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Research article

Metabolic alterations in conventional and genetically modified soybean plants with GmDREB2A;2 FL and GmDREB2A;2 CA transcription factors during water deficit

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ABSTRACT

Water deficit is one of the main abiotic stress that affects plant growth and productivity. The *GmDREB2A;2* (*Glyma14g06080*) gene is an important transcription factor involved in regulating the plants' responses under water deficit. In previous studies, soybean plants overexpressing full-length (GmDREB2A;2 FL) and constitutively active (GmDREB2A;2 CA) forms of the *GmDREB2A;2* gene, presented higher tolerance to water deficit when compared with the conventional cultivar BRS 283. Therefore, identifying the changes in metabolite profile in these tolerant genotypes can contribute to the understanding of the metabolic pathways involved in the tolerance mechanism. In this work, the metabolic changes in roots and leaves of genetically modified (GM) soybean plants subjected to water deficit were elucidated by ^{1}H-NMR spectroscopy. Three events were analyzed, one containing the gene in FL form (GmDREB2A;2 FL) and two presenting its CA form (GmDREB2A;2 CA-1 and GmDREB2A;2 CA-2) and compared with the conventional cultivar BRS 283. The results indicated different responses between leaves and roots for all genotypes. Most of these metabolic variations were related to carbohydrate and amino acid pathways. BRS 283 stood out with higher accumulation of amino acids in leaves under water deficit. The results also showed that the events GmDREB2A;2 FL and GmDREB2A;2 CA-1 presented higher concentrations of β-glucose and fructose in leaves, whereas BRS 283 accumulated more sucrose and pinitol. In roots, the GM events accumulated higher β-glucose, fructose, asparagine and phenylalanine, when compared with the conventional cultivar. These insights can add information on how the transcription factor (TF) DREB2A acts in soybean plants triggering and controlling a network of complex responses to drought.

1. Introduction

Environmental stresses, such as drought and heat, are constant threats to soybean cultivation causing losses of billions of dollars each year (Das et al., 2017). Stress results in reduced plant growth and development due to the deregulation of metabolic homeostasis (Shulaev et al., 2008). Therefore, changes in cellular metabolic pathways occur, altering the metabolism of carbohydrates, amino acids and peptides (Huang and Gao, 2000) in an effort to maximize growth and development processes, with the purpose of reestablishing cellular homeostasis (Guy et al., 2008). Understanding the global response of plants to abiotic stresses at metabolite level is crucial to define biotechnological and genetically-enhancing strategies to increase the tolerance of soybean plants to stress (Jorge et al., 2015).

Nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry have been the classical analytical tools in metabolomics. Mass spectrometry has the advantage of very low detection and quantification limits. Conversely, NMR is simpler, rapid and detects several

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Abbreviations

ABA	abscisic acid
AREB	ABA-responsive element binding protein
ABF	ABRE binding factor
ANOVA	analysis of variance
bZIP	basic leucine zipper
C	control
CA	constitutively active GmDREB2A;2
CBF	c-repeat binding factor
DRE	dehydration responsive element
DREB	dehydration responsive element binding
FL	full-length GmDREB2A;2
G	genotype
GABA	gamma-aminobutyric acid
GM	genetically modified
gs	stomatal conductance
MYB/MYC	myeloblastosis/myelocystomatosis
N	nitrogen
NAC, NAM, ATAF1/2	CUC2 family
ND	not detected
NMR	nuclear magnetic resonance
PCA	principal component analysis
PEG	polyethylene glycol
ROS	reactive oxygen species
TCA	tricarboxylic acid
TF	transcription factor
TPS	trehalose-6-phosphate synthase
WC	water condition
WD	water deficit

metabolites in a single spectrum. Therefore, NMR have been used to monitor the metabolic variation in plants induced by drought or other environmental stresses (Bligny and Douce, 2001).

Plant cells perceive stress stimuli through several sensors which, in turn, activate the signaling pathways involving secondary messengers, plant hormones, signal transducers and transcription regulators (Zandalinas et al., 2018). Therefore, several signals converge to regulate stress-induced genes that encode proteins and enzymes directly involved in the metabolism of stress, contributing to the specificity of the acclimation response to a particular stress stimulus (Casaretto et al., 2016). Among the main cellular mechanisms that regulate the acclimatization of plants to adverse environmental conditions are the stress-responsive transcription factors (TFs) (Zandalinas et al., 2018). Transcriptome studies in plants under drought and high salinity stress showed that dependent and independent pathways on abscisic acid (ABA) can be mediated by several TFs (Yoshida et al., 2014; Trivedi et al., 2016). The bZIP (basic leucine zipper) and MYB/MYC (myeloblastosis/myelocystomatosis) families are important TFs in ABA-responsive signaling networks, whereas ABA-independent pathways can be mediated by TFs from the CBF/DREB family (C-repeat Binding Factor/Dehydration Responsive Element Binding) and also from NAC (NAM, ATAF1/2, CUC2) family (Trivedi et al., 2016). Recent progress has been made and identifying a crosstalk in ABA-dependent and ABA-independent signaling pathways during osmotic stress response (Yoshida et al., 2014).

DREB TFs play a key role in the plant's stress signal transduction pathway, as they can bind specifically to the DRE element (G/ACCGAC - Dehydration Responsive Element) and activate the expression of many stress-induced genes (Agarwal et al., 2006). *GmDREB2A* (*Glyma14g06080*) is an orthologous gene of *AtDREB2A* identified in soybean that has regulatory function and machinery conserved between *Arabidopsis thaliana* and soybean. In Arabidopsis plants, *GmDREB2A* was activated by drought, heat and cold (Mizoi et al., 2013). However, as noted in previous studies with Arabidopsis, overexpression of *GmDREB2A* did not induce expression of target genes, indicating that post-transcriptional modifications are necessary to activate DREB2A proteins (Sakuma et al., 2006). Experiments have identified a domain region of negative regulation in *DREB2A* and that a modification in this domain (*DREB2ca*) induced stress-responsive genes and improves drought tolerance in Arabidopsis (Mizoi et al., 2013; Sakuma et al., 2006).

In previous studies of our group, transgenic soybean events containing the *GmDREB2A;*2 FL gene (*Glyma14g06080*) and its active form *GmDREB2A;*2 CA presented better performance to water deficit when compared with the conventional background (unpublished data). Some works have already been carried out in order to investigate the physiological and molecular changes resulting from the insertion of the *DREB* gene in other species (Dubouzet et al., 2003; Mizoi et al., 2013). On the other hand, the metabolic profile of these tolerant genotypes has not yet been determined. Therefore, a metabolomics study of these genetically modified plants (GMs) compared with the conventional background under water deficit stress can provide useful information for the definition of new strategies to develop more tolerant plants.

The aim of this study was to determine the main metabolic changes in tissues of GM soybean plants overexpressing the *GmDREB2A;*2 FL gene and its active form *GmDREB2A;*2 CA compared with the conventional cultivar BRS 283 in response to water deficit.

2. Materials and methods

2.1. Plant material and experimental conditions

Two expression cassettes were inserted separately into a soybean conventional cultivar, BRS 283, using *Agrobacterium tumefaciens* methodology. One contains the target gene in full-length (FL) (*pCambia3300J-35S:GmDREB2A;*2 FL) and the other presents its in constitutively active form (CA) (*pCambia3300J-35S:GmDREB2A;*2 CA) forms. The main difference between the constructions is a deletion at GmDREB2A;2 negative regulation domain which resulted in the CA form of the TF (Mizoi et al., 2013). *GmDREB2A;*2 FL and *GmDREB2A;*2 CA genes were under the control of the constitutive promoter *CaMV 35S* (Cauliflower mosaic virus) and *TNOS* terminator (*A. tumefaciens* nopaline synthase). Confirmation of the positive events was performed through conventional PCR. Positive plants were grown in a greenhouse for selection of those events with 3:1 Mendelian segregation in the T1 generation, and generation progress was made to obtain homozygous seeds. Three independent events were obtained, one FL (GmDREB2A;2 FL) and two CA (GmDREB2A;2 CA-1 and GmDREB2A;2 CA-2). The performance of these plants was evaluated under water deficit and the events GmDREB2A;2 FL and GmDREB2A;2 CA-1 presented higher tolerance to the stress compared with the conventional cultivar BRS 283 and the GmDREB2A;2 CA-2. (unpublished data).

In the current study, seeds in the T_3 generation from these three GM (genetically modified) events (GmDREB2A;2 CA-1, GmDREB2A;2 CA-2, GmDREB2A;2 FL) and from the cultivar BRS 283 (conventional background) were germinated on paper moistened with a volume of water equivalent to two and a half times the weight of dry paper for 96 h in a germination chamber at 25 ± 1 °C and relative humidity of 100%. Then, each seedling was transferred to a 1 L pot filled with a mixture of substrate and sand (1:1), with the substrate consisting of soil, sand and organic compound (3:2:2). The randomized blocks experimental design was used, using a 4 × 2 factorial design (four genotypes and two water conditions: control – C and water deficit – WD). Three biological repetitions were used, each repetition consisting of a bulk of two plants.

