Appendix 12

Analysis of Expression Levels of Arabidopsis Acetohydroxyacid Synthase (AHAS) Protein, by ELISA, in the BPS-CV127-9 Soybean, Plants Grown in Brazilian Field Trials during the 2007 Season

The Chemical Company

CLA 0009

GLOBAL ENVIRONMENTAL AND CONSUMER SAFETY LABORATORY

Report Number

RF-1383-07

Report Title

Analysis of Expression Levels of *Arabidopsis* Acetohydroxyacid Synthase (AHAS) Protein, by ELISA, in the BPS-CV127-9 Soybean, Plants Grown in Brazilian Field Trials during the 2007 season.

Field Experiment Numbers

CVSOY-07-002-001
CVSOY-07-002-002
CVSOY-07-002-003
CVSOY-07-002-005
CVSOY-07-002-006
CVSOY-07-002-007

Author

[redacted]

Study Completion Date

April 08, 2008

Sponsor

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Test Facility

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Phone.: [redacted]
Fax: [redacted]

Were issued t[illegible]nal Reports, being this the Number 02.

CERTIFICATES

a. Swiss Federal Office of Public Health

The Swiss GLP Monitoring Authorities

Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

Swiss Confederation

Federal Department of Home Affairs DHA
Federal Office of Public Health FOPH

Federal Department of the Environment, Transport, Energy and Communications DETEC
Federal Office for the Environment FOEN

swissmedic
Swiss Agency for Therapeutic Products

Statement of GLP Compliance

According to Article 14 paragraph 3 Ordinance on Good Laboratory Practice [OGLP, SR 813.112.1]

The notification authority for chemicals confirms that the following test facility was inspected with respect to the compliance with the Swiss Ordinance on Good Laboratory Practice, adopted on 18th May 2005 [OGLP, SR 813.112.1]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted on 26th November 1997 by decision of the OECD Council [C(97)186/Final].

Unequivocal name and address of the test facility:	Areas of expertise according to article 3 paragraph 1 letter d OGLP:
BASF SA Global Environmental and Consumer Safety Laboratory – GENCS Av. Brasil 791 Bairro Eng. Nelva 12521-900 Guaratinguetá, SP Brazil	5./ Studies on behaviour in water, soil and air; bioaccumulation, 6./ Residue studies.

Inspection authority: Federal Office of Public Health (FOPH)

Date of inspection: 18th to 22nd June 2007

Date of decision: 12th September 2007

Based on the above mentioned decision it can be confirmed that the above mentioned test facility is able to conduct studies according to the aforementioned areas of expertise in compliance with the principles of GLP. The above mentioned test facility is listed in the register and GLP list according to the Article 14 OGLP and is inspected on a regular basis according to Article 6 paragraph 2 OGLP.

Swiss Federal Office of Public Health
Consumer protection directorate
Notification authority for chemicals
CH-3003 Bern

Dag Kappes

FEDERAL OFFICE OF PUBLIC HEALTH

Bern, 2nd November 2007, The Head, Dr. Dag Kappes. *

The notification authority for chemicals is the coordination and decision authority for the good laboratory praxis (GLP) for the FOEN, the FOPH and Swissmedic.
Swiss Federal Office of Public Health, Consumer protection directorate, Notification authority for chemicals, CH-3003 Bern.
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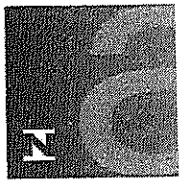

República Federativa do Brasil
Ministério do Desenvolvimento, Indústria e Comércio Exterior
Instituto Nacional de Metrologia, Normalização e Qualidade Industrial – Inmetro

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 Antônio Lima de Oliveira
Coordenador Geral de Acreditação

