Study Title

Assessment of the Cry1Ac Protein Levels in Soybean Tissues Collected from MON 87701 Produced in U.S. Field Trials During 2007

This report reflects data generated and reported in Study REG-08-187

Authors

Katherine E. Niemeyer
Andre Silvanovich, Ph.D.

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Sponsor and Performing Laboratory

Monsanto Company
Biotechnology Regulatory Sciences
800 North Lindbergh Boulevard
Saint Louis, Missouri 63167

Laboratory Project ID

MSL0021531

The text below applies only to use of the data by the United States Environmental Protection Agency (U.S. EPA) in connection with the provisions of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA).

The inclusion of this page in all studies is for quality assurance purposes and does not necessarily indicate that this study has been submitted to the U.S. EPA.

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Statement of Compliance

This document describes the protein expression analyses of the Cry1Ac protein in soybean tissues collected from MON 87701 produced in U.S. field trials during 2007 performed under Monsanto study REG-08-187. The Statement of Compliance from Monsanto study REG-08-187 is provided below.

Statement of Compliance

This study meets the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160 with the following exceptions: The storage stability of the Cry1Ac protein in pollen tissues has not been determined. The storage stability of the CP4 EPSPS protein in processed root has not been determined.

Submitter — Date

Eddie L — 8/28/2008

Sponsor Representative — Date

8/28/08

Study Director — Date

Quality Assurance Statement

Study Title: Assessment of the Cry1Ac and CP4 EPSPS Protein Levels in Soybean Tissues Collected from MON 87701 and MON 87701 × MON 89788 Produced in U.S. Field Trials During 2007

Study Number: REG-08-187

Following is a list of reviews conducted by the Monsanto Regulatory Quality Assurance Unit on study REG-08-187.

Reviews conducted by the Quality Assurance Unit confirm that the final report for study REG-08-187 accurately describes the methods and standard operating procedures followed and accurately reflects the raw data of the study.

Dates of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
07/30/2008	ELISA	07/30/2008	07/30/2008
08/19/2008	Moisture Analysis	08/19/2008	08/19/2008
08/25/2008	Raw Data Audit	08/27/2008	08/27/2008
08/26/2008	Draft Report Review	08/27/2008	08/27/2008

Additionally, the Quality Assurance Unit reviewed this report, MSL 0021531, and confirmed that this report accurately reflects the portions of the final report for study REG-08-187 that are reported in MSL 0020530.

Dates of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
08/26/2008	Draft Report Review	08/27/2008	08/27/2008

Niki K Scanlon

Niki Scanlon
Quality Assurance Unit
Monsanto Regulatory, Monsanto Company

8/27/2008
Date

Report Certification

This report is an accurate and complete representation of a portion of the work conducted in study REG-08-187

Signatures of Approval:

Report Author — Date: 8/28/08

Sponsor Representative — Date: 8/28/2008

Study Information

Study Number:	REG-08-187
Study Title:	Assessment of the Cry1Ac and CP4 EPSPS Protein Levels in Soybean Tissues Collected from MON 87701 and MON 87701 × MON 89788 Produced in U.S. Field Trials During 2007
Report Title:	Assessment of the Cry1Ac Protein Levels in Soybean Tissues Collected from MON 87701 Produced in U.S. Field Trials During 2007
Facility:	Monsanto Company Biotechnology Regulatory Sciences 800 North Lindbergh Boulevard Saint Louis, Missouri 63167
Study Director:	Katherine E. Niemeyer
Contributors:	William Planer, Louise Lee, Michelle Glaspie, and Aster Beyene
Team Lead:	Andre Silvanovich, Ph.D.
Study Initiation Date:	May 2, 2008
Report Date:	August 28, 2008
Records Retention:	All study specific raw data (including rejected data and data not reported), protocols, final reports, and facility records will be retained at Monsanto Company, St. Louis, Missouri.