The seedlings were kept in a growth chamber with light at a programmed temperature of 28 ± 2 °C and received irrigation to maintain

the substrate around 80% of the field capacity until the stress treatments were imposed. Stress by water deficit was imposed by suspension of irrigation when the plants reached the vegetative stage V3 (Fehr, 1977). After the onset of stress, the plants were monitored daily in relation to stomatal conductance (*gs*) until they reached *gs* values of less than 200 mmol H_2O m^{-2} s^{-1} (Flexas et al., 2004), totaling eight days under water deficit. A set of plants representing the control group was kept under irrigation. At the end of the stress period, samples from the third fully-expanded trifoliate leaf (apex-base direction) and from the roots were collected, packed separately in aluminum foil, immediately immersed in liquid nitrogen, stored at −80 °C, lyophilized and milled for the metabolic analyses.

2.2. Sample preparation for nuclear magnetic resonance (NMR) analyses

Extracts from the tissues (roots and leaves) were obtained from 30 mg of the lyophilized tissue and 1.00 mL of a mixture of CD_3OD:D_2O (80:20 v/v) containing 0.264 mmol of 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt (TSP-d_4). The suspension was homogenized for 30 s in a benchtop vortex and then heated at 50 °C for 10 min. The samples were subsequently centrifuged for 5 min, and 600 µL of the supernatant was cooled at 4 °C for 30 min. Then, 20 µL of phosphate buffer at 2.40 mM were added to each sample (Coutinho et al., 2018). The prepared samples were stored overnight at 4 °C before the NMR analyses, when 600 µL of each extract were transferred to 5 mm NMR tubes.

2.3. NMR analyses

^{1}H-NMR spectra of the extracts were acquired at 298 K in an *Avance III HD* spectrometer at 600 MHz equipped with a 5 mm BBO probe. The NOESY 1D pulse sequence was used, with pre-saturation time of 1.50 s and acquisition time of 3.64 s (64 K points), accumulation of 256 transients and spectral width of 15 ppm. All FID underwent Fourier transformation after application of the exponential window function with 0.3 Hz line broadening. Phase and baseline fixes were performed in the *TopSpin* software. The ^{1}H-NMR chemical shifts were referenced to TSP-d_4 (δ 0.00). The metabolites were identified from data described in the literature (Coutinho et al., 2018).

2.4. Data analysis

The ^{1}H NMR data ranging from 0.00 to 10.00 ppm were converted to ASCII files using Bruker TopSpin 3.5. The NMR data were aligned using the *icoshift* algorithm (Savorani et al., 2010). Then, the region corresponding to residual signal of water and TSP was excluded. The data preprocessing and Principal Component Analysis (PCA) from ^{1}H NMR were performed using MATLAB R2016b and PLS-Toolbox from normalized and mean-centring data. Chenomx software was used to calculate the levels of 25 metabolites as measured by ^{1}H NMR and the relative concentration of pinitol was determined based of signal area at chemical shift in 3.60 ppm.

The relative concentration data analysis showed a normal distribution and was submitted to the analysis of variance (ANOVA,

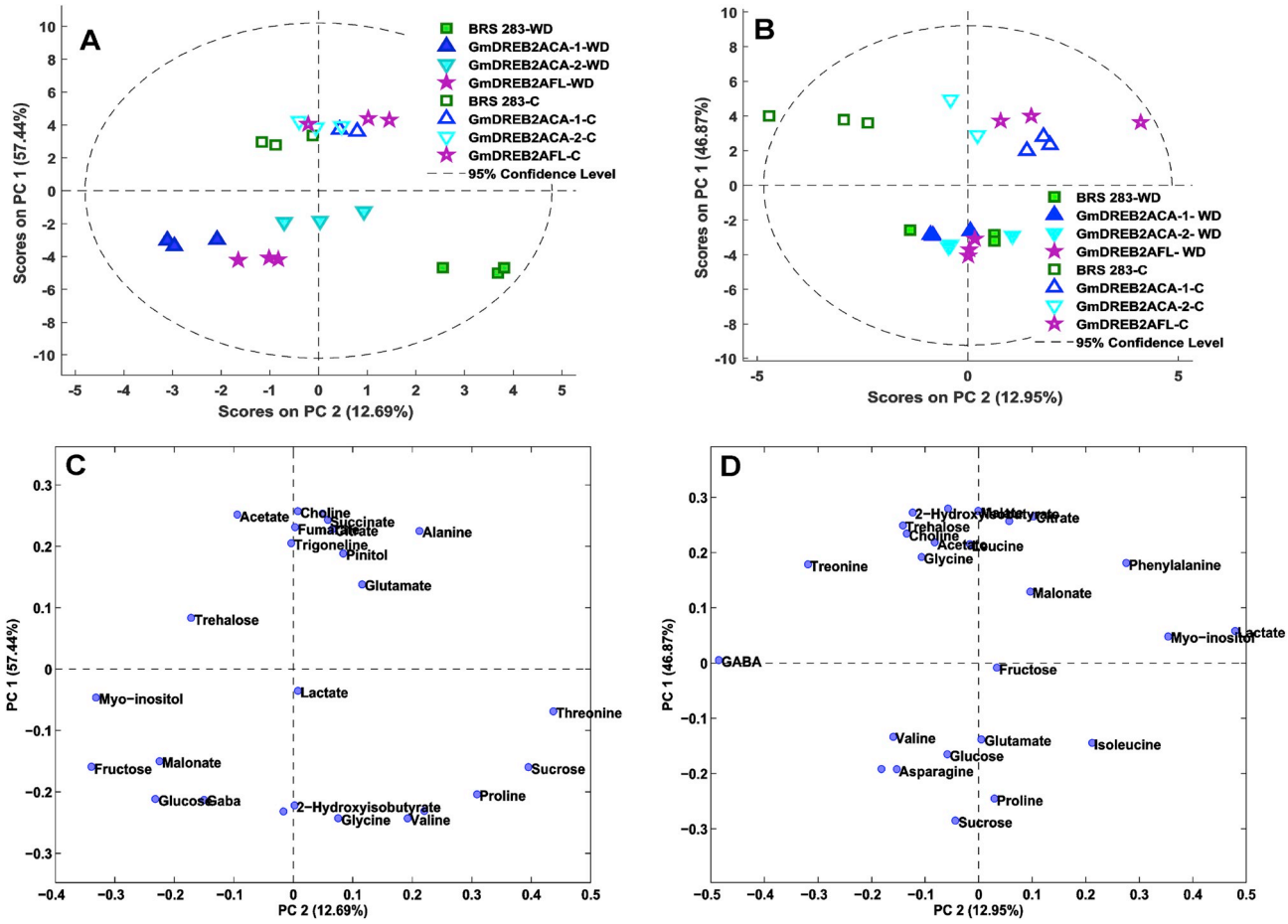

Fig. 1. Principal components analysis (PCA) of 27 metabolites from genetically modified soybean events (GMs) GmDREB2A;2 FL, GmDREB2A;2 CA-1, GmDREB2A;2 CA-2 and conventional cultivar BRS 283 under control (C) and water deficit (WD) conditions. Score (A) and loading plot (C) of leaf samples. Score (B) and loading plot (D) of root samples.

Appendix B and Appendix C). The comparison of means was performed by the Tukey test ($p \leq 0.05$), using R Software, with the interface "RStudio" (Estudio Team, 2013) and package assistance "ExpDes.pt" (Ferreira et al., 2018). The main effects of genotype (G) or water condition (WC) are shown in tables whereas interaction effects (G x WC) are shown in graphics.

3. Results

3.1. Identified metabolites by ^{1}H-NMR analysis in soybean under water deficit

Twenty-seven metabolites (11 amino acids, 7 organic acids, 6 sugars and 3 others of different classification) were identified in the NMR spectra, of the root and leaf extracts, of the GM and control plants in response to water deficit stress. The chemical shifts and coupling constants of the identified metabolites are in Appendix A.

3.2. Principal Components Analysis (PCA)

Principal Components Analysis (PCA) was applied to determine the effect of water deficit on the metabolic levels in leaves and roots of the GM plants and the respective conventional background the cultivar BRS 283. The results of the two principal components (PC1 and PC2) were considered (Fig. 1). PC1 and PC2 explained respectively, 57.44% and 12.69% of the variance of the leaf spectra (Fig. 1A), and 46.87% and 12.95% of the roots (Fig. 1B). PC1 separated the leaf samples in two groups, one consisting of plants in the control condition and the other of plants under water deficit condition. On the other hand, PC2 separated plants by their genotypes. Genotype discrimination was observed only in water deficit condition, and the conventional cultivar BRS 283 was the farthest from the other GM genotypes (Fig. 1A).

