Valine amino acid determination in transgenic soybean exposed to glyphosate

Determinação de aminoácidos da valina em soja transgênica exposta ao glifosato

DOI: 10.55905/rdelov16.n45-021

André Luiz de Souza Lacerda
PhD in Agricultural Engineering
Institution: Flextronics Instituto de Pesquisa (FIT)
Address: Sorocaba - SP, Brasil
E-mail: andre.lacerda@fit-tecnologia.org.br

ABSTRACT
With the increase of glyphosate tolerant transgenic soybean cultivation areas, this substance has become the main herbicide for this crop, its use may interfere in the metabolism and nutritional aspects of transgenic plants. In this scenario, the objective of this work was to verify concentrations of amino acid valine and in glyphosate tolerant soybean cultivar. Comparing the averages between protein and total amino acid levels in transgenic soybean exposed and not exposed to glyphosate, it was found that there was no significant difference by Tukey test at 5% probability. In conclusion, the data indicate that although the transgenic event affects some routes, amino acid synthesis was not affected nor in nutritional terms at the glyphosate doses studied.

Keywords: soybean, amino acids, transgenics, glyphosate.

RESUMO
Com o aumento das áreas de cultivo de soja transgênica tolerante ao glifosato, substância que se tornou o principal herbicida para essa cultura, seu uso pode interferir no metabolismo e nos aspectos nutricionais das plantas transgênicas. Nesse cenário, o objetivo deste trabalho foi verificar as concentrações do aminoácido valina e em cultivar de soja transgênica exposta e não exposta ao glifosato. Comparando as médias entre os níveis de proteína e aminoácidos totais na soja transgênica exposta e não exposta ao glifosato, verificou-se que não houve diferença significativa pelo teste de Tukey a 5% de probabilidade. Em conclusão, os dados indicam que, embora o evento transgêncio afete algumas rotas, a síntese de aminoácidos não foi afetada nem em termos nutricionais nas doses de glifosato estudadas.

Palavras-chave: soja, aminoácidos, transgênicos, glifosato.
1 INTRODUCTION

Amino acids are molecules with structural characteristics in common, formed by a central carbon, almost always asymmetric, linked to a carboxyl group (COOH), an amino group (NH2) and a hydrogen atom. In addition to these three structures, amino acids have a radical generically called “R”, which differentiates them (Figure 1). A wide variety of amino acids is theoretically possible, but from a practical point of view, we are only interested in L-alpha-amino acids. Only 20 types are used for building proteins [6]. Of these 20 amino acids, nine amino acids are essential: Phenylalanine, Valine, Tryptophan….

Valine (symbol Val or V) [11] is an α-amino acid that is used in the biosynthesis of proteins. It contains an α-amino group (which is in the protonated −NH$_3^+$ form under biological conditions), an α-carboxylic acid group (which is in the deprotonated −COO$^-$ form under biological conditions), and a side chain isopropyl group, making it a non-polar aliphatic amino acid. It is essential in humans, meaning the body cannot synthesize it: it must be obtained from the diet. Human dietary sources are foods that contain protein, such as meats, dairy products, soybean products, beans and legumes. It is encoded by all codons starting with GU (GUU, GUC, GUA, and GUG).

Several hypotheses are attributed to the effects of amino acids on plants. The main functions of amino acids would be: protein synthesis; intermediate compounds of endogenous plant hormones; complexing effect on nutrients and other agrochemicals. Amino acids and their applications in agriculture: greater resistance to water stress and high temperature, greater tolerance to attack by diseases and pests.

Figure 1 – General structure of amino acid (A) and valine amino acid (B).

![Figure 1](source: Author (2023))
However, such statements lack scientific foundations. The effect of amino acids on plants has been investigated by some authors, however there are still basic questions such as: absorption of amino acids by plants; use of exogenous amino acids by the plant, sites of action in the plant metabolism have not been found, however, works that effectively demonstrate the positive action of the direct application of amino acids in plants. The difficulty in absorbing amino acids, the plants need for specific amino acids and their intermediate position in secondary metabolism are aspects that interfere with the correct interpretation of their modes of action.

