:50 T:B ratio. Tissues were extracted by homogenization in the specified buffer using a Polytron® homogenizer (Model PT3000, Brinkmann Instrument Company, Westbury, NY) set at ≈17,500 rpm with two bursts of about 30 seconds each. The insoluble material was removed by filtration using 0.5 µm Serum Filter System (Fisher Scientific, Pittsburg, PA). In instances where sample size prevented the use of a Polytron®, (e.g. pollen and silk), tissues were homogenized with a Wheaton 2000 (Wheaton Science Products, Millville, NJ) set at 3,000 rpm. The insoluble material for these samples was removed by centrifugation at ≈20,000 × g for about 10 minutes at ≈4°C. The supernatant was stored as aliquots in the −80°C freezer until ELISA analysis. The extraction efficiency for these tissue types was summarized in Appendix 2 for both proteins.

4.2 Cry3Bb1 and NPTII Protein ELISA Methods

For each protein, Cry3Bb1 and NPTII, a direct double antibody sandwich ELISA has been developed and validated (Dudin et al., 1999 and Bhakta et al., 1999, respectively). The ELISA validation summary for both proteins is attached as Appendix 2. The Cry3Bb1 and NPTII protein levels in tissue extracts were measured using ELISA methods according to their respective SOPs (Appendix 1).

Cry3Bb1 or NPTII protein levels in extracts were measured by a validated sandwich ELISA based on purified rabbit antisera and HRP-conjugated detection antibodies. A sensitive HRP substrate, TMB (3,3'5,5'-tetramethylbenzidine) was used for color development in both ELISA assays. Quantitation of the Cry3Bb1 or NPTII protein levels was accomplished by interpolation from a Cry3Bb1 or NPTII protein standard curves. In each assay, the range for the standard curve was 1 to 64 ng/mL.

4.3 Control of Bias

The test events and control lines in the field trial were planted in a non-systematic manner at all field sites using randomized block system as described in the Production Plans # 99-01-39-08 and # 99-04-39-01. When appropriate, corn tissues were processed by thorough grinding before extraction to minimize tissue bias.

The accuracy of each analytical method was evaluated in terms of bias during ELISA validation process. Accuracy was defined by two components of the system: recovery of the standard protein spiked at several levels into control matrix and the extraction efficiency using positive tissue samples (Appendix 2). Therefore, all reported Cry3Bb1 and NPTII protein levels (µg/g fwt) were corrected for method bias which was calculated as follows:

$$Method\ Bias = \frac{(\%\ Extraction\ Efficiency)}{(100)} \times \frac{(\%\ Spike\ and\ Recovery)}{(100)}$$

Method bias was summarized for each ELISA in Appendix 2. The limit of quantitation (LOQ) was 1 ng/mL for each protein ELISA. Negative and positive quality control (QC) samples were included in each ELISA assay to verify performance according to the established assay specifications.

4.4 Data Reduction and Statistical Analyses

The absorbance (Abs) density of each reaction was measured by dual wavelength using a Molecular Devices SPECTRAmax® PLUS photometer (450nm - 650 nm). Standard protein concentrations and the corresponding absorbance readings were plotted by SOFTmax® PRO, version 2.4.1 (Molecular Devices Corporation, Sunnyvale, CA) using a 4-parameter curve fit. Following the interpolation from the standard curve, the concentrations of Cry3Bb1 or NPTII proteins in the corn tissue extracts were converted to microgram protein per gram fresh weight of tissue using the respective extraction tissue to buffer volume ratios and were subsequently divided by a tissue-specific factor to account for method bias (Section 4.3). Microsoft Excel™ Version 7.0 was used to calculate the average protein levels and standard deviations (SD) across sites where multiple number of samples were analyzed.

