Appendix 11

Analysis of Expression Levels of Arabidopsis Acetohydroxyacid Synthase (AHAS) Protein, by ELISA, in the Cultivance Soybean Event 127, Plants Grown in Brazilian Field Trials during the Summer 2006/2007 Season.

BASF
The Chemical Company

Ensaios BPL
CLA 0009

GLOBAL ENVIRONMENTAL AND CONSUMER SAFETY LABORATORY

Report Number

RF-1247-07

Report Title

Analysis of Expression Levels of Arabidopsis Acetohydroxyacid Synthase (AHAS) Protein, by ELISA, in the Cultivance Soybean Event 127, Plants Grown in Brazilian Field Trials during the summer 2006/2007 season.

Field Experiment Numbers

CVSOY-06-002-008
CVSOY-06-002-010
CVSOY-06-002-011
CVSOY-06-002-012
CVSOY-06-002-013
CVSOY-06-002-014
CVSOY-06-002-016

Author

[redacted]

Study Completion Date

November 13, 2007

Sponsor

BASF Plant Science, L.L.C.
Regulatory Affairs Manager
26 Davis Drive
Research Triangle Park, North Carolina, USA
27709
Phone: [redacted]
Fax: [redacted]

Test Facility

BASF S.A.
Global Environmental and Consumer Safety
Laboratory - GENCS
Avenida Brasil, 791 – C10/C20
Bairro Engenheiro Neiva
Guaratinguetá – São Paulo – Brasil
CEP: 12521-000
Phone.: [redacted]
Fax: [redacted]

Were issued two Final Reports, being this the Number 02.

CERTIFICATES

a. Swiss Federal Office of Public Health

The Swiss GLP Monitoring Authorities

Swiss Federal Office of Public Health
Swiss Agency for the Environment, Forests and Landscape
swissmedic Swissmedic Swiss Agency for Therapeutic Products

Statement of GLP Compliance

It is hereby confirmed that

during the period of April 4 - 6, 2005

the following Test Facility of **BASF**

27.537-000 Resende,RJ
Brazil

was inspected by the Federal Office of Public Health with respect to the compliance with the Swiss legislation on Good Laboratory Practice.

Test Facility	**Areas of expertise**
Laboratório Agro de Resíduos da America Latina (LARAL)	**Residue studies (analytical part)**

The inspection was performed in agreement with the OECD Guidelines for National GLP Inspections and Audits. It was found that the aforementioned test facility was operating in compliance with the Swiss Ordinance relating to Good Laboratory Practice [RS 813.016.5] at the time it was inspected.

Swiss Federal Office of Public Health
The Director

[redacted]

Berne, June 2005 Prof. [redacted]

Note: *The new test facility GENCS has been inspected by the SFOPH GLP monitoring unit from Switzerland. The compliance with GLP principles was certified, nevertheless the new certificate has not been issued yet, therefore this one is still being used.*

República Federativa do Brasil
Ministério do Desenvolvimento, Indústria e Comércio Exterior
Instituto Nacional de Metrologia, Normalização e Qualidade Industrial – Inmetro

Coordenação Geral de Acreditação

Certificado de Acreditação

Acreditação n° CLA 0009

Acreditação inicial: 14-08-2001

LABORATÓRIO GLOBAL DE MEIO AMBIENTE E SEGURANÇA ALIMENTAR
GLOBAL ENVIRONMENTAL & CONSUMER SAFETY LABORATORY/GENCS
BASF S. A.
AVENIDA BRASIL, 791 - ENGENHEIRO NEIVA - GUARATINGUETÁ - SP

A Coordenação Geral de Acreditação do Inmetro - CGCRE/INMETRO - concede acreditação ao Laboratório acima identificado, segundo os requisitos estabelecidos na NIT-DICLA-028. Rev.01 e NIT-DICLA-034, Rev. 00 Esta acreditação constitui a expressão formal do reconhecimento da sua competência para realizar os estudos constantes no Escopo de Acreditação.

Marcos Aurélio Lima de Oliveira
Coordenador Geral de Acreditação

Emissão: 17-07-2007

Validade: 14-08-2009

IDENTIFICATION AND SIGNATURES OF PERSONNEL INVOLVED IN THE STUDY

13/11/2007
dd / mm / yyyy

[redacted] Cabral [redacted]
Study Director*

13/11/2007
dd / mm / yyyy

[redacted]
Analist *

13 11 2007
dd / mm / yyyy

[redacted]
Test Facility Management*

29/11/2007
dd / mm / yyyy

[redacted]
Sponsor Representative*

* Address: Test Facility and Sponsor (see front page).

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

I, the undersigned, declare that the Study identified as 1247-07 entitled "Analysis of Expression Levels of Arabidopsis Acetohydroxyacid Synthase (AHAS) Protein, by ELISA, in the Cultivance Soybean Event 127, Plants Grown in Brazilian Field Trials during the summer 2006/2007 season" , carried out in accordance with the OECD Guidelines (Accredited by Swiss Federal Office of Public Health) and NIT-DICLA-028 Rev.01 (Accredited by Brazilian National Metrology Institute – Inmetro) Principles of Good Laboratory Practice (GLP) being it reproducible.

I hereby declare that this Study was performed under my supervision according to the procedures described herein, and that this Report presents a true and accurate record of the results obtained.

The results obtained in the GENCS Study 1247-07 and described on this Report RF-1247-07 are applied only to the test system identified in 2.1.4.

Field Report and sampling procedures were the responsibility of BASF Plant Science Technical Department.

The original Final Report, Number 02, was sent to the Sponsor.

[redacted]

13 / 11 / 2007
dd mm yyyy

Chemistry Bachelor
CRQ-RJ Number 3112125
Study Director*
Phone: [redacted]

* Address: Test Facility (see front page).

QUALITY ASSURANCE UNIT STATEMENT

This Final Report was inspected by the Quality Assurance Unit (QAU) of Global Environmental and Consumer Safety Laboratory (GENCS) in accordance with the OECD Guidelines (Accredited by Swiss Federal Office of Public Health) and NIT-DICLA-028 Rev.01 (Accredited by Brazilian National Metrology Institute – INMETRO) Principles of Good Laboratory Practice (GLP).

The dates of inspections made by the QAU, including the phases inspected, and the dates any inspection results were reported to Management and Study Director are listed below:

		Dates of Inspection (*dd/mm/yyyy*)	Report of Inspection Results (*dd/mm/yyyy*)
Study Plan		06 / 08 / 2007	06 / 08 / 2007
Laboratory Phase(s) Inspected	Preparation of Standard Stock Solution	07 / 08 / 2007 (Study 1244-07)	07/08/2007
	Weighing of samples	13/ 08 / 2007	14 / 08 / 2007
	Clean-up	13-14/08/2007	14/08/2007
	Quantification	14-20/08/2007	20/08/2007
Raw Data		16-18/10/2007	22/10/2007
Final Report		19/10/2007	22/10/2007

Receipt, Storage and Samples Preparation (processing with dry ice) inspections are verified by process-based, being the data available at the Quality Assurance Unit archives.

Records of all inspections performed by the Quality Assurance Unit are retained in the GENCS archives for at least thirty years after approval of the Final Report.

The data presented in this Final Report reflect exactly the Raw Data generated during the conduction of the study 1247-07. The Raw Data were inspected by QAU, according to the Standard Operating Procedure SOP-GE.018.

[redacted]

13 / 11 / 2007
dd mm yyyy

[redacted]
Chemical Technician
QAU Leader*
CRQ-RJ Number 03417656
Quality Assurance Unit
Phone: [redacted]

* Address: Test Facility (see front page).

ARCHIVES

The Study Plan and the Final Report, Number 01, will be stored at Global Environmental and Consumer Safety Laboratory (GENCS) Central Archives, at least for 30 years after approval of the Final Report.

Retention Soybean samples are kept in appropriate GENCS -80°C Freezer, until the Sponsor approves its disposal.

Reference Items are stored at GENCS, under the appropriate conditions in order to guarantee their stability, until their expiration date.

