JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Changes in Amino Acid Profile in Roots of Glyphosate Resistant and Susceptible Soybean (*Glycine max*) Induced by Foliar Glyphosate Application

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ABSTRACT: Amino acid profiles are useful to analyze the responses to glyphosate in susceptible and resistant soybean lines. Comparisons of profiles for 10 amino acids (Asp, Asn, Glu, Gln, Ser, His, Gly, Thr, Tyr, Leu) by HPLC in soybean roots were performed in two near isogenic pairs (four varieties). Foliar application of glyphosate was made to soybean plants after 5 weeks of seeding. Roots of four varieties were collected at 0 and 72 h after glyphosate application (AGA) for amino acid analysis by HPLC. Univariate analysis showed a significant increase of several amino acids in susceptible as well as resistant soybean lines; however, amino acids from the major pathways of carbon (C) and nitrogen (N) metabolism, such as Asp, Asn, Glu and Gln, and Ser, increased significantly in susceptible varieties at 72 h AGA. Multivariate analysis using principal component analysis (2D PCA and 3D PCA) allowed different groups to be identified and discriminated based on the soybean genetic origin, showing the amino acid responses on susceptible and resistant varieties. Based on the results, it is possible to infer that the increase of Asn, Asp, Glu, Gln, and Ser in susceptible varieties would be related to the deregulation of C and N metabolism, as well as changes in the growth mechanisms regulated by Ser.

KEYWORDS: *glyphosate, amino acid profile, soybean, transgenic, multivariate analysis*

1. INTRODUCTION

The analysis of free amino acids involved in connected metabolic pathways (i.e., amino acids from N assimilation such as Asp, Asn, Glu, and Gln) can be useful to understand how it extends the effect of stressors in metabolism, including the comparison of conventional and transgenic counterparts of plant species after herbicide application.

Glyphosate is a broad spectrum herbicide whose mode of action is the inhibition of 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) from the shikimate pathway, responsible for the synthesis of aromatic amino acids (phenylalanine, tryptophan, and tyrosine).[1−4] Although the mode of action of glyphosate does not involve other targets in metabolism than EPSPS, its application can induce increases in the majority of individual amino acids[4,5] especially when dealing with susceptible varieties.[6,7]

Multivariate methods can explain complex relationships among big data collections, which are difficult to reveal by univariate analysis;[8] these tools have been used for a wide spectrum of analysis to obtain genotypical classification.[6,9,10] Furthermore, chemometrics appears as an appropriate statistical tool, considering the complex nature of biochemical responses to stress and their interrelationships.[11] Chemometrics has been successfully applied to biological systems in order to analyze several types of interactions and effects: (a) in the stress response using data of antioxidant enzymes activity in cotton plants fertilized by silicon;[12] (b) to show differential antioxidant responses in wheat under pathogenic fungus incidence;[8] (c) to obtain indicators for changes in processed and stored vegetables;[13] and (d) to elucidate the metabolic pathway participants in development of plant pathogenicity.[14]

The present work aims to study the free amino acid contents in roots of soybean lines resistant and susceptible to glyphosate, focusing on amino acid profile responses after glyphosate applications for the discrimination of soybean lines. Furthermore, it intends to explore the use of chemometric tools for interpretation of metabolic consequences of glyphosate application, describing the behavior and transport of amino acids from N assimilation, as well as the signaling of serine in soybean roots.

Received: August 10, 2017
Revised: September 13, 2017
Accepted: September 18, 2017
Published: September 18, 2017

DOI: 10.1021/acs.jafc.7b03676
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 1. Statistical Analysis of Free Amino Acid Content in Roots of Susceptible (DC = DM48 and MC = MSOY7501) and Resistant (DR = DM4800RG and MR = MSOY7575RR) Soybean at 0 and 72 h after Glyphosate Application, Using the Tukey Means Test[a]