5.0 Results and Discussion

5.1 Test and Control Substance Characterization

The identity of each test and control substance was confirmed by the Study Director prior to their use in the study by reviewing the chain-of-custody documentation supplied with the samples. For Production Plan # 99-01-39-08, the test and control substances were characterized at the DNA level and test events were positively identified during this study. DNA analyzed from leaf and grain samples indicated event identity was maintained at all sites in each replicate of the MON 863 event. DNA analyses also confirmed the expected absence of the *cry3Bb1* and *nptII* expression cassettes in the MON 846 control line at all sites in each replicate. Results of the test and control substance characterization on leaf and grain samples from U.S.

Production Plan # 99-01-39-08 are retained with the study data. Due to the limited number of tissue samples analyzed from Argentina Production Plan # 99-04-39-01, pollen tissue samples were not confirmed by molecular analyses prior to being used in this study, as was documented by a protocol amendment. However, the identity of each test and control substance was confirmed prior to their use in the study by reviewing the chain-of-custody documentation supplied with these pollen tissues.

In addition, leaf, forage, root and grain tissue samples and extracts thereof were analyzed within the timeframe of the demonstrated Cry3Bb1 and NPTII protein stability (Dudin et al., 1999; Bhakta et al., 1999).

5.2 Protein Levels in Corn Plant Tissue Samples of MON 863

Protein values were reported as micrograms (µg) of protein per gram (g) on a fresh weight basis of collected tissues (fwt). Young leaf, forage, mature root and grain were analyzed using extracts prepared from tissue samples collected from four U.S. field sites. Young leaf of the MON 863 event was collected approximately at the V-4 growth stage or 21 days post-planting and the level of the Cry3Bb1 protein averaged 81 µg/g. The levels of the Cry3Bb1 protein in grain averaged 70 µg/g. The Cry3Bb1 protein levels in mature root and forage averaged 41 µg/g and 39 µg/g, respectively. The levels of this protein in pollen were estimated using tissue samples collected from four field sites (one field site in U.S. and three sites in Argentina), and averaged 62 µg/g. Finally, Cry3Bb1 protein level was approximately 10 µg/g in silk collected as a single composite from one U.S. field site.

Site to site variation at four U.S. locations of the Cry3Bb1 protein levels in the MON 863 event was observed to be approximately two-fold as demonstrated by the following ranges: leaf (65 - 93 µg/g), forage (24 - 45 µg/g), root (25 - 56 µg/g) and grain (49 - 86 µg/g).

In addition, various tissues of the MON 863 event were collected during the corn growing season at three U.S. field sites. The level of the Cry3Bb1 protein in leaves decreased from 81 µg/g at the V-4 stage to about 43 µg/g prior to pollination (approximately 49 days post-planting). The level of the Cry3Bb1 protein in whole plants followed the same pattern: 46 µg/g (at two weeks prior to pollination) decreased to 25 µg/g at senescence or post-harvest. Similarly, root tissues collected at the same sampling times as whole plant tissues, indicated a decrease in the Cry3Bb1 protein levels from 58 µg/g to 24 µg/g.

The NPTII protein levels in young leaf across four U.S. field sites ranged from 0.74 to 1.4 µg/g and the levels in forage tissues ranged from 0.17 to 0.23 µg/g. The NPTII

protein levels in grain were below the LOD (<0.076 µg/g) in all of the samples analyzed from four U.S. field sites.

Tissues from the non-transgenic corn line (MON 846) of comparable genetic background were also collected and analyzed. Results of analyses of MON 846 for Cry3Bb1 and NPTII protein levels were not reported since these control tissues were utilized to verify the limit of detection for each tissue type established during validation. DNA analyses confirmed the expected absence of the *cry3Bb1* and *nptII* expression cassettes in the control samples of MON 846 line. The limit of detection for each tissue type in the Cry3Bb1 protein ELISA varied from 0.08 µg/g in silk to 0.76 µg/g in root tissues. The limit of detection for the NPTII protein ELISA varied from 0.075 µg/g in forage to 0.093 µg/g in leaf tissues.