[redacted]

13 / 11 / 2007
dd mm yyyy

Chemistry Bachelor
CRQ-RJ Number 3112125
Study Director*
Phone: [redacted]

* Address: Test Facility (see front page).

GUIDELINES COVERED

- INMETRO NIT-DICLA-028 Rev.01 (Sep/2003), Brazilian Normative.
- OECD Principles of Good Laboratory Practice, revised on 1997.

ABSTRACT

Soybean (*Glycine max L.*) plants have been developed that are tolerant to the imidazolinone class of agricultural herbicides. The herbicide-tolerant soybean plants were derived from a single transformation event, referred to as Cultivance Soybean Event 127 (CV Soy 127), that was produced by the introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*csr1-2*) gene from Arabidopsis thaliana into the soybean plant genome under the control of its endogenous promoter. The level of acetohydroxyacid synthase (AHAS) protein in different tissues of both CV Soy 127 and a closely related conventional (nontransgenic) variety grown at two different locations in Brazil during the 2006/2007 growing season was measured by enzyme-linked immunosorbent assays (ELISA). The tissues analyzed included whole plants, first trifoliate, leaves, roots, pod, grain and flowers. The developmental stages which were examined were the V2 (emergence of the second trifoliate), R2 (full bloom) and R8 (maturity). Plants were harvested and shipped overnight on wet ice to GENCS where they were separated into parts and processed prior to analysis. In addition, the level of AHAS in young leaves from the V2 stage and grain from plants grown during the 2006/2007 growing season at seven different locations in Brazil was quantified.

Samples were analyzed according to SOP-PA.0273:"Determination of Protein Residues in Vegetal Tissues," based on Methods BASF SOP No. BPS 510.06.00 and SOP No. BPS 510.16.00. This method quantified the total amount of AHAS and it does not distinguish between the endogenous and modified AHAS. The limit of detection for the AHAS ELISA was determined experimentally to be 3 ng/g fresh weight of tissue. The lower limit of quantification (LOQ) was determined experimentally based on the calibration curve to be 13 ng/g fresh weight of tissue. Fortification experiments were conducted to determine the dilution recovery of the method using concentrations corresponding to 51 – 510 ng/g fresh weight of tissue and the overall recoveries (n=55) were 85% with a coefficient of variation of 17% for the soybean tissues analyzed. Furthermore, the extraction efficiency of the method was performed for all soybean tissues by monitoring total protein by BCA analysis and for AHAS, specifically, in tissues containing quantifiable levels. AHAS extraction efficiency was found to be approx. 97, 90 and 63 % for soybean whole plants (V2 stage), leaves (V2 stage) and pods, respectively.

In general, the levels of AHAS protein in leaf tissues was found to be very low and in the majority of the samples was below the LOQ. Cultivance Soybean Event 127 leaf samples from the V2 stage had AHAS levels between 53 and 128 ng AHAS/g fresh wt., corresponding to 335 and 714 ng AHAS/g dry weight. CV 127 whole plants from the V2 stage had 40 – 61 ng/g fresh wt. corresponding to 214 -314 ng AHAS/g dry wt. AHAS levels in the CV 127 first trifoliate leaf from the V2 stage ranged from 55 – 60 ng AHAS/g fresh wt. or 278 – 300 ng AHAS/g dry wt. In R2 the highest level of AHAS protein in leaf tissues was 24 ng AHAS/g fresh wt. or 106 ng AHAS/g dry wt. Except for non-GMO leaf tissues in V2 that showed 14-16 ng AHAS/g fresh wt or 73-86 ng AHAS/g dry wt., all other leaf samples from CV Soy 127 or from the conventional variety, the AHAS protein was barely detectable at levels lower than the LOQ. Using the mean levels of AHAS found in whole plants from Santo Antonio de Posse and Londrina or the LOQ when the AHAS level was too low to be quantified, the overall amount of AHAS present per hectare, assuming 260,000 plants per hectare, was estimated for the three developmental stages examined. The highest level was found at the R8 stage at < 355 mg AHAS/hectare (<144 mg AHAS/acre). At the R2 stage the value was estimated to be < 331 mg AHAS/hectare (<134 mg AHAS/acre) and at the V2 stage 82 mg AHAS/hectare (33 mg AHAS/acre).

STUDY SCHEDULE

Study Initiation Date:	August 09th, 2007	Experimental Completion Date:	August 30th, 2007
Experimental Starting Date:	August 10th, 2007	Study Completion Date:	November 13th, 2007

TABLE OF CONTENTS

LIST OF TABLES

ABBREVIATIONS AND DEFINITIONS

ahasl	Imidazolinone-tolerant acetohydroxyacid synthase large subunit gene
AHASL	Acetohydroxyacid synthase large subunit
BCA	Bicinchonic Acid
BSA	Bovine Serum Albumin
csr1-2	*Arabidopsis thaliana* acetohydroxyacid synthase large subunit gene containing a single mutation (Ser653Asn) which confers resistance to imidazolinone herbicides
ELISA	Enzyme linked immunosorbent assay
S653N	Serine residue at position 653 of *Arabidopsis thaliana* acetohydroxyacid synthase large subunit replaced with asparagines
STDC	Standard of Calibration Solution
STDF	Standard of Fortification Solution
STDS	Standard Stock Solution
TBST	Tris buffered saline with Tween

1. INTRODUCTION

Soybean (*Glycine max* L.) plants have been developed that are tolerant to the imidazolinone class of agricultural herbicides. The herbicide-tolerant soybean plants, referred to as Cultivance Soybean Event 127, were produced by introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*ahasl*) gene from *Arabidopsis thaliana* into the soybean plant genome via biolistics. Acetohydroxyacid synthase (AHAS) is a key enzyme in plants, bacteria and fungi that is required for the biosynthesis of the branched chain amino acids valine, leucine, and isoleucine. Herbicides of the imidazolinone class function by binding near the active site of the catalytic AHAS large subunit, thereby preventing normal functioning of the enzyme (Pang et al., 2002). Several AHAS genes encoding AHAS enzymes that are tolerant to imidazolinone herbicides have been discovered in plants through mutagenesis and selection and have been used to create imidazolinone-tolerant maize (*Zea mays* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), oilseed rape (*Brassica napus* and *B. juncea* L.), and sunflower (*Helianthus annuus* L.). These crops were developed through mutagenesis, selection, and conventional breeding technologies and have been commercialized under the Clearfield® brand name since 1992. There are five single point mutations in AHAS genes that have been found to result in tolerance to imidazolinones in plants (Tan et al., 2005). One of these, a mutation that results in a substitution of a serine residue with an asparagine at position 653 (relative to the AHAS enzyme from *Arabidopsis thaliana*), is known to result in good tolerance to imidazolinone herbicides with no cross-tolerance to other AHAS inhibitors (Lee et al., 1999). The imidazolinone-tolerant AHAS large subunit *csr1-2* gene (Sathasivan et al., 1990) from *Arabidopsis thaliana* that has the S653N mutation was transformed into soybean (*Glycine max* L.) plants with the native *A. thaliana* promoter to create soybean plants that are tolerant to imidazolinone herbicides. This has led to the development by BASF and Embrapa of a genetically modified soybean named Cultivance® Soybean Event 127 (CV Soy 127). BASF is seeking regulatory approvals for CV Soy 127 from the regulatory authorities in appropriate countries worldwide.

To assess the range of expression of the transgenic protein in imidazolinone tolerant soybean plants derived from Cultivance Soybean Event 127 as compared to non-transgenic isoline Conquista, the amount of AtAHAS protein was determined in the GENCS Study 1247-07 by enzyme-linked immunosorbent assays (ELISAs) in the following samples:

1) various plant tissues and whole plants at three developmental stages from two field locations in Brazil from the 2006/2007 growing season. Based on these results the level of AHAS present on a per-acre and per hectare basis were also estimated for the various developmental stages.

2) young leaves (V2 stage) from five field locations from the 2006/2007 growing season.

3) grain harvested from six field locations from the 2006/2007 growing season.

The results obtained in this study will be used as part of the regulatory dossier used to obtain approval worldwide.

