Study Title

Assessment of CP4 EPSPS Protein Levels in Leaf, Seed, Root, and Forage Tissues from MON 87705 Soybean Grown in 2007/2008 Chile Field Trials

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Sponsor and Performing Laboratory

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Laboratory Project ID

MSL Number: MSL0021832

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Statement of Compliance

This study meets the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160.

Submitter — Date

5-15-09

Daniel J. Jenkins — Date
Sponsor Representative

5/15/09

Tao Geng, Ph.D. — Date
Study Director

Quality Assurance Statement

Study Title: Assessment of CP4 EPSPS Protein Levels in Leaf, Seed, Root, and Forage Tissues from MON 87705 Soybean Grown in 2007/2008 Chile Field Trials

Study Number: REG-08-363

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

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

Dates of Inspection /Audit	Phase	Date Reported to Study Director	Date Reported to Management
10/21/2008	Protein Extraction and/or Quantitation	10/29/2008	10/29/2008
11/05/2008	ELISA	11/11/2008	11/11/2008
05/04/2009	Draft Report and Data Audit	05/14/2009	05/14/2009

Date: 5/14/09

Quality Assurance Unit
Monsanto Regulatory
Monsanto Company

Study Certification Page

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

Signatures of Final Report Approval:

5/15/09

Tao Geng, Ph.D.
Study Director

Date

14 May 2009

Katherine Niemeyer
GLP Study Execution Team Lead

Date

Study Information Page

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Study Director:	Tao Geng, Ph.D.
Contributors:	Seth Tauchman, Loreen Stillwell, and Aster Beyene, Ph.D.
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Table of Contents

Abbreviations[1] and Definitions

cp4 epsps	coding sequence for 5-enolpyruvylshikimate-3-phosphate synthase derived from *Agrobacterium* sp. strain CP4
CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase derived from *Agrobacterium sp.* strain CP4
CV	coefficient of variation
DWCF	dry weight conversion factor
dwt	dry weight
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FATB	Fatty acid thioesterase B
FAD2	Fatty acid desaturase 2
fwt	fresh weight
HRP	horseradish peroxidase
LOD	limit of detection
LOQ	limit of quantitation
OSL	over season leaf
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline containing Tween-20
PCR	polymerase chain reaction
SD	standard deviation
SOP	standard operating procedure
TBA	tris-borate containing L-ascorbic acid
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 biotechnology-derived soybean, MON 87705, to generate nutritionally-improved soybean oil with decreased levels of saturated fats (16:0 palmitic acid and 18:0 stearic acid) and increased levels of oleic acid (18:1). Specifically, MON 87705 uses gene suppression technology to decrease the levels of two key fatty acid biosynthetic enzymes, FATB and FAD2. Suppression of the FATB enzyme results in a decrease in the levels of saturated fats (16:0 palmitic acid and 18:0 stearic acid), while suppression of the FAD2 enzyme results in an increase of oleic acid (18:1). MON 87705 also contains the 5-enolpyruvylshikimate-3-phosphate synthase gene derived from *Agrobacterium sp.* strain CP4 (*cp4 epsps*). Expression of the gene product, CP4 EPSPS protein, renders the soybean plant tolerant to glyphosate which is the active ingredient in the Roundup® family of agricultural herbicides.

The purpose of this study was to assess levels of CP4 EPSPS protein in leaf, seed, root, and forage tissues from MON 87705 soybeans produced in 2007/2008 Chile field trials.

By using ELISA analysis, the study showed that the mean CP4 EPSPS protein levels in OSL-1, OSL-2, OSL-3, OSL-4, forage, root, and seed tissues were 200, 530, 220, 210, 120, 77 and 110 µg/g dwt, respectively.

2.0 Introduction

2.1 Background

Monsanto Company has developed biotechnology-derived soybean, MON 87705, to generate nutritionally-improved soybean oil with decreased levels of saturated fats (16:0 palmitic acid and 18:0 stearic acid) and increased levels of oleic acid (18:1). Specifically, MON 87705 uses gene suppression technology to decrease the levels of two key fatty acid biosynthetic enzymes, FATB and FAD2. Suppression of the FATB enzyme results in a decrease in the levels of saturated fats (16:0 palmitic acid and 18:0 stearic acid), while suppression of the FAD2 enzyme results in an increase of oleic acid (18:1).