Several commercial products containing amino acids also contain mineral nutrients and other compounds, making it difficult to characterize their specific effect on plants. It is considered, based on some evidence, that some amino acids can act as plant protectors from the action of mineral salts and other agrochemicals or, on the contrary, increase the absorption and effect of these products [5].

Aboute glyphosate is a non-selective product that controls large numbers of narrow broadleaved plants by inhibiting EPSP synthetase, an enzyme that participates in the metabolic pathway of aromatic amino acid biosynthesis (tryptophan, tyrosine and lysine), which are essential for growth of the plant [8].

Companies have developed genetically modified soybean cultivars, where the gene (CP4) that encodes the enzyme EPSPs [5-enolpyruvate-shikimate-3-phosphate synthase] inhibits the action of glyphosate. Thus, it is possible that glyphosate tolerant soybeans develop even after application of the glyphosate herbicide [8].

To understand what has been accomplished, it is necessary to comment on the mode of action of glyphosate.Glyphosate, active ingredient of Roundup® (N-phosphonomethyl-glycine) herbicide, binds and blocks the activity of the enzyme EPSP (5-enolpyruvylshikimate-3-phosphate) synthase, which participates in the aromatic amino acid biosynthesis in plants. In the absence of glyphosate, the enzyme EPSPS acts by catalyzing the reaction of S3P (or shikimate-3-phosphate) and PEP (phosphoenolpyruvate), giving rise to the production of EPSP (5-enolpyruvylshikimate-3-phosphate), which are the substances responsible for the synthesis of aromatic amino acids. Thus, the presence of glyphosate in the plant, restricting the manufacture of aromatic amino acids, makes it impossible to synthesize various proteins, causing the plant to paralyze growth of both the root system and the shoot.
In 2010, an experiment conducted in Maringá, brought to light more evidence that reinforces the potential use of amino acids in the prevention of injuries caused by glyphosate [13]. In this research, the products were applied on RR soybean plants (Glycine max cultivars BRS 242 RR and Embrapa 58), and composed the following treatments: a) without amino acids; b) seed treatment with amino acids; c) seed treatment with amino acids + foliar application of amino acids and d) only foliar application of amino acids combined with different doses of glyphosate (1,200 and 2,400 g i.a. ha\(^{-1}\)). The application of the commercial product based on amino acids: AminoPlus, which is composed of alanine (1.16%), arginine (0.18%), aspartic acid (1.94%), glutamic acid (3.31%), glycine (0.20%), isoleucine (0.17%), leucine (0.26%), lysine (0.24%), phenylalanine (0.14%), serine (0.17%), threonine (0.18%), tryptophan (0.17%), tyrosine (0.12%), valine (0.28%) and the nutrients: N - 11% and K\(_2\)O - 1%, via seed treatment (5 mL kg\(^{-1}\) of seeds) and/or foliar spraying (2 L ha\(^{-1}\)) reduced the phytotoxic effects caused by glyphosate. Foliar spraying (with or without seed treatment) stood out, as it prevented the decrease in height, shoot and total dry biomass, as well as avoided the reduction in dry mass and number of nodules per plant, which are caused by the application of glyphosate [13].

2 MATERIAL AND METHODS

**Field experiment:** This experiment was conducted in a randomized block design with nine treatments and four replications. The cultivar analyzed was genetically modified soybean BRS Valiosa RR. Sowing was carried with density of 14 to 16 linear plants per meter and spacing of 0.5 m between rows. The experimental plots consisted of six 5 m long lines with four replications. It was considered as useful area, the four central lines with 4 m in length, disregarding 0.5 m from the ends of each plot.