2. MATERIALS AND METHOD

2.1 Field Procedures

Detailed information of the test system and the trials can be found in the sections below.
The field portions of this study were not conducted under GLP and was the responsibility of BASF Plant Science. However the trails were conducted under Good Agricultural Practice (GAP).
The field experimental phase was described in the Experimental plan CV-SOY-06-002, submitted to the Brazilian Authorities (CTNBio) by BASF Plant Science. This section provides a brief summary of this phase.

Field Technicians	*Field Stations*
Danilo Antônio Rinaldi Cel: (43)9113-2519 Email: dnrinaldi@yahoo.com.br	SNT – EMBRAPA Biasness Office of Ponta Grossa Address: Rodovia do Talco, km 03 – Distrito Industrial Caixa Postal 97, CEP 84001-970 – Ponta Grossa, PR
	CNPSo – EMBRAPA Soybean Address: Rodovia Carlos João Strass – Acesso Orlando Amaral – Caixa Postal 231 – CEP 86001-970 – Londrina, PR
	BASF EEA – BASF Agricultural Experimental Field Station Address: Rodovia SP 340, km 144 – Sítio São João Quinhão – Caixa Postal 42 – CEP 13830-000 – Santo Antonio de Posse, SP
Nelson Orasmo Filho Cel: (34) 9168-0134 Email: nofilho@hotmail.com	EPAMIG CTTP – Technological Center of Triângulo e Alto Paranaíba –Experimental Farm Getulio Vargas Address: Rua Afonso Rato, 1301 – Caixa Postal 351 – CEP 38001-970 – Uberaba, MG
	CNPMS – EMBRAPA Corn and Sorgo Address: Rodovia MG 424, km 65, Caixa Postal 151 – CEP: 35701-970 – Sete Lagoas, MG
Catarina Villas de Souza Cel: (61)9214-3244 Email: catarinavillas@brturbo.com.br	CNPAF – EMBRAPA Rice and Beans Address: Rodovia Goiânia/Nova Veneza, km 12 – Fazenda Capivara – Caixa Postal 179 – CEP: 75375-000 – Santo Antonio de Goiás, GO
	EMBRAPA CNPH Address: Br 060 km 09 – Rodovia Brasília/Anápolis – Caixa Postal 218 – CEP 70359-970 – Brasília, DF

2.1.1 Field Specimen List

Location	*Experiment number*	*Plot number*	*Specimen Description*
EMBRAPA SNT, Ponta Grossa, PR	CVSOY-06-002-008	*21 and 23*	*GMO, Cultivance soybean*
		22 and 24	non *GMO, control soybean*
EMBRAPA CNPSo, Londrina, PR	CVSOY-06-002-010	*21 and 23*	*GMO, Cultivance soybean*
		22 and 24	non *GMO, control soybean*
BASF EEA, Sto. Antonio de Posse, SP	CVSOY-06-002-011	*21 and 23*	*GMO, Cultivance soybean*
		22 and 24	non *GMO, control soybean*
EPAMIG CTTP, Uberaba, MG	CVSOY-06-002-012	*21 and 23*	*GMO, Cultivance soybean*
		22 and 24	non *GMO, control soybean*
EMBRAPA CNPMS, Sete Lagoas, MG	CVSOY-06-002-013	*21 and 23*	*GMO, Cultivance soybean*
		22 and 24	non *GMO, control soybean*
EMBRAPA CNPAF, Santo Antonio de Goiás, GO	CVSOY-06-002-014	*21 and 23*	*GMO, Cultivance soybean*
		22 and 24	non *GMO, control soybean*
EMBRAPA CNPH, Brasília, DF	CVSOY-06-002-016	*21 and 23*	*GMO, Cultivance soybean*
		22 and 24	non *GMO, control soybean*

2.1.2 Field Experimental Design

Country	*Plot area (≥)*	*Minimum Distance between plots (borders)*	*Plants/Hectare*
Brazil	**16 m^2**	**2,0 m**	**260.000**

2.1.3 Trial Treatment

Each trial location had two replications where each one contained one plot of the imidazolinone-tolerant Cultivance Soybean Event 127 and one plot of an imidazolinone-sensitive near isogenic control soybean. All plots of the Cultivance Soybean Event 127 were sprayed with an imidazolinone herbicide. All plots of the imidazolinone-sensitive control were treated with conventional herbicides as necessary to control weeds.

2.1.4 Sampling/ Test System Details

The samples destined to be analyzed for AHAS protein levels were collected at three different growth stages from the different locations as summarized below:

Growth Stage	Location	Samples Collected
V2	- EEA Sto. Antonio de Posse, SP - CNPSo , Londrina, PR	- Whole Plants - 1st Trifoliates
	- Other 5 locations	- Leaves
R2	- EEA Sto. Antonio de Posse, SP - CNPSo , Londrina, PR	- Whole Plants
R8	- EEA Sto. Antonio de Posse, SP - CNPSo , Londrina, PR	- Whole Plants
	- All locations, except EMBRAPA SNT, Ponta Grossa, PR	- Grains

Depending on the kind of tissue to be analyzed, the samples of the test system, Cultivance Soybean Event 127, and conventional control soybean variety, were collected and separated into parts, when desired, as described below:

Leaves from all sites

Six plants per plot from each location were collected at the V2 stage, placed in a single sample bag and shipped to GENCS. Upon receipt, the leaves were removed and pooled to generate one sample per plot for processing, extraction and analysis.

Whole Plants from two locations

Six whole plants per plot, including roots, were collected from two locations, Santo Antonio de Posse and Londrina, at three different developmental stages, V2 (emergence of second trifoliate), R2 (beginning pod set) and R8 (maturity). Each whole plant was shipped in a separate, labeled sample bag. Upon receipt, the plants from three samples from each location were separated into parts (leaves, stems, roots, flowers and pods when present). The different parts from each individual plant were treated as a single sample, with the following exceptions. There was insufficient flower tissue in the R2 Stage and root tissue in the V2 Stage to analyze by individual plant. Therefore, the roots of three plants per plot in V2 were kept together as were the flowers in R2 to get a representative amount of sample for analysis. The whole plants from the other three samples were processed as whole plants and each individual plant comprised a single sample.

Grains

A minimum of 500 g of seed from each plot at two locations, Santo Antonio de Posse and Londrina, were shipped in sample bags to GENCS. These were processed as one sample per plot.

Packing of Samples:

All samples were clearly identified and securely packed, in order to assure the traceability and integrity of the samples.

Transport of samples

The samples were shipped overnight on wet ice in styrofoam boxes and received by GENCS within 24 hours (maximum) after sampling.

Receiving and Storage of Samples:

The soybean samples received by GENCS, detailed in the following table, were stored in freezers at (– 80 ± 6°C). Each field trial received an entrance register for protein expression (REEP) number as listed below. After being logged in, the samples were placed into the freezers (operational temperature: (-80 ± 6)°C). Samples were processed in the presence of dry ice by using an appropriated blender.

Experiment number	Growth Stage	Samples Receiving Date	REEP
CVSOY-06-002-010	V2	09th November, 2006	0015
	R2	15th December, 2006	0022
	R8	16th March, 2007	0030
CVSOY-06-002-011	V2	23rd November, 2006	0016
	R2	22nd December, 2006	0025
	R8	16th March, 2007	0031
CVSOY-06-002-008	V2	29th November, 2006	0017
CVSOY-06-002-012	V2	13th December, 2006	0020
	R8	15th June, 2007	0041
CVSOY-06-002-013	V2	01st December, 2006	0019
	R8	15th June, 2007	0042
CVSOY-06-002-014	V2	29th November, 2006	0018
	R8	15th June, 2007	0043
CVSOY-06-002-016	V2	13th December, 2006	0021
	R8	15th June, 2007	0044

2.2. Analytical Phase - Expression Levels Analysis of AHAS Protein

SOP-PA.0273 Rev.02 is the method to determine amounts of AHAS Proteins in Vegetal Tissues using ELISA assays.

2.2.1 Sample Preparation

Grind the soybean tissue sample (whole plants, leaves, roots, flowers, pods or grains) with dry ice in a mill to obtain homogenous samples for analysis. After the dry ice has sublimed, pack into separate cups and store into a -80°C Freezer (operational temperature: (-80 ± 6)°C).