MON 87705 contains the 5-enolpyruvylshikimate-3-phosphate synthase gene derived from *Agrobacterium sp.* strain CP4 (*cp4 epsps*). Expression of the gene product, CP4 EPSPS, renders the plant tolerant to glyphosate, which is the active ingredient in the Roundup family of agricultural herbicides. Glyphosate binds to the endogenous

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plant EPSPS enzyme and blocks the biosynthesis of shikimate-3-phosphate, thereby depriving plants of aromatic amino acids (Steinrücken and Amrhein, 1980; Haslam, 1993). The CP4 EPSPS protein is structurally similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate relative to endogenous plant EPSPS (Padgette et al., 1996). Introduction of the *cp4 epsps* gene into soybean allows for the production of aromatic amino acids and other metabolites even in the presence of glyphosate (Padgette et al., 1996).

2.2 Purpose

The purpose of this study was to assess the levels of CP4 EPSPS protein in leaf, seed, root, and forage tissues of MON 87705 soybeans produced in 2007/2008 Chile field trials.

3.0 Materials

3.1 Test, Control, and Reference Substances

3.1.1 Test Substance

The test substance was MON 87705 soybean harvested from Chile (Material code 18254). Test substance tissue samples were collected as outlined in Production Plan REG-07-170, from starting seed lot GLP-0702-18254-S.

3.1.2 Control Substance

The control substance A3525 was conventional soybean (Material code 18252) with a similar genetic background to the test substance. Control substance tissue samples were collected as outlined in Production Plan REG-07-170, from starting seed lot GLP-0702-18252-S.

3.1.3 Reference Substance

The *E. coli* produced CP4 EPSPS (lot 20-100015) protein was used as the analytical reference standard. The purity was 97% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and densitometric analysis. The purity-corrected total protein concentration of the standard was 3.7 mg/ml. A copy of the certificate of analysis was included in the study file, which addresses the characterization and stability of the standard.

3.1.4 Characterization of Test and Control Substances

The identities of the test and control substances were confirmed by analysis of the harvested seed DNA by an event-specific polymerase chain reaction (PCR) method and the resulting Verifications of Identity (VOIs) were archived in the Monsanto Regulatory Archives. The copies of VOIs were also included in the study file. Any test or control substance, with three or more pools tested unexpectedly positive or negative during PCR verification were not used in this study.

4.0 Methods

4.1 Generation of Tissue Samples

Tissues of the test and control substances were collected during the 2007/2008 growing season from five field sites in Chile as follow: Quilapilum, CHACABUCO (site code QUI); Melipilla, MELIPILLA (site code MEL); Calera de Tango, MAIPO (site code CdT); Rancagua, CACHAPOAL (site code RAN); and San Fernando, COLCHAGUA (site code SFR), as described in production plan REG-07-170 and production report (Rowland, 2009). These field sites were representatives of soybean producing regions suitable for commercial production. At each site, three replicated plots of plants containing MON 87705, as well as the 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 OSL-1, OSL-2, OSL-3 OSL-4 samples were collected approximately at V3 – V4, V6 – V8, V10 – V12; and V14 – V16 stages, respectively. The forage and root were collected approximately at R6 stage. And the seed was collected at R8 stage. Throughout the field production, sample identity was maintained by using unique sample identifiers and proper chain-of-custody documentation. All tissues used in this study, except seed, were stored in a -80°C freezer prior to shipment. All tissue samples, except seed, were shipped on dry ice to the Monsanto processing facility in Saint Louis, Missouri and stored in a -80°C freezer. Seed samples were stored and shipped at an ambient temperature.

4.2 Tissue Processing and Protein Extraction Methods

4.2.1 Tissue Processing Method

Tissue samples were processed by the Monsanto Sample Management team. Processed tissue samples were transferred into tubes and stored in a -80°C freezer until shipped on dry ice to the analytical facility.

