Physiological effects of glyphosate over amino acid profile in conventional and transgenic soybean (*Glycine max*)

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ABSTRACT

This paper compares the responses of conventional and transgenic soybean to glyphosate application in terms of the contents of 17 detectable soluble amino acids in leaves, analyzed by HPLC and fluorescence detection. Glutamate, histidine, asparagine, arginine + alanine, glycine + threonine and isoleucine increased in conventional soybean leaves when compared to transgenic soybean leaves, whereas for other amino acids, no significant differences were recorded. Univariate analysis allowed us to make an approximate differentiation between conventional and transgenic lines, observing the changes of some variables by glyphosate application. In addition, by means of the multivariate analysis, using principal components analysis (PCA), cluster analysis (CA) and linear discriminant analysis (LDA) it was possible to identify and discriminate different groups based on the soybean genetic origin.

1. Introduction

Metabolite profiling is the analysis of a small number of known metabolites on specific compound classes (for example, lipid, amino acid or sterol) [1]. Profiling (or shotgun) procedures can be very informative in investigating the substantial equivalence of a genetically modified organism (GMO) and its isogenic counterpart. One such procedure is a comparison of contents of metabolites; as Garcia-Villaba et al. [2] state, such studies, done so far for soybean, have focused on the analysis of a given family of compounds. For example, isoflavones, the main secondary metabolites in soybean, have been determined by HPLC [3] and LC/MS [4]; in both of the previous works, differences between GM and non-GM soybeans were non-significant. However, Mounts et al. [5] reported differences in the phospholipids fraction of the contents of tocopherols, sterols, and phospholipids in oils obtained from GM soybeans and non-GM soybeans (determined by normal- and reversed-phase HPLC and GC).

Functions of many metabolites depend on their concentrations, determined by synthesis and turnover. Therefore, as Last et al. [1] mention, changes in the amount of some plant metabolites indicate the extent to which their functions are induced or repressed.

Resistance to stress in plants may – to some extent – be determined by amino acid metabolism, in which osmotic adjustment and the accumulation of compatible osmolytes, detoxification of active oxygen species and risk elements, and intracellular pH regulation [6] play a special role.

Glyphosate is used to control annual and perennial grasses and broad-leaved weeds, either non-selectively in fruit orchards, vineyards, rubber and oil palm plantations, ornamental trees and brushes, non cropland and post-planting/pre-emergence in cereals, vegetables and other crops, or selectively in genetically modified glyphosate-tolerant crops [7]. It inhibits 5-enolpyruvylshikimate-3-phosphate synthase, a plant specific enzyme required for the production of aromatic amino acids (phenylalanine, tryptophan and tyrosine) in the shikimate pathway [8,9].

Glyphosate treatment increases concentration of amino acids [8,10]. Even though it should not have any direct effect on the amino acids of the non-aromatic biosynthetic families, for some of these compounds a general increase in concentration is seen, subject to increasing concentrations of glyphosate [10].

The objective of the present work was to study the free amino acid contents of soybean lines – resistant and susceptible to glyphosate – with the focus on amino acids profiles' responses to glyphosate applications. Amino acid profile and differentiation of soybean lines by amino acid contents in leaves were also analyzed.

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2. Material and methods

2.1. Reagents

The water used in all studies was ultrapure water (18.2 MΩ cm) obtained from Millipore Milli-Q Integral Water Purification System (Billerica, MA). Amino acid standards and o-phthaldialdehyde (OPA) were bought from Sigma (St. Louis, MO). Methanol, sodium acetate, disodium phosphate, acetic acid and tetrahydrofuran were obtained from Merck (Darmstadt, Germany).

2.2. Instrumental

The HPLC data were obtained by AKTA Purifier FPLC (Amersham Pharmacia Biotech, Piscataway, NJ). The amino acids were separated on a Spherisorb ODS-2 C18 column (5 μm, 4 × 250 mm). The column effluent was monitored by a fluorescence detector Shimadzu, model RF350 (Kyoto, Japan) operating with an excitation wavelength of 250 nm and an emission wavelength of 480 nm.

2.3. Plant samples

Two glyphosate-resistant lines (designated DM4800RG and Msoy7575RR) and two glyphosate-susceptible lines (DM48 and Msoy7501) were used for the experiments [8] and were brought from INTA Marcos Juarez (Argentina) and CENA, USP (Brazil). DM varieties are nearly isogenic pairs and Msoy varieties are isogenic.