6.0 Conclusion

In summary, it was observed that the mean concentrations of Cry3Bb1 protein in the MON 863 event were highest in leaf (81 µg/g), followed by grain (70 µg/g), pollen (62 µg/g), root (41 µg/g), forage (39 µg/g), and silk (10 µg/g).

Field trials were conducted using agronomic practices and field conditions typical of commercial corn cultivation and provided a variety of environmental situations representative of the various geographical regions where CRW-protected corn lines would be grown. Therefore, data generated should be representative of the expected protein levels in the commercial corn cultivation of the MON 863 event.

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Table 1. Cry3Bb1 and NPTII Proteins Levels in Leaf, Forage, Root, Grain, Pollen, and Silk Samples of MON 863 Tissues Collected in 1999 Field Trials

Tissue Type	No. of Days Post-Planting	Average µg/g fwt (SD)[1] Range[2] Cry3Bb1 Protein	NPTII Protein
Young Leaf[3]	21 days	81 (11) 65 - 93	0.98 (0.27) 0.74 - 1.4
Forage[4]	90 days	39 (10) 24 - 45	0.19 (0.03) 0.17 - 0.23
Mature Root[4]	90 days	41 (13) 25 - 56	Not Done[8]
Grain[5]	125 days	70 (17) 49 - 86	<0.076[9]
Silk[6]	58 days	10 (N/A) (N/A)	Not Done[8]
Pollen[7]	60 days	62 (18) 30 - 93	Not Done[8]

1: Protein levels are expressed as microgram (µg) of protein per gram (g) fresh weight of tissue (fwt). The average and standard deviation (SD) values were based on one replicated sample from four field sites where (n=4). See notes 6 and 7 for silk and pollen, respectively.
2: Minimum and maximum values from the analyses of samples across sites.
3: The samples were a pool of tissues ranging from 37 to 50 plants collected at approximately V-4 stage.
4: Forage (OSWP-3; above ground portion only) and mature root (OSR-3) were composited of two plants and collected at early dent stage.
5: Processed grain samples were composited from 28-41 corn ears collected at plant maturity and dried to ≈15% moisture content; (n=4).
6: Silk was composited (n=1) from five plants at ≈50% pollen shed from one field site.
7: In U.S., one sample of pollen tissue was composited over a period of ≈7 days (≈60 days post-planting or ≈50% pollen shed). Samples of pollen from Argentina were composited as four replicates per site (three sites total) and collected ≈ 65 days post-planting over ≈5 days; (n=13).
8: Not Done: these tissue samples were collected but not analyzed as per study protocol.
9: The Limit of Detection (LOD) for the grain method is 0.076 µg/g fwt.

Table 2. Cry3Bb1 Protein Levels in Leaf, Whole Plant, and Root Samples of MON 863 Tissues Collected During the Growing Season in 1999 Field Trials

Tissue Type / No. of Days Post-Planting[3]	Average µg/g fwt (SD)[1] (Range)[2] 21	35	49	90	126
Leaf	81 (14) 65 - 93	79 (6.4) 72 - 84	43 (18) 30 - 56[6]	Not Collected[4]	Not Collected[4]
Whole Plant[5]	Not Collected[4]	46 (7.8) 38 - 54	31 (3.3) 28 - 33[7]	37 (12) 24 - 45	25 (11) 13 - 35
Root[6]	Not Collected[4]	58 (10) 46 - 66	57 (3.8) 54 - 59[7]	37 (11) 25 - 47	24 (18) 3.2 - 36

1: Protein levels are expressed as microgram (µg) of protein per gram (g) fresh weight of tissue (fwt). The mean and standard deviation (SD) were calculated from the analyses of one replicate collected from three field sites (n=3). Leaf at 21 days; root and whole plant at 90 days were collected at four field sites, but only three sites corresponding to the field sites where overseason sample were collected were included in the calculations shown in this table.

2: Minimum and maximum values from the analyses of samples across three field sites.