4.2.2 Extraction Methods

The CP4 EPSPS protein was extracted from soybean tissues as described in Monsanto Standard Operating Procedure (SOP) BR-ME-1087-01 and BR-ME-1087-02. Extraction parameters for each tissue type are described in Appendix 1. The CP4 EPSPS protein was extracted from all leaf, forage, root and seedtissues using a Harbil Mixer with the appropriate amount of Tris-borate buffer with L-ascorbic acid (TBA) [0.1 M Tris, 0.1 M $Na_2B_4O_7 \cdot 10H_2O$, 0.01 M $MgCl_2$, 0.05% (v/v) Tween-20 at pH 7.8, and 0.2% (w/v) L-ascorbic acid]. Insoluble material was removed from all tissue extracts using a serum filter (Fisher Scientific, Pittsburgh, PA). The extracts were aliquotted and stored frozen in a -80°C freezer until ELISA analysis.

4.3 ELISA Reagents and Methods

Protein extracts prepared from test and control substances were analyzed by a validated ELISA method to assess the CP4 EPSPS protein levels. Sample extraction and ELISA analysis of CP4 EPSPS protein levels were conducted according to SOP BR-ME-1087-01 and BR-ME-1087-02.

4.3.1 Anti-CP4 EPSPS Antibodies

Mouse monoclonal antibody clone 39B6.1 (IgG2a isotype, kappa light chain; lot 7022111) specific for the CP4 EPSPS protein was purified from mouse ascites fluid using Protein-A Sepharose affinity chromatography and was used as the capture antibody in the CP4 EPSPS ELISA. The concentration of the purified IgG was determined to be 2.3 mg/ml by spectrophotometric methods. Production of the 39B6 monoclonal antibody was performed by Strategic Biosolutions (Newark, DE). The purified antibody was stored in a buffer (pH 7.2) containing 20mM sodium phosphate, 150 mM sodium chloride, and 15 ppm Proclin 300 (Sigma-Aldrich, St. Louis, MO).

The detection reagent was goat anti-CP4 EPSPS antibody, otherwise known as anti-protein 4 (Sigma-Aldrich, catalog number P-5867) conjugated to HRP.

4.3.2 CP4 EPSPS ELISA Method

The CP4 EPSPS ELISA was performed manually according to SOP BR-ME-1087-01 and BR-ME-1087-02. Mouse anti-CP4 EPSPS antibody was diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 150 mM NaCl, pH 9.6) and immobilized onto 96-well microtiter plates at 2.0 µg/ml by dispensing 100 µl per well, followed by incubation in a 4°C refrigerator for greater than 12 h. Plates were washed 3 times with 1X phosphate buffered saline (PBS) with 0.05% (v/v) Tween-20 (1X PBST) followed by the addition of 100 µl per well of CP4 EPSPS

protein standard or sample extract and incubated at 37°C for 1 h. Plates were washed 3 times with 1X PBST, followed by the addition of 100 µl per well of goat anti-CP4 EPSPS peroxidase conjugate and incubated at 37°C for 1 h. Plates were washed 3 times with 1X PBST, and developed by adding 100 µl per well of HRP substrate, 3,3',5,5'- tetramethylbenzidine (TMB, Kirkegaard & Perry, Gaithersburg, MD). The enzymatic reaction was terminated by the addition of 100 µl per well of 6 M H_3PO_4 after 10 min development. Quantitation of CP4 EPSPS protein was accomplished by interpolation from a CP4 EPSPS protein standard curve that ranged in concentration from 0.456 – 14.6 ng/ml.

4.3.3 CP4 EPSPS ELISA Validation

Appendix 1 summarizes the results of validation of the ELISA used to assess the CP4 EPSPS protein levels in soybean tissues.

4.4 Control of Bias

The test and control substances were planted in three replicated plots arranged in a randomized block design at five field sites to minimize the bias as described in Production Plan REG-07-170. Representative tissues from each plot were collected as described in the production plans. All tissues 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 CP4 EPSPS protein standard and inter-assay negative and positive controls.

4.5 Moisture Analysis

4.5.1 Sample Preparation

One tissue-specific site pool (TSSP) was prepared for each tissue type per site. To prepare the TSSPs, comparable amounts (on a volumetric basis) from a minimum of 4 samples representing various test and control substances/site were mixed. A minimum of two grams of sample combined for each TSSP sample was prepared. The IDs of the samples used to prepare the TSSP were documented and a unique ID for each of the TSSP samples was assigned and archived with the raw data. All TSSP samples were stored in a -80°C freezer until analysis.

4.5.2 Moisture Analysis Methods

Leaf, seed, root and forage tissues were analyzed for moisture content according to SOP BR-ME-1238-01 using the IR-200 Infrared Moisture Analyzer (Denver

Instruments Company, Arvada, CO). The drying parameters used for seed tissues were the same as the drying parameters used for leaf tissues.