	amino acid content in roots (ηmol gr^{-1} FW)			
	DC0	DC72	DR0	DR72
Asp	0.00428 ± 0.00042 c	0.03637 ± 0.01100 a	0.00913 ± 0.00269 c	0.01087 ± 0.00300 c
Glu	0.00524 ± 0.00195 b	0.01979 ± 0.00521 a	0.00585 ± 0.00043 b	0.00980 ± 0.00233 b
Asn	0.09779 ± 0.06534 b	0.49014 ± 0.32486 a	0.29894 ± 0.05952 ab	0.41349 ± 0.24721 ab
Ser	0.00426 ± 0.00092 cd	0.01685 ± 0.00319 a	0.00531 ± 0.00090 cd	0.01029 ± 0.00217 b
Gln	0.00900 ± 0.00224 c	0.04471 ± 0.00579 a	0.00725 ± 0.00203 c	0.01090 ± 0.00222 c
His	0.01536 ± 0.00410 cb	0.02040 ± 0.00628 ab	0.02594 ± 0.00523 a	0.02959 ± 0.00574 a
Gly	0.28409 ± 0.04923 cb	0.24856 ± 0.07380 cb	0.32584 ± 0.02036 cb	0.41479 ± 0.12878 ab
Thr	0.06466 ± 0.01611 de	0.11182 ± 0.01080 ab	0.09384 ± 0.01259 bcd	0.10853 ± 0.02887 abc
Tyr	0.08698 ± 0.01948 ab	0.15686 ± 0.09658 a	0.08977 ± 0.00898 ab	0.08310 ± 0.00700 ab
Leu	0.00812 ± 0.00264 d	0.05935 ± 0.01059 bc	0.00668 ± 0.00102 d	0.06377 ± 0.01537 bc
	amino acid content in roots (ηmol gr^{-1} FW)			
	MC0	MC72	MR0	MR72
Asp	0.01081 ± 0.00129 c	0.02465 ± 0.00147 b	0.00466 ± 0.00103 c	0.00656 ± 0.00087 c
Glu	0.00641 ± 0.00158 b	0.01636 ± 0.00271 a	0.00425 ± 0.00063 b	0.00789 ± 0.00071 b
Asn	0.17362 ± 0.02111 ab	0.37761 ± 0.06344 ab	0.21780 ± 0.01835 ab	0.16078 ± 0.02344 ab
Ser	0.00868 ± 0.00209 bc	0.01839 ± 0.00266 a	0.00403 ± 0.00102 d	0.00708 ± 0.00099 bcd
Gln	0.01476 ± 0.00242 c	0.02861 ± 0.00784 b	0.01293 ± 0.00163 c	0.00758 ± 0.00219 c
His	0.00986 ± 0.00222 c	0.02067 ± 0.00480 ab	0.00864 ± 0.00109 c	0.01199 ± 0.00329 cb
Gly	0.17620 ± 0.04995 c	0.40580 ± 0.08581 ab	0.31300 ± 0.01176 cb	0.52471 ± 0.07639 a
Thr	0.04212 ± 0.00964 e	0.11512 ± 0.02244 ab	0.06977 ± 0.01512 cde	0.14442 ± 0.01058 a
Tyr	0.04673 ± 0.00972 b	0.15364 ± 0.03336 a	0.08280 ± 0.01050 ab	0.15108 ± 0.00918 a
Leu	0.08409 ± 0.01860 b	0.08759 ± 0.02836 b	0.04144 ± 0.01767 cd	0.13925 ± 0.00924 a

[a]Means of $n = 4$ ± standard deviation. Means obtained from four varieties at 0 and 72 h AGA (eight treatments in total) for each amino acid were analyzed by Tukey means test. Means containing the same letter are not significantly different according to Tukey means test ($P < 0.05$).

2. MATERIALS AND METHODS

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

2.2. Instrumental. Amino acid profiles were obtained from an AKTA Purifier FPLC (Amersham Pharmacia Biotech, Piscataway, NJ). Separation of amino acids was carried out on a Spherisorb ODS-2 C18 column (5 μm, 4 × 250 mm). Amino acids were detected by a Shimadzu fluorescence detector, model RF350 (Kyoto, Japan), operating with an excitation wavelength of 250 nm and an emission wavelength of 480 nm.

2.3. Plant Samples. Two pairs of nearly isogenic glyphosate susceptible and resistant soybean varieties were used: pair DM48-DM4800RG obtained from INTA Marcos Juarez (Argentina) and pair Msoy7501-Msoy7575RR from CENA, USP (Brazil).

