

Report Title

Assessment of CP4 EPSPS Protein Levels in Canola Pollen Tissues from MON 88302
Produced in United States Greenhouse Trials during 2010

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Study Completed On

September 30, 2011

Sponsor

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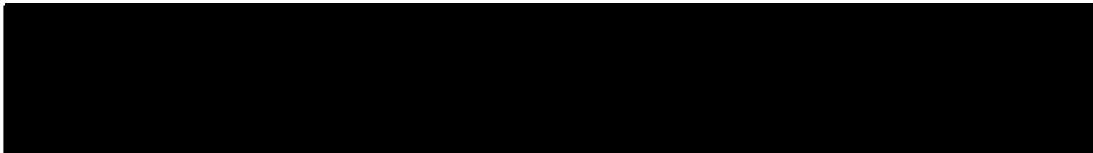
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Statement of Compliance

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

Submitter

Date



Sponsor Representative



Study Director

Quality Control Statement

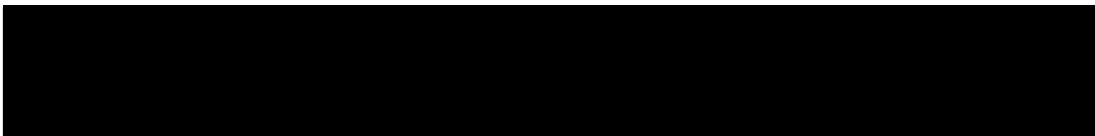
Study Title: Assessment of CP4 EPSPS Protein Levels in Canola Pollen
Tissues from MON 88302 Produced in United States Greenhouse
Trials during 2010

Study Number: RAR-2011-0427

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
09/21/2011	Report and Data Audit	09/21/2011	09/26/2011

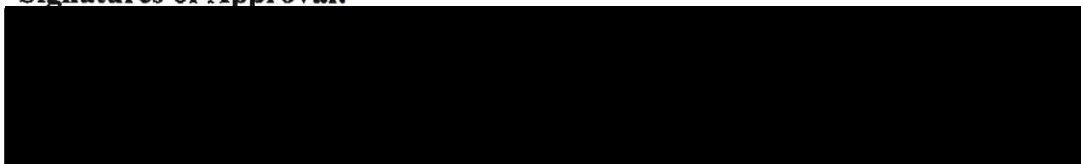


Monsanto Regulatory, Monsanto Company

Study Certification

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

Signatures of Approval:



Study Director

Study Information

Study Number: RAR-2011-0427

Report Number: MSL0023598

Report Title: Assessment of CP4 EPSPS Protein Levels in Canola Pollen Tissues from MON 88302 Produced in United States Greenhouse Trials during 2010

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Study Initiation Date: September 1, 2011

Study Completion Date: September 30, 2011

Records Retention: The protocol, all raw data, documentation, records, and the final report for this study are retained at Monsanto Company.

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Abbreviations, Acronyms, and Definitions

BBCH	Bayer, BASF, Ciba-Geigy and Hoechst cereal grain growth scale
CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase derived from <i>Agrobacterium</i> sp. strain CP4
CV	coefficient of variation
DWCF	dry-weight conversion factor
dwt	dry-weight of tissue
<i>E. coli</i>	<i>Escherichia coli</i>
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
fw	fresh-weight of tissue
GRR	Guidelines for Research Records
HRP	horseradish peroxidase
IgG	Immunoglobulin G
LOQ	limit of quantitation
n	number of samples
PBST	phosphate-buffered saline containing Tween-20
PCR	polymerase chain reaction
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOP	standard operating procedure
SQT	Seed Quality Technologies Group
TBA	Tris-Borate with L-Ascorbic Acid
TMB	3, 3', 5, 5'- tetramethylbenzidine
Tris	tris(hydroxymethyl)aminomethane
TSSP	tissue-specific site pool
QAU	Quality Assurance Unit

1.0 Summary

Monsanto Company has developed a second-generation glyphosate-tolerant canola product, MON 88302, designed to provide growers with improved weed control through greater flexibility for glyphosate herbicide application. MON 88302 produces the same 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein that is produced in commercial Roundup Ready[®] crop products, via the incorporation of a *cp4 epsps* coding sequence. The CP4 EPSPS protein confers tolerance to the herbicide glyphosate, the active ingredient in the family of Roundup[®] agricultural herbicides. The purpose of this study was to determine the level of CP4 EPSPS protein in canola pollen tissue of MON 88302 grown in United States greenhouse trial under Production Plan PPN-10-285.

The expression levels of CP4 EPSPS protein were determined by enzyme-linked immunosorbent assay (ELISA) in tissues collected from MON 88302 produced in a U.S. greenhouse trial during 2010 with three plots under Production Plan PPN-10-285. The protein level for pollen tissue was calculated on a microgram (µg) per gram (g) fresh weight (fwt) basis. Moisture content was then measured and the protein level was converted and reported on a dry weight (dwt) basis.