In roots, PC1 separated the samples in two groups, one containing the control samples and the other containing the samples under water deficit. PC2 discriminated genotypes in the control condition, where the

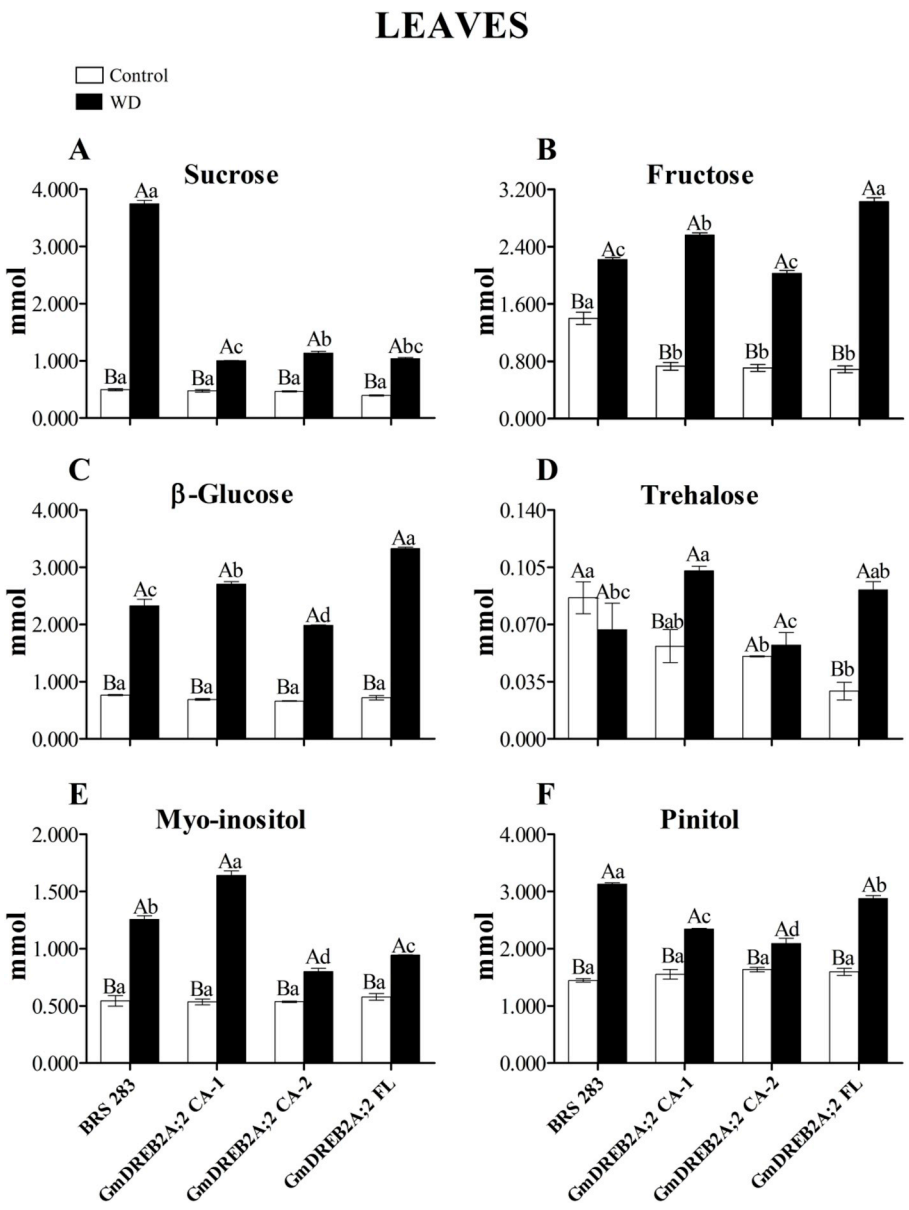

Fig. 2. Carbohydrate content in leaves of genetically modified genotypes (GmDREB2A;2 FL, GmDREB2A;2 CA-1 and GmDREB2A;2 CA-2) and conventional cultivar (BRS 283) under control and water deficit (WD) conditions. The means (n = 6) and standard deviation, represented by columns and bars, respectively, followed by similar capital letters (between water conditions) and lowercase letters (between genotypes) do not differ by Tukey's test ($p \leq 0.05$). The relative concentration of metabolites was estimated at 600 μL of extract, which was prepared from 30 mg of powder tissue.

conventional cultivar was the farthest from the other genotypes, showing metabolic differences among the BRS 283 cultivar and the GM events (Fig. 1B).

In Fig. 1C and D the metabolites are distributed according to their contribution to the formation of these clusters. As shown in the loadings plot (Fig. 1C), the separation of control and stressed leaf samples along the PC1 axis was mainly attributed to acetate, choline, succinate, glycine, valine, β-glucose, GABA (gamma-aminobutyric acid), 2-hydroxyisobutyrate and proline. The separation of the GM genotypes and cultivar BRS 283 in leaves under water deficit was along the PC2 axis. Myo-inositol, fructose, β-glucose, malonate, threonine, sucrose and proline were the metabolites responsible for this separation (Fig. 1C). In roots, 2-hydroxyisobutyrate, citrate, malate, trehalose, sucrose, proline and asparagine metabolites were relevant in the separation of samples in stressed condition from the control (Fig. 1D). On the other hand, the separation of genotypes in the control condition is due to the variation in the levels of the metabolites GABA, threonine, lactate, myo-inositol and phenylalanine (Fig. 1D).

3.3. Analysis of variance of identified metabolites

The water deficit produced varied effects on the metabolism of amino acids, carbohydrates, organic acids and other metabolites in both leaves and roots of GM soybean genotypes and conventional cultivar BRS 283. Analysis of variance was carried out to identify the main effects of genotype, water condition and interaction (genotype x water condition) in leaves (Appendix B) and roots (Appendix C) for the metabolites evaluated. The variables in which interaction between factors was observed were presented in graphs, while main effects of genotype and water condition were described in tables.

3.3.1. Water deficit effect in carbohydrate metabolism

In leaf tissues, interaction effect was significant for the compounds belonging to the carbohydrate metabolism: sucrose, fructose, β-glucose, trehalose, myo-inositol and pinitol (Appendix B). During water deficit there was a significant increase in the quantities of sucrose, fructose, β-glucose, myo-inositol and pinitol in soybean leaves for all GM genotypes and also for the cultivar BRS 283, when compared to the control condition (Fig. 2). Higher accumulation of fructose (Fig. 2B) and β-glucose (Fig. 2C) was observed for the GM events GmDREB2A;2 CA-1 and GmDREB2A;2 FL compared with the GM GmDREB2A;2 CA-2 event and the conventional cultivar BRS 283. On the other hand, the cultivar BRS 283 presented higher sucrose (Fig. 2A) and pinitol (Fig. 2F) content when compared with GM events.

Under control condition, sucrose, β-glucose, myo-inositol and pinitol levels remained stable in the leaves of all genotypes, while fructose (Fig. 2B) and trehalose (Fig. 2D) levels were higher in genotype BRS 283 relative to GM genotypes.

The main effect of water condition was detected in leaves for glutamic acid, lactate and malonate, whose concentrations increased under water deficit (Table 1).

In roots, only β-glucose and trehalose showed interaction effect (Appendix C). Under water deficit condition, β-glucose level increased significantly in GM events GmDREB2A;2 CA-1 and GmDREB2A;2 FL (Fig. 3A). These genotypes accumulated higher level of this sugar compared with the conventional cultivar BRS 283 and the event GmDREB2A;2 CA-2, which showed no changes after stress (Fig. 3A). The trehalose concentration in roots was lower under water deficit condition when compared to the control, for all genotypes (Fig. 3B). Control plants presented significantly lower trehalose level in the event GmDREB2A;2 CA-1, while stressed plants showed lower level of this sugar in the conventional cultivar BRS 283 (Fig. 3B).

The main effect of water condition was also observed in roots for sucrose accumulation and all the studied genotypes showed higher level of this metabolite under water deficit (Table 1). For fructose the main effect genotype was significant in roots and plants of the event GmDREB2A;2 FL had a higher concentration of this sugar compared with the event GmDREB2A;2 CA-2 and the cultivar BRS 283 (Table 2). The other sugars (sucrose, fructose, myo-inositol) showed no significance differences (Appendix C).

3.3.2. Water deficit effect on amino acid metabolism

For the amino acids class, the variables that showed interaction effects in leaves are presented in Fig. 4. The leaves of all GM genotypes and cultivar BRS 283 showed increased in proline, glycine, isoleucine, leucine, valine and GABA levels under stress condition (Fig. 4). Only the BRS 283 cultivar under water deficit showed significantly higher level of alanine when compared to control (Fig. 4A). Threonine (Fig. 4F), also increased in BRS 283 cultivar and the event GmDREB2A;2 CA-2 compared to the control condition. Furthermore, under water deficit, BRS 283 cultivar was superior to the GM events to the amino acids levels quantified in leaves (Fig. 4), except for leucine (Fig. 4E) and GABA (Fig. 4H).

In roots, the interaction effect was observed only for the amino acid asparagine (Appendix C). Under water deficit condition, the roots of the events GmDREB2A;2 CA-1 and GmDREB2A;2 FL showed an increase in the asparagine concentration compared to the control condition, also differing significantly from the conventional cultivar BRS 283 and the event GmDREB2A;2 CA-2 (Fig. 5). Under control conditions, it was observed that roots of the event GmDREB2A;2 CA-2 presented a lower level of asparagine compared with GmDREB2A;2 CA-1 (Fig. 5).

The water condition effect was significant for the amino acids leucine, threonine, phenylalanine and alanine. A reduction of leucine, threonine and phenylalanine concentration and an increase in alanine level was observed in the roots of all genotypes under water deficit (Table 1). The genotype effect was observed for valine and phenylalanine (Table 2). The events GmDREB2A;2 CA-1 and GmDREB2A;2 FL showed a higher content of phenylalanine and the event GmDREB2A;2 CA-1, a higher concentration of valine compared with the cultivar BRS 283 (Table 2).