2.2.2 Principle of the method SOP-PA.0273 Rev.02

Lyophilization

Using a Freeze Dryer lyophilize an aliquot of powdered sample as described in SOP-PA.0202 and calculate the % of water (or the % dry weight) in the samples.

Extraction Step

Weight a lyophilized sample amount corresponding to (1 ± 0.001) g of fresh material and place in a centrifuge tube. Add 3 mL extraction solution (14.8 mM KH_2PO_4, 35.2 mM K_2HPO_4, 100 mM Sodium Pyruvate, 5 mM $MgCl_2$, pH 7.2) and mix nearly 10 seconds using a Mechanic Agitator. After being kept for 30 minutes on ice and the sample was extracted[*1] using a homogenizer at 24000 rpm with up and down movements for nearly 10 seconds to ensure a good homogeneity. The extract was centrifuged at 3000 rpm at (5 ± 3)°C for 10 minutes. The supernatant was filtered using filter paper (miracloth). This filtrate was transferred to a fresh centrifuge tube and centrifuged at 14000 rpm at (5 ± 3)°C for 15 minutes. The supernatant was reserved at (5 ± 3)°C until analyzed as described below. Extracts were analyzed for AHAS the day prepared.

Note: [*1]
To determine the extraction efficiency, three consecutive extractions on the same transgenic sample were performed as described above, reserving the extracts separately.

ELISA Step (AHAS quantification):

Each extract was quantitatively analyzed for AHAS protein by a sandwich enzyme-linked immunosorbent assay (ELISA) [Tijssen, 1985] using immunoaffinity-purified polyclonal rabbit anti-AHAS peptide 2 antibody and Protein G-purified goat antibodies specific for AHAS. NUNC 96-well plates (VWR; West Chester, PA) were coated with rabbit anti-peptide 2 and incubated at 37°C for 1 hr. The plate was washed two times with wash buffer and then blocked with 1% BSA in Tris buffered saline (25 mM Tris-HCl, 3 mM KCl, 0.14 M NaCl, pH 7.4) with 0.05% Tween for 60 min. at 37°C. After washing twice, samples and standards were applied in triplicate. Plates were incubated overnight at (5±3)°C, and then washed five times prior to the addition of the goat anti-*At*AHAS followed by incubation for 1 hr at 37°C. Plates were then washed three times and donkey anti-goat-horseradish peroxidase (HRP) was added. After incubation at 37°C for 1 hr, the plates were washed three times and HRP substrate was added (TMB Substrate Kit; Pierce). After 20 min at room temperature 1 M HCl was added to stop the reaction. The absorbance at 450 nm was measured using a Tecan Sunrise® multiwell plate reader. The results were analyzed using DeltaSoft PC software (Version1.71.4; Biometallics, Inc.; Princeton, NJ). The four-parameters algorithm was used to generate a curve. The AHAS protein was quantified from the standard concentration curve generated from highly purified AtAHAS protein.

Lower Limit of Quantitation (LOQ):
The Lower Limit of Quantitation for this method in soybean tissues samples was 13 ng of AHAS/ g of fresh tissue.

Limit of Detection (LOD):
The Limit of Detection for AHAS in this method in soybean tissues samples was 3 ng of AHAS/ g of fresh tissue.

The LOQ and LOD calculated for each tissue on a dry weight basis can be found in the Table 2.

BCA Step (Total Protein Determination):

Note: This step were used to help in the determination of the efficiency of extraction. It just measures the total protein present in the extract for direct comparison with a standard using Bovine Serum Albumin (BSA) Standards.

Total protein in each extract was quantified by the BCA™ procedure (bicinchoninic acid procedure; Pierce Biotechnology, Inc.; Rockford, IL) in accordance with the manufacturer's instructions, using bovine serum albumin as the standard. Samples (25 µl) were loaded onto a multiwell plate in triplicate, reacted with 200 µl of a 1:50 (B:A) mixture of BCA reagent, incubated at 37°C for 30 min and allowed to cool at room temperature for 10 min. The absorbance at 562 nm was measured using a Tecan Sunrise® multiwell plate reader. The results were analyzed using DeltaSoft PC software (Version 1.71.4, Biometallics, Inc.; Princeton, NJ) using the linear regression curve.

2.2.3 Apparatus, Materials, Reagents and Solvents

2.2.3.1 Apparatus and Materials

Microplate Reader (TECAN, Sunrise Basic)	
Microplate Washer (TECAN, Columbus / Plus Basic)	
Analytical Balance (Mettler Toledo, 0.00000g)	
Semi Analytical Balance (Mettler Toledo, 0.000g)	
Centrifuge with temperature control (FANEN and Hettich Mikro)	
Incubator (Nova Ética)	
Water Purification System (Millipore)	
pHmeter (Mettler Toledo)	
Ultrasound bath (Branson)	
Homogenizer (Ultra Turrax Ika)	
Mechanic Agitator for tubes/ "Vortex" (Scientific Industries)	
Freeze Dryer (Terroni Eq. Científicos)	
Refrigerator / Operation interval: 5±3 °C (Vidy)	
Automatic Pippet / Multichannel 20-300µL, 100-1500µL; Monochannel 25µL. (Gilson and Thermo Electron Corporation)	
Volumetric flask	10, 20, 25, 100, 200, 250, 500 e 2000 mL
Calibrated Volumetric flask	5, 10 mL
Becker	25 e 250 mL
Volumetric Pipette	3, 10 mL
Graduated Pipette	10 mL
Volumetric Cylinder Flask	10, 25 e 500mL
Centrifuge tube	10 mL
Plastic reservoir	50 mL
Filter Paper	Miracloth
Microplate (NUNC)	96 wells – Flat Bottom
Microplate (FALCON)	96 wells – "U" Bottom
Centrifuge Microtubes	1.7 mL, 2 mL
Amber flask	10, 25, 100, 250, 500 e 1000 mL

2.2.3.2 Reagents and Solvents

Milli-Q Water Grade 1, Millipore.
Potassium Phosphate Monobasic(KH_2PO_4), J.T. Baker.
Potassium Phosphate Dibasic(K_2HPO_4) , J.T. Baker.
Sodium Piruvate, J.T. Baker.
Magnesium Chloride, Sigma-Aldrich.
20X TBS – Tween (TBST), USB
Bovine Serum Albumine (BSA), Jackson Immuno Research.
TMB Substrate Kit: Buffer Hidrogen Peroxide (H_2O_2) 0.02% + Peroxidase Substrate Solution (TMB), PIERCE.
Rabbit anti-peptide 2 antibody (R&pep 2)
Goat anti-AtAHAS IgG (G&AtAHAS) antibody
Enzyme-Antibody conjugate: Donkey anti-goat IgG horseradish Peroxidase(D&G-HRP), Jackson ImmunoResearch.
Chloridric acid 37% (HCl), J.T. Baker.
Sodium Carbonate (Na_2CO_3), J.T. Baker.
Sodium Bicarbonate ($NaHCO_3$), J.T. Baker.
BCA Reagente A, PIERCE
BCA Reagente B, PIERCE

2.2.4 Calibration and Fortification Standard Solutions

2.2.4.1 Fortification Standard Solutions of AtAHAS

Standard Stock Solution (STDS)

To prepare a 10^4 ng/mL AtAHAS stock solution, a volume corresponding to $5*10^4$ ng of *At*AHAS standard was placed into a 5 mL volumetric flask. It was dissolved and diluted to mark with diluent (1% BSA in 1X TBST). This solution was set valid during 3 months when stored at (5±3) °C.

Standard of Fortification Solutions (STDF)

Standard solutions were prepared for fortification as described in the list below.

Solution	Stock Solution Concentration (ng/mL)	Aliquot Taken (µL)	Dilute to (mL)	Standard of Fortification Solution Concentration (ng/mL)	Standard of Fortification Solution Identification
STDS	10^4	50	5	100	STDI
STDS	10^4	85	5	170	STDF B
STDF B	170	500	5	17,0	STDF A

These Standard of Fortification Solutions were prepared with diluent (1% BSA in 1X TBST).