3: Approximate collection dates : V-4 stage leaf was collected at ≈21days post-planting; 50% pollen shed was at ≈50 to 60 days post-planting; early dent was at ≈90 days post-planting; and stover or senescent plant was collected at 126-130 days post-planting.

4: Not Done: not all overseason samples were collected. Overseason leaf collected until pollination only (≈49 days post-planting). Collection of root and whole plant samples over the growing season began at approximately two weeks prior to pollination or 35 days post-planting.

5: Whole plant samples are aerial (above ground) portion only. Whole plant collection corresponds to the root collection. The last overseason sample at 126 days is collected from a senescent plant or at stover stage.

6: Root samples correspond to the whole plant samples which are collected at the same time. Samples collected at 90 days are at the forage stage. The last overseason sample at 126 days is collected from a senescent plant or at stover stage.

7: Samples were collected only at two sites, samples from one site arrived thawed and were deleted from the study by a protocol amendment.

Appendix 1

Standard Operating Procedures (SOP)

GEN-EQP-019-01	Operation and Use of a Brinkman Polytron
BtM-PRO-067-01	Preparation of Protein Extracts of Corn Tissues
BR-ME-0059-04	Extraction and Direct ELISA Analysis of Cry3Bb1 in Corn Tissue
BR-ME-0050-03	Extraction and Direct ELISA Analysis of NPTII in Corn Leaf Tissue
BR-ME-0115-02	Extraction and Direct ELISA Analysis of NPTII in Corn Forage and Grain Tissue

Appendix 2

Cry3Bb1 ELISA Validation Summary

I. Accuracy

Tissue Type	Tissue to Buffer Ratio	Extraction Variability[1] (CV%)	Extraction Efficiency[2] (%)	Spike and Recovery[3] (%)	Method Bias Correction Factor[4]
Leaf	1:100	13.6	97.1	95.9	0.93
Grain	1:100	21.1	87.7	70.9	0.62
Forage	1:50	16.4	89.1	105	0.94
Root	1:100	21.7	77.2	91.8	0.71
Silk	1:40	10.6	≈100[2]	68.9	0.69
Pollen	1:40	22.5	38.8	94.4	0.37

II. Precision

Standard Curve Precision Range[5]:	4.4 – 14.6% CV
Intra-Assay Variability[5]:	6.1% CV
Inter-Assay Variability[5]:	8.4% CV

III. Range of Quantitation

1.0 – 64 ng/mL

IV. Sensitivity

Tissue Type	Dilution or Max Sample Volume (µL/well)	LOD (ng/mL)[6]	LOD (µg/g fwt)[7]
Leaf	Neat / (50)	0.87	0.087
Forage	Neat / (50)	4.4	0.22
Grain	Neat / (50)	0.96	0.096
Root	Neat / (50)	7.64	0.76
Silk	1:2 / (25)	N/A	0.08[8]
Pollen	1:2 / (25)	N/A	0.26[8]