2.4. Experimental design and treatments

Transgenic characteristics of plant material were determined by verifying the presence of CP4 EPSPS protein, as described by Moldes et al. [8], with positive results for DM4800RG and MSoy7575RR, and negative ones for DM48 and MSoy7501. Seeds of each soybean line were superficially sterilized using hypochlorite solution (2%) soaked in water and placed in sterile plates. Pre-germination was carried out in the dark at 30 °C for 48 h. Pre-germinated seeds were sown in sterile sand:vermiculite (3:1) in 3000 cm^3 pots. Three seedlings per pot were grown in a glasshouse at 15–30 °C, 30–60% humidity, under a natural light regime. Plants were supplied twice a week with 100 cm^3 of nutrient solution without the addition of vitamins [11] to minimize the effects of nutrient starvation. No insecticide and fungicide application was necessary to be applied during the experiment. Glyphosate (Agrisato 480 CS manufactured by ALKAGRO) was sprayed on 5-week-old plants, in an application chamber. The herbicide was diluted in water at 2:100 proportion and applied on the foliar surface using a precision sprayer device, equipped with continuous-deposition tips (XR110015), placed 0.50 m from the pot's upper surface. A 200 kPa work pressure allowed an intake corresponding to 200 dm^3 ha^{-1} of mix. The dose of glyphosate applied was the recommended by manufacturers for field applications. Leaves were harvested 0 and 72 h after glyphosate application, frozen in liquid nitrogen and stored at −80 °C. Experiments were laid out in a complete randomized design with four replications.

2.5. Extraction of amino acids and quantification

Frozen tissue (0.100 g) was homogenized in 2 cm^3 MCW extraction solution (12 cm^3 methanol, 5 cm^3 chloroform and 3 cm^3 water) and centrifuged at 2500g for 20 min at 4 °C. The supernatant was collected and added to 0.5 cm^3 chloroform and 0.75 cm^3 Milli-Q water. The water-soluble phase was used for further analysis. For the quantification of soluble amino acids in the leaves, three samples of each plant replicate were analyzed by HPLC as o-phthaldialdehyde (OPA) derivates, as described by Puiatti and Sodek [12]. The elution of the amino acids was performed with a linear gradient formed by solutions of 65% methanol and phosphate buffer pH 7.25 (50 mmol dm^{-3} sodium acetate, 50 mmol dm^{-3} disodium phosphate, 1.5 cm^3 acetic acid, 20 cm^3 tetrahydrofuran, 20 cm^3 methanol in 1000 cm^3 ultrapure water). The gradient increased the proportion of 65% methanol from 20% to 60% between 0 and 25 min, 60% to 75% from 25 to 31 min and 75% to 100% from 31 to 50 min at a 1 cm^3 min^{-1} flow rate. Data obtained from this process are expressed as nmol amino acid g^{-1} leave of fresh weight (excluding proline which does not form an OPA derivate).

2.6. Statistical analysis

The data presented correspond to mean values and standard deviation (Tables 1–3) of the amino acid contents on leave extracts with four replications of each one. Variance analysis was performed on experimental data. Significant differences between the responses of cultivars to glyphosate were determined by ANOVA and Tukey's multiple range test using the SAS statistical program (SAS Institute Inc., Cary, NC, USA, 1999).

Principal component analysis (PCA) was carried out using the Unscrumbler 6.11 software package (CAMO AS, Norway). Cluster analysis (CA) was carried out using the Multivariado software (Argentina). Linear discriminant analysis (LDA) was carried out using the InfoStat software (Argentina). In all cases the original data matrix was auto-scaled for column, subtracting the media of each column from every sample and dividing it for their standard deviation.

3. Results

3.1. Univariate statistical assessment

The amino acid content in the leaves of conventional and transgenic soybean was performed by HPLC on 17 detectable amino acids, resulting in most cases in a general increase after glyphosate foliar application. Univariate analysis of amino acid contents on leaf extracts was performed by ANOVA and Tukey's means test. Leaf extracts were collected at 0 and 72 h after glyphosate foliar application.

Three amino acids, Ser, Val and Asp, did not show differences in the extracts of the four soybean lines at 0 h (Table 1). Gln content in conventional lines (DM48 and Msoy7501) was significantly lower than in the transgenic lines (DM4800RG and Msoy7575RR). Table 1 shows differences in the content of several amino acids (Asn, Hys, Gly + Thr, Arg + Ala, Phe and Leu) for the pair DM48-DM4800RG. However, the Msoy7501-Msoy7575RR pair has no differences in almost every amino acid analyzed (except for Tyr, and Gln mentioned before). Gln was the only amino acid presenting such a decrease in both conventional lines.