2.2.4.2 Standard of Calibration Solutions

- ***Suggested AtAHAS Standard of Calibration Solutions (STDC) for ELISA analysis***

Solution	Solution Concentration (ng/mL)	Aliquot Taken (µL)	Diluted to (µL)	Calibration Solution Concentration (ng/mL)	Calibration Solution Identification
STDI	100	100	200	50,0	STDC H
STDC H	50,0	100	200	25,0	STDC G
STDC G	25,0	100	200	12,5	STDC F
STDC F	12,5	100	200	6,25	STDC E
STDC E	6,25	100	200	3,12	STDC D
STDC D	3,12	100	200	1,56	STDC C
STDC C	1,56	100	200	0,781	STDC B
STDC B	0,781	100	200	0,390	STDC A

These calibration standard solutions were diluted with diluent (1% BSA in 1X TBST).

- ***Suggested BSA Standards of Calibration Solutions (STDC) for BCA analysis***

Calibration Solution Identification	Calibration Solution Concentration (µg/mL)
STDC G1	2000
STDC F1	1500
STDC E1	1000
STDC D1	750
STDC C1	500
STDC B1	250
STDC A1	125

2.2.5 Instrumental Conditions

2.2.5.1 For ELISA:

Parameters - Microplate Reader

Detector: Spectrophotometer Wavelength: 450 nm

Parameters - Microplate Washer

Final Aspiration: Time: 4 s; Speed: 10 mm/s.

Aspiration Position: Bottom

Wash Speed : 10 mm/s

Dispense Position: Overflow

Wash Volume: 300 µL

2.2.5.2 For BCA:

Parameters - Microplate Reader

Detector: Spectrophotometer Wavelength: 562 nm

2.2.6 Calculations

Excel is used to calculate the AHAS ng/g and percent recovery and to present the data in a report format. The following equations are the basis for all calculations:

Determination of Specific Protein (AHAS)

$$C_{Asp}\ (ng/mL) = C * \left(\frac{(A - D)}{(Response - D)} - 1 \right)^{\wedge 1/B}$$, 4-Parameter curve[*1].

Note:[*1] (Log x ; y), where x = concentration (ng/mL) and y = Response (OD).

$$R_{sp}\ (ng/g) = \frac{C_{Asp} * V_E * (1-\%H_2O/100)}{W_{LF}} * DF$$

$$\%\ Recovery = \frac{R_{sp}\ (ng/g)_{\text{Spiked Sample}}}{Fortification\ (ng/g)} * 100$$

$$Fortification\ (ng/g) = \frac{V_{STDF} * C_{STDF} * V_E * (1-\%H_2O\ /100)}{W_{LF} * V_{SAMPLE}}$$

Determination of Total Protein

$$C_{Atp}\ (\mu g/mL) = \frac{Response - Interception}{Slope}$$

$$R_{tp}\ (mg/g) = \frac{C_{Atp} * V_E * (1-\%H_2O/100)}{W_{LF} * 1000} * DF$$

Where:
C_{Asp} = Analytical Concentration of specific protein (AHAS) calculated through the formula above using the response.
C_{Atp} = Analytical Concentration of total protein calculated through the formula above using the response.
Response = Response of sample (OD).
A, B, C e D = Parameters of the 4-Parameter curve.
R_{sp} = Specific Protein (AHAS) amount per Fresh Sample amount. (ng/g)
R_{tp} = Total Protein amount per Fresh Sample amount. (mg/g)
V_E = Volume of Extraction. (3,0 mL)
W_{LF} = Weight of Lyophilized sample taken to analysis. (g)
DF = Dilution Factor to the sample (when it is applicable).
$\%H_2O$ = % of water in the sample.
C_{STDF} = Concentration of Standard used to fortification.(Level 1: 17,0ng/mL; Level 2: 170ng/mL)
V_{STDF} = Volume of standard solution used to spike.
(1-% H_2O /100) = Factor that considers the % of water in the sample.
V_{SAMPLE} = Aliquot taken of V_E to make the fortification.

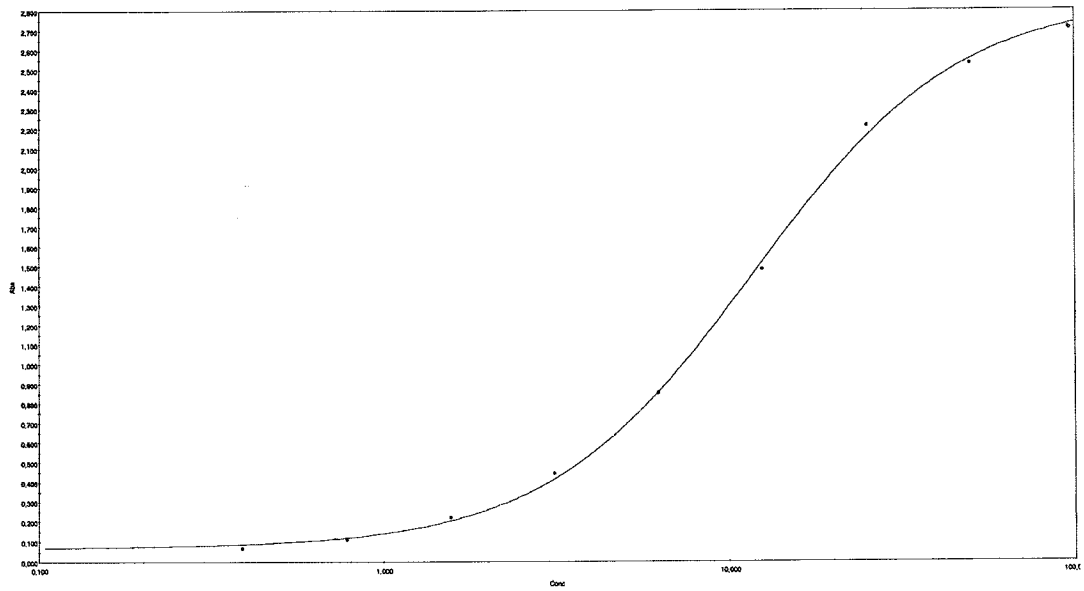
Note: To calculate R_{sp} in spiked samples, the response of non-GMO sample used to fortification was subtracted of the spiked response.

2.2.7 Calibration Curves

In this section are presented the Typical Calibration curves reached during the whole analysis to ELISA and BCA analysis.

- **Typical Calibration Curve obtained in the ELISA assays for determining AHAS Protein**

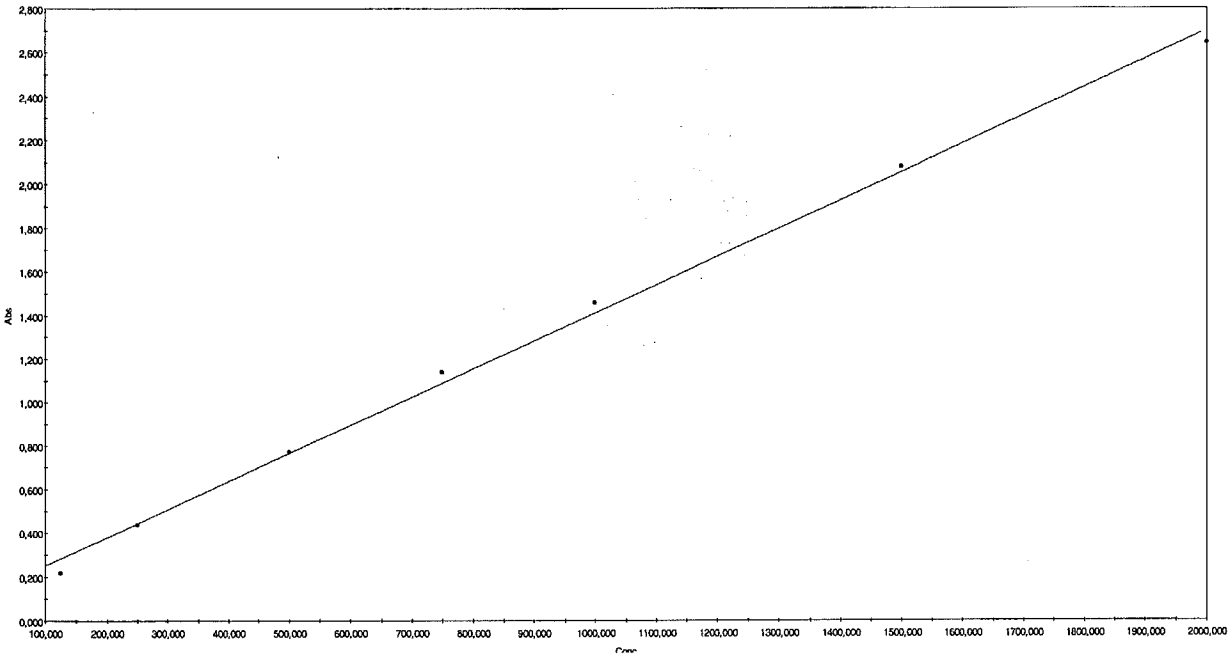
1st Set of ELISA Analysis (August 13th to 14th), Plate 4