3.3.3. Water deficit effect on the organic acid levels and other identified metabolites

In leaves, the interaction effect was significant for acetate, citrate, fumarate, succinate, choline, 2-hydroxyisobutyrate and trigonelline metabolites (Appendix B). Under the control condition, leaves of the event GmDREB2A;2 FL presented significantly lower acetate content compared with GmDREB2A;2 CA-1, not differing from the other genotypes (Fig. 6A). After water deficit imposition, lower level of acetate

Table 1
Metabolites level (mmol) in leaves and roots of genetically modified genotypes (GmDREB2A;2 FL, GmDREB2A;2 CA-1 and GmDREB2A;2 CA-2) and conventional cultivar (BRS 283) that presented main effect of water condition.

	METABOLITES	MEANS	
		C	WD
Leaves	Glutamic acid	0,217 ± 0,021B	0,306 ± 0,031[a]
	Lactate	0,018 ± 0,002B	0,035 ± 0,003[a]
	Malonate	0,070 ± 0,036B	0,230 ± 0,044[a]
Roots	Sucrose	0,798 ± 0,056B	3,056 ± 0,125[a]
	Alanine	0,026 ± 0,001B	0,029 ± 0,001A
	Phenylalanine	0,029 ± 0,003A	0,017 ± 0,001B
	Leucine	0,051 ± 0,002A	0,037 ± 0,002B
	Threonine	0,058 ± 0,005A	0,040 ± 0,004B
	Citrate	0,606 ± 0,069A	0,181 ± 0,010B
	Choline	0,283 ± 0,005A	0,177 ± 0,006B
	Malate	4,475 ± 0,309A	2,937 ± 0,100B
	Malonate	0,938 ± 0,199A	0,407 ± 0,090B
	Succinate	0,251 ± 0,006A	0,083 ± 0,004B

C = control; WD = water deficit. Means (number of repetitions = 6) ± standard errors followed by the same letters do not differ from each other by the Tukey test ($p \leq 0.05$). The relative concentration of metabolites was estimated at 600 μL of extract, which was prepared from 30 mg of powder tissue.

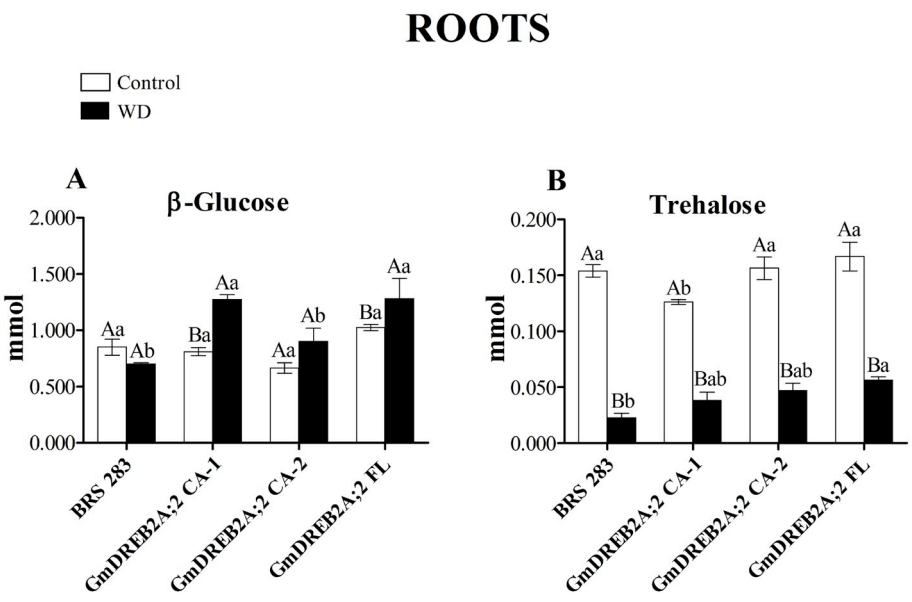

Fig. 3. Carbohydrate content in roots of genetically modified genotypes (GmDREB2A;2 FL, GmDREB2A;2 CA-1 and GmDREB2A;2 CA-2) and conventional cultivar (BRS 283) under control and water deficit (WD) conditions. The means (n = 6) and standard deviation, represented by columns and bars, respectively, followed by similar capital letters (between water conditions) and lowercase letters (between genotypes) do not differ by Tukey's test ($p \leq 0.05$). The relative concentration of metabolites was estimated at 600 µL of extract, which was prepared from 30 mg of powder tissue.

Table 2
Metabolites level (mmol) in roots of genetically modified genotypes (GmDREB2A;2 FL, GmDREB2A;2 CA-1 and GmDREB2A;2 CA-2) and conventional cultivar (BRS 283) that presented main effect of genotype.

METABOLITES	MEANS			
	BRS 283	GmDREB2A;2 CA-1	GmDREB2A;2 CA-2	GmDREB2A;2 FL
Fructose	0,994 ± 0,080c	1,468 ± 0,073 ab	1,149 ± 0,053bc	1,555 ± 0,131a
Phenylalanine	0,015 ± 0,002b	0,027 ± 0,004a	0,023 ± 0,003 ab	0,026 ± 0,005a
Valine	0,023 ± 0,001b	0,032 ± 0,002a	0,030 ± 0,003 ab	0,025 ± 0,001 ab
Malonate	0,706 ± 0,290 ab	1,156 ± 0,206a	0,256 ± 0,081b	0,571 ± 0,099 ab

Means (number of repetitions = 6) ± standard errors followed by the same letters do not differ from each other by the Tukey test ($p \leq 0.05$). The relative concentration of metabolites was estimated at 600 µL of extract, which was prepared from 30 mg of powder tissue.

was detected compared to the control condition, except for the event GmDREB2A;2 FL, which did not present change and, together with GmDREB2A;2 CA-1 and GmDREB2A;2 CA-2, presented significantly higher amounts when compared with the conventional cultivar BRS 283 (Fig. 6A).

The event GmDREB2A;2 CA-2 showed a higher content of citrate in leaves under control condition compared with the cultivar BRS 283 and to the events GmDREB2A;2 CA-1 and GmDREB2A;2 FL (Fig. 6B). After water deficit imposition, conventional cultivar BRS 283 presented a significantly higher citrate content compared to the control condition and the events GmDREB2A;2 CA-2 and GmDREB2A;2 FL which, in contrast, greatly reduced the citrate level under water deficit (Fig. 6B). Water deficit also led to reduction in the fumarate level in leaves of the cultivar BRS 283 and the event GmDREB2A;2 CA-1. Moreover, under such water conditions the event GmDREB2A;2 FL presented an increased content of this organic acid, relative to the two events GmDREB2A;2 CA and did not differ statistically from the conventional cultivar BRS 283 (Fig. 6C).

The content of the organic acid succinate increased significantly in leaves of all genotypes under water deficit, relative to the control condition, and cultivar BRS 283 stood out with significantly higher levels than the GM genotypes (Fig. 6D). For lactate and malonate, the interaction was not significant in leaves (Appendix B); however, the main effect of water condition indicated higher levels of these compounds under water deficit in all genotypes compared to the control condition (Table 1).

Choline (Figure 6E), 2-hydroxyisobutyrate (Fig. 6F) and trigonelline (Fig. 6G) were accumulated under water restriction in leaves of all genotypes. BRS 283 leaves showed a higher choline content in both water conditions imposed (Fig. 6E) and a higher 2-hydroxyisobutyrate content under water deficit compared with the GM events (Fig. 6F). However, the event GmDREB2A;2 FL presented a significantly higher trigonelline content compared with the other genotypes (Fig. 6G).

In roots, significant interaction between genotype and water condition was observed for fumarate, lactate and 2-hydroxyisobutyrate (Appendix C). Roots of the GM soybean plants and the conventional cultivar BRS 283 showed a sharp reduction of fumarate content after water deficit (Fig. 7A). In addition, under control condition, roots of the events GmDREB2A;2 CA-2 and GmDREB2A;2 FL showed a higher fumarate content compared with the event GmDREB2A;2 CA-1 (Fig. 7A). Under water deficit, only the roots of the cultivar BRS 283 and of the event GmDREB2A;2 CA-2 did not show reduction in lactate content relative to the control condition (Fig. 7B).

The main effect of water condition affected organic acids levels in roots of the GM soybean plants and of the conventional cultivar BRS 283. Significant reduction in acetate, malate, succinate, citrate, and malonate accumulation in the roots of plants under water deficit conditions was observed (Table 1). The genotype effect found for malonate showed that the event GmDREB2A;2 CA-1 differed from GmDREB2A;2 CA-2, but it did not differ from GmDREB2A;2 FL and from the conventional cultivar BRS 283 (Table 2).

In roots, a significant reduction was observed for choline (Table 1) and 2-hydroxyisobutyrate (Fig. 8) concentrations compared to the control condition for all genotypes. The event GmDREB2A;2 CA-2 under control condition presented the lowest 2-hydroxyisobutyrate content compared with all other genotypes (Fig. 8).