Emissão: 17-07-2007

Validade: 14-08-2009

IDENTIFICATION AND SIGNATURES OF PERSONNEL INVOLVED IN THE STUDY

08/04/2008
dd / mm / yyyy

[redacted]
Study Director / Analist *

08/04/2008
dd / mm / yyyy

[redacted]
Test Facility Management*

18/04/2008
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 1383-07 entitled "Analysis of Expression Levels of *Arabidopsis* Acetohydroxyacid Synthase (AHAS) Protein, by ELISA, in the BPS-CV127-9 Soybean Plants Grown in Brazilian Field Trials during the 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 – Sept. 2003) 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 1383-07 and described on this Report RF-1383-07 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]

08 / 04 / 2008
dd mm yyyy

Chemistry Bachelor, MS
CRQ-SP Number 05201582
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-035 (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		08 / 10 / 2007	08 / 10 / 2007
Laboratory Phase(s) Inspected	Preparation of Standard Stock Solution	27/ 10 / 2007	27/10/2007
	Weighing of samples	30/ 10 / 2007	30 / 10/ 2007
	Clean-up	30-31/10/2007	31/10/2007
	Quantification	31/10 – 07/11/2007	07/11//2007
Raw Data		12-13/03/2008	17/03/2008
Draft of Final Report		14/03/2008	17/03/2008

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 the Raw Data generated during the conduction of the study 1383-07. The Raw Data were inspected by QAU, according to the Standard Operating Procedure SOP-GE.018.

[redacted]

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

03 /04 /2008
dd mm yyyy

* 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]

08 / 04 / 2008
dd mm yyyy

Chemistry Bachelor, MS
CRQ-SP Number 05201582
Study Director*
Phone: [redacted]

* Address: Test Facility (see front page).

GUIDELINES COVERED

- INMETRO NIT-DICLA-028, 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 BPS-CV127-9 (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 2007 growing season was measured by enzyme-linked immunosorbent assays (ELISA). The tissues analyzed included whole plants, first trifoliates, leaves, roots, pods, grains 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 2007 growing season at six 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 14 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=69) were 93% with a coefficient of variation of 9% 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. 100 and 88% for soybean whole plants (V2 stage) and leaves (V2 stage), 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. BPS-CV127-9 soybean leaf samples from the V2 stage had AHAS levels between 18 and 27 ng AHAS/g fresh wt., corresponding to 113 and 133 ng AHAS/g dry wt. CV 127 whole plants from the V2 stage showed non quantifiable amounts of AHAS. AHAS levels in the CV 127 first trifoliates leaf from the V2 stage ranged from <14 – 59 ng AHAS/g fresh wt. or <76 – 254 ng AHAS/g dry wt. In R2 there were no quantifiable amounts of AHAS in any tissue, and as was the case for the R8 growth stage, except for roots that showed between 15 and 17 ng AHAS/ g fresh wt. (or 42 and 48 ng AHAS/ g dry wt.). The AHAS level in all tissues for the other time points from both CV Soy and the the conventional variety was detectable at levels lower than the LOQ. Using the mean levels of AHAS found in whole plants from Santo Antonio de Goiás and Brasília 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 estimated value for the R8 stage was <293 mg AHAS/hectare (<118 mg AHAS/acre). At the R2 stage the value was estimated to be < 267 mg AHAS/hectare (<108 mg AHAS/acre) and at the V2 stage <33 mg AHAS/hectare (<13 mg AHAS/acre).

STUDY SCHEDULE

Study Initiation Date:	October 09th, 2007	Experimental Completion Date:	January 31st,2008
Experimental Starting Date:	October 12nd,2007	Study Completion Date:	April 08th, 2008

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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 to be tolerant to the imidazolinone class of agricultural herbicides. The herbicide-tolerant soybean plants, referred to as BPS-CV127-9 soybeans, 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 BPS-CV127-9 (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 BPS-CV127-9 as compared to a non-transgenic isoline variety, the amount of AtAHAS protein was determined in the GENCS Study 1383-07 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 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) 1st trifoliates (V2 stage) from six field locations from the 2007 growing season.