2.4. Experimental Design and Treatments. The presence of CP4 EPSPS gene was determined in seeds of the varieties mentioned above by PCR as described by Moldes et al.[4] to verify the characteristic of glyphosate susceptibility−resistance, with positive results for DM4800RG and MSoy7575RR, and negative ones for DM48 and MSoy7501. Seeds of each soybean variety were superficially sterilized using hypochlorite solution (2%) soaked in water and placed in sterile plates. Pregermination was carried out in the dark at 30 °C for 48 h. Pregerminated 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.[15] No insecticide and fungicide application was made during all 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 rates and applied on the foliar surface using a precision sprayer device, equipped with continuous-deposition tips (XR110015) to 0.50 m from the pot's upper surface. A pressure of 200 kPa allowed an intake corresponding to 200 dm^3 ha^{-1} of solution, which is the dose of glyphosate recommended by manufacturers for field applications. Plants were harvested at 0 and 72 h after glyphosate application (AGA), frozen in liquid nitrogen, and stored at −80 °C. Experiments were carried out in a completely randomized design with four replications. For simplicity, names of conventional (C) and resistant (R) varieties were designated as follows: DM48, DC; DM4800RG, DR; Msoy7501, MC; Msoy7575RR, MR. Treatments at 0 and 72 AGA were designated for variety DM48 at 0 h AGA as DC0; DM48 at 72 h AGA as DC72; DM4800RG at 0 h AGA as DR0; DM4800RG at 72 h AGA as DR72; Msoy7501 at 0 h AGA as MC0; Msoy7501 at 72 h AGA as MC72; Msoy7575RR at 0 h AGA as MR0; Msoy7575RR at 72 h AGA as MR72.

2.5. Extraction of Amino Acids and HPLC Quantification. Amino acids were obtained by MCW extraction solution (12 cm^3 of methanol, 5 cm^3 of chloroform, and 3 cm^3 of water). Frozen root tissue (0.100 g) was homogenized in 2 cm^3 of MCW solution and centrifuged at 2500*g* for 20 min at 4 °C. The supernatant was collected and added to 0.5 cm^3 of chloroform and 0.75 cm^3 of Milli-Q water to immiscible phase separation. The water-soluble phase was used for further analysis. Three replicate samples of each plant were analyzed by HPLC as *o*-phthaldialdehyde (OPA) derivatives as described by Puiatti and Sodek.[16] Eluent solution was performed with a solution 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 of acetic acid, 20 cm^3 of tetrahydrofuran, 20 cm^3 of methanol in 1000 cm^3 of ultrapure water) increasing 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 10 amino acids detected by HPLC (Asp, Glu, Asn, Ser, Gln, His, Gly, Thr, Tyr, and Leu) were expressed as ηmol amino acid g^{-1} root fresh weight.

DOI: 10.1021/acs.jafc.7b03676
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