The same comparison was performed with amino acids leaf extracts obtained 72 h after glyphosate foliar application (Table 2). Ser and Asp contents did not show significant differences in both pairs. Nevertheless, glyphosate effects on amino acids' metabolism were much evident 72 h after application because several free amino acids showed significant differences in both soybean pairs. The DM48-DM4800RG pair shows significant differences in eight amino acids (Glu, Asn, Hys, Gly + Thr, Arg + Ala, Met, Ile and Lys), whereas the Msoy7501-Msoy7575RR pair has differences in five amino acids (Glu, Asn, Hys, Arg + Ala and Ile).

Furthermore, the change of free amino acids content before (0 h) and after (72 h) glyphosate application was compared by Tukey's mean test. It was thus possible to observe a much more

Table 1
Statistical analysis of free amino acids contents in leaves of conventional (c) and transgenic (d) soybean immediately before glyphosate application (0 h), using Tuckey means test.

	Amino acids content in leaves (nmol g^{-1} FW)			
	DM48[a,b,c]	DM4800RG[a,b,d]	Msoy7501[a,b,c]	Msoy7575RR[a,b,d]
Asp	0.299 ± 0.071a	0.349 ± 0.040a	0.331 ± 0.080a	0.389 ± 0.077a
Glu	0.234 ± 0.073b	0.444 ± 0.079ab	0.533 ± 0.150a	0.500 ± 0.085a
Asn	1.511 ± 0.443b	2.649 ± 0.401a	1.813 ± 0.155b	2.104 ± 0.459ab
Ser	0.309 ± 0.096a	0.348 ± 0.062a	0.329 ± 0.074a	0.440 ± 0.089a
Gln	0.212 ± 0.044c	0.334 ± 0.041ab	0.301 ± 0.039bc	0.408 ± 0.070a
Hys	0.044 ± 0.012b	0.084 ± 0.020a	0.066 ± 0.014ab	0.070 ± 0.019ab
Gly + Thr	0.239 ± 0.019b	0.369 ± 0.082a	0.220 ± 0.058b	0.217 ± 0.043b
Arg + Ala	0.197 ± 0.038b	0.495 ± 0.059a	0.425 ± 0.177ab	0.387 ± 0.134ab
Tyr	0.336 ± 0.064a	0.218 ± 0.045ab	0.118 ± 0.031b	0.290 ± 0.083a
Met	0.110 ± 0.021a	0.074 ± 0.016b	0.054 ± 0.012b	0.060 ± 0.014b
Val	0.065 ± 0.011a	0.096 ± 0.022a	0.090 ± 0.015a	0.105 ± 0.032a
Phe	0.025 ± 0.006b	0.153 ± 0.054a	0.114 ± 0.032ab	0.189 ± 0.058a
Ile	0.022 ± 0.005b	0.034 ± 0.008ab	0.034 ± 0.009ab	0.045 ± 0.011a
Leu	0.011 ± 0.002b	0.027 ± 0.006a	0.022 ± 0.005a	0.026 ± 0.006a
Lys	0.057 ± 0.026b	0.200 ± 0.016a	0.045 ± 0.006b	0.059 ± 0.017b

Letters within a row indicate significant differences by Tukey means test.
 [a] Mean ± standard deviation.
 [b] n = 4 replicates.
 [c] Conventional varieties.
 [d] Transgenic varieties.

Table 2
Statistical analysis of free amino acids contents in leaves of conventional (c) and transgenic (d) soybean 72 h after glyphosate application, using Tuckey means test.