4-Parameter Fit: Y = (A-D)/(1+(X/C)^B) + D
A = 0,068 B = 1,469 C = 11,743 D = 2,846
20 / 50 / 80%: x = 4,569 / 11,743 / 30,178 y = 0,624 / 1,457 / 2,290
RMS = 0,043, r = 0,998, r^2 = 0,995 , Basic weighting

- **Typical Calibration Curve obtained in the BCA assays for determining Total Protein**

1st Set of BCA Analysis (August 13 to 14th), Plate 3



Linear Fit: Y = Intercept + Slope * X
Slope = 0,001 +/- 0,000, Intercept = 0,121 +/- 0,032
RMS = 0,050, r = 0,999, r^2 = 0,997 , Basic weighting

2.2.8 Reference Substances Information

Reference Substance of *At*AHAS was used for fortification experiments and for calibration purposes in ELISA assays. These Reference Substance specifications are described below.

Name	Code	Nominal Concentration	Purity %
Arabidopsis thaliana Acetohydroxyacid Synthase	*At*AHAS	753 µg/mL	85.2
Expiration Date	**Origin**	**Lot-No.**	
February 06th, 2008.	BASF Plant Science L.L.C	1393101-0202	
Stability	Expected to be stable at least for 1 year stored at ≤ - 20°C.		

Reference Substance of BSA was used for calibration purposes in BCA assays. These Reference Substance specifications are described below.

Name	Code	Nominal Concentrations
Bovine Serum Albumin	BSA	2000 µg/mL
Expiration Date	**Supplier**	1500 µg/mL
13/06/2012	Pierce	1000 µg/mL
Lot-No.	**Pierce Product Number**	750 µg/mL
IB 110264	23208	500 µg/mL
Stability		250 µg/mL
Expected to be stable at least for 5 years if stored at 5±3°C.		125 µg/mL

2.2.9 Recovery and Extraction Efficiency

For fortification experiments, samples were analyzed from six analytical sets of control samples from non-GMO plots.

Control samples were fortified with *At*AHAS standard solutions after extraction, at concentration levels of 51 ng/g and 510 ng/g. The fortified samples were analyzed simultaneously with the other samples in order to ensure the reliability of the method. These results are summarized below.

Analyte	Test System	Number of recoveries	Fortification Level (ng/g)	Mean Recovery (%)	Overall Recovery ± *C.V. (%)
AtAHAS	Soybean (Whole Plant, Leaves, Roots, Pods, Flowers and Grains)	34	51	80	85 ± 17
		21	510	92	

* Coefficient of Variation

The extraction efficiency was also evaluated, where it has been checked the percentage of AHAS protein extracted in the first extraction of a series of three extractions on at least three samples of GMO soybean tissues, pods, young leaves and young whole plants, the only tissues that showed relevant amounts of AHAS for quantification. A second check for efficiency of the extraction was performed by the determination of the percentage of total protein extracted in the first extraction of a series of three extractions on at least three samples for all soybean tissues. The results for the extraction efficiency evaluation are given in the following Table 1.

Table 1. Extraction Efficiency Results for AHAS and/or Total Protein in different Soybean tissues.

Tissue	Line	Replicate	Mean % of AHAS and/or Total Protein found in the first extraction of three Consecutive Extractions ± SD (N = 3; 3 samples per tissue per growth stage)																	
			V2 (2nd trifoliate emerged)						R2 (Fill bloom)						R8 (Maturity)					
			AHAS	Mean	SD	Total Protein	Mean	SD	AHAS	Mean	SD	Total Protein	Mean	SD	AHAS	Mean	SD	Total Protein	Mean	SD
Leaves	CV 127	1	86			82			-			79			-			-		
		2	92	90	3	84	84	2	-	-	-	80	80	1	-	-	-	-	-	-
		3	92			86			-			81			-			-		
Whole Plants	CV 127	1	91			83			-			81			-			68		
		2	100	97	5	83	84	2	-	-	-	80	81	0,5	-	-	-	72	69	3
		3	100			85			-			81			-			67		
Roots	CV 127	1	-			86			-			83			-			42		
		2	-	-	-	82	79	9	-	-	-	75	79	4	-	-	-	56	52	8
		3	-			68			-			78			-			57		
Pods	CV 127	1	-			-			-			-			63			75		
		2	-	-	-	-	-	-	-	-	-	-	-	-	64	63	0,9	75	74	2
		3	-			-			-			-			62			71		
Flowers	CV 127	1	-			-			-			77			-			-		
		2	-	-	-	-	-	-	-	-	-	76	78	2	-	-	-	-	-	-
		3	-			-			-			79			-			-		
Grains	CV 127	1	-			-			-			-			-			81		
		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	82	81	0,6
		3	-			-			-			-			-			82		

Values of AHAS were determined by ELISA and values of Total Protein were determined by BCA Assay. "-" = Tissue not available at this stage or non-quantifiable amounts found that made the calculation for efficiency of extraction non relevant for AHAS.

3. RESULTS

The AHAS protein levels at various soybean developmental stages in various tissues are presented in detailed summary tables those can be found below. Values of AHAS amounts were determined by ELISA and were not corrected for extraction efficiency or procedural recoveries.

Table 2. Calculated LOQ and LOD used for AHAS results expression.

Mean Concentration from all calibration curves corresponding to 20% of Linear range of the Curve (ng/mL) ± SD		4,321 ± 0,754					
LOQ Curve (AHAS ng/g fresh Wt.)		13	**LOD (AHAS ng/g fresh Wt.)**	3			
Tissue	**Growth Stage**	**LOQ Curve (AHAS ng/g Dry Wt.)**	**LOD (AHAS ng/g Dry Wt.)**	**LOQ Estimated (mg AHAS/ Hectare)**	**LOQ Estimated (mg AHAS/ Acre)**	**LOD Estimated (mg AHAS/ Hectare)**	**LOD Estimated (mg AHAS/ Acre)**
Whole Plants	V2	68	16	20	8	5	2
	R2	65	15	331	134	76	31
	R8	15	3	355	144	71	29
Leaves*	V2	70	16				
	R2	62	14				
Roots	V2	41	9				
	R2	38	9				
	R8	17	4				
Flowers	R2	77	18				
Pods	R8	15	3				
Grains	R8	15	3				

*1st trifoliate was included in the calculation for leaves.

Table 3. AHAS Protein Levels on a Fresh/ Dry Weight Basis during development of Cultivance Soybean Event 127 derived plants in the Londrina/PR location.