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Abbreviations[1] and Definitions

ANOVA	Analysis of variance
BSA	Bovine serum albumin
B.t.	*Bacillus thuringiensis*
CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase derived from *Agrobacterium sp.* strain CP4
Cry1Ac	Class I (lepidoptera-specific) crystal protein from *B.t.* subsp. *kurstaki* (*B.t.k.)* HD-1
CV	Coefficient of variation
DCA	Deoxycholic acid
DTT	dithiothreitol
DWCF	Dry weight conversion factor
dwt	Dry weight of tissue
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
fwt	Fresh weight of tissue
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
LOD	Limit of detection
LOQ	Limit of quantitation
M19	Mouse monoclonal antibody clone Cry1Ac-M19-N4-A6
NA	Non-applicable
OSL	Over-season leaf
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween 20
PCR	Polymerase chain reaction
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	Standard operating procedure
TBA	Tris-borate buffer with L-ascorbic acid
TMB	3,3',5,5'-tetramethylbenzidine
Tris	Tris(hydroxymethyl)aminomethane
TSSP	Tissue-specific site pool

[1] Standard abbreviations, e.g., units of measure, were used in this report according to format described in "Instructions to Authors" in the Journal of Biological Chemistry.

1.0 Summary

Monsanto Company has developed insect-protected soybean MON 87701, which contains the *cry1Ac* gene and expression of the Cry1Ac protein confers protection to certain lepidopteran insects.

The purpose of this study was to assess the expression levels of Cry1Ac proteins in MON 87701 soybean tissues produced in U.S. field trials during 2007. Over-season leaf (OSL), root, seed, and forage tissue samples were collected as outlined in Production Plan 07-01-71-01 and pollen/anther samples were collected as outlined in Production Plan 07-01-71-02. In this study, over season leaf (OSL-1-4), root, pollen (anther), forage, and seed tissues were assessed using a validated, protein-specific ELISA assay. The over season leaf samples were collected at four different growth stages: (1) V3 – V4 stage, (2) V6 – V8 stage, (3) V10 – V12 stage, and (4) V14 – V16 stage. Target protein levels for all tissue types were calculated on a microgram (µg) per gram (g) fresh weight (fwt) basis. Moisture content was then measured for all tissue types, except pollen, and protein levels were converted and reported on a dry weight (dwt) basis. Moisture analysis was not performed on pollen due to insufficient sample volume and therefore the protein levels for pollen will be reported in µg/g fwt.

ELISA results determined that the mean Cry1Ac protein levels in MON 87701 across all U.S. sites were 4.7 µg/g dwt in harvested seed, 34 µg/g dwt in forage, and less than the limit of detection (<LOD) in root. The mean Cry1Ac protein level in pollen (anther) from replicate samples collected at a single site (Jackson county, IL) was 2.3 µg/g fwt. In leaf tissue samples harvested throughout the growing season, mean Cry1Ac protein levels in MON 87701 across all sites ranged from 220 - 340 µg/g dwt.

2.0 Introduction

2.1 Background

Monsanto Company has developed insect-protected soybean MON 87701, which contains the *cry1Ac* gene and expression of the Cry1Ac protein confers protection to certain lepidopteran insects.

Cry1Ac protein levels were determined in soybean plants produced at five U.S. field sites in 2007 under Production Plan 07-01-71-01. Field sites were selected to represent the geographical regions where soybean is commercially grown. At each field site, soybean was planted using a randomized complete block design consisting of three replicate blocks. Cry1Ac protein levels were determined in soybean pollen tissues produced at a single site (Jackson County, IL) in 2007 under Production Plan 07-01-71-02. Soybean was planted using a single plot design.

2.2 Purpose

The purpose of this study was to assess the quantities of the Cry1Ac proteins in MON 87701 soybean tissues produced in U.S. field trials during 2007. Over-season leaf (OSL), root, seed, and forage tissue samples were collected as outlined in Production Plan 07-01-71-01 and pollen samples were collected as outlined in Production Plan 07-01-71-02.

3.0 Materials

3.1 Test, Control, and Reference Substances

3.1.1 Test Substances

The test substance was MON 87701 grown in U.S. field trials during 2007. Over-season leaf (OSL), root, seed, and forage tissue samples were collected as outlined in Production Plan 07-01-71-01 and pollen samples were collected as outlined in Production Plan 07-01-71-02 from plants grown from starting seed lot GLP-0612-17898-S.

3.1.2 Control Substances

The negative control substance was a conventional soybean with a similar genetic background to the test plants grown in 2007 U.S. field trials. OSL, root, seed, and forage tissue samples of the negative control substance were collected as outlined in Production Plan 07-01-71-01 and pollen samples were collected as outlined in Production Plan 07-01-71-02 from plants grown from starting seed lot GLP-0612-17895-S.