4.5.3 Determining Dry Weight Conversion Factor

The mean percent moisture for each TSSP was calculated from the three moisture analyses of a given pool. A TSSP Dry Weight Conversion Factor (DWCF) reported in two significant figures was established for each production as follows:

$$DWCF = 1 - [(Mean\ Percent\ TSSP\ Moisture)/100]$$

The sample mean, standard deviation (SD), and coefficient of variance were calculated and retained in the raw data for each TSSP.

4.5.4 Application of Dry Weight Conversion Factor (DWCF)

The DWCF was only applied to samples with protein quantities greater than or equal to the assay limit of quantitation (LOQ) on a fresh weight (fwt) basis. Mean protein quantities assessed on a fresh weight basis were converted into quantities reported in dry weight (dwt) using the following calculation:

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

4.6 Data Analyses

The CP4 EPSPS ELISA plates were analyzed on a SPECTRAmax Plus (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 nm. Data reduction analyses were performed using Molecular Devices SOFTmax PRO GxP version 5.0.1 software. 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 converted to a "µg/g fwt" basis. For all proteins, this conversion utilized a sample dilution factor and a buffer-to-tissue ratio. The protein values in "µg/g fwt" were also converted to "µg/g dwt" by applying the DWCF. Microsoft Excel 2007 (12.0.6324.5001) SP1 MSO (12.0.6320.5000) (Microsoft, Redmond, WA) was used to calculate the CP4 EPSPS protein levels in soybean tissues.

4.7 Protocol amendments

The protocol of the study was amended to clarify the VOIs of harvested seeds and the location of stability data in archive. These changes have no impact on the study.

5.0 Results and Discussions

5.1 CP4 EPSPS Protein Levels in MON 87705 Tissues

Summaries of mean, standard deviation (SD), and range of the CP4 EPSPS protein levels reported on µg/g fwt and µg/g dwt bases in MON 87705 soybean tissues are presented in Table 1. The mean protein levels of CP4 EPSPS in OSL-1, OSL-2, OSL-3, and OSL-4 tissues across five sites for MON 87705 were 200 ± 72, 530 ± 230, 220 ± 94, and 210 ± 92 µg/g dwt, respectively. Mean levels of CP4 EPSPS in forage, root, and seed tissues across five sites for MON 87705 were 120 ± 24, 77 ± 24, and 110 ± 44 µg/g dwt, respectively. The CP4 EPSPS levels in all control substances were less than LOD or LOQ.

Two OSL-2 samples of MON 87705 from CDT site and one OSL-2 sample of MON 87705 from QUI site had CP4 EPSPS levels that were less than the LOD in the ELISA analysis. These three samples were re-extracted twice (two separate extractions for each sample). Two separate extractions of each sample were reanalyzed by ELISA, whose results were still less than the CP4 EPSPS ELISA LOD. These samples were excluded from cross site calculations. The non-inclusion of three samples with unexpected results from fifteen total samples had no impact on the final conclusions of this study as there were sufficient samples of the tissue type to adequately represent the growing regions.

5.2 Stability of Test and Control Substances

All of the test and control substances were extracted and analyzed by ELISA within the time frame of verified tissue stability for the CP4 EPSPS protein.

6.0 Conclusions

MON 87705 was grown in Chilean field trials at five field sites in 2007/2008. Tissue samples were collected at various growth stages throughout the growing season and analyzed for CP4 EPSPS protein levels using a validated ELISA method. The mean CP4 EPSPS protein levels in OSL-1, OSL-2, OSL-3, OSL-4, forage, root, and seed tissues were 200, 530, 220, 210, 120, 77 and 110 µg/g dwt, respectively.