Experiment / Location											
CVSOY-06-002-010 / Londrina - PR											
Stage	Tissue	Plots	Line	N° of Plants	N° of Samples	Mean ng AHAS/ g Fresh wt.	SD	CV%	Mean ng AHAS/ g Dry wt.	SD	CV%
V2 (2nd trifoliate emerged)	Whole Plants	21 and 23	Event 127	6	6	61	10	16	314	59	19
	Leaves	21 and 23	Event 127	6	6	103	24	24	511	116	23
	Roots	21 and 23	Event 127	6	2	<13	1	28	<41	3	17
	1st trifoliate	21 and 23	Event 127	6	2	55	5	9	278	27	10
	Whole Plants	22 and 24	Non GM Control	6	6	<13	2	19	<68	10	19
	Leaves	22 and 24	Non GM Control	6	6	15	3	19	78	17	21
	Roots	22 and 24	Non GM Control	6	2	<13	4	141	<41	13	141
	1st trifoliate	22 and 24	Non GM Control	6	2	<13	4	61	<70	22	60
R2 (Full Bloom)	Whole Plants	21 and 23	Event 127	6	6	ND	3	156	ND	15	156
	Leaves	21 and 23	Event 127	6	6	<13	4	120	<62	24	122
	Roots	21 and 23	Event 127	6	6	<13	2	41	<38	7	38
	Flowers	21 and 23	Event 127	6	2	<13	1	24	<77	7	19
	Whole Plants	22 and 24	Non GM Control	6	6	ND	0	-	ND	0	-
	Leaves	22 and 24	Non GM Control	6	6	ND	1	159	ND	7	159
	Roots	22 and 24	Non GM Control	6	6	<13	2	33	<38	5	32
	Flowers	22 and 24	Non GM Control	6	2	<13	1	35	<77	12	48
R8 (Maturity)	Whole Plants	21 and 23	Event 127	6	6	<13	3	26	<15	3	26
	Pods	21 and 23	Event 127	6	6	24	1	5	26	1	5
	Roots	21 and 23	Event 127	6	6	<13	4	117	<17	4	117
	Grains	21 and 23	Event 127	-	2	<13	1	28	<15	2	29
	Whole Plants	22 and 24	Non GM Control	6	6	<13	2	44	<15	2	44
	Pods	22 and 24	Non GM Control	6	6	<13	0,5	12	<15	0,6	12
	Roots	22 and 24	Non GM Control	6	6	ND	3	245	ND	4	245
	Grains	22 and 24	Non GM Control	-	2	<13	0,7	20	<15	0,8	20

Values were determined by ELISA and were not corrected for extraction efficiency. "< LOQ" = sample had detectable non-quantifiable amounts below the lower limit of quantification specified in the Table 2. "ND" = AHAS was considered non-detectable because the result generated during ELISA did not exceed the LOD of the method.

Table 4. AHAS Protein Levels on a Fresh/ Dry Weight Basis during development of Cultivance Soybean Event 127 derived plants in Santo Antonio de Posse/SP location.

Experiment / Location											
CVSOY-06-002-011 / Sto Antonio de Posse - SP											
Stage	Tissue	Plots	Line	N° of Plants	N° of Samples	Mean ng AHAS/ g Fresh wt.	SD	CV%	Mean ng AHAS/ g Dry wt.	SD	CV%
V2 (2nd trifoliate emerged)	Whole Plants	21 and 23	Event 127	6	6	40	6	15	214	37	17
	Leaves	21 and 23	Event 127	6	6	128	47	37	714	284	40
	Roots	21 and 23	Event 127	6	2	<13	0,7	7	<41	1	4
	1st trifoliate	21 and 23	Event 127	6	2	60	12	20	300	47	16
	Whole Plants	22 and 24	Non GM Control	6	6	<13	2	22	<68	12	24
	Leaves	22 and 24	Non GM Control	6	6	14	1	10	73	8	11
	Roots	22 and 24	Non GM Control	6	2	<13	1	24	<41	9	41
	1st trifoliate	22 and 24	Non GM Control	6	2	<13	0,7	7	<70	3	7
R2 (Full Bloom)	Whole Plants	21 and 23	Event 127	6	6	34	6	18	160	33	20
	Leaves	21 and 23	Event 127	6	6	24	4	17	106	19	18
	Roots	21 and 23	Event 127	6	6	17	4	24	50	13	26
	Flowers	21 and 23	Event 127	6	2	22	9	43	125	45	36
	Whole Plants	22 and 24	Non GM Control	6	6	<13	3	27	<65	14	28
	Leaves	22 and 24	Non GM Control	6	6	<13	2	33	<62	11	34
	Roots	22 and 24	Non GM Control	6	6	<13	3	49	<38	9	52
	Flowers	22 and 24	Non GM Control	6	2	<13	2	28	<77	11	27
R8 (Maturity)	Whole Plants	21 and 23	Event 127	6	6	<13	5	51	<15	6	47
	Pods	21 and 23	Event 127	6	6	26	2	8	30	2	8
	Roots	21 and 23	Event 127	6	6	<13	6	140	<17	10	140
	Grains	21 and 23	Event 127	-	2	<13	0,7	11	<15	0,8	9
	Whole Plants	22 and 24	Non GM Control	6	6	<13	2	31	<15	2	31
	Pods	22 and 24	Non GM Control	6	6	<13	0,9	18	<15	1	18
	Roots	22 and 24	Non GM Control	6	6	ND	2	245	ND	2	245
	Grains	22 and 24	Non GM Control	-	2	<13	0	0	<15	0,02	0,5

Values were determined by ELISA and were not corrected for extraction efficiency. "< LOQ" = sample had detectable non-quantifiable amounts below the lower limit of quantification specified in the Table 2. "ND" = AHAS was considered non-detectable because the result generated during ELISA did not exceed the LOD of the method.

Table 5. AHAS Protein Levels in Soybean Event 127 leaves on a Fresh/ Dry Weight Basis at different locations.

Experiment N° / Location	Stage	Tissue	Plots	Line	N° of Plants	N° of Samples	Mean ng AHAS/ g Fresh wt.	SD	CV%	Mean ng AHAS/ g Dry wt.	SD	CV%
CVSOY-06-002-008 / Ponta Grossa - PR	V2 (2nd trifoliate emerged)	Leaves	21 and 23	Event 127	12	2	92	29	32	478	136	28
			22 and 24	Non GM Control	12	2	<13	3	28	<70	21	36
CVSOY-06-002-012 / Uberaba - MG	V2 (2nd trifoliate emerged)	Leaves	21 and 23	Event 127	12	2	80	12	15	427	73	17
			22 and 24	Non GM Control	12	2	16	0	0	86	9	11
CVSOY-06-002-013 / Sete Lagoas - MG	V2 (2nd trifoliate emerged)	Leaves	21 and 23	Event 127	12	2	53	31	59	335	189	56
			22 and 24	Non GM Control	12	2	<13	1	20	<70	12	25
CVSOY-06-002-014 / Sto Antonio de Goias - GO	V2 (2nd trifoliate emerged)	Leaves	21 and 23	Event 127	12	2	61	13	22	337	53	16
			22 and 24	Non GM Control	12	2	<13	3	31	<70	16	31
CVSOY-06-002-016 / Brasilia - DF	V2 (2nd trifoliate emerged)	Leaves	21 and 23	Event 127	12	2	61	42	70	363	257	71
			22 and 24	Non GM Control	12	2	<13	2	18	<70	16	23

Values were determined by ELISA and were not corrected for extraction efficiency. "< LOQ" = sample had detectable non-quantifiable amounts below the lower limit of quantification specified in the Table 2. "ND" = AHAS was considered non-detectable because the result generated during ELISA did not exceed the LOD of the method.

Table 6. AHAS Protein Levels in Soybean Event 127 grains on a Fresh/ Dry Weight Basis at different locations.

Experiment N° / Location	Stage	Tissue	Plots	Line	N° of Plants	N° of Samples	Mean ng AHAS/ g Fresh wt.	SD	CV%	Mean ng AHAS/ g Dry wt.	SD	CV%
CVSOY-06-002-012 / Uberaba -MG	R8 (Maturity)	Grains	21 and 23	Event 127	-	2	<13	1	14	<15	2	15
			22 and 24	Non GM Control	-	2	<13	0,7	13	<15	0,8	13
CVSOY-06-002-013 / Sete Lagoas -MG	R8 (Maturity)	Grains	21 and 23	Event 127	-	2	13	0	0	<15	0	0
			22 and 24	Non GM Control	-	2	<13	0	0	<15	0	0
CVSOY-06-002-014 / Sto Antonio de Goias - GO	R8 (Maturity)	Grains	21 and 23	Event 127	-	2	<13	2	25	<15	2	25
			22 and 24	Non GM Control	-	2	<13	4	64	<15	4	65
CVSOY-06-002-016 / Brasilia - DF	R8 (Maturity)	Grains	21 and 23	Event 127	-	2	<13	0	0	<15	0	0
			22 and 24	Non GM Control	-	2	<13	0,7	16	<15	0,8	16

Values were determined by ELISA and were not corrected for extraction efficiency. "< LOQ" = sample had detectable non-quantifiable amounts below the lower limit of quantification specified in the Table 2. "ND" = AHAS was considered non-detectable because the result generated during ELISA did not exceed the LOD of the method.