3) grain harvested from six field locations from the 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 were the responsibility of BASF Plant Science. However the trials were conducted under Good Agricultural Practice (GAP).
The field experimental phase was described in the Experimental plan CV-SOY-07-002, submitted to the Brazilian Authorities (CTNBio - Comissão Técnica Nacional de Biossegurança) 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	EMBRAPA Soja – Campo Experimental de Vilhena Address: Rodovia BR 364, Km 06 Caixa Postal 405, CEP 84001-000 – Vilhena, RO
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Catarina Villas Braga de Souza Cel: (61)9214-3244 Email: catarinavillas@brturbo.com.br	EMBRAPA CNPAF – Embrapa Arroz e Feijão 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
	EMBRAPA Meio Norte Address: Av. Duque de Caxias, 56502 – Bairro Buenos Aires – Caixa Postal 01 – CEP: 64006-220 – Teresina, PI

2.1.1 Field Specimen List

Location	Experiment number	Plot number	Specimen Description
EPAMIG CTTP, Uberaba, MG	CVSOY-07-002-001	21 and 23	GMO, BPS-CV127-9 soybean
		22 and 24	non GMO, control soybean
EMBRAPA CNPMS, Sete Lagoas, MG	CVSOY-07-002-002	21 and 23	GMO, BPS-CV127-9 soybean
		22 and 24	non GMO, control soybean
EMBRAPA CNPAF, Santo Antonio de Goiás, GO	CVSOY-07-002-003	21 and 23	GMO, BPS-CV127-9 soybean
		22 and 24	non GMO, control soybean
EMBRAPA CNPH, Brasília, DF	CVSOY-07-002-005	21 and 23	GMO, BPS-CV127-9 soybean
		22 and 24	non GMO, control soybean
EMBRAPA SOJA, Vilhena, RO	CVSOY-07-002-006	21 and 23	GMO, BPS-CV127-9 soybean
		22 and 24	non GMO, control soybean
EMBRAPA MEIO NORTE, Teresina, PI	CVSOY-07-002-007	21 and 23	GMO, BPS-CV127-9 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 BPS-CV127-9 and one plot of an imidazolinone-sensitive near isogenic control soybean. All plots of the BPS-CV127-9 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	- EMBRAPA CNPAF, Santo Antonio de Goiás, GO - EMBRAPA CNPH, Brasília, DF	- Whole Plants - 1st Trifoliates
	- Other 4 locations	- 1st Trifoliates
R2	- EMBRAPA CNPAF, Santo Antonio de Goiás, GO - EMBRAPA CNPH, Brasília, DF	- Whole Plants
R8	- EMBRAPA CNPAF, Santo Antonio de Goiás, GO - EMBRAPA CNPH, Brasília, DF	- Whole Plants - Grains
	- Other 4 locations	- Grains

Depending on the kind of tissue to be analyzed, the samples of the test system, BPS-CV127-9, and conventional control soybean variety, were collected and separated into parts, when desired, as described below:

Leaves (1st Trifoliates) 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 1st trifoliates 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 Goiás and Brasília, 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 (1st trifoliates, 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.

Grain

A minimum of 500 g of seed from each plot from six locations 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-07-002-003	V2	28th March, 2007	0032
	R2	04th May, 2007	0039
	R8	03rd July, 2007	0045
	R8	13rd September, 2007	0047
CVSOY-07-002-005	V2	28th March, 2007	0033
	R2	04th May, 2007	0040
	R8	03rd July, 2007	0046
	R8	13rd September, 2007	0048
CVSOY-07-002-001	V2	14th April, 2007	0036
	R8	13rd September, 2007	0049
CVSOY-07-002-002	V2	14th April, 2007	0037
	R8	13rd September, 2007	0050
CVSOY-07-002-006	V2	04th April, 2007	0034
	R8	13rd September, 2007	0051
CVSOY-07-002-007	V2	14th April, 2007	0035
	R8	13rd September, 2007	0052

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, stems, 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 bags 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