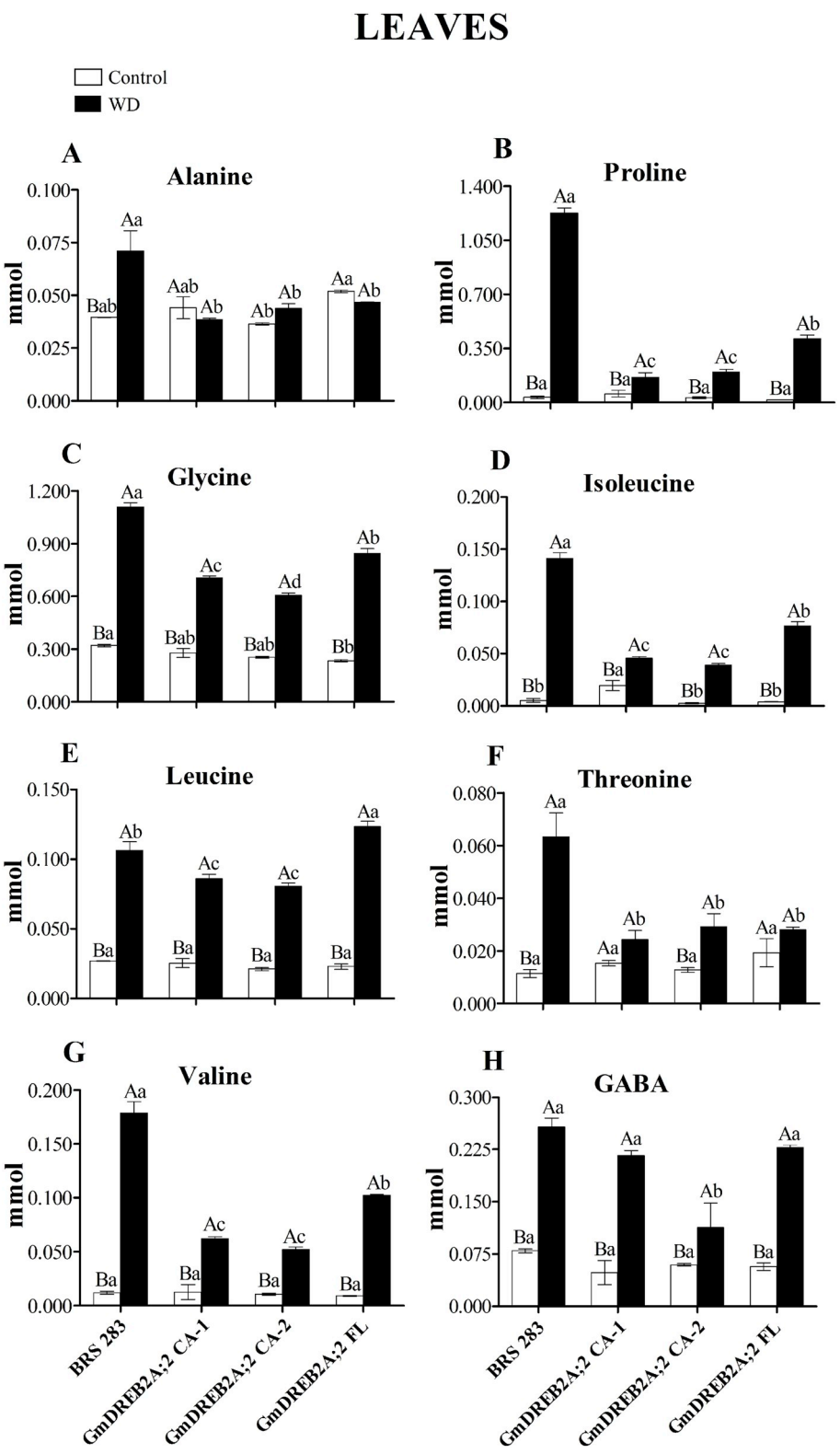

Fig. 4. Amino acid content in leaves of genetically modified genotypes (GmDREB2A;2 FL, GmDREB2A;2 CA-1 and GmDREB2A;2 CA-2) and conventional cultivar (BRS 283) under control and water deficit (WD) conditions. The means (n = 6) and standard deviation, represented by columns and bars, respectively, followed by similar capital letters (between water conditions) and lowercase letters (between genotypes) do not differ by Tukey's test ($p \leq 0.05$). The relative concentration of metabolites was estimated at 600 μL of extract, which was prepared from 30 mg of powder tissue.

3.4. Metabolic profiles of GM events and cultivar BRS 283 affected by water deficit

Fig. 9 summarizes the main metabolic pathways altered by water deficit condition in leaves and roots of the different soybean genotypes. Our results demonstrated that water stress induced marked changes in the primary metabolism of sugars, amino acids and organic acids of the tricarboxylic acid (TCA) cycle. The effect of water deficit stress on the

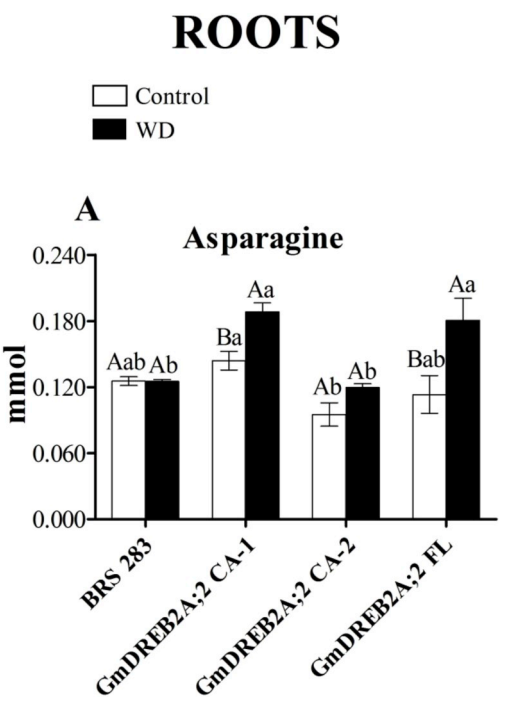

Fig. 5. Asparagine content in roots of genetically modified genotypes (GmDREB2A;2 FL, GmDREB2A;2 CA-1 and GmDREB2A;2 CA-2) and conventional cultivar (BRS 283) under control and water deficit (WD) conditions. The means (n = 6) and standard deviation, represented by columns and bars, respectively, followed by similar capital letters (between water conditions) and lowercase letters (between genotypes) do not differ by Tukey's test ($p \leq 0.05$). The relative concentration of metabolites was estimated at 600 µL of extract, which was prepared from 30 mg of powder tissue.

metabolites accumulation was more pronounced in leaves than in roots, so that all genotypes in the study showed remarkable accumulation for most of the metabolites after water deficit imposition (Fig. 9). In roots, after water deficit, the metabolic changes that stood out for the genotypes in this study were those representing a significant reduction or no changes in the profile of several metabolites (Fig. 9).

4. Discussion

^{1}H-NMR analyses of soybean leaf and root extract showed that water deficit led to significant changes in the primary metabolism of soybean tissues. The metabolites identified in the leaves showed a higher relative content variation in all genotypes under water deficit, while in the roots led primarily to the reduction of the relative concentrations of some metabolites. Similar results were observed in C3 grass plants subjected to water deficit, in which the concentrations of several metabolites showed opposite trends in shoot and root, meaning that the metabolism was activated in the leaves, at the same time it was deactivated in the roots (Gargallo-Garriga et al., 2014).

Among the metabolic pathways affected by water deficit, three groups stood out: carbohydrates, amino acids and organic acids of the TCA cycle. The carbohydrate metabolism led to a great accumulation of sucrose, β-glucose, fructose, myo-inositol and pinitol in the leaves of all genotypes. Previous studies have shown that sugars (such as oligosaccharides of the raffinose family, sucrose, trehalose and sorbitol), sugar alcohols, amino acids and amines usually accumulate under drought conditions (Moore et al., 2009; Tripathi et al., 2016). The accumulation of soluble sugars in plant tissues under drought or salinity stresses are responsible for higher tolerance to low water availability (Gupta and Kaur, 2005) due to the increase in the synthesis of osmoprotectants or compatible solutes that are part of the normal metabolism (Chen and Murata, 2002). The accumulation of these compounds helps the stressed cells to retain water and maintain the structural integrity of cellular membranes (Hare et al., 1998).

Sucrose accumulation has been widely documented under drought condition (Lee et al., 2008; La et al., 2019). Sugars, especially sucrose are important signals in the regulation of stress responses and tolerance mechanism (Zheng et al., 2010; Ma et al., 2017). In this study, a striking difference among tested genotypes was the accumulation of sucrose under water deficit. Although all genotypes have accumulated sucrose under stress, the conventional cultivar BRS 283 stood out from GM genotypes by demonstrating a steep increase in the concentration of this sugar under water deficit. A possible explanation for this result is the reduction in the growth of plants under water deficit, meaning lower consumption of this sugar for the formation of new tissues. The reduction of growth, mainly in shoot, is a response commonly observed in water deficit situations (Skirycz, and Inzé, 2010). Therefore, these processes would be more drastically affected in the sensitive cultivar compared with the GM events.

In this study, after water deficit stress, the levels of β-glucose and fructose were significantly higher in leaves of the events GmDREB2A;2 FL and GmDREB2A;2 CA-1 compared with conventional cultivar BRS 283, unlike sucrose level, which were higher in cultivar BRS 283. In plants, sucrose may be converted into fructose and glucose by the action of two enzymes: invertase and sucrose synthase (Winter and Huber, 2000). The smaller accumulation of glucose and fructose in some genotypes may be due to the lower activity of these enzymes, since González et al. (1995) and Andersen et al. (2002) observed, respectively, reduced activity of the enzymes sucrose synthase in soybean nodules and invertase in maize plants after water deficit. Similarly, González et al. (1995) detected a lower activity of sucrose synthase associated with the increase of sucrose in maize after water deficit. In previous studies that evaluated the physiological responses of these GM events, the cultivar BRS 283 and the event GmDREB2A;2 CA-2 were more sensitive to water deficit compared with the events GmDREB2A;2 FL and GmDREB2A;2 CA-1 (unpublished data). Thus, this observed sucrose accumulation pattern may be associated with the tolerance level of these genotypes.