**Treatments:** consisted of glyphosate applied only once T1 and T2, respectively at doses: 1.5 and 2.0 L ha\(^{-1}\) p.c., glyphosate sequentially applied T3, T4 and T5, respectively at doses: 1.5 / 1.5; 2.0 / 1.5 and 2.0 / 1.5 / 1.5 L ha\(^{-1}\) of p.c, with intervals of 15 to 20 days between applications and T6 clean weeded control (without glyphosate application).

**Glyphosate application:** The commercial product used was Roundup Ready® in the formulation of 480 g L\(^{-1}\) acid equivalent made between 15 and 20 days after emergence of soybean seedlings, from physiological stage V2, when the edges leaves of the second trifolium
no longer touch, according to the classification of [7]. Applications have been always done in the morning, without wind to avoid drift, with the aid of a constant pressure (CO2) costal sprayer, adjusted to syrup volume of 200 L ha⁻¹ and with fan nozzles (110° - SF - 02) following the manufacturers recommendations.

Separation and analysis of amino acid composition and quantification of seed soluble lysine by HPLC: High performance Liquid Chromatography (HPLC) was employed for the separation and quantitative determination of free amino acids using a Spherisorb ODS-2 reverse phase column (C18) after derivatization with o-ophydialdehyde (OPA) [9]. OPA derivatives were detected by fluorescence, for which 1 g of flour obtained from the grinding of mature seeds in 10 ml of MCW (methanol, chloroform and water in the ratio 12:5:3) was used. The mixture was left overnigh at 4 °C. The supernatant was centrifuged at 6000 rpm for 20 min, then 1 mL chloroform and 1.5 mL water were added to each 4 mL MCW. It was centrifuged again by carefully removing the formed aqueous phase and then lyophilized. The pellet was resuspended in 300 mL of water and the free amino acid solution frozen at -20 °C. After thawing, the samples were filtered on a 0.22 µ pore PVDF Millipore filter to remove fractions of reserve proteins, albumin and globulin, which are water soluble and then analyzed by HPLC. OPA derivatives were detected by fluorescence or UV absorption. To a 20 mL aliquot (standard or sample) was added 60 mL of the OPA reagent. OPA reagent was prepared by dissolving 50 mg of OPA in 1 mL of methanol and mixing 6.5 mL of borate NaOH buffer (2.4% w v⁻¹ boric acid in H₂O; pH adjusted with 2 M NaOH). On the day of the analyzes, 5 mL of 2-mercaptoethanol was added to 625 mL of OPA. After exactly 2 minutes, 10 mL corresponding to each genotype or standard was injected into the HPLC, starting the elution of the mixture on a linear gradient (20-100% B [methanol 65%]) in buffer A (50 mM Na Oac, 50 mM Na 2 HPO 4, tetrahydrafuran, methanol, pH 7.25). The flow rate was 0.8 mL min for 50 min. The gradient has been programmed to linearly increase the ratio of “B” to “A”. OPA amino acid derivatives were detected by a fluorescence monitor, 265 nm excitation and 480 nm emission. Amino acid concentrations in the samples were determined by the area of the integrated peaks, compared to the peaks of a standard at 250 nmol mL⁻¹. For quantification of lysine specifically, the gradient was repeated, but at a 75-100% gradient, to allow the appearance of the lysine peak prior to amino acid degradation. Results were expressed as a percentage of moles of amino acids recovered in relation to total amino acids (mol%).
**Total free soluble amino acid (ALT) dosage:** After extraction of amino acids, an aliquot of this solution was analyzed to verify total soluble amino acids. A calibration curve was constructed using a leucine standard at concentrations of 40, 80, 120, 160 and 200 nmol mL\(^{-1}\). A 100 mL fraction of the amino acid solution was placed in a test tube by adding 900 mL of water. For both unknown sample and standard curve analysis, 0.5 mL of sodium citrate buffer (0.2 M, pH 5.0), 0.2 mL of ninhydrin reagent (5% in methylglycol) and 1 mL KCN (2% 0.01 M solution in methyl glycol). The test tube was covered with glass balls to prevent evaporation and placed in a water bath at 100 °C for 20 min. It was allowed to reach room temperature and was completed with 60% ethanol. Spectrophotometer reading of standards and samples against white was taken at 570 nm.