Weigh 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 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 Study in soybean tissues samples was 14 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 was used to help in the determination of the efficiency of extraction. It 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)	
pH meter (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, 2000 mL
Calibrated Volumetric flask	5, 10 mL
Beaker	25 and 250 mL
Volumetric Pipette	3, 10 mL
Graduated Pipette	10 mL
Volumetric Cylinder Flask	10, 25 and 1000mL
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, 100, 250, 1000 mL
Calibrated Volumetric Pipette	1, 2, 3 mL

2.2.3.2 Reagents and Solvents

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

2.2.4 Standard of Calibration and Fortification Solutions

2.2.4.1 Standard of Fortification Solutions of AtAHAS

Standard Stock Solution (STDS)

To prepare a 10^4 ng/mL *At*AHAS 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 standard of calibration 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 Lens: 400-700 nm 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 Lens: 400-700 nm 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

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

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 & 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.

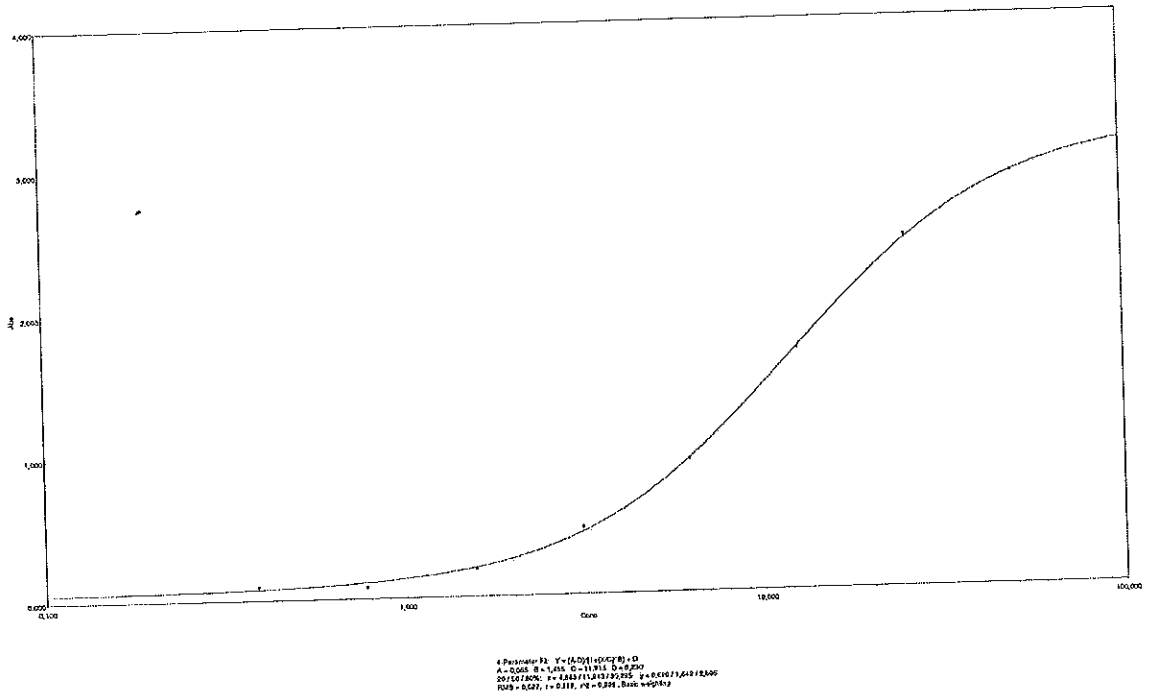
Note: To calculate R_{sp} in spiked samples, the response of non-GMO sample used in the fortification experiment was subtracted from the spiked response.

2.2.7 Calibration Curves

In this section examples of typical calibration curves obtained throughout the study for ELISA and BCA protein analyses are presented.