Table 7. Estimation of AHAS Protein Levels in Soybean Event 127 Whole plants per Hectare and Acre in two different Locations.

Experiment / Location							
CVSOY-06-002-010 / Londrina - PR							
Stage	**Line**	**Mean ng AHAS/ g Dry wt.**	**Mean g Dry wt./ Plant**	**N° Plants/ Hectare**	**N° Plants/ Acre**	**Estimated mg AHAS/ Hectare**	**Estimated mg AHAS/ Acre**
V2 (2nd trifoliate emerged)	Event 127	314	1,20	260000	105221	98	40
	Non GM Control	<68	1,04	260000	105221	<20	<8
R2 (Full Bloom)	Event 127	ND	16,36	260000	105221	ND	ND
	Non GM Control	ND	29,78	260000	105221	ND	ND
R8 (Maturity)	Event 127	<15	73,94	260000	105221	<355	<144
	Non GM Control	<15	88,00	260000	105221	<355	<144
CVSOY-06-002-011 / Santo Antonio de Posse - SP							
Stage	**Line**	**Mean ng AHAS/ g Dry wt.**	**Mean g Dry wt./ Plant**	**N° Plants/ Hectare**	**N° Plants/ Acre**	**Estimated mg AHAS/ Hectare**	**Estimated mg AHAS/ Acre**
V2 (2nd trifoliate emerged)	Event 127	214	1,18	260000	105221	66	27
	Non GM Control	<68	1,20	260000	105221	<20	<8
R2 (Full Bloom)	Event 127	160	14,67	260000	105221	610	247
	Non GM Control	<65	17,58	260000	105221	<331	<134
R8 (Maturity)	Event 127	<15	77,60	260000	105221	<355	<144
	Non GM Control	<15	124,76	260000	105221	<355	<144

	Growth Stage	**Line**	**Estimated mg AHAS/ Hectare**	**Estimated mg AHAS/ Acre**
Mean of two locations	V2	Event 127	**82**	**33**
		Non GM Control	**<20**	**<8**
	R2	Event 127	**<331**	**<134**
		Non GM Control	**<331**	**<134**
	R8	Event 127	**<355**	**<144**
		Non GM Control	**<355**	**<144**

Values were determined by ELISA and were not corrected for extraction efficiency. "< LOQ" = sample had detectable non-quantifiable amounts below the lower limit of quantification specified in the Table 2. "ND" = AHAS was considered non-detectable because the result generated during ELISA did not exceed the LOD of the method.

4. DISCUSSION

The mean of estimated levels of AHAS in two locations, Santo Antonio de Posse/ SP and Londrina/ PR, in Cultivance Soybean Event 127 whole plants were 82, <331 and <355 mg/Hectare for the growth stages V2 (2nd trifoliate emerged), R2 (Full Bloom) and R8 (Maturity), respectively. The non-GMO control used in these same filed trials showed the mean of estimated Levels of AHAS in Soybean Whole Plants of <20, <331 and <355 mg/Hectare for the same growth stages V2, R2 and R8, respectively.

The AHAS protein expression in Cultivance Soybean Event 127 leaves, whole plants and 1st trifoliate of V2 Growth Stage from Santo Antonio de Posse/ SP showed 128, 40 and 60 ng of AHAS/ g fresh weight corresponding to 714, 214 and 300 ng/ g dry weight of these tissues, respectively. Similar results for the trial from Londrina/PR; the same tissues in V2, leaves, whole plants and 1st trifoliate, showed 103, 61 and 55 ng of AHAS/ g fresh weight corresponding to 511, 314 and 278 ng/ g dry weight of these tissues, respectively. The other GMO and non-GMO tissues in this growth stage showed non-quantifiable or non-detectable amounts of AHAS (values less than the LOQ -13 ng AHAS/g fresh weight tissue or less than the LOD- 3 ng AHAS/g fresh weight tissue), or values very closer to the LOQ and less or equal than 16 ng AHAS/g fresh weight tissue.

In R2 growth Stage from Santo Antonio de Posse/SP, all Cultivance Soybean Event 127 plant parts analyzed (whole plants, leaves, roots and flowers) had quantifiable amounts of AHAS. They were 34, 24, 17 and 22 ng AHAS/ g fresh weight, that correspond to 160, 106, 50 and 125 ng AHAS/ g dry weight, for whole plants, leaves, roots and flowers, respectively. The other non-GMO tissues from this location as the GMO and non-GMO tissues from Londrina had non-quantifiable amounts of AHAS.

At maturity stage (R8 stage) only Cultivance Soybean Event 127 pods showed AHAS expression over the LOQ in both Locations, Santo Antonio de Posse/SP and Londrina/PR. The results were 24 ng AHAS/ g fresh weight (or 26 ng AHAS/ g dry weight) from Londrina/PR and 26 ng AHAS/ g fresh weight (or 30 ng AHAS/ g dry weight) from Santo Antonio de Posse/SP. The other GMO and non-GMO tissues in this growth stage showed non-quantifiable amounts of AHAS.

Consistent levels of AHAS expression were found in leaf tissues from five different sites. The leaf material collected from Cultivance Soybean Event 127 leaves at V2 from Ponta Grossa/ PR, Uberaba/ MG, Sete Lagoas/ MG, Sto. Antonio de Goiás/ GO and Brasília/ DF, showed 92, 80, 53, 61 and 61 ng of AHAS / g fresh weight (or 478, 427, 335, 337 and 363 ng of AHAS / g dry weight), respectively. Except for Uberaba/MG, the non-GM soybean leaves from all locations had levels of AHAS less than the LOQ. The AHAS expression in Uberaba/MG non-GM leaves was 16 ng/g fresh tissue or 86 ng/g dry tissue.

The Cultivance Soybean Event 127 and non-OGM mature grain from all locations had levels of AHAS less or equal than the LOQ.

5. REFERENCES

a. SOP No. BPS 510.06.00 - General Procedure for Enzyme Linked Immunosorbent Assay (ELISA) – BASF Plant Science.

b. SOP No. BPS 510.16.00 - *Arabidopsis* Acetohydroxyacid Synthase (*At*AHAS) ELISA, BASF Plant Science.

c. SOP No. BPS 510.04.00 - Protein Determination Using the BCA Procedure – BASF Plant Science.

d. OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, Document Number 1. Paris/1997.

e. Brazilian GLP Norm NIT-DICLA-028 (Rev.01) – Critérios para o Credenciamento de Laboratórios de Ensaios Segundo os Princípios BPL – Boas Práticas de Laboratório – Inmetro -Setembro /2003.

f. Lee, Y., A. K. Chang, and R. G. Duggleby. 1999. Effect of mutagenesis at serine 653 of Arabidopsis thaliana acetohydroxyacid synthase on the sensitivity to imidazolinone and sulfonylurea herbicides. FEBS Letters 452:341-345.

g. Pang, S. S., R. G. Duggleby, and L. W. Guddat. 2002. Crystal structure of yeast acetohydroxyacid synthase: a target for herbicidal inhibitors. J. Mol. Biol. 317:249-262.

h. Sathasivan, K., Haughn, G. W., and Murai, N. (1990) Nucleotide sequence of a mutant acetolactate synthase gene from an imidazolinone-resistant Arabidopsis thaliana var. Columbia. Nucl. Acids Res. 18:2188.

i. Tan, S., R. R. Evans, M. L. Dahmer, B. K. Singh, and D. L. Shaner. 2005. Imidazolinone-tolerant crops: history, current status and future. Pest Manag. Sci. 61:246-257.