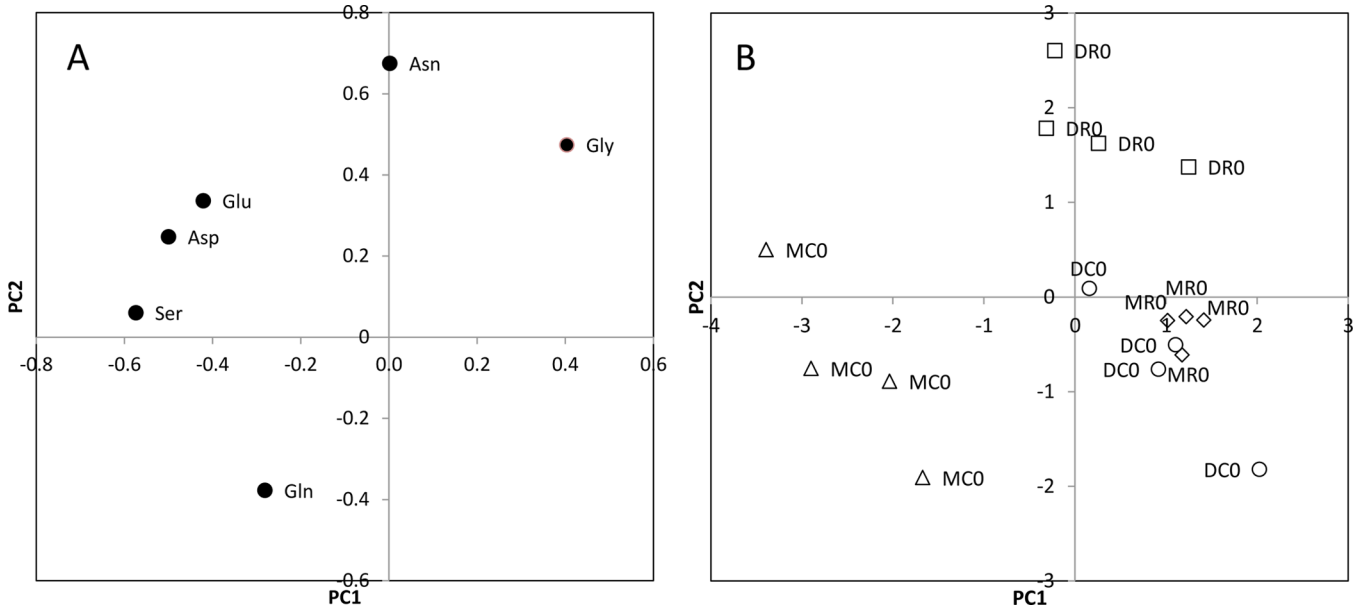

Figure 1. Loading (A) and score (B) plots for 0 h AGA showing the discrimination of susceptible and resistant varieties. DC0 = DM48; DR0 = DM4800RG; MC0 = MSOY7501; MR0 = MSOY7575RR.

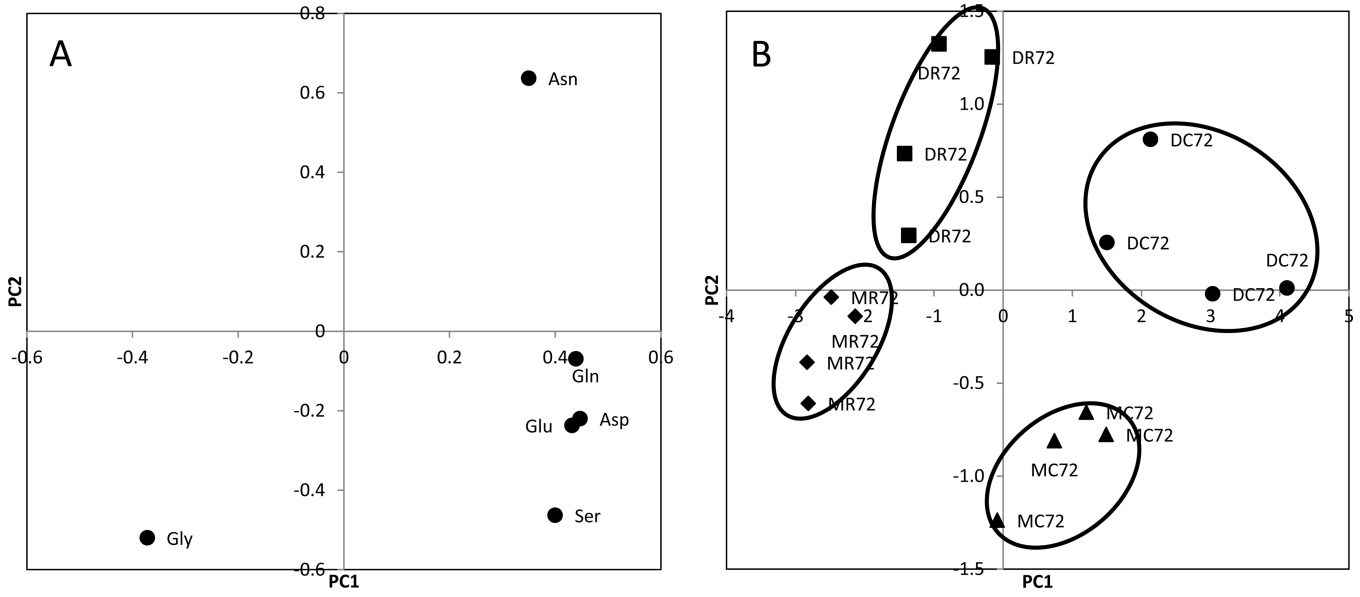

Figure 2. Loading (A) and score (B) plots for 72 h AGA showing the discrimination of susceptible and resistant varieties. DC72 = DM48; DR72 = DM4800RG; MC72 = MSOY7501; MR72 = MSOY7575RR.