**3 RESULTS AND DISCUSSION**

In Table 1, mean total amino acid values in genetically modified soybean seeds are tolerated and exposed to glyphosate as glyphosate applications, and it was verified that there was no significant difference at the Tukey test level of 5% without total amino acid levels (nMol ml\(^{-1}\)) for glyphosate doses studied in relation to the control as glyphosate herbicide applications that do not influence the metabolism of soybean plants after total amino acid levels.

Evaluated the activity of some key enzymes involved in combating reactive oxygen species (ROS) as well as differential protein species in the leaves of both soybean genotypes, transgenic (T) and non-transgenic (NT). The results revealed that all the evaluated enzymes presented higher activity in T soybean leaves when compared to NT. Higher concentrations of hydrogen peroxide and malondialdehyde were also observed, clearly indicating an oxidative stress condition established in the transgenic genotype. In addition, 47 proteins were differentially abundant when comparing the leaves of both plants, 26 species were accurately identified, including proteins involved in genetic modification (CP4 EPSP\(_5\)) [1].

The differential evaluation of enzymes and proteins expressed in T and NT soybean seeds. Analysis of malondialdehyde, ascorbate peroxidase (EC 1.11.1.11), glutathione reductase (EC 1.6.4.2) and catalase (EC 1.11.1.6) revealed higher levels in transgenic seeds (29.8; 30.6; 71.4 and 35.3%, respectively). Protein separation in soybean seeds was performed by two-dimensional polyacrylamide gel electrophoresis and 192 proteins were obtained by laser matrix desorption / ionization (MALDI) in flight time quadrupole (MSQ) analyzed and electrospray
ionization (ESI). In addition, CP4 EPSPS enzyme, involved in genetic modification, was identified by enzymatic digestions using trypsin or chymotrypsin and ESI-QTOF MS / MS. Among the identified proteins, cytosolic glutamine synthetase, glycinin subunit G1 and glycine-rich RNA-binding were differentially expressed after analysis using the two-dimensional electrophoresis technique and the application of a regulatory factor of 1.5 or greater [2].

The results obtained in the above study indicated that the genetic modification itself could be a stress factor, causing changes in the activity of some enzymes. Changes in soybean seed proteome were corroborated with the production of MDA, which is an indicator of lipid peroxidation and oxidative stress and was higher in soybean seeds. In addition, higher enzymatic activities were observed for APX, GR and CAT in transgenic seeds. This set of results suggests a higher stress level in soybean T seeds, even when no herbicides were used, since the herbicide resistance gene was inserted into the seed DNA. This fact explains some results previously reported in the literature, stating that transgenic seeds have a greater ability to transport metals from the soil and have greater bioavailability of metals in seeds when compared to non-transgenic soybeans.

In addition, the enzyme involved in genetic modification (CP4 EPSPS) was also easily identified by ESI-QTOF MS / MS using trypsin as a cleavage enzyme. Among the 192 proteins identified, four of them were differentially expressed by 2-D DIGE analysis with a regulatory factor of 0.5 or higher. Given the correlation between differentially expressed proteins and the enzymatic activities found, it is not difficult to rationalize that genetic modification induces a condition of oxidative stress in seeds.

Tables 1 and 2 show the average values of free amino acids (asparagine, glutamine, tyrosine, methionine, valine, phenylalanine, isoleuciona, leuciona and lysine) in glyphosate tolerant soybean seeds after exposure to glyphosate applications and none.