	Amino acids content in leaves (nmol gr^{-1} FW)			
	DM48[a,b,c]	DM4800RG[a,b,d]	Msoy7501[a,b,c]	Msoy7575RR[a,b,d]
Asp	0.569 ± 0.076a	0.462 ± 0.059a	0.473 ± 0.079a	0.461 ± 0.116a
Glu	1.819 ± 0.121a	0.569 ± 0.112d	1.282 ± 0.126b	0.794 ± 0.043c
Asn	6.322 ± 1.318a	3.833 ± 0.280b	5.684 ± 0.492a	3.857 ± 0.626b
Ser	0.522 ± 0.054a	0.439 ± 0.069a	0.393 ± 0.108a	0.424 ± 0.108a
Gln	0.205 ± 0.018b	0.246 ± 0.041ab	0.178 ± 0.051b	0.356 ± 0.096a
Hys	0.424 ± 0.041a	0.133 ± 0.039b	0.318 ± 0.080a	0.100 ± 0.028b
Gly + Thr	1.063 ± 0.040a	0.462 ± 0.103b	0.471 ± 0.073b	0.347 ± 0.042b
Arg + Ala	1.493 ± 0.411ab	0.792 ± 0.179c	1.855 ± 0.195a	1.001 ± 0.128bc
Tyr	0.457 ± 0.045a	0.453 ± 0.106a	0.243 ± 0.063b	0.291 ± 0.056b
Met	0.523 ± 0.111a	0.104 ± 0.016b	0.087 ± 0.026b	0.118 ± 0.033b
Val	0.087 ± 0.014b	0.105 ± 0.014b	0.569 ± 0.055a	0.122 ± 0.019b
Phe	0.487 ± 0.076a	0.458 ± 0.127a	0.050 ± 0.014b	0.379 ± 0.107a
Ile	0.516 ± 0.131a	0.089 ± 0.020b	0.516 ± 0.076a	0.088 ± 0.021b
Leu	0.032 ± 0.008b	0.038 ± 0.009b	0.322 ± 0.085a	0.031 ± 0.004b
Lys	0.568 ± 0.160a	0.134 ± 0.036c	0.204 ± 0.037bc	0.335 ± 0.069b

Letters within a row indicate significant differences by Tukey means test.
 [a] Mean ± standard deviation.
 [b] n = 4 replicates.
 [c] Conventional varieties.
 [d] Transgenic varieties.

significant increase in individual free amino acids content in conventional rather than in transgenic varieties (Table 3). We found that Glu, Asn, Gly + Thr, Arg + Ala and Ile had a significant increase due to glyphosate application in the conventional lines. DM48 was the most susceptible one, and it suffered the most important alteration in amino acid content. On the other hand, Gln did not show differences before (0 h) and after (72 h) glyphosate application for all soybean varieties and a similar result was observed for Asp and Ser (except for DM48).

3.2. Multivariate statistical assessment

Multivariate analysis was used to obtain classifications based on genetic origin. In order to perform a comparative analysis of the different soybean lines PCA, CA and LDA were used as discrimination tools. For this purpose, the multivariate analysis was carried out on the samples taken 72 h after glyphosate application. In all cases, the concentration of Ser, Hys, Gly + Thr, Tyr, Met, Val, Ile in soybean leaves was the original variable used to obtain the

models. For PCA, a score plot was obtained by using the first and second principal components, which included 82% of the total original information since, usually, they contain the major information of the system [13]. For the PCA model, five principal components were needed, which represent 98.93% of the total information. The classification through the PCA analysis was found by means of a score plot (Fig. 1). The presence of four groupings: two transgenic groups and two non transgenic groups can be observed; every sub group corresponds to the different soybean varieties: Conventional varieties DM48 and Msoy7501 and transgenic varieties DM4800RG and Msoy7575RR. The loading plot (Fig. 2) indicates the influence of every original variable on the principal components of the PCA model.

The same classification results were obtained by CA using the same variables that were used in the PCA analysis. The amalgamation criterion used in CA was complete linkage, whereas the selected distance was the Pearson distance [14]. Fig. 3 shows the grouping for the different lines of soybean. There are 3 big groups: the upper group (conventional line DM48), the middle group

Table 3
Changes of free amino acids content in leaves of conventional (a) and transgenic (b) soybean between 0 h and 72 h after glyphosate application, using Tuckey means test.

| | Amino acids content in leaves (nmol g^{-1} FW) | | | | | | | |
| | DM48[a] | | DM4800RG[b] | | Msoy7501[a] | | Msoy7575RR[b] | |
	0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
Asp	0.299b	0.569a	0.349b	0.462a,b	0.331b	0.473a,b	0.389a,b	0.461a,b
Glu	0.234e	1.819a	0.444d,e	0.569c,d	0.533c,d	1.282b	0.500d	0.794c
Asn	1.511c	6.322a	2.649c,b	3.833b	1.813c	5.684a	2.104c	3.857b
Ser	0.309b	0.522a	0.348a,b	0.439a,b	0.329a,b	0.393a,b	0.440a,b	0.424a,b
Gln	0.212c,d	0.205d	0.334a,b,c	0.246b,c,d	0.301a,b,c,d	0.178d	0.408a	0.356a,b
Hys	0.044b	0.424a	0.084a,b	0.133a,b	0.066b	0.318a,b	0.070b	0.100a,b
Gly + Thr	0.239c,d	1.063a	0.369c,b	0.462b	0.220d	0.471b	0.217d	0.347b,c,d
Arg + Ala	0.197d	1.493a	0.495c,d	0.792c,b	0.425c,d	1.855a	0.387c,d	1.001b
Tyr	0.336a,b	0.457a	0.218b,c	0.453a,b	0.118c	0.243b,c	0.290b	0.291b
Met	0.110b	0.523a	0.074b	0.104b	0.054b	0.087b	0.060b	0.118b
Val	0.065b	0.087b	0.096b	0.105b	0.090b	0.569a	0.105b	0.122b
Phe	0.025b	0.487a	0.153b	0.458a,b	0.114b	0.050b	0.189b	0.379a,b
Ile	0.022b	0.516a	0.034b	0.089b	0.034b	0.516a	0.045b	0.088b
Leu	0.011b	0.032b	0.027b	0.038b	0.022b	0.322a	0.026b	0.031b
Lys	0.057c	0.568a	0.200c,b	0.134c	0.045c	0.204c,b	0.059c	0.335b