Under water deficit, the accumulation profile for trehalose in leaves of the GM events GmDREB2A;2 CA-1 and GmDREB2A;2 FL also proved to be higher relative to the GM event GmDREB2A;2 CA-2 and to the cultivar BRS 283. High levels of trehalose were also observed in *Capparis ovata* (Ilhan et al., 2015), GM maize (Agreda-Laguna et al., 2018), GM rice (Garg et al., 2002), and it was found that this contributed to higher tolerance to water deficit stress. Studies reported that plants overexpressing the trehalose-6-phosphate synthase gene (TPS) presented higher amounts of trehalose-6-phosphate, which helped in the perception of the carbon deficit and in regulation of photosynthesis (Lyu et al., 2013; Rathod et al., 2016).

Our results showed myo-inositol and pinitol accumulation under water deficit conditions in the leaves of all genotypes. In tobacco plants, under salt stress, it was found that the presence of myo-inositol could protect sensitive enzymes and membranes from damaged by reactive oxygen species (ROS) (Sheveleva et al., 1997). According to Silvente et al., (2012), under drought conditions, higher level of pinitol were found in the leaves of a sensitive soybean cultivar, while lower level was present in the drought tolerant cultivar, supporting this possibility. Several research reports have been demonstrated that the sugar alcohol plays an important role in osmotic adaptation and confers enhanced tolerance to high salinity or water stress (Krasensky and Jonak, 2012; Foster et al., 2015; Shi et al., 2015).

Amino acids are essential components of proteins and the main metabolites involved in nitrogen (N) metabolism in plants (Ohyama et al., 2017). In this study, soybean plants of all genotypes submitted to water deficit showed an increased concentration of glycine, leucine, valine, proline, glutamic acid, GABA and isoleucine amino acids in leaves and alanine in roots. Similarly, Witt et al. (2012) found high concentrations of some amino acids, such as proline, tryptophan,

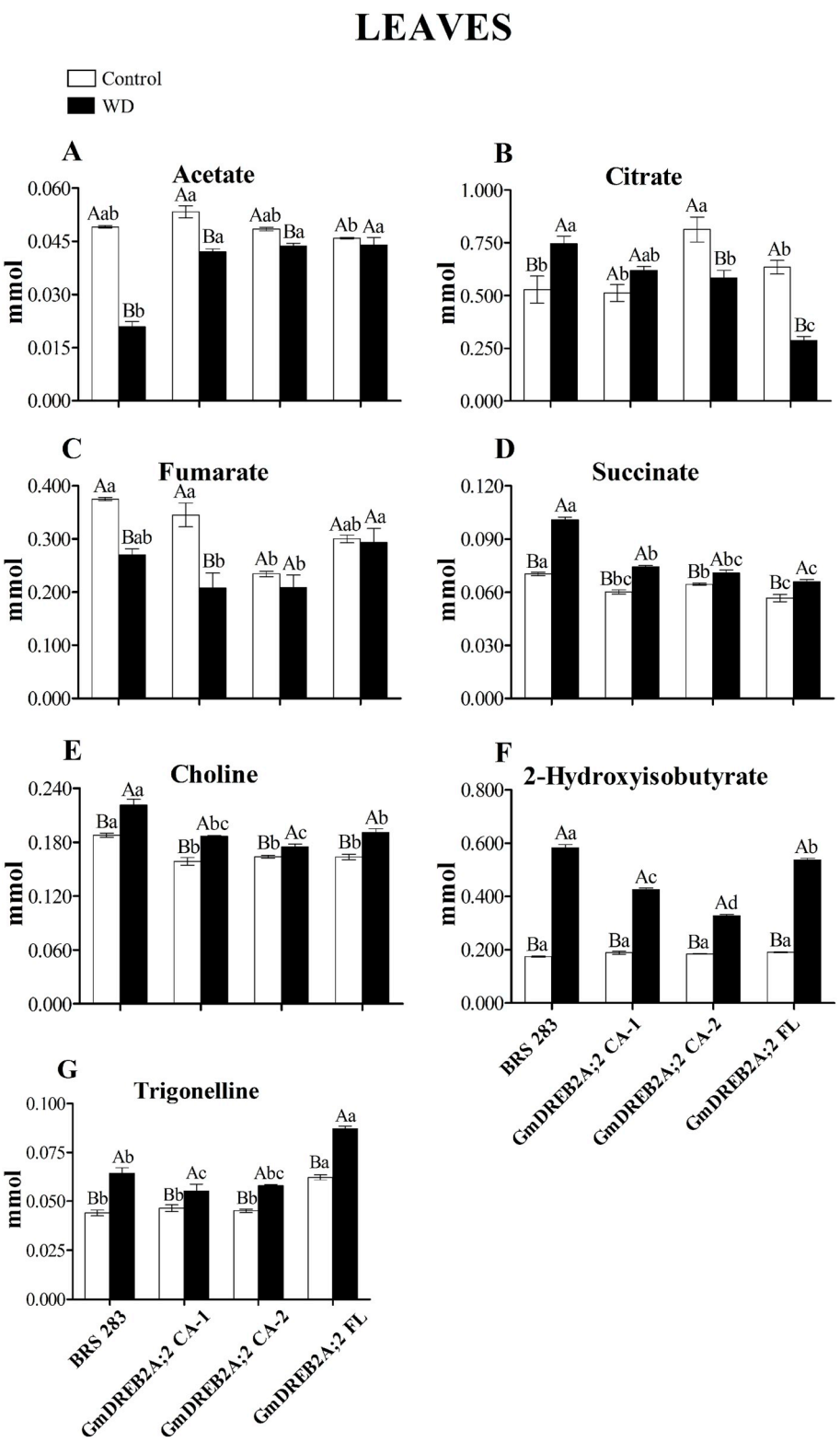

Fig. 6. Organic acids, choline, 2-hydroxyisobutyrate and trigonelline content in leaves of genetically modified genotypes (GmDREB2A;2 FL, GmDREB2A;2 CA-1 and GmDREB2A;2 CA-2) and conventional cultivar (BRS 283) under control and water deficit (WD) conditions. The means (n = 6) and standard deviation, represented by columns and bars, respectively, followed by similar capital letters (between water conditions) and lowercase letters (between genotypes) do not differ by Tukey's test ($p \leq 0.05$). The relative concentration of metabolites was estimated at 600 µL of extract, which was prepared from 30 mg of powder tissue.

phenylalanine and histidine, in maize hybrids under drought. Muscolo et al., (2015) also detected increased levels of several amino acids, such as methionine, isoleucine, valine, arginine, proline, histidine, phenylalanine, tyrosine, ornithine and asparagine in lentil genotypes submitted to osmotic stress induced by polyethylene glycol (PEG).

The increase in amino acid content is related to a higher stress tolerance in plants (Sanchez et al., 2008), which occurs through osmotic adjustment and intracellular pH regulation (Alia and Mohanty,

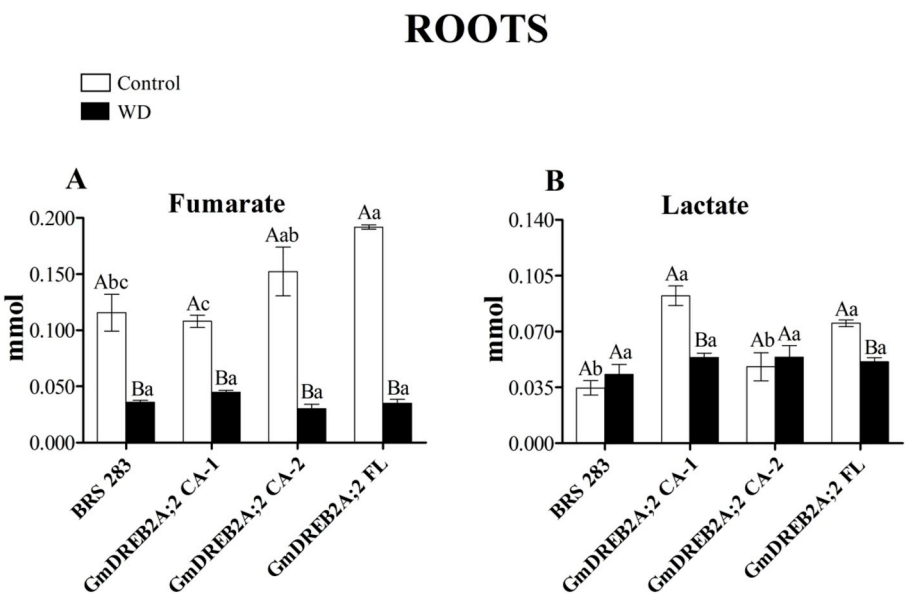

Fig. 7. Organic acids content in roots of genetically modified genotypes (GmDREB2A;2 FL, GmDREB2A;2 CA-1 and GmDREB2A;2 CA-2) and conventional cultivar (BRS 283) under control and water deficit (WD) conditions. The means (n = 6) and standard deviation, represented by columns and bars, respectively, followed by similar capital letters (between water conditions) and lowercase letters (between genotypes) do not differ by Tukey's test ($p \leq 0.05$). The relative concentration of metabolites was estimated at 600 µL of extract, which was prepared from 30 mg of powder tissue.