1. Extraction variability was assessed by determining the %CV (standard deviation, SD divided by the average and multiplied by 100%) of the Cry3Bb1 protein levels based on extractions performed at least twice for each tissue type.
2. Extraction efficiency for each tissue type was determined by extracting three replicates and re-extracting the same tissue several successive times. Silk extraction efficiency is reported as ≈100% due to rounding of calculations.
3. Spike and recovery: tissues from control corn plants were extracted with assay buffer spiked with known quantities of Cry3Bb1 protein at three concentrations spanning the range of quantitation. Each spike was performed in three replicates.
4. Method Bias Correction Factor: was defined as two components of accuracy. More specifically, mean recovery of the standard protein from spiked control tissues (see note 3) and extraction efficiency for transgenic tissues (see note 2) comprised the method bias which was calculated from the combined measurement of these two parameters. The following formula was applied to all the Cry3Bb1 protein measurements after conversion to µg/g fwt values: Total Cry3Bb1 protein (µg/g fwt) = µg/g fwt / [(% extraction efficiency)/100 × (% spike and recovery)/100]. All of the protein data was corrected by the tissue specific factors, unless values were below LOD.
5. The intra-assay precision was assessed by determining the %CV of the protein level in a particular sample within a single assay in triplicate wells. The inter-assay precision was assessed by measuring the concentration of a positive sample in 10 ELISA assays. Intra- and inter-assay variability were calculated using a one way analysis of variance (ANOVA).
6. The limit of detection (LOD) was calculated using the non-transgenic sample extracts for the respective tissue types. The LOD was calculated at the dilution listed as follows: LOD = mean (ng/mL) + 3 standard deviations.
7. LOD (µg/g fwt): the ng/mL values were converted to µg/g fwt by multiplying the LOD (ng/mL; see note 6) by the tissue weight to buffer volume ratio (g/mL) and dividing by a conversion factor of 1000 ng/µg.
8. Pollen and silk LODs were calculated from each ELISA assay using data from this study. LODs listed are based on the field control tissues collected for this study. LOD for silk tissues was calculated at a 1:4 dilution and for pollen tissues at a 1:20 dilution.

NPTII ELISA Validation Summary

I. Accuracy

Tissue Type	Tissue to Buffer Ratio	Extraction Variability[1] (CV%)[1]	Extraction Efficiency[2] (%)	Spike and Recovery[3] (%)	Method Bias Correction Factor[4]
Leaf	1:100	4.4	97.9	91.4	0.89
Forage	1:50	10.1	94.8	104.9	0.99
Grain	1:100	8.0	78.4	71.8	0.56

II. Precision

Standard Curve Precision Range[5]: 7.0 – 15.8% CV
Intra-Assay Variability[5]: 8.3% CV
Inter-Assay Variability[5]: 15.5% CV

III. Range of Quantitation

1.0 – 64 ng/mL

IV. Sensitivity

Tissue Type	Dilution or Max Sample Volume (µL/well)	LOD (ng/mL)[6]	LOD (µg/g fwt)[7]
Leaf	Neat / (50)	0.93	0.093
Forage	Neat / (50)	1.50	0.075
Grain	Neat / (50)	0.76	0.076

1. Extraction variability was assessed by determining the %CV (standard deviation, SD of a set of numbers divided by the average and multiplied by 100%) of the NPTII protein levels based on extractions repeated 5 times.
2. Extraction efficiency for each tissue type was performed by extracting three replicates and re-extracting the same tissue several successive times.
3. Spike and recovery: tissues from control corn plants were extracted with assay buffer spiked with known quantities of NPTII protein at three concentrations spanning the range of quantitation. Each spike was performed in three replicates.
4. Method Bias Correction factor: was defined as two components of accuracy. More specifically, mean recovery of the standard protein from spiked control tissues (see note 3) and extraction efficiency for transgenic tissues (see note 2) comprised the method bias which was calculated from the combined measurement of these two parameters. The following formula was applied to all the NPTII protein measurements after conversion to µg/g fwt values: Total NPTII protein (µg/g fwt) = µg/g fwt / [(% extraction efficiency)/100 × (% spike and recovery)/100]. All protein data was corrected by the tissue specific factors, unless values were below LOD.
5. The intra-assay precision was assessed by determining the %CV of NPTII protein level in a particular sample within a single assay in triplicate wells. The inter-assay precision was assessed by measuring the concentration of a positive sample in 8 ELISA assays. Intra- and inter-assay variability were calculated using a one way analysis of variance (ANOVA).
6. The limit of detection (LOD) was calculated using the non-transgenic sample extracts for the respective tissue types. The LOD was calculated at the dilution listed as follows: LOD = mean (ng/mL) + 3 standard deviations.
7. LOD (µg/g fwt): the ng/mL values were converted to µg/g fwt by multiplying the LOD (ng/mL; see note 6) by the tissue weight to buffer volume ratio (g/mL) and dividing by a conversion factor of 1000 ng/µg.