2.6. Statistical Analysis. Data analysis was performed by ANOVA, and significant differences between the responses of cultivars to glyphosate were determined by Tukey's multiple range test using the SAS statistical program (SAS Institute Inc., Cary, NC, USA, 1999) ($P < 0.05$).

Principal component analysis (PCA) was carried out using the Unscrambler X 10.3 software package (CAMO AS, Norway) using autoscaled data.

3. RESULTS

3.1. Univariate Statistical. The present work revealed a significant increase for Asp, Glu, Gln, Thr, and Tyr in DC72, while DR72 did not show such an increase (Table 1): Asn, Ser, and Leu increased significantly in both varieties although it was more drastic in DC72 for Asn and Ser, whereas His and Gly did not exhibit significant differences in both varieties. On the other hand, the MC−MR pair showed similar behavior to the DC−DR pair, but Asp, Glu, Asn, Ser, Gln, and His increased in DR72 (Table 1). Thr and Tyr increased in both varieties, and Leu increased only in the resistant variety. In both pairs, the most abundant amino acids measured were Asn and Gly but only Asn exhibited a similar behavior in comparison to total amino acids in roots. In adition, Tyr increased in both susceptible varieties DC72 and MC72.

3.2. Multivariate Analysis. Multivariate analysis was carried out for evaluation of glyphosate effects in two PCA models (2D and 3D). In the 2D PCA score plots, varieties are classified according to individual amino acid contents, obtaining two multivariate models, one at 0 h (Figure 1) and another at 72 h (Figure 2) after glyphosate application (AGA): this model was obtained using 5 principal components (PC), wich explain

DOI: 10.1021/acs.jafc.7b03676
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