The mean CP4 EPSPS protein level in MON 88302 pollen across the three plots was 9.0 µg/g dwt.

2.0 Introduction

2.1 Background

Monsanto Company has developed a second-generation glyphosate-tolerant canola product, MON 88302, designed to provide growers with improved weed control through greater flexibility for glyphosate herbicide application. MON 88302 produces the same 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein that is produced in commercial Roundup Ready crop products, via the incorporation of a *cp4 epsps* coding sequence. The CP4 EPSPS protein confers tolerance to the herbicide glyphosate, the active ingredient in the family of Roundup agricultural herbicides.

The CP4 EPSPS protein level was determined in canola pollen tissue produced in a U.S. greenhouse during 2010. Canola was planted in a three replicate, randomized, complete block design.

2.2 Purpose

The purpose of this study was to determine the level of CP4 EPSPS protein in canola pollen tissue of MON 88302 grown in a 2010 U.S. greenhouse trial under Production Plan PPN-10-285.

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3.0 Materials

3.1 Test and Reference Substances

3.1.1 Test Substance

Tissue samples from MON 88302 were harvested from a U.S greenhouse trial during 2010 from plants grown from starting seed lot 11266363.

3.1.2 Control Substance

Tissue samples from a conventional control were harvested from a U.S greenhouse trial during 2010 from plants grown from starting seed lot 11266676.

3.1.3 Characterization of Test Substance

The identities of the test and control substances were confirmed by analysis of the leaf DNA extracted from individual plant leaf punches using an endpoint Taqman[®] PCR analysis method. The molecular identity analysis was performed by the Seed Quality Technologies Group (SQT). The results were stored in the scoring tools of the SQT group under the request ID and a copy of the results were archived with this study data. Any plant that tested unexpectedly was excluded from the study.

3.1.4 Reference Substances

An *Escherichia coli* (*E. coli*)-produced CP4 EPSPS protein standard (lot 10000739) was used as the reference substance for analysis of CP4 EPSPS protein levels. The total protein concentration of the standard was determined by amino acid analysis. The purity was 97% as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometric analysis. The purity-corrected protein concentration of the standard was 3.7 mg/ml.

4.0 Methods

4.1 Generation of Plant Samples

4.1.1 Summary of Field Design

The greenhouse trial was initiated during 2010 to generate MON 88302. Pollen was collected from three plots planted in a randomized complete block field design. Test tissue samples were collected from each replicated plot while the control samples were collected from only one plot.

4.2 Tissue Processing and Protein Extraction Methods

4.2.1 Processing Method

All tissue samples harvested were shipped to Monsanto's Sample Management Team on dry ice. The tissue samples were stored in a -80 °C freezer until transferred on dry ice to the analytical facility.

4.2.2 Extraction Methods

CP4 EPSPS protein was extracted from the pollen tissue samples as described in the current version of SOP AG-ME-1362 following the leaf extraction parameters

listed in the table below. The extracts were aliquotted and stored frozen in a -80 °C freezer until ELISA analysis.

MON 88302 Extraction Parameters¹

Sample Type	Tissue-to-Buffer Ratio	Extraction Buffer ²
Pollen	1:100	1× TBA

¹CP4 EPSPS protein was extracted from each tissue by adding the appropriate volume of CP4 EPSPS extraction buffer, and shaking in a Harbil mixer. The extracted sample was clarified using a serum filter.

²Tris-borate buffer with L-ascorbic acid (1× TBA) [0.1 M Tris, 0.1 M Na₂B₄O₇, 0.005 M MgCl₂, 0.05% (v/v) Tween-20 at pH 7.8, 0.2% (w/v) L-ascorbic acid]

4.3 ELISA Reagents and Methods

4.3.1 CP4 EPSPS Antibodies

Mouse monoclonal antibody clone 39B6.1 specific for the CP4 EPSPS protein was purified from mouse ascites fluid using Pharmacia Protein-A affinity first flow chromatography. The concentration of the purified IgG was determined to be 5.9 mg/ml by spectrophotometric methods. Production of the 39B6.1 monoclonal antibody was performed by Strategic Diagnostics Inc (SDIX, Newark, DE). The purified antibody was stored in a buffer (0.02 M NaH₂PO₄, 0.15 M NaCl, and 0.05% NaN₃).

The detection reagent was goat anti-CP4 EPSPS antibody lot G-857792, otherwise known as anti-protein 4 (Sigma-Aldrich, catalog number P-5867) conjugated to horse radish peroxidase (HRP).