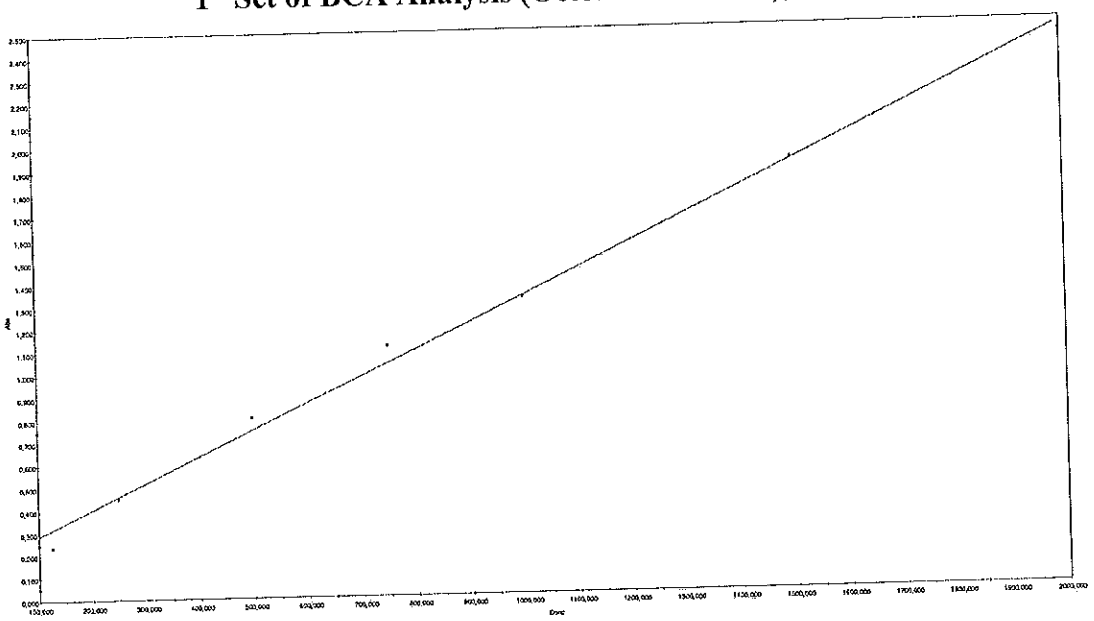
- **Typical Calibration Curve obtained in the ELISA assays for determining AHAS Protein**

1st Set of ELISA Analysis (October 30 to 31st), Plate 1



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

1st Set of BCA Analysis (October 30 to 31st), Plate 1



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 06, 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. The 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)	43	51	97	93 ± 9
		26	510	94	

* Coefficient of Variation

The extraction efficiency defined as 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) was also evaluated. 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 (Full 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	83			81			75			82			-			-		
		2	80	88	11	79	80	1	69	69	6	81	80	2	-	-	-	-	-	-
		3	100			81			63			78			-			-		
Whole Plants	CV 127	1	100			81			-			85			-			76		
		2	100	100	0	85	83	2	-	-	-	75	81	5	-	-	-	72	74	2
		3	100			83			-			82			-			74		
Roots	CV 127	1	-			82			58			77			-			72		
		2	-	-	-	79	78	5	77	68	10	82	76	7	-	-	-	73	74	2
		3	-			73			69			69			-			77		
Pods	CV 127	1	-			-			-			-			-			84		
		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	81	83	2
		3	-			-			-			-			-			83		
Flowers	CV 127	1	-			-			-			76			-			-		
		2	-	-	-	-	-	-	-	-	-	79	77	2	-	-	-	-	-	-
		3	-			-			-			76			-			-		
Grains	CV 127	1	-			-			-			-			-			76		
		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	71	73	2
		3	-			-			-			-			-			74		

Values of AHAS were determined by ELISA and values of Total Protein were determined by BCA Assay. "-" = Tissue not available at this stage or amounts 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 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		4,759 ± 0,744					
LOQ Curve (AHAS ng/g fresh Wt.)		14	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	78	17	33	13	7	3
	R2	66	14	267	108	57	23
	R8	28	6	293	118	63	25
Leaves*	V2	76	16				
	R2	59	13				
Roots	V2	51	11				
	R2	41	9				
	R8	37	8				
Flowers	R2	83	18				
Pods	R8	17	4				
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 BPS-CV127-9 derived pPlants in the Santo Antônio de Goiás / GO location.