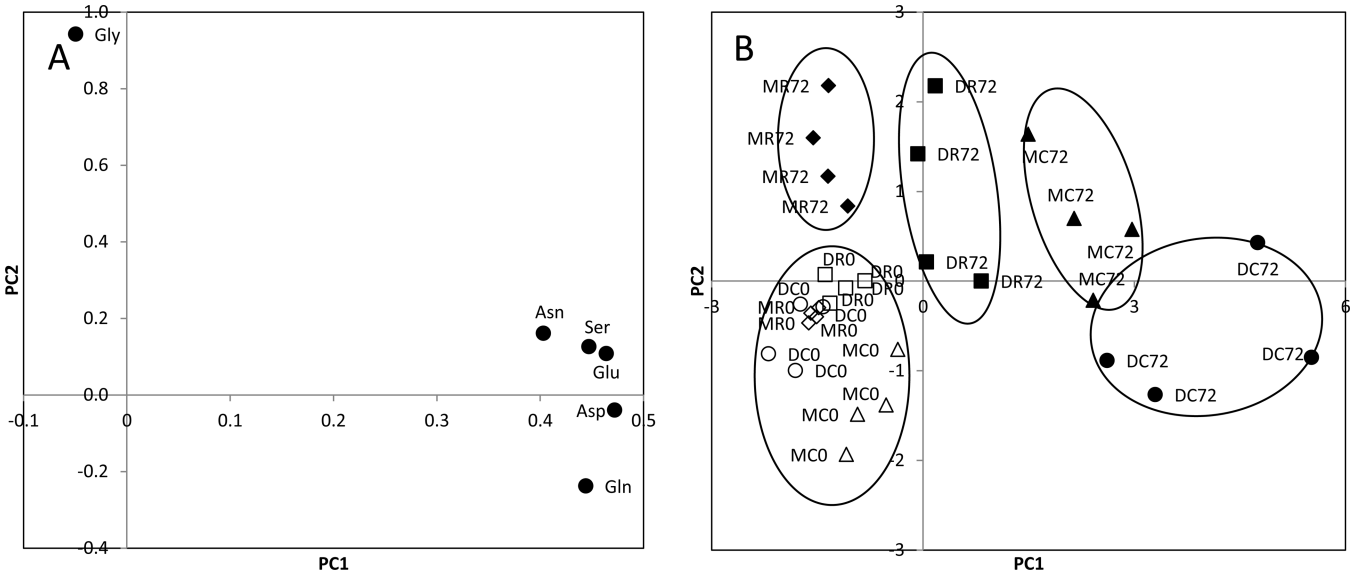

Figure 3. Loading (A) and score (B) plots for 0 and 72 h AGA using 2 PCs. Score plot shows changes from 0 h AGA (white symbols), which performs a unique group, to 72 h AGA (black symbols), which shows four differential responses to glyphosate by each variety. DC = DM48; DR = DM4800RG; MC = MSOY7501; MR = MSOY7575RR.

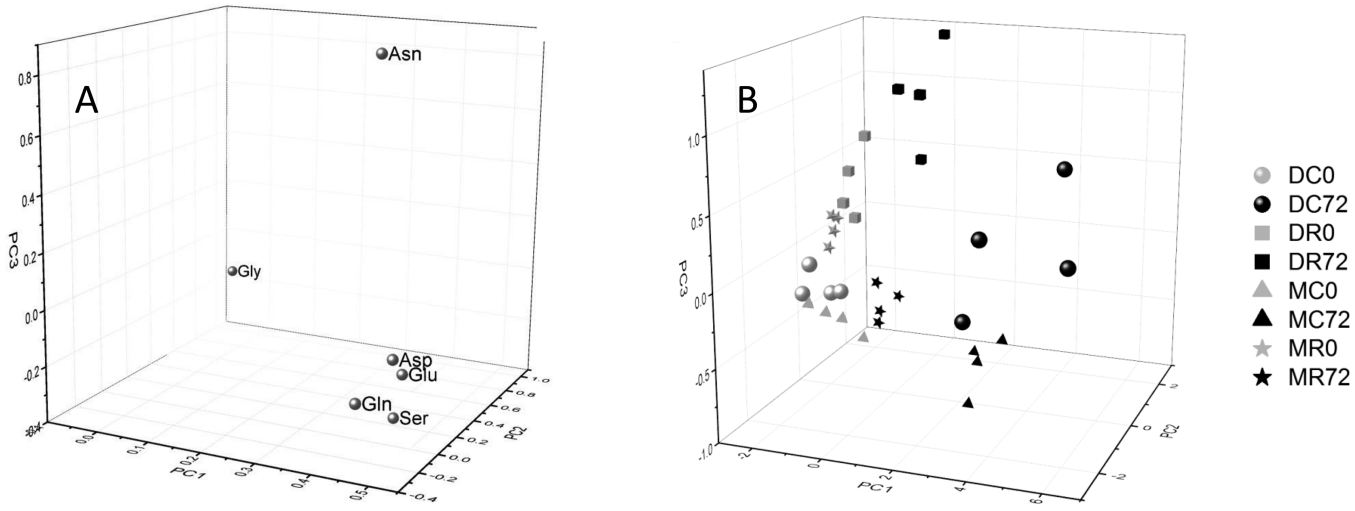

Figure 4. Loading (A) and score (B) plots for 0 (white symbols) and 72 (black symbols) h AGA using 3 PCs. DC = DM48; DR = DM4800RG; MC = MSOY7501; MR = MSOY7575RR.

97.70% of the original information. Loading plot for 0 h AGA (Figure 1A) showed homogeneous influence of 6 variables and score plot (Figure 1B) indicating similar basal level of amino acids for three varieties, DC0, DR0, and MR0, and differential grouping only for MC0. At 0 h, it was not expected for varieties to show differences between groups because plants still were not under herbicide stress. However, at 72 h, under the influence of Glu, Gln, Asp, and Asn (from the N assimilation metabolism), and Ser (Ser/Gly metabolism), which is shown in the loading plot (Figure 2A), the score plot (Figure 2B) showed that DC72, DR72, MC72, and MR72 performed in four groups and amino acid contents in DC72 and MC72 are higher than in DR72 and MR72. For this model, 4 principal components (PC) explain 97.60% of the data matrix. In addition, variable Gly was higher in resistant varieties than the susceptible ones. Furthermore, the pair DC72−DR72 were differentiated from MC72−MR72 by the influence of the Asn variable in PC2, showing that Asn contents in the DC72−DR72 pair were higher than in the MC72−MR72 pair.