Letters within a row indicate significant differences by Tukey means test.
[a] Conventional varieties.
[b] Transgenic varieties.
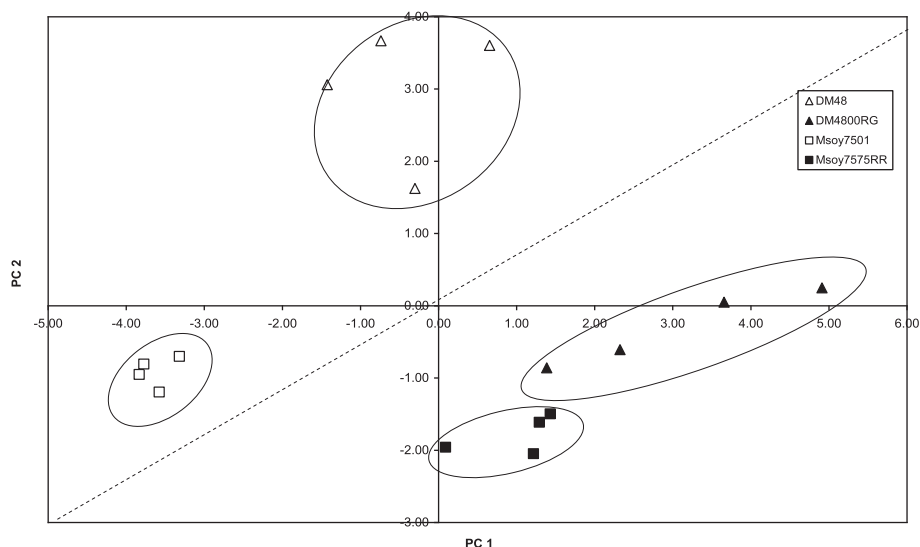
Fig. 1. Scores plot for the classification of soybean by means of PCA, showing the discrimination of conventional (white symbols) and transgenic (black symbols) varieties.

(transgenic group DM4800RG and Msoy7575RR) and the lower group (conventional line Msoy7501). In Fig. 3 it can be seen that the classification between conventional and transgenic lines is clear. Almost all samples within the transgenic group were adequately classified, except for one sample of the Msoy7575RR line. This result is in agreement with the PCA analysis, which showed that the transgenic varieties are close between them (Fig. 1).

The results obtained by LDA were similar to those obtained by PCA and CA: four sub groups (varieties) and two groups (transgenic and non transgenic soybean). For the LDA classification, the discriminant model was built using the same variables of PCA and CA. The canonical function (CF) found was the following:

$$CF = -10.74 + 0.16Ser - 2.10Hys - 0.16Gly \& Thr - 0.38Tyr + 3.53Met - 1.34Val - 3.25Ile.$$

The classification of varieties using the LDA canonical functions 1 and 2, was performed (Fig. 4). Again, the transgenic groups are close between them, whereas the conventional lines are away, as

occurred in PCA and CA. Table 4 shows the results obtained by the LDA model. It shows that all samples were adequately classified with an error of 0 % in all cases, which indicates an adequate fit of the LDA model.