4.3.2 CP4 EPSPS ELISA Method

The CP4 EPSPS ELISA was performed according to the current version of SOP AG-ME-1362. Mouse anti-CP4 EPSPS capture antibody was diluted in coating buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, and 0.15 M NaCl) and immobilized onto 96-well microtiter plates at 2 µg/ml followed by incubation in a 4 °C refrigerator for ≥8 h. Prior to each step in the assay, plates were washed with 1× phosphate-buffered saline (PBS) containing 0.05% Tween-20 (1 × PBST). CP4 EPSPS protein standard or sample extract was added at 100 µl per well and incubated for 60 to 70 minutes at 37 °C. Goat anti-CP4 EPSPS HRP conjugate was added at 100 µl per well and incubated for 60 to 70 minutes at 37 °C. Plates were developed by adding 100 µl per well of 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 6 M H₃PO₄. Quantification of the

CP4 EPSPS protein was accomplished by interpolation on a CP4 EPSPS protein standard curve that ranged from 0.456-14.6 ng/ml.

4.4 Moisture Analysis

Moisture content was determined using an IR-200 Moisture Analyzer (Denver Instrument Company, Arvada, CO) according to the current version of SOP BR-ME-1238. The drying parameters used are documented and archived with the study. A homogeneous tissue-specific site pool (TSSP) was prepared consisting of pollen samples grown in the greenhouse under Production Plan PPN-10-285. The average percent moisture for the TSSP was calculated from triplicate analyses. A TSSP Dry Weight Conversion Factor (DWCF) was calculated as follows:

$$\text{DWCF} = 1 - \left(\frac{\text{Mean \% TSSP Moisture}}{100} \right)$$

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:

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

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

4.5 Data Analyses

CP4 EPSPS ELISA plates were analyzed on a SPECTRAmax Plus 384 (Molecular Devices, Sunnyvale, CA) microplate spectrophotometer, using a dual wavelength detection method. 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 5.4 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 reported on a “µg/g fwt” basis for data that were greater than or equal to the LOQ. 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. Microsoft Excel 2007 (Microsoft, Redmond, WA) was used to calculate the CP4 EPSPS protein levels in canola pollen tissue. The sample mean, standard deviation (SD), and range was also calculated by Microsoft Excel 2007. All protein expression levels were rounded to two significant figures as described in AG-BP-1098.

4.6 Quality Measures

All data generated was reviewed by the Quality Assurance Unit (QAU) to ensure adherence to the protocol and study reconstructability. All raw data were

documented in accordance with Monsanto's Guidelines for Research Records (GRRs), and all study data will be retained in Monsanto's Regulatory archives. All of these measures were taken to ensure the integrity of the study.

5.0 Results

The across-plot mean, standard deviation (SD), and range are reported in Table 1 for CP4 EPSPS protein levels on a $\mu\text{g/g}$ fwt and $\mu\text{g/g}$ dwt basis in canola pollen tissue collected during 2010 from a U.S. greenhouse trial.

5.1 CP4 EPSPS Protein Levels in MON 88302

The mean CP4 EPSPS protein level in MON 88302 was 9.0 $\mu\text{g/g}$ dwt in pollen.

5.2 Stability of Test Substances

Tissue storage stability for CP4 EPSPS in canola pollen tissue has not been determined.

6.0 Conclusions

MON 88302 was grown in a U.S. greenhouse trial during 2010. Tissue samples were collected and analyzed for CP4 EPSPS protein levels using an ELISA method. These data provides an estimation of the protein levels for CP4 EPSPS protein on a fresh weight and dry weight basis in canola pollen tissue.

Table 1. Summary of CP4 EPSPS Protein Levels in Pollen Tissue Collected from MON 88302 Produced in a 2010 U.S. Greenhouse

Tissue Type	Development Stage¹	Mean (SD) Range (µg/g fwt)²	Mean (SD) Range (µg/g dwt)³	LOQ (µg/g fwt)⁴
Pollen	60-69 BBCH Growth Stage	8.1 (0.64) 7.4 –8.6	9.0 (0.71) 8.2 –9.6	0.91

¹The crop development stage at time of pollen collection.

²Protein levels are expressed as the arithmetic mean and standard deviation (SD) in microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fwt). The mean, SD, and range (minimum and maximum values) was calculated for pollen across all plots (n=3).

³Protein levels are expressed as the arithmetic mean and standard deviation (SD) in microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dwt). The dry weight values were calculated by dividing the µg/g fwt by the dry weight conversion factor obtained from moisture analysis data.

⁴LOQ=limit of quantitation.

Appendices

Appendix 1. Standard Operating Procedures

AG-ME-1362-01	Extraction and Direct ELISA Analysis of CP4 EPSPS in Canola Tissues
AG-BP-1098- 01	Significant Figures and Rounding in Data
BR-ME-1238-01	Analysis of Moisture Content Using the Denver Instrument IR-200 Moisture Analyzer