A second PCA study aimed to evaluate the effect of glyphosate showing differential behavior of amino acid content in both susceptible and resistant pair between 0 and 72 h AGA in the same PCA model made on 2D (Figure 3) and 3D (Figure 4). For the present model it was necessary to use four principal components (PC), which explained 98.0% of the data matrix. PCA 2D loading plot (Figure 3A) showed that variables Asp, Asn, Glu, Gln, and Ser influenced over PC1 and Gly over PC2. Score plot (Figure 3B) grouped all the soybean varieties at 0 h AGA in a unique group (white symbols), indicating that amino acid contents were no different for DC0, DR0, MC0, and MR0. Nevertheless, the 72 h AGA score plot showed clear differentiation of four groups where each group belonged to each soybean variety assayed. Gly had more influence on DR72 and MR72, while Asn, Asp, Gln, Ser, and Glu had more influence on DC72 and MR72. In addition, the difference of

DOI: 10.1021/acs.jafc.7b03676
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

grouping between resistant DR72−MR72 and conventional DC72−MC72 was influenced by PC1, while the differences between DC0−DR0−MC0−MR0 and DC72−DR72−MC72−MR72 were influenced by PC2 (Figure 3B).

PCA 3D loading plot (Figure 4A) showed the influence of variables Asp, Glu, Gln, and Ser over PC1, Gly over PC2, and Asn over PC2 and PC3. Four varieties at 0 h AGA maintained similar levels of amino acid contents performing a single group like PCA 2D. Meanwhile, at 72 h AGA, four varieties were grouped differentially in score plot (Figure 4B). Susceptible varieties at 72 h AGA (DC72 and MC72) were grouped by principal influence of Glu, Gln, Asp, Asn (from the N assimilation metabolism), and Ser (Ser/Gly metabolism). DR0 and DR72 showed displacement in PC3 influenced by Asn making a difference with behavior of DC0 and DC72 whose displacement was in PC1 influenced by Asp, Glu, Gln, and Ser (Figure 4B). Therefore, DM and Msoy varieties at 72 h AGA showed a displacement in PC3 because DC72 and DR72 are differentiated from MC72 and MR72 by influence of Asn.

4. DISCUSSION

The analysis of information collected from statistical studies carried out can be divided in several issues. The effect of glyphosate exposure on amino acids from N assimilation metabolism such as Asn, Asp, Glu, and Gln can be seen in Table 1. Since Asn is involved in N storage and transport,[17] then Asn accumulation 72 h AGA in roots of both susceptible varieties could indicate diminishing use of Asn as substrate in further metabolic processes producing alterations in assimilation and distribution of N. This is supported by the increase of Asp, Glu, and Gln in the roots, substrates of asparagine synthetase, glutamine synthase (GS), and glutamate synthase (GOGAT), which are fundamental actors in N assimilation.[18] Nevertheless, this work does not allow distinguishing if accumulation of Asn is due to the interruption of transport of N assimilates, proteolysis proliferation, or inhibition of protein synthesis. The increase of Tyr, when it was expected to decrease due to the glyphosate mode of action, suggests a proteolysis proliferation, although a study that determined the N^{14}−N^{15} amino acid profile in glyphosate susceptible biotypes of *Amaranthus palmieri* indicated that glyphosate induced synthesis *de novo* of several amino acids (Asn, Glu, Ser, and Ala).[19] Thus, catabolism and anabolism of amino acids plus inhibition of protein synthesis could induce the observed increase in amino acid contents under glyphosate stress.