4. Discussion

Noctor et al. [15] defined major amino acids as those normally present in high concentration (Glu, Gln, Asp, Asn, Gly, Ser, Ala and Thr) and the minor amino acids as generally less abundant (His, Arg, Tyr, Trp, Met, Val, Phe, Ile, Leu and Lys). In the present work, when the major amino acid levels are considered, there are specific differences between conventional and transgenic soybean. Minor amino acids do not show a correlated behavior to glyphosate application that may permit to differentiate conventional and transgenic soybean. Phe, Tyr and Trp were reported as the first amino acids biosynthesis inhibited by glyphosate because of the interference on shikimate pathway [16]. But, in some plant species, aromatic amino acids increase instead of being inhibited. Trenkamp
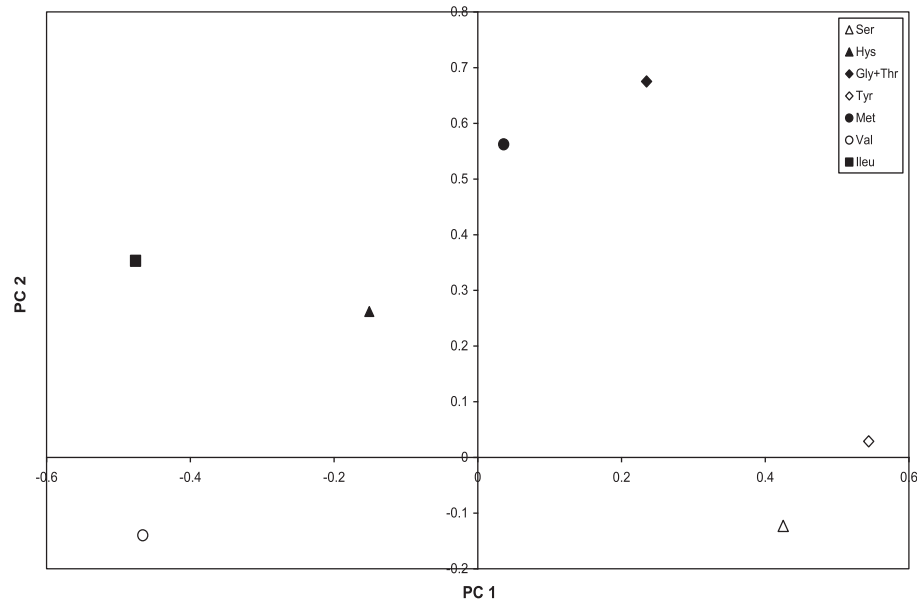
Fig. 2. Loading plot showing the influence of eight selected amino acids onto PC1 and PC2 in the final PCA model.
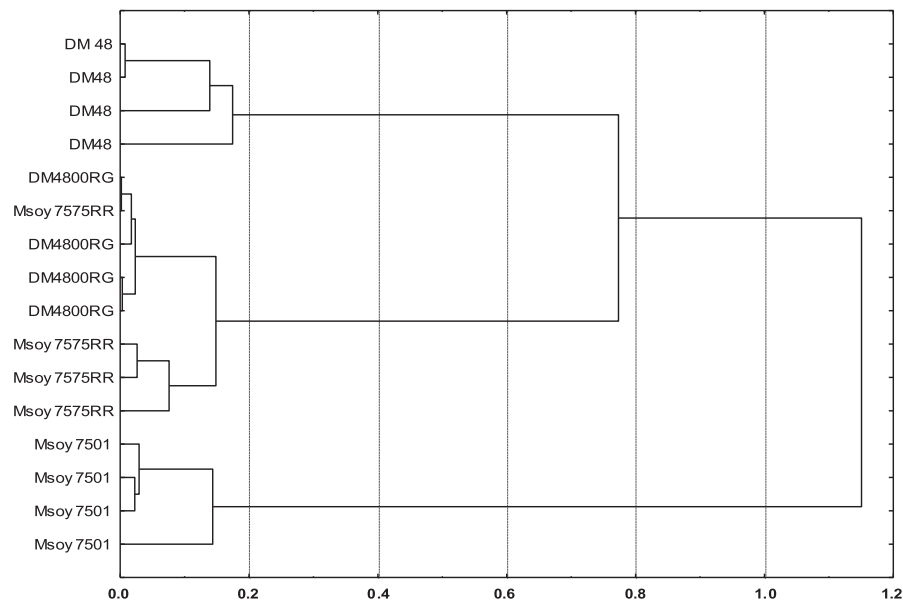

Fig. 3. Dendogram obtained by cluster analysis, showing four groups corresponding to the different transgenic and conventional soybean varieties.

et al. [17] found Phe and Tyr increase by glyphosate application in *Arabidopsis thaliana*. Other cases of increased aromatic amino acids by glyphosate were mentioned by Cooley and Foy [18].