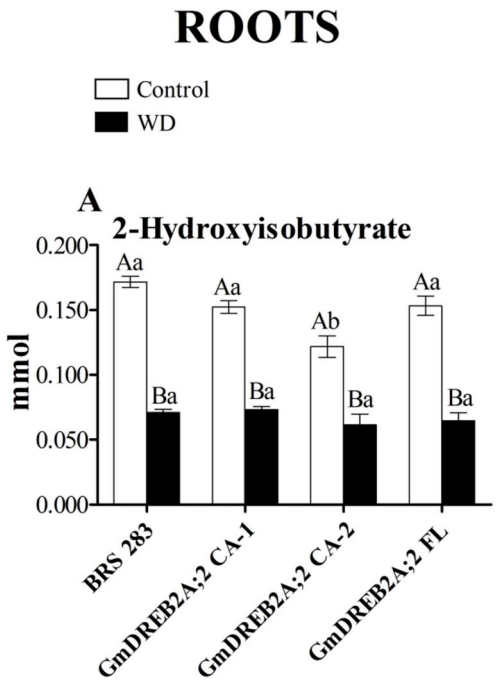

Fig. 8. 2-hydroxyisobutyrate content in roots of genetically modified genotypes (GmDREB2A;2 FL, GmDREB2A;2 CA-1 and GmDREB2A;2 CA-2) and conventional cultivar (BRS 283) under control and water deficit (WD) conditions. The means (n = 6) and standard deviation, represented by columns and bars, respectively, followed by similar capital letters (between water conditions) and lowercase letters (between genotypes) do not differ by Tukey's test ($p \leq 0.05$). The relative concentration of metabolites was estimated at 600 µL of extract, which was prepared from 30 mg of powder tissue.

1991). Studies demonstrate that such adverse situations, as osmotic stress, trigger an autophagy process that remobilizes nutrients from the leaves to sustain the physiological needs of the plant (Marshall and Vierstra, 2018). This coping mechanism may involve protein degradation to obtain energy in an alternative respiratory pathway, leading to amino acids accumulation (Izumi et al., 2013). Therefore, our results suggest that cultivar BRS 283 could have presented this intensification in protein degradation to obtain energy, since it presented a higher accumulation of most amino acids in leaves, compared with the GM genotypes.

In soybean, asparagine is one of the main forms of soluble nitrogen synthesized in the leaf and transported to the root (Ohyama et al., 2017), and is essential for the plant development, given the role of nitrogen in the synthesis of nucleic acids, proteins, hormones, chlorophyll and several other compounds (Marschner et al., 1995). Ramos et al. (2005) detected higher amounts of asparagine in nodules of soybean plants in a water deficit situation compared to level in irrigated plants. Similarly, the present study observed higher level of asparagine in the roots of the GMs GmDREB2A;2 CA-1 and GmDREB2A;2 FL under drought, relative to BRS 283 and GmDREB2A;2 CA-1.

Organic acids present multiple functions in plants, including pH and osmotic potential regulation, defense against pathogens, energy metabolism (TCA cycle) and antioxidant activity in response to different environmental stresses (Jones, 1998; Ma et al., 2001) such as drought (Fang and Xiong, 2015). Studies with soybean genotypes showed that leaves exposed to drought presented higher levels of succinate, malate and 2-oxoglutarate (Silvente et al., 2012). Similarly, we found an increase in succinate, malonate and lactate concentrations in the leaves of all genotypes after water deficit stress. The accumulation of such metabolites as organic acids can serve as a strategy for energy supply throught the alternative pathway of TCA (Zandalinas et al., 2017).

On the other hand, the roots of soybean plants under water deficit showed a decrease of some organic acids including fumarate, citrate, succinate, malate and acetate in all genotypes in this experiment. These results are in accordance to previous studies that observed a decrease in the quantities of organic acids in roots of lentil plants under osmotic stress (Muscolo et al., 2015).

An increase in levels of such metabolites, such as choline and trigonelline was detected under water deficit for all genotypes studied. The highest level observed for choline was in BRS 283, and for trigonelline, in GmDREB2A;2 FL. This may be related to the function of these compounds as osmoprotectants, since higher level of choline was observed in Arabidopsis (Zhang et al., 2010) and trigonelline in soybean plants (Malencic et al., 2003) in response to osmotic stress by dehydration, to which an osmoprotectant function was attributed.

Among the metabolic pathways affected by water deficit in this study, those involving metabolism of carbohydrates and amino acids stood out due to the accumulation of several metabolites, in relation to the accumulation of intermediates of the TCA cycle. The high levels of most metabolites observed in leaves suggest that this was a metabolic defense strategy used by all genotypes under stress conditions. Different concentrations of several metabolites analyzed were also observed

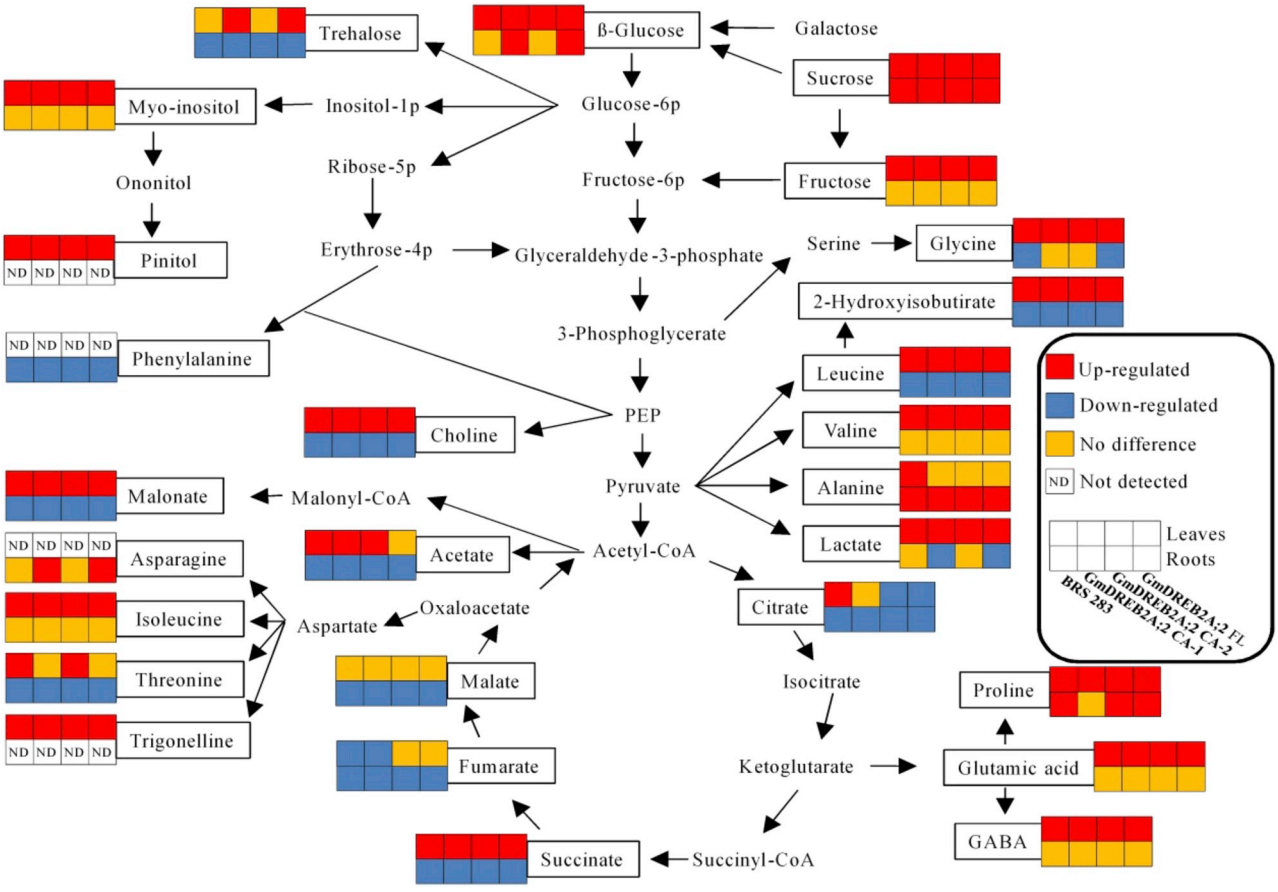

Fig. 9. Schematic representation of metabolic pathways, indicating the main changes after water deficit imposition (WD) in leaves and roots of genetically modified genotypes (GmDREB2A;2 FL, GmDREB2A;2 CA-1 and GmDREB2A;2 CA-2) and conventional cultivar (BRS 283). Boxes around the metabolites indicate that they were detected; squares represent the genotypes, from left to right: BRS 283, GmDREB2ACA-1, GmDREB2ACA-2 and GmDREB2AFL; the upper row of squares refers to the leaves, and the lower, to the roots. Red squares represent significantly higher (up-regulated) levels of the metabolite under water deficit compared to the irrigated control, blue squares represent significantly lower (down-regulated) levels of the metabolite under water deficit compared to the irrigated control, yellow squares represent similar levels of the metabolite in both conditions, white squares containing the acronym ND (not detected) indicate that the metabolite was not detected. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

between the GM events and the conventional cultivar BRS 283, indicating that the overexpression of GmDREB2A;2 FL and GmDREB2A;2 CA transcription factors promoted metabolic adjustments in soybean.