7.0 Acknowledgments

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Table 1. Summary of CP4 EPSPS Protein Levels in Leaf, Forage, Root, and Seed Tissues from MON 87705 Soybean Grown in 2007/2008 Chile Field Trial

Tissue Type[1] (sample size n)	LOQ/LOD (µg/g fwt)	CP4 EPSPS Protein Levels	
		Mean (SD)[2] Range[3] (µg/g fwt)[4]	Mean (SD)[2] Range[3] (µg/g dwt)[5]
OSL-1 (n=15)	0.57/0.26	36 (14) 16-65	200 (72) 84-340
OSL-2 (n=12)	0.57/0.26	110 (51) 60-230	530 (230) 290-1000
OSL-3 (n=19)	0.57/0.26	51 (21) 11-84	220 (94) 47-350
OSL-4 (n=15)	0.57/0.26	51 (21) 27-94	210 (92) 110-410
Forage (n=15)	0.57/0.10	32 (5.3) 22-40	120 (24) 77-160
Root (n=15)	0.57/0.11	24 (6.4) 14-34	77 (24) 41-120
Seed (n=15)	0.34/0.26	100 (39) 35-190	110 (44) 40-210

1. The OSL-1, OSL-2, OSL-3 OSL-4 samples were collected approximately at V3 – V4, V6 – V8, V10 – V12; and V14 – V16 stages, respectively. The forage and root were collected approximately at R6 stage. And the seed was collected at R8 stage.
2. The means and standard deviations were calculated for each tissue type across all sites. The "n" values for the calculated mean and standard deviations represent the number of analyses figured into the calculation.
3. Minimum and maximum values were determined each tissue type across all sites.
4. Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
5. Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a dry weight (dwt) basis. The dry weight values were calculated by dividing the µg/g fwt by the dry weight conversion factors obtained from moisture analysis data.

Appendix 1. CP4 EPSPS ELISA Validation Summary

I. Accuracy

Extraction Efficiency acceptance criteria = 70 – 100%
Spike and Recovery acceptance criteria = 70 – 130%

Soybean Tissue	Tissue-to-Buffer Ratio	Extraction Buffer	Extraction Efficiency (%)	Spike and Recovery (%)
Leaf	1:50	TBA	100	103
Seed	1:50	TBA	100	101
Root	1:50	TBA	100	96
Forage	1:50	TBA	100	95

Matrix Effects (Leaf, Forage, and Root): No matrix interferences (non-specific binding) noted after samples diluted at least 1:25.
Matrix Effects (Seed): No matrix interferences (non-specific binding) noted after samples diluted at least 1:15.
Matrix Effects acceptance criteria = 70 - 130%

Tissue Matrix	Average Percent Recovery
Leaf	99%
Seed	100%
Root	109%
Forage	105%

Parallelism: Parallelism exists, meaning that the plant produced CP4 EPSPS protein is immunologically equivalent to the *E. coli* produced CP4 EPSPS protein standard.
Parallelism acceptance criteria = 70-130%

Tissue Matrix	Average Percent Recovery Range
Leaf	94 – 105%
Seed	93 – 100%
Root	100 – 102 %
Forage	93 – 102%

II. Precision

Range of Quantitation: 0.456 – 14.6 ng/ml
Method Curve Fit: 4-parameter

Intra-Assay Precision: 2.9 % CV
Inter-Assay Precision: 13 % CV

III. Sensitivity

Limits of Quantitation and Detection:

Soybean Tissue	Matrix Dilution	LOD (ng/ml)	LOD (µg/g fwt)	LOQ (ng/ml)	LOQ (µg/g fwt)
Leaf	1:25	0.212	0.26	0.456	0.57
Seed	1:15	0.346	0.26	0.456	0.34
Root	1:25	0.091	0.11	0.456	0.57
Forage	1:25	0.079	0.10	0.456	0.57

IV. QC Range

QC (+) Range (ng/ml)	QC (-) Range (ng/ml)
2.983 to 6.589	<LOQ

V. Other Assay Criteria

Criteria	Value/Range
Mean absorbance of the buffer blank: (L_1) + 3 SD	≤0.066
Mean absorbance of the highest standard with blank subtracted: (L_1) - 3 SD	≥1.589
% residual of back-calculated concentration of the standards	• **0.913-14.6 ng/ml standards:** ≤15.0% • **0.456 ng/ml standard:** ≤25.0%
R^2 of the standard curve	≥0.98

Appendix 2. Standard Operating Procedures

BR-ME-1087-01	Extraction and Direct ELISA Analysis of CP4 EPSPS Protein in Soybean Tissues Using the Tecan Genesis Workstation
BR-ME-1087-02	Extraction and Direct ELISA Analysis of CP4 EPSPS Protein in Soybean Tissues
BR-ME-1238-01	Analysis of Moisture Content Using the Denver Instrument IR-200 Moisture Analyzer