Literature reports that glyphosate effect includes a general increase in the amino acid pool [8,10,18]. Such alteration can be due to metabolic regulations of amino acid biosynthesis and protein hydrolysis in response to the glyphosate inhibition of the shikimate pathway and its metabolites [19]. Jaworski [20] proposed that glyphosate effect on the shikimate pathway results in a slow-down in protein synthesis; as a consequence, the demand of amino acids for protein synthesis diminishes while the free amino acids pool increases. Ravn et al. [21] found similar increasing trends in the amounts of the free amino acids Glu, Gln, Pro, Val, Leu/Ile, Thr and Lys in *Apera spica-venti* in response to a foliar applied gly-phosate and they considered proteolysis should be the cause of a general increase of individual amino acids contents. Results in

the present work showed that none of the amino acids decreased in content; proteolysis could be responsible for the general in-crease of free amino acids (Table 3). Nevertheless, Gln did not suf-fer significant alterations owing to glyphosate application. Then, Gln seems to be metabolized in spite of the general protein hydro-lysis caused by glyphosate in conventional soybean lines. Asp and Ser show a similar behavior.

Gln is an amide nitrogen donor in the reaction catalyzed by asparagine synthetase (AS) (EC 6.3.5.4). Asp is the acceptor of the amide group and the reaction yielded Asn and Glu [22–25]. Several stress factors like drought, salt stress, nutrient starvation, toxic metals and pathogens can be responsible for an increase of the asparagine level as stress response [26]. The increase of Asn implies Gln and Asp expense. Because it was possible to follow the behavior of four amino acids involving this reaction, present re-sults could suggest glyphosate as a possible inductor of AS (directly
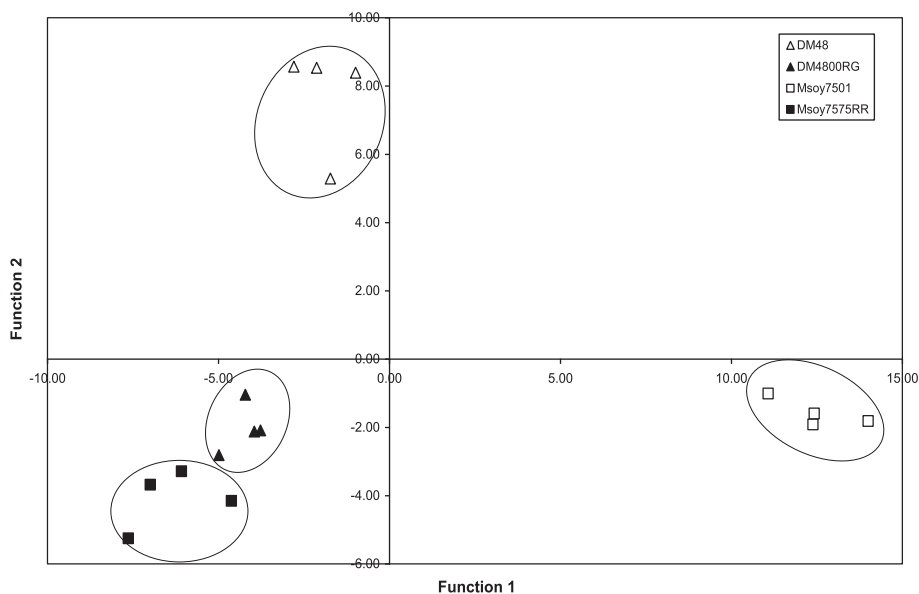
Fig. 4. LDA plot obtained by plotting the canonical functions 1 and 2, which shows the discrimination between conventional (white symbols) and transgenic (black symbols) soybean varieties.

Table 4
Linear discriminant analysis of conventional (DM48 and Msoy7501) and transgenic (DM4800 RG and Msoy7575RR) soybean varieties.

Groups	DM48	Msoy7501	DM4800RG	Msoy7575RR	Total	Error (%)
DM48	4	0	0	0	4	0.00
MS7501	0	4	0	0	4	0.00
DM4800RG	0	0	4	0	4	0.00
MS7575RR	0	0	0	4	4	0.00
Total	4	4	4	4	16	0.00

or indirectly) in conventional soybean. Furthermore, no significant alterations in Asp, Gln, Asn and Glu on resistant lines indicate that glyphosate does not initiate a stress response and does not induce significant alterations in gene expression on transgenic soybean [27].

Mineral starvation like potassium, sulfur, phosphorous, magnesium and micro nutrients (in particular zinc) can stimulate large increases in Asn concentration [26]. Then, it is possible to link ion deficiencies to an increase of Asn as side-effects of glyphosate in conventional soybean. Cakmak et al. [28] report that leaf concentrations of calcium, manganese, magnesium and iron were reduced by glyphosate application, particularly in young leaves of non glyphosate resistant soybean.