Data from the leaves indicated that, under water deficit, the events GmDREB2A;2 FL and GmDREB2A;2 CA-1 were superior to the conventional cultivar BRS 283 regarding the concentrations of such metabolites involved in the carbohydrates metabolism as fructose, β-glucose and trehalose. On the other hand, the leaves of the conventional cultivar prioritized amino acid metabolism. In roots, in addition to the increased accumulation of β-glucose for the events GmDREB2A;2 FL and GmDREB2A;2 CA-1, the larger content of asparagine and phenylalanine found in these events may have also acted as osmoprotectants, improving the responses of these plants under water deficit stress.

Therefore, the present study showed a large-scale alteration in the soybean plants' metabolism when exposed to drought, revealing specific genotype responses to the accumulation of sugars, amino acids, organic acids and other compounds. The variation in the metabolic profile detected among genotypes can partially explain the best performance of the GM soybean plants with the GmDREB2A;2 CA-1 and GmDREB2A;2 FL genes, in accordance with previous studies that showed the higher tolerance of these events in drought conditions.

Author contributions

J.P.M. and S.R.M. carried out all the biological experiments and helped to drafted the manuscript. I.D.C. and R.F.L. carried RMN analysis and helped to drafted the manuscript. KN and KY-S participated in the critical revision of the article. L.M.M-H, A.L.N. and L.A.C. participated in the design of the study and edited the manuscript.

Conflicts of interest

The authors declare no conflict of interest to declare.

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Appendices

Appendix A
Chemical shifts (δ) and coupling constants of the primary metabolites identified in the hydroalcoholic extract of leaves and roots of soybean plants.

METABÓLITES	Chemical shifts (δ,ppm) and coupling constants (*J*, Hz)
CARBOHYDRATES	
β-Glucose	5.21 (3.7), 4,62 (8.0)
Fructose	
Myo-inositol	3.62 (t, 4.2)
Pinitol	3.60 (s)
Sacarose	5.40 (3.8)
Trehalose	5.17 (d, 1.7)
AMINO ÁCIDS	
Glutamic acid	2,12 (m)
Alanine	1,46 (7.3)
Glycine	3,55 (s)
GABA	1,88 (m), 2,29 (t, 7.4), 3.01 (t, 3.4)
Isoleucine	0,93 (t, 7.6), 0,98 (d, 7.5)
Leucine	0,95 (t, 6.7)
Proline	2,03 (m), 2.34 (m)
Threonine	1,32 (6.6)
Valine	0,98 (6.90)
ORGANIC ACIDS	
Acetate	1.91 (s)
Citrate	2.53 (d, 16.1)
Fumarate	6.50 (s)
Lactate	1,32 (d,6.3)
Malate	2.34 (dd, 10.1), 2.65 (2.9)
Malonate	3.10 (s)
Succinate	2.38 (s)
OTHERS	
Choline	3.2 (s)
2- Hydroxyisobutyrate	1,36 (s)
Trigonelline	8,85 (t, 6,3)

Appendix B
– Summary of the analysis of variance of the metabolic compounds detected by 1H-NMR in leaves of the genetically modified soybean events (GMs) GmDREB2A;2 FL, GmDREB2A;2 CA-1, GmDREB2A;2 CA-2 and the conventional cultivar BRS 283 under control (C) and water deficit (WD) conditions.

METABÓLITES	MEAN SQUARE						
	Blocks	G	WC	GxWC	Residue	CV (%)	Mean
DF	2	3	1	3	14		
CARBOHYDRATES							
β-Glucose	0,014	0,514*	21,109*	0,480*	0,006	4,63	1,646
Fructose	0,014	0,292*	14,902*	0,643*	0,007	5,02	1,671
Myo-inositol	0,005	0,199*	2,247*	0,217*	0,002	5,58	0,855
Pinitol	0,012	0,262*	6,700*	0,437*	0,009	4,49	2,085
Sucrose	0,004	2,817*	9,730*	2,615*	0,002	3,96	1,096
Trehalose	0,000	0,001*	0,003*	0,002*	0,000	20,71	0,068
AMINO ÁCIDS							
Glutamic acid	0,010	0,015	0,048*	0,008	0,007	32,39	0,262
Alanine	0,000	0,000*	0,000*	0,000*	0,000	13,44	0,047
GABA	0,001	0,007*	0,122*	0,006*	0,001	19,47	0,132
Glycine	0,000	0,089*	1,781*	0,057*	0,001	5,83	0,543
Isoleucine	0,000	0,003*	0,027*	0,004*	0,000	12,5	0,042
Leucine	0,000	0,001*	0,034*	0,001*	0,000	9,53	0,062
Proline	0,002	0,365 *	1,304*	0,377*	0,001	13,79	0,267
Threonine	0,000	0,000*	0,003*	0,001*	0,000	30,95	0,025
Valine	0,000	0,005*	0,046*	0,005*	0,000	13,86	0,055
ORGANIC ACIDS							
Acetate	0,000	0,000*	0,001*	0,000*	0,000	4,85	0,043
Citrate	0,014	0,063*	0,024*	0,109*	0,004	10,42	0,572
Fumarate	0,000	0,011*	0,028*	0,006*	0,001	11,76	0,280
Lactate	0,000	0,000	0,002*	0,000	0,000	30,45	0,027
Malate	0,008	0,244*	0,000	0,184*	0,001	3,68	1,974
Malonate	0,019	0,038	0,154*	0,002	0,019	92,03	0,150
Succinate	0,000	0,001*	0,001*	0,000*	0,000	3,03	0,070

(*continued on next page*)

Appendix B (*continued*)

METABÓLITES	MEAN SQUARE						
	Blocks	G	WC	GxWC	Residue	CV (%)	Mean
OTHERS							
2- Hydroxyisobutyrate	0,000	0,019*	0,486*	0,021*	0,000	2,9	0,327
Choline	0,000	0,001*	0,004*	0,000*	0,000	3,32	0,181
Trigoneline	0,000	0,001*	0,002*	0,000*	0,000	5,68	0,058

G = genotypes; WC = water condition; CV = coefficient of variance; DF = degrees of freedom; GABA = gamma-aminobutyric acid. * Significant according to the Tukey test ($p \leq 0.05$).

Appendix C

Summary of the analysis of variance of the metabolic compounds detected by 1H-NMR in roots of the genetically modified soybean events (GMs) GmDREB2A;2 FL, GmDREB2A;2 CA-1, GmDREB2A;2 CA-2 and the conventional cultivar BRS 283 under control (C) and water deficit (WD) conditions.

METABOLITES	MEAN SQUARE						
	Blocks	G	WC	GxWC	Residue	CV (%)	Mean
DF	2	3	1	3	14		
CARBOHYDRATES							
β-Glucose	0,034	0,213*	0,243*	0,098*	0,020	15,08	0,939
Fructose	0,006	0,419*	0,036	0,050	0,054	17,94	1,291
Myo-inositol	0,008	0,028	0,029	0,007	0,010	21,48	0,478
Sucrose	0,130	0,006	30,583*	0,248	0,103	16,65	1,927
Trehalose	0,000	0,001*	0,072*	0,000*	0,000	12,15	0,096
AMINO ACIDS							
Glutamic acid	0,001	0,000	0,002	0,001	0,001	27,01	0,119
Alanine	0,000	0,000	0,000*	0,000	0,000	10,67	0,028
Asparagine	0,001	0,004*	0,007*	0,001*	0,000	12,05	0,136
GABA	0,000	0,004*	0,000	0,006*	0,000	14,39	0,088
Glycine	0,006	0,014	0,097*	0,017*	0,005	24,12	0,291
Isoleucine	0,000	0,000*	0,000	0,000	0,000	15,7	0,025
Leucine	0,000	0,000	0,001*	0,000	0,000	15,2	0,044
Proline	0,001	0,001	0,105*	0,012*	0,001	26,41	0,125
Threonine	0,000	0,000	0,002*	0,000	0,000	30,88	0,049
Valine	0,000	0,000*	0,000	0,000	0,000	17,43	0,027
ORGANIC ACIDS							
Acetate	0,000	0,000	0,030*	0,001*	0,000	8,03	0,173
Citrate	0,039	0,018	1,082*	0,014	0,033	46,05	0,394
Fumarate	0,000	0,002*	0,067*	0,003*	0,000	18,93	0,089
Lactate	0,000	0,001*	0,001*	0,001*	0,000	16,38	0,057
Malate	0,886	0,613	14,186*	0,249	0,684	22,31	3,706
Malonate	0,195	0,838*	1,693*	0,235	0,191	65,02	0,672
Succinate	0,000	0,001	0,169*	0,000	0,000	9,53	0,167
OTHERS							
2-Hydroxyisobutyrate	0,000	0,001*	0,040*	0,000*	0,000	8,95	0,109
Choline	0,000	0,000	0067*	0,001	0,000	7,55	0,230
Phenylalanine	0,000	0,000*	0,001*	0,000	0,000	24,06	0,023

G = genotypes; WC = water condition; CV = coefficient of variance; DF = degrees of freedom; GABA = gamma-aminobutyric acid. * Significant according to the Tukey test ($p \leq 0.05$).

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