Data indicated that although the transgenic event affects some routes, amino acid synthesis was not affected. From the results obtained, the studied organism apparently maintained a similar balance to conventional soybean, so that the genetic modification did not produce significant alterations to maintain its metabolism. This fact is moving researchers working with genetically modified organisms to better understand the specific aspects of genetically modified plant metabolism and to identify key proteins that may eventually be subject to genetic modification so that soybeans can have higher tolerances to the glyphosate herbicide.
Others researchers concluded that isolated or sequential applications of glyphosate did not affect the growth and yield of GM cultivar BRS Valiosa RR soybean, as well as the oil content and protein of GM cultivar were not altered by glyphosate, regardless of its time and dose application [8].
Table 1 - Mean values of free amino acids (asparagine, glutamine, tyrosine, methionine, valine, phenylalanine, isoleucina, leuciona and lysine) in glyphosate tolerant genetically modified soybean seeds exposed to glyphosate applications, Esalq / USP.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Doses (g.La ha⁻¹)</th>
<th>Asp</th>
<th>Glu</th>
<th>Tyr</th>
<th>Met</th>
<th>Val</th>
<th>Phe</th>
<th>Ile</th>
<th>Leu</th>
<th>Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 – Glyphosate</td>
<td>720</td>
<td>166.4 a</td>
<td>334.3 a</td>
<td>163.6 a</td>
<td>127.9 a</td>
<td>146.0 a</td>
<td>76.1 a</td>
<td>83.1 a</td>
<td>78.4 a</td>
<td>0</td>
</tr>
<tr>
<td>T2 – Glyphosate</td>
<td>960</td>
<td>140.5 a</td>
<td>346.6 a</td>
<td>134.3 a</td>
<td>107.6 a</td>
<td>129.7 a</td>
<td>49.6 a</td>
<td>54.9 a</td>
<td>53.7 a</td>
<td>0</td>
</tr>
<tr>
<td>T3 - Glyphosate/glyphosate</td>
<td>720/720</td>
<td>151.0 a</td>
<td>326.3 a</td>
<td>129.0 a</td>
<td>79.6 a</td>
<td>137.6 a</td>
<td>49.0 a</td>
<td>54.0 a</td>
<td>55.8 a</td>
<td>0</td>
</tr>
<tr>
<td>T4 - Glyphosate/glyphosate</td>
<td>960/720</td>
<td>137.5 a</td>
<td>338.6 a</td>
<td>130.7 a</td>
<td>79.8 a</td>
<td>153.5 a</td>
<td>45.9 a</td>
<td>52.4 a</td>
<td>52.2 a</td>
<td>0</td>
</tr>
<tr>
<td>T5- Glyphosate/glyphosate/glyphosate</td>
<td>960/720/720</td>
<td>145.7 a</td>
<td>339.9 a</td>
<td>127.2 a</td>
<td>118.3 a</td>
<td>141.7 a</td>
<td>49.4 a</td>
<td>53.1 a</td>
<td>53.6 a</td>
<td>0</td>
</tr>
<tr>
<td>T6 – Witness</td>
<td>---</td>
<td>131.1 a</td>
<td>348.6 a</td>
<td>132.8 a</td>
<td>111.5 a</td>
<td>117.8 a</td>
<td>50.3 a</td>
<td>52.8 a</td>
<td>51.7 a</td>
<td>0</td>
</tr>
<tr>
<td>DMS</td>
<td>---</td>
<td>111.4</td>
<td>291.7</td>
<td>105.3</td>
<td>114.5</td>
<td>107.7</td>
<td>42.3</td>
<td>44.6</td>
<td>43.8</td>
<td>---</td>
</tr>
<tr>
<td>CV (%)</td>
<td></td>
<td>30.9</td>
<td>34.6</td>
<td>31.6</td>
<td>44.0</td>
<td>31.0</td>
<td>32.7</td>
<td>31.4</td>
<td>31.1</td>
<td>---</td>
</tr>
</tbody>
</table>

Means followed by the same letter in the column do not differ statistically from each other by the Tukey test at 5% probability. Source: Author (2007).
4 CONCLUSION

In conclusion, the data indicate that although the transgenic event affects some routes, amino acid synthesis was not affected nor in nutritional terms by glyphosate applications at the studied doses.
REFERENCES