Beyond the reasons why amino acid levels increase due to glyphosate action, it must be taken into consideration that the effect of such increase should interfere in feedback regulation of enzymes that transform and drive metabolites to other metabolic pathways. Glu and Asp participate in C metabolism regulation as allosteric effectors for phosphoenolpyruvate carboxylase (PEPC) (Glu and Asp negatives effectors) and pyruvate kinase (Asp positive and Glu negative) that regulate the demand of C skeletons—oxalacetate and 2-oxoglutarate—for assimilation of N.[20] The increase of Asp and Glu observed in susceptible soybean varieties in the present work could induce the imbalance of the C metabolism, which would have undesirable effects, not only in the supply of C skeletons for amino acid synthesis and N assimilation but also to obtain energy from mitochondrial respiration for the assimilation of mineral nutrients in the root. In addition, the analysis of amino acid profiles in roots allows assessment about processes related to nitrogen assimilation and transport of amino acid to other plant organs, where Asp, Glu, Asn, and Gln have crucial roles, while Ser has relevance in the development of non-photosynthetic tissues.

On the other hand, Ser inhibits some isoforms of 3-phosphoglyceraldehyde hydrogenase (PGDH, EC 1.1.1.95) from phosphorylated pathway acting in non-photosynthetic tissues for Ser biosynthesis, which also brings 2-oxoglutarate as restitution in tricarboxylic acid cycle as glutamate synthesis.[21,22] Increase of Ser is also observed in susceptible soybean; therefore increase of Ser, Asp, and Glu could contribute in different ways to the deregulation of C metabolism and N assimilation.

The association of score and loading plots has been useful for suggesting classifications of vegetable lines according to their geographic distribution based on their elemental composition.[9,23,24] Nevertheless, in the present work we try to associate score and loading plots from multivariate analysis to try to suggest how physiological and metabolic responses to glyphosate application are differentiated on glyphosate susceptible and resistant soybeans. Since amino acid contents were analyzed in roots, we performed the multivariate analysis with the critical variables that intervene in amino acid metabolism of non-photosynthetic tissues such as Asp, Asn, Glu, Gln, Ser, and Gly. PCA 2D as well as PCA 3D, which were useful to show the behavior of each soybean variety before (0 h) and after (72 h) glyphosate application and their respective responses. PCA 2D showed that both susceptible varieties are displaced on PC1 by influence of variables Asp, Asn, Glu, Gln, and Ser, indicating that there exists a trend for susceptible varieties to increase the content of these amino acids. As mentioned above, Asp and Glu increases are responsible for deregulation of C and N metabolisms,[19,20] while Ser is responsible for signaling mechanism of root growth regulation.[21,22] On the other hand, the displacement of resistant varieties was observed in PC2 by influence of Gly variable, indicating a trend of resistant soybean to increase Gly content under glyphosate application. In addition, it was observed that differential grouping of resistant varieties indicated that they are not totally resilient to glyphosate. PCA 3D allowed further differentiation of four soybean varieties due to the influence of Asn variable in PC3. In this way, it is possible to confirm that the response of four soybean varieties to glyphosate is different for each variety, but the influence of variables Asp, Glu, Gln, and Ser still allows differentiating the behavior of susceptible and resistant soybeans.

Through the use of univariate analysis we can establish a differentiation between conventional and transgenic lines, observing the changes in the content of some amino acids to glyphosate application. Furthermore, the comparison of glyphosate effects over amino acid profiles between conventional and transgenic soybean allowed us to consider glyphosate effects over C and N metabolism, which indicated that a rigorous analysis of the amino acid profile could give information about other side effects of glyphosate. By using multivariate analyses it was possible to achieve the correct classification of the four studied soybean varieties. In addition, the chemometric tool permits differentiation of the response of the four soybean varieties used in the study to glyphosate exposure, allowing similar interpretation of results as univariate statistical analysis, but using an integrated statistical analysis.

DOI: 10.1021/acs.jafc.7b03676
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

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Funding

This work was funded by the Fundação de Amparo à Pesquisa do Estado de Sao Paulo (FAPESP, Grant No. 04/08444-6). The authors also want to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de La Pampa, and the Coordenaçao de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Brazil) for grants.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We would like to thank Dr. Diego Soldini (INTA-Marcos Juarez) and Dr. Valdemar L. Tornisielo (CENA, USP, Piracicaba) for the plant material used in the present work.

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DOI: 10.1021/acs.jafc.7b03676
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