Experiment / Location											
CVSOY-07-002-003 / Sto Antônio de Goiás - GO											
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	<14	2	22	<78	12	24
	Leaves	21 and 23	Event 127	6	6	18	4	21	113	31	28
	Roots	21 and 23	Event 127	6	2	<14	1	7	<51	4	12
	1st trifoliates	21 and 23	Event 127	6	2	21	4	20	126	23	19
	Whole Plants	22 and 24	Non GM Control	6	6	<14	2	36	<78	8	28
	Leaves	22 and 24	Non GM Control	6	6	<14	1	17	<76	7	17
	Roots	22 and 24	Non GM Control	6	2	<14	1	8	<51	1	3
	1st trifoliates	22 and 24	Non GM Control	6	2	<14	0	0	<76	3	6
R2 (Full Bloom)	Whole Plants	21 and 23	Event 127	6	6	<14	3	32	<66	11	32
	Leaves	21 and 23	Event 127	6	6	<14	3	22	<59	11	25
	Roots	21 and 23	Event 127	6	6	<14	3	29	<41	10	40
	Flowers	21 and 23	Event 127	6	2	-	-	-	-	-	-
	Whole Plants	22 and 24	Non GM Control	6	6	<14	1	-	<66	3	-
	Leaves	22 and 24	Non GM Control	6	6	<14	1	15	<59	4	17
	Roots	22 and 24	Non GM Control	6	6	<14	3	55	<41	7	56
	Flowers	22 and 24	Non GM Control	6	2	-	-	-	-	-	-
R8 (Maturity)	Whole Plants	21 and 23	Event 127	6	6	<14	1	8	<28	3	16
	Pods	21 and 23	Event 127	6	6	<14	2	18	<17	2	15
	Roots	21 and 23	Event 127	6	6	17	8	48	48	27	57
	Grains	21 and 23	Event 127	-	2	<14	1	14	<15	1	14
	Whole Plants	22 and 24	Non GM Control	6	6	<14	1	21	<28	3	24
	Pods	22 and 24	Non GM Control	6	6	<14	1	16	<17	1	15
	Roots	22 and 24	Non GM Control	6	6	<14	3	30	<37	7	30
	Grains	22 and 24	Non GM Control	-	2	<14	0	0	<15	0	0

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.

Table 4. AHAS Protein Levels on a Fresh/ Dry Weight Basis during Development of BPS-CV127-9 derived Plants in Brasília / DF location.

Experiment / Location											
CVSOY-07-002-005 / Brasília - DF											
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	<14	4	31	<78	20	32
	Leaves	21 and 23	Event 127	6	6	27	9	35	133	51	39
	Roots	21 and 23	Event 127	6	2	<14	3	26	<51	18	41
	1st trifoliate	21 and 23	Event 127	6	2	22	8	36	111	40	37
	Whole Plants	22 and 24	Non GM Control	6	6	<14	1	18	<78	9	23
	Leaves	22 and 24	Non GM Control	6	6	<14	1	21	<76	8	22
	Roots	22 and 24	Non GM Control	6	2	<14	1	7	<51	1	1
	1st trifoliate	22 and 24	Non GM Control	6	2	<14	0	0	<76	1	4
R2 (Full Bloom)	Whole Plants	21 and 23	Event 127	6	6	<14	1	24	<66	6	22
	Leaves	21 and 23	Event 127	6	6	<14	2	29	<59	9	30
	Roots	21 and 23	Event 127	6	6	<14	4	33	<41	11	32
	Flowers	21 and 23	Event 127	6	2	<14	1	6	<83	6	9
	Whole Plants	22 and 24	Non GM Control	6	6	<14	1	28	<66	8	31
	Leaves	22 and 24	Non GM Control	6	6	<14	1	25	<59	7	25
	Roots	22 and 24	Non GM Control	6	6	<14	2	28	<41	7	30
	Flowers	22 and 24	Non GM Control	6	2	<14	0	0	<83	2	5
R8 (Maturity)	Whole Plants	21 and 23	Event 127	6	6	<14	3	23	<28	6	28
	Pods	21 and 23	Event 127	6	6	<14	3	39	<17	4	39
	Roots	21 and 23	Event 127	6	6	15	5	34	42	13	31
	Grains	21 and 23	Event 127	-	2	<14	1	8	<15	1	9
	Whole Plants	22 and 24	Non GM Control	6	6	<14	2	19	<28	4	27
	Pods	22 and 24	Non GM Control	6	6	<14	1	26	<17	1	26
	Roots	22 and 24	Non GM Control	6	6	15	3	23	41	8	20
	Grains	22 and 24	Non GM Control	-	2	<14	1	16	<15	1	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.