Similar to Asn, Glu increased more in conventional soybean than in transgenic lines. Glu is also involved in the synthesis of Asp [23–25], Ile, Ala and Arg [29,30], δ-aminolevulinate (belonging to the tetrapyrrols biosynthesis pathway) [16], and photorespiration pathway [31]. The accumulation of Glu in conventional soybean could be due to the inhibition of pathways that use glutamate. Nevertheless, the present work shows no diminishing of Asp, Ile, Ala or Arg biosynthesis to indicate their inhibition. Literature reports that δ-aminolevulinate synthesis is strongly inhibited by glyphosate in several plant species like maize, barley and soybean among others. In this case, interferences in glutamate activation for δ-aminolevulinate synthesis could be caused by Glu accumulation [16].

Ser and Gly are involved in the photorespiration pathway [32]. The Gly biosynthesis is carried out by several enzymes like serine:glyoxylate aminotransferase and glutamate:glyoxylate aminotransferase, situated in peroxisome, transaminating glyoxylate to Gly, using Ser or Glu as amino donor respectively [31]. Gly accumulation and no significant alteration of Ser contents in glyphosate susceptible soybean could be indicating interferences in the

transport of Gly to the mitochondrion. In this organelle, two molecules of Gly yield one molecule of Ser by combined action of glycine decarboxylase and serine hydroxymethyltransferase [31]. Some reports have shown that glyphosate triggers oxidative stress in plants [8,33–35], while other reports show that this stress inhibits glycine decarboxylase and increases Gly/Ser ratio [36,37].

4.1. Multivariate analysis

Previous works have studied multivariate classification in different samples to determine denomination of origin for honey [38] and wines [39]; quality of edible oils [40], classification of crops [41] and varieties of fruits [42]. More recently, multivariate tools were successfully used for the classification of propolis samples [43] and South American herbs [44].

Despite the multivariate analysis can be influenced for other factors as environmental conditions, temperature, hydration of the plants, time after application and dose of glyphosate, etc, these conditions were carefully controlled to avoid mistakes in the interpretation of model.

All multivariate tools have shown a similar ability for the classification of soybean lines. It can be seen that PCA, CA and LDA were able to classify soybean based on its genetic origin. For PCA, a straight line which separates the conventional groups from the transgenic groups can be observed in Fig. 1. The principal differentiation between varieties in Fig. 2 is given by Hys and Ile. Among conventional varieties, DM48 is separated by Gly + Thr and Met influence, whereas Val influenced Msoy7501 separation. Among transgenic varieties, Tyr contents influenced their differentiation.

Cluster analysis (Fig. 3) shows a similar behavior to that of PCA, because it was able to classify two big groups: transgenic and non-transgenic. Inside every group there are two small groups: the

transgenic group contains the DM4800RG and MSOY7575RR lines; the non transgenic group DM48 and MSOY7501. In the transgenic group, almost all samples (with exception of one sample of DM4800RG) were correctly classified. In the non-transgenic group, all samples were correctly classified. This indicates that CA is an important multivariate tool for a satisfactory classification.

LDA is a supervised method, which means that it involves both, calibration and prediction steps. In both cases, LDA produced 0% error (Table 4) which means that the supervised classification was free from error. Moreover, Fig. 4 shows the classification of varieties using the LDA canonical functions 1 and 2. In this plot the four groupings can be seen in a similar way to that observed in the score plot (Fig. 1) which allowed obtaining another graphic classification. In this case, the transgenic varieties (DM4800RG and Msoy 7575RR) are close between them, whereas the conventional varieties are away, as shown in the PCA and CA. In addition, all multivariate tools were capable of providing a correct classification, which involves new and fast methods to discriminate conventional from genetically modified lines.

5. Conclusions

This paper showed a complete statistical analysis carried out in conventional and transgenic soybean varieties. Through the univariate analysis we can establish a differentiation between conventional and transgenic lines, observing the changes of some variables by glyphosate application. Furthermore, the comparison of glyphosate effects over amino acids profile between conventional and transgenic soybean allowed us to consider possible effects of glyphosate over functionality of key enzymes like asparagine synthetase, side-effects as mineral nutrient starvation, and altered photorespiration, which indicate that a rigorous analysis of the amino acid profile could give information about other secondary target of glyphosate. Using the PCA, CA and LDA multivariate analyses, it was possible to achieve the correct classification of the four studied soybean varieties. For these reasons, this paper is useful both, to evaluate the amino acid profile behavior and to differentiate conventional or transgenic soybean varieties by the application of glyphosate.

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