3.1.3 Characterization of Test and Control Substances

The identities of the test and control substances were confirmed by analysis of the starting seed DNA by an event-specific polymerase chain reaction (PCR) method and the results were archived under the seed lot numbers. The seed samples harvested from the field were also verified by PCR and the resulting Verification of Identity was archived under the starting seed lot numbers, following the Monsanto standard operating procedure (SOP) BR-PO-0573-02. Any test or control seed substance, and its associated tissues (i.e. leaf, forage, etc.), which had three or more pools test unexpectedly positive or negative during PCR verification, were not reported in this study.

3.1.4 Reference Substance

A Cry1Ac protein standard (Orion lot # 10000780) was used as the reference substance for the Cry1Ac ELISA. The protein concentration of the protein standard was determined by amino acid composition analysis and the purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometric analysis. The purity of the Cry1Ac protein standard was 82% and the purity-corrected concentration was 1.1 mg/ml.

Copies of the certificate of analysis for the Cry1Ac protein standard were archived with the study file, which address each standard's characterization and stability.

4.0 Methods

4.1 Generation of Plant Samples

4.1.1 Summary of Field Design

Production Plan 07-01-71-01 (Armstrong, 2008) was initiated during the 2007 planting season to generate test and control substances at various soybean-growing locations in the U.S. The field sites were as follows: Baldwin County,

AL (site code AL); Jackson County, AR (site code AR); Clarke County, GA (site code GA); Jackson County, IL (site code IL); and Wayne County, NC (site code NC). These field sites were representative of soybean producing regions suitable for commercial soybean production. At each site, three replicated plots of soybean plants containing MON 87701, as well as the negative control, were planted using a randomized complete block field design. Over season leaf (OSL 1-4), root, forage, and seed tissues were collected from each replicated plot at all field sites. The over season leaf samples were collected four times at different growth stages: : (1) V3 – V4 stage, (2) V6 – V8 stage, (3) V10 – V12 stage, and (4) V14 – V16 stage. Production Plan 07-01-71-02 (Armstrong, In progress) was initiated during the 2007 planting season to generate test and control substances at Jackson County, IL (site code IL) in the U.S. At this site, plots of plants containing MON 87701, as well as the negative control, were planted using a single plot field design. Pollen/anther tissues were collected from each plot. Throughout both field productions, sample identity was maintained by using unique sample identifiers and proper chain-of-custody documentation. All tissue samples, except seed, were stored in a -80ºC freezer and shipped on dry ice to the Monsanto processing facility in Saint Louis, Missouri. Seed samples were stored and shipped at ambient temperature.

4.2 Tissue Processing and Protein Extraction Methods

4.2.1 Processing Method

All tissue samples produced at the field sites were shipped to Monsanto's processing facility. During the processing step, dry ice was combined with the individual samples, and vertical cutters or mixers were used to thoroughly grind and mix the tissues. Processed samples were transferred into capped 15 ml tubes and stored in a -80°C freezer until use.

4.2.2 Extraction Methods

The Cry1Ac proteins were extracted from soybean tissues, as described in Appendix 2. All processed tissues were kept on dry ice during extract preparation. All tissues were extracted using a Harbil mixer (Harbil Industries, Compton, CA). Insoluble material was removed from the extracts by using a Serum Filter System (Fisher Scientific, Pittsburgh, PA), except pollen, which were centrifuged. The extracts were aliquoted and stored in a -80°C freezer until analyzed.

4.3 ELISA Reagents and Methods

4.3.1 Cry1Ac Antibodies

Mouse monoclonal antibody clone M19-N4-A6, also known as M19 (IgG1 isotype, kappa light chain; lot 7495955) specific for the Cry1Ac protein was purified from mouse ascites fluid using Protein-A Sepharose affinity chromatography and was used as the capture antibody in the Cry1Ac ELISA. The concentration of the purified IgG was determined to be 6.0 mg/ml by spectrophotometric methods. Production of the M19 monoclonal antibody was performed by Strategic Biosolutions (Newark, DE). The purified antibody was

stored in a buffer (pH 7.2) containing 10 mM sodium phosphate, 150 mM sodium chloride, and 15 mM sodium azide.

Goat antibodies (lot G-805044) specific for Cry1Ac were purified using Protein-G Agarose affinity chromatography. The concentration of the purified IgG was determined to be 3.7 mg/ml by spectrophotometric methods. The purified antibody was stored in 1X phosphate-buffered saline (PBS), pH 7.4, and coupled with biotin (Pierce, Rockford, IL) according to the manufacturer's instructions, and assigned lot number G-805045. The detection reagent was NeutrAvidin (Pierce, Rockford, IL) conjugated to horseradish peroxidase (HRP).