Table 5. AHAS Protein Levels in BPS-CV127-9 1st Trifoliates on a Fresh/ Dry Weight Basis at different locations.

Experiment No / Location	Stage	Tissue	Plots	Line	N° of Plants	N° of Samples	Mean ng AHAS/ g Fresh wt. / range	SD	CV%	Mean ng AHAS/ g Dry wt. / range	SD	CV%
CVSOY-07-002-001 / Uberaba - MG	V2 (2° trifoliate emerged)	1st Trifoliates	21 and 23	Event 127	12	2	59 (18 - 99)	57	98	254 (93 - 414)	227	89
			22 and 24	Non GM Control	12	2	<14 (10 - 12)	1	13	<76 (50 - 82)	8	14
CVSOY-07-002-002 / Sete Lagoas - MG	V2 (2° trifoliate emerged)	1st Trifoliates	21 and 23	Event 127	12	2	40 (24 - 56)	23	57	220 (120 - 320)	141	64
			22 and 24	Non GM Control	12	2	<14 (11 - 11)	0	0	<76 (62 - 66)	3	5
CVSOY-07-002-006 / Vilhena - RO	V2 (2° trifoliate emerged)	1st Trifoliates	21 and 23	Event 127	12	2	39 (32 -46)	10	25	207 (168 - 247)	56	27
			22 and 24	Non GM Control	12	2	14 (11 - 17)	4	30	<76 (56 - 89)	23	32
CVSOY-07-002-007 / Teresina - PI	V2 (2° trifoliate emerged)	1st Trifoliates	21 and 23	Event 127	12	2	<14 (11 - 14)	2	17	<76 (67 - 84)	12	16
			22 and 24	Non GM Control	12	2	<14 (5 - 6)	1	13	<76 (30 - 36)	4	13

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.

Table 6. AHAS Protein Levels in BPS-CV127-9 Grain on a Fresh/ Dry Weight Basis at Different Locations.

Experiment No / Location	Stage	Tissue	Plots	Line	N° of Plants	N° of Samples	Mean ng AHAS/ g dFresh wt.	SD	CV%	Mean ng AHAS/ g Dry wt.	SD	CV%
CVSOY-07-002-001 / Uberaba - MG	R8 (maturity)	Grains	21 and 23 22 and 24	Event 127 Non GM Control	- -	2 2	<14 <14	1 0	14 0	<15 <15	1 0	13 0
CVSOY-07-002-002 / Sete Lagoas - MG	R8 (maturity)	Grains	21 and 23 22 and 24	Event 127 Non GM Control	- -	2 2	<14 <14	0 1	0 24	<15 <15	0 2	0 24
CVSOY-07-002-006 / Vilhena - RO	R8 (maturity)	Grains	21 and 23 22 and 24	Event 127 Non GM Control	- -	2 2	<14 <14	0 1	0 13	<15 <15	0 1	0 13
CVSOY-07-002-007 / Teresina - PI	R8 (maturity)	Grains	21 and 23 22 and 24	Event 127 Non GM Control	- -	2 2	<14 <14	0 1	0 13	<15 <15	0 1	1 13

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.

Table 7. Estimation of AHAS Protein Levels in BPS-CV127-9 Whole plants per Hectare and Acre in two different Locations.