4.3.2 Cry1Ac ELISA Method

The Cry1Ac ELISA was performed according to SOP BR-ME-1214 (draft). Mouse anti-Cry1Ac antibody was diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 150 mM NaCl, pH 9.6) to a final concentration of 2.0 µg/ml, and immobilized onto 96-well microtiter plates followed by incubation in a 4°C refrigerator for $\geq$8 h. Prior to each step in the assay, plates were washed with 1X phosphate-buffered saline with Tween-20 (PBST). Cry1Ac protein standard or sample extract was added at 100 µl per well and incubated for 1 h at 37°C. The captured Cry1Ac protein was detected by the addition of 100 µl per well of biotinylated goat anti-Cry1Ac antibodies and NeutrAvidin-HRP (Pierce, Rockford, IL). Plates were developed by adding 100 µl per well of HRP substrate, 3,3',5,5'-tetramethyl-benzidine (TMB; Kirkegaard & Perry, Gaithersburg, MD). The enzymatic reaction was terminated by the addition of 100 µl per well of 3 M H_3PO_4. Quantification of the Cry1Ac protein was accomplished by interpolation from a Cry1Ac protein standard curve that ranged from 1.0 – 32 ng/ml.

4.4 Control of Bias

The test and control substances were planted using a randomized complete block design as described in Production Plan 07-01-71-01 and a single plot design as described in Production Plan 07-01-71-02 to prevent any sampling bias. Representative tissues from each plot were collected as described in the production plans. All tissues, except pollen/anther, were processed by thoroughly grinding to produce a homogeneous sample before extraction to minimize sampling bias. All of the ELISA methods used were optimized to minimize method bias. Protein extracts from the test and control substances were analyzed by ELISA with the appropriate Cry1Ac protein standard and inter-assay negative and positive controls.

4.5 Moisture Analysis

All tissues, except pollen, were analyzed for moisture content using an IR-200 Moisture Analyzer (Denver Instrument Company, Arvada, CO). Analyses were analyzed according to BR-ME-1238-01. A homogeneous tissue-specific site pool (TSSP) was prepared using the test and control samples of a given tissue type grown

at a given site. These pools were prepared for all tissues, except pollen, in this study. The average percent moisture for each TSSP was calculated from triplicate analyses. A TSSP Dry Weight Conversion Factor (DWCF) was calculated as follows:

$$DWCF = 1 - [Mean\ \%\ TSSP\ Moisture / 100]$$

The DWCF was used to convert protein levels assessed on a µg/g fresh weight (fwt) basis into levels reported on a µg/g dry weight (dwt) basis using the following calculation:

$$Protein\ Level\ in\ Dry\ Weight = \frac{(Protein\ Level\ Fresh\ Weight)}{(DWCF)}$$

The protein levels that were reported to be less than or equal to the limit of detection (LOD) or less than the limit of quantitation (LOQ) on a fresh weight basis were not reported on a dry weight basis.

4.6 Data Analyses

All Cry1Ac ELISA plates were analyzed on a SPECTRAmax Plus (Molecular Devices, Sunnyvale, CA) or SPECTRAmax Plus 384 (Molecular Devices, Sunnyvale, CA) microplate spectrophotometer, using a dual wavelength detection method. All protein concentrations were determined by optical absorbance at a wavelength of 450 nm with a simultaneous reference reading of 620-650 nm. Data reduction analyses were performed using Molecular Devices SOFTmax PRO GXP version 5.0.1. Absorbance readings and protein standard concentrations were fitted with a four-parameter logistic curve fit. Following the interpolation from the standard curve, the amount of protein (ng/ml) in the tissue was reported on a "µg/g fwt" basis. For all proteins, this conversion utilized a sample dilution factor and a tissue-to-buffer ratio. The protein values in "µg/g fwt" were also converted to "µg/g dwt" by applying the DWCF, except for pollen which was not analyzed for moisture content due to insufficient sample volume. Microsoft Excel 2002 (Version 10.68241.6839 SP3, Microsoft, Redmond, WA) was used to calculate the Cry1Ac protein levels in soybean tissues.

4.7 Protocol Amendments and Deviations

The study protocol was amended to change the study director assignment. This amendment had no impact on the data.

The protocol was amended to clarify that pollen tissues used in this study were from production plan 07-01-71-02 and were only harvested from one site, Jackson County, IL. This amendment also clarified that pollen tissues would only be tested for Cry1Ac protein levels. This amendment had no impact on the study as it only provides clarification.