Experiment / Location							
CVSOY-07-002-003 / Santo Antônio de Goiás - GO							
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	<78	1,79	260000	105221	<33	<13
	Non GM Control	<78	1,91	260000	105221	<33	<13
R2 (Full Bloom)	Event 127	<66	9,27	260000	105221	<267	<108
	Non GM Control	<66	11,41	260000	105221	<267	<108
R8 (Maturity)	Event 127	<28	42,18	260000	105221	<293	<118
	Non GM Control	<28	30,89	260000	105221	<293	<118
CVSOY-07-002-005 / Brasília - DF							
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	<78	1,36	260000	105221	<33	<13
	Non GM Control	<78	1,45	260000	105221	<33	<13
R2 (Full Bloom)	Event 127	<66	19,3	260000	105221	<267	<108
	Non GM Control	<66	22,3	260000	105221	<267	<108
R8 (Maturity)	Event 127	<28	40,85	260000	105221	<293	<118
	Non GM Control	<28	46,85	260000	105221	<293	<118

	Growth Stage	**Line**	**Estimated mg AHAS/ Hectare**	**Estimated mg AHAS/ Acre**
Mean of two locations	V2	Event 127	**<33**	**<13**
		Non GM Control	**<33**	**<13**
	R2	Event 127	**<267**	**<108**
		Non GM Control	**<267**	**<108**
	R8	Event 127	**<293**	**<118**
		Non GM Control	**<293**	**<118**

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.

4. DISCUSSION

The mean of estimated levels of AHAS in two locations, Santo Antonio de Goiás/GO and Brasília/DF, in BPS-CV127-9 whole plants were <33, <267 and <293 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 field trials showed the mean of estimated Levels of AHAS in Soybean Whole Plants of <33, <267 and <293 mg/Hectare for the same growth stages V2, R2 and R8, respectively.

The AHAS protein expression in BPS-CV127-9 leaves and 1st trifoliate of V2 Growth Stage from Santo Antonio de Goiás/GO showed 18 and 21 ng of AHAS/ g fresh weight corresponding to 113 and 126 ng/ g dry weight of these tissues, respectively. Similar results for the trial from Brasília /DF the same tissues in V2, leaves and 1st trifoliate, showed 27 and 22 ng of AHAS/ g fresh weight corresponding to 133 and 111 ng/ g dry weight of these tissues, respectively. The other GMO and non-GMO tissues in this growth stage showed non-quantifiable (values less than the LOQ -14 ng AHAS/g fresh weight tissue) or non-detectable amounts of AHAS for the two locations.

In R2 growth stage from Santo Antonio de Goiás/GO and from Brasília/DF locations, all BPS-CV127-9 and non-GMO plant tissues analyzed (whole plants, leaves, roots and flowers) had non-quantifiable amounts of AHAS.

At maturity stage (R8 stage) only BPS-CV-127-9 roots showed quantifiable AHAS expression in both locations. The results were 17 ng AHAS/ g fresh weight (or 48 ng AHAS/ g dry weight) from Santo Antônio de Goiás/GO and 15 ng AHAS/ g fresh weight (or 42 ng AHAS/ g dry weight) from Brasília/DF. The other GMO and non-GMO tissues in this growth stage showed non-quantifiable amounts of AHAS, except for the non-GMO roots from Brasília, showing 15 ng AHAS/ g fresh weight (or 41 ng AHAS/ g dry weight).

The 1st trifoliate material collected from BPS-CV127-9 soybeans at V2 from Uberaba/ MG, Sete Lagoas/ MG, Vilhena/RO and Teresina/PI, showed 59, 40, 39, and <14 ng of AHAS / g fresh weight (or 254, 220, 207, and <76 ng of AHAS / g dry weight), respectively. Except for Vilhena/RO, the non-GM soybean leaves from all locations had levels of AHAS less than the LOQ. The AHAS expression in Vilhena/RO non-GM leaves was 14 ng/g fresh tissue (<76 ng/g dry tissue).

The BPS-CV-127-9 and non-GM mature grain from all locations had levels of AHAS lower than the LOQ.

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