The protocol was amended to correct the stated wavelength of the reference readings used to determine protein concentrations. The protocol listed that protein concentrations would be determined by optical absorbance at a wavelength of 450 nm with a simultaneous reference reading of 650 nm. The protocol was amended to state the reference reading would be at a wavelength of 620 - 650 nm. This amendment

had no impact, as the amendment is within the specifications of SOP BR-ME-1214 (draft).

The protocol was amended to state that a draft version of the SOP BR-ME-1214 would be used to analyze Cry1Ac protein levels. Samples were analyzed before the method SOP was signed. However, the method had been validated and internally reviewed for use before analyzing samples. A copy of the draft method used will be archived with the study.

The protocol was amended to correct the number of replicates for pollen. The protocol originally stated there would be three replicates for pollen, but the correct number was four. This amendment had no impact as it reflects what was analyzed in the study.

A protocol deviation was written to address the extraction and analysis of samples that should not have been analyzed in the study due to three or more pools testing unexpectedly positive or negative by PCR. All data generated from these analyses were not reported, resulting in no impact on the study data.

A protocol deviation was written to address pollen samples being extracted by a method other than what was stated in the protocol. The number of beads and the type of bead was different than was stated in the protocol. Also, samples were centrifuged versus serum filtered as stated in the protocol. These deviations occurred due to insufficient sample volume. The method was scaled down in order to perform the extraction successfully. The same extraction buffer and tissue-to-buffer ratio was used, therefore there was no impact on the study.

A protocol deviation was written to address the field design for Production Plan 07-01-71-02. The protocol stated that the test and control substances were planted in a non-systematic manner at all field sites using a randomized complete block design. However, Production Plan 07-01-71-02 was planted in a single plot design. This deviation has no impact as it was written to reflect what field design was actually used in the production.

Additional protocols amendments occurred in study REG-08-187 that do not pertain to the work described in this break-out report and therefore are not included in this report.

4.8 Data Not Reported

All of the data generated using the samples that should not have been analyzed because they had three or more pools test unexpectedly positive or negative by PCR, as well as their associated tissues, were not reported, but were archived with the study.

5.0 Results

Summaries of means, standard deviations (SD), and ranges are reported for Cry1Ac protein levels on a μg/g fwt and μg/g dwt basis in soybean tissues collected from five U.S. field sites in 2007. Pollen samples from one U.S. site are only reported in μg/g fwt and only Cry1Ac protein levels were analyzed.

5.1 Cry1Ac Protein Levels in Soybean

The Cry1Ac protein levels for MON 87701 are presented in Tables 1-2. Results showed that the mean Cry1Ac protein levels in MON 87701 across all U.S. sites were 4.7 µg/g dwt in harvested seed, 34 µg/g dwt in forage and less than the limit of detection (<LOD) in root. The mean Cry1Ac protein level in MON 87701 pollen (anther) from a single site, was 2.3 µg/g fwt. In leaf tissues harvested throughout the growing season, the mean Cry1Ac protein levels in MON 87701 across all sites ranged from 220 - 340 µg/g dwt.

The levels of Cry1Ac protein in tissue samples from the conventional soybean negative control substance were below the Cry1Ac assay LOQ or LOD for each tissue type.

5.2 Stability of Test Materials

All of the test and control substances for leaf, root, seed, and forage were extracted and analyzed by ELISA within the timeframe of verified tissue stability for the Cry1Ac protein. Storage stability of the Cry1Ac protein in pollen tissue has not been determined. The storage stability of the Cry1Ac protein in extracts has not been determined.

6.0 Conclusions

MON 87701 was grown in U.S. field trials at five field sites in 2007. Tissue samples were collected at various growth stages throughout the growing season and analyzed for Cry1Ac protein levels in MON 87701 using a validated ELISA method. These data provide an estimation of the Cry1Ac protein levels on a fresh weight and dry weight basis in seven tissue types (OSL1-4, seed, forage, and root) collected during different stages of the growing season. These data also provide an estimation of the Cry1Ac protein levels on a fresh weight basis in pollen tissue from a single site in the U.S.

7.0 Acknowledgments

The authors would like to acknowledge Jack Milligan and the Agronomy and Sample Processing Center for processing the tissue samples and Andre Van Oyen, Jr. and John Lake from the Sample Dispensary for the sample distributions.

8.0 References

Armstrong, Thomas F. Field Production of Large Bulk Soybean Seed Samples from Insect-Protected Soybean MON 87701 and Insect-Protected Soybean MON 87701 × Glyphosate Tolerant Soybean MON 89788 Grown in the United States during 2007. Monsanto Production Plan, St. Louis, MO, Production Plan 07-01-71-02 (In Progress).

Armstrong, Thomas F. 2008. Field Production of Tissues from Insect Protected Soybean MON 87701 and Insect Protected MON 87701 Glyphosate Tolerant Soybean MON 89788 Grown in the United States during 2007. Monsanto Technical Report, St. Louis, MO, MSL0021397.

Table 1. Summary of Cry1Ac Protein Levels in Soybean Leaf Tissues Collected from MON 87701 Produced in U.S. Field Trials Conducted in 2007

Tissue Type[1]	Mean (SD)[2,4] Range[5] (µg/g fwt)	Mean (SD)[3] Range (µg/g dwt)
OSL-1	30 (8.5) 12 – 40	220 (70) 110 – 350
OSL-2	38 (16) 18 – 80	260 (100) 130 – 500
OSL-3	34 (17) 14 – 77	240 (110) 94 – 480
OSL-4	53 (36) 15 – 110	340 (290) 78 – 960

1. Tissues were collected at the following growth stages:
   a. OSL-1: V3 – V4 c. OSL-3: V10 – V12
   b. OSL-2: V6 – V8 d. OSL-4: V14 – V16
2. Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
3. Protein levels are expressed as µg/g on a dry weight (dwt) basis. The dry weight values were calculated by dividing the fwt by the dry weight conversion factors obtained from moisture analysis data.
4. The mean and standard deviation were calculated across sites (n=15, except OSL-1 where n=13).
5. Minimum and maximum values were determined for each tissue type across sites.

Table 2. Summary of Cry1Ac Protein Levels in Soybean Tissues Collected from MON 87701 Produced in U.S. Field Trials Conducted in 2007

Tissue Type[1]	Mean (SD)[2,4] Range[5] (µg/g fwt)	Mean (SD)[3] Range (µg/g dwt)
Root	<LOD (–) –	n/a[6] (–) –
Forage	9.0 (8.8) 2.5 – 32	34 (36) 8.2 – 140
Seed	4.2 (0.73) 3.1 – 5.0	4.7 (0.79) 3.4 – 5.7
Pollen	2.3 (0.58) 1.8 – 3.1	NA NA

1. Tissues were collected at the following growth stages:
   a. Root: R6 c. Seed: R8, Harvested at or Dried to ~10-15% moisture
   b. Forage: R6 d. Pollen: R2
2. Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
3. Protein levels are expressed as µg/g on a dry weight (dwt) basis. The dry weight values were calculated by dividing the fwt by the dry weight conversion factors obtained from moisture analysis data. Moisture analysis was not performed on pollen tissues.
4. The mean and standard deviation were calculated across sites (n=15; except pollen where n=4).
5. Minimum and maximum values were determined for each tissue type across sites.
6. Protein levels that were <LOD on a fwt basis were not converted to dwt values.

Appendices

Appendix 1. Standard Operating Procedures

BR-ME-1214 (draft)	Extraction and Indirect ELISA Analysis of Full Length Cry1Ac in Soybean Tissues
BR-ME-1238-01	Analysis of Moisture Content Using the Denver Instrument IR-200 Moisture Analyzer
BR-PO-0573-02	Test, Control, and Reference Material Confirmation of Identity

Appendix 2. Cry1Ac Extraction Procedures From Different Soybean Tissues

Tissue	Extraction Buffer	T:B Ratio	No. of ¼" Chromesteel beads	Shake time (minutes)	Sample Clarification method
Leaf[1]	Na_2CO_3/DTT[2]	1:100	8	7.0	Serum Filter
Root	Na_2CO_3/DTT	1:20	8	7.0	Serum Filter
Pollen	Na_2CO_3/DTT	1:20	6[3]	7.0	Centrifugation
Forage	Na_2CO_3/DTT	1:100	8	7.0	Serum Filter
Seed	Na_2CO_3/DTT	1:50	8	7.0	Serum Filter

1 Overseason leaf (OSL-1, OSL-2, OSL-3, and OSL-4)
2 Na_2CO_3 + 10mM DL-Dithiothreitol + 0.07% Tween 20™
3 Pollen used 2.3 mm beads, not ¼" beads.