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Characterization of biochemical behavior of sorghum (*Sorghum bicolor* [Moench.]) under saline stress conditions using multivariate analysis

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The aim of this research was to characterize the biochemical behavior of sorghum plants under saline stress using multivariate statistical analysis methods for efficient management of *Sorghum bicolor* [Moench.]). The experimental design was completely randomized design composed of three saline concentrations (0, 1.5 and 2.0 M) in 10 replications. In the multivariate analysis (hierarchical method), there were distinct and sub-groups in the sorghum plant treatments. Group 1 consisted of the root parts and under this group there were two subgroups: 1.5 to 2.0 concentration (Group 1) and 2 concentration (Group 2). The increase of NaCl concentration in the roots and leaves has inverse correlation with decrease of nitrate reductase, amino acids, protein and starch. The amounts of amino acids, carbohydrates, sucrose and proline in the roots and carbohydrates, sucrose and proline in the leaves of sorghum plants are reliable biological indicators of saline stress conditions in the soil. The nitrate compound differed ($p \leq 0.05$) in the sorghum plant roots; it had an average value of 0.04 µmol kg^{-1} of nitrate in the control treatment dry matter. The nitrate average was between 0.04 and 0.06 µmol kg^{-1}, but without statistical difference for all concentrations.

Key words: Multivariate statistics, salt concentration, proline, carbohydrate.

INTRODUCTION

Knowing the effects of salts on plants and soil, as well as the phenomena involved, is essential when the aim is to adopt appropriate water management practices and cultivation aimed at higher production. In addition, it causes an improvement in quality of the final product and benefits the families that make a living marketing this

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crop, especially in regions with salinity problems. The increased concentration of salts in soil decreases soil productivity state (Iqbal et al., 2014).The occurrence of excess salts in the soil can directly impair nitrogen absorption by plants, especially when they occur in the form of nitrate (NO^{3-}) or ammonium (NH^{4+}), limiting the growth of forage (Debouba et al., 2006; Lea and Azevedo, 2006; Grattana and Grieveb, 1998). Soils with excess sodium (Na^{+}) in the root environment affect plant growth because it alters membrane integrity (Silva et al., 2010) and causes changes in nutrient absorption, thus leading to great photosynthetic changes (Reddy et al., 2015).

Studies related to the effect of salinity on the biochemical behavior of sorghum (*Sorghum bicolor* [Moench.]) are still incipient, especially in relation to the nitrogen metabolism in the plant physiology. In Brazil, sorghum is used in human and animal nutrition, and as raw material for production of anhydrous alcohol, alcoholic drinks, colas and sugar extraction (Ribas, 2003; Cruz et al., 2015). Approximately 10 to 15% of the area planted with sorghum in Brazil is used as silage and has stood out due to its high production (Avelino et al., 2011). The good sorghum productivity results are due to its moderate tolerance to abiotic stresses, among which are saline stresses (Aquino et al., 2007). It is a crop that has bromatological characteristics similar to corn. This leads to its suitable fermentation and accumulation in silage, high concentration of soluble carbohydrates and crude protein in some varieties, and agronomic characteristics that present high biomass productivity (Von Pinho et al., 2006).

The assumption is that under high saline soils, some biochemical compounds present in the sorghum plants are significantly altered, and thus affect crop production. The present study aimed to characterize the biochemical behavior of sorghum plants under saline stress using multivariate statistical analysis methods, to efficiently manage *S. bicolor* [Moench.]).

MATERIALS AND METHODS

Experimental set up

The experiment was conducted in a greenhouse at the Federal Rural University of Amazonia (UFRA), Capitão Poço Decentralized Unit located between geographic coordinates of 01°44’04’’S and 47°03’28’’W, with an average altitude of 96 m asl. A completely randomized design (CRD) was established in the experiment in 2013, consisting of three saline concentrations of NaCl (0, 1.5 and 2.0 M), with 10 repetitions using forage sorghum plants (*Sorghum bicolor* [Moench.]). Plant Max variety totaling 30 experimental units was made up of two plants per pot^{-1}. The pots were placed in a spacing of 0.60 m between rows and 0.40 m between plants in a random distribution. The sorghum plants were grown in Leonard pots containing modified silica substrate: vermiculite (1:2) and irrigated with Hoagland and Arnon (1950) nutrient solution.

Increase in the doses of sodium chloride (NaCl) was done from 18th day after seedling germination (11 days) until plant collection. The preliminary test with this species (sorghum) showed that it could not tolerate sodium chloride (NaCl) after 11 days.

Collections of destructive plants in the vegetative stage were carried out (33 days), at 9:00 am, where the plants were separated into roots and leaves. Samples of each part were taken to a forced-air oven at 70°C (±5°C), and were subsequently crushed in a mill to perform the analysis.

Variables analyzed

The nitrate reductase activity was recorded using the method described by Hageman and Hucklesb (1971). To obtain the total soluble proteins, the method described by Bradford (1976) was used. For soluble amino acids, the method described by Peoples et al. (1989) was used and the free ammonia was obtained by the method described by Weatherburn (1967). The concentration of proline was obtained via the method described by Bates et al. (1973); nitrate, by Cataldo et al. (1975); total soluble carbohydrates, by Dubois et al. (1956); glycine betaine, by Grieve and Grattan (1983); and sucrose, by Van Handel (1968).

Statistical analysis

Initially, the variability in the data was evaluated using descriptive statistics. This involved calculating the average, standard deviation of the mean and coefficients of variation. The associations between biochemical compounds and saline concentrations applied in different parts of the plant (leaf and root) were analyzed by multivariate techniques. Thus, the data were subjected to multivariate exploratory cluster analyses by hierarchical method and principal components. The hierarchical cluster analysis method is an exploratory multivariate technique which gathers the sample units in groups, so that there is uniformity and heterogeneity within the groups. The structure of the groups contained in the data is seen on a graph called dendrogram, constructed from the similarity matrix between samples (Sneath and Sokal, 1973). The similarity matrix was constructed with Euclidean distance and the connection of the groups was performed using the Ward method.

The principal component analysis is a multivariate exploratory technique that condenses the information contained in a set of original variables into a set of smaller dimensions consisting of new latent variables, preserving a relevant amount of original information. The new variables are the eigenvectors (principal components) generated by linear combinations of the original variables constructed from the eigenvalues of the covariance matrix (Hair et al., 2005). After the standardization of data (zero mean and unit variance), the analyses were conducted in the program STATISTICA 7.0 (StatSoft. Inc., Tulsa, OK, USA). Simultaneously with the statistical analyses, the basic assumptions of analysis of variance, normality of errors and homogeneity of variances were tested for all variables (data not shown).

RESULTS AND DISCUSSION

Biochemical compounds in the leaf and root of sorghum plants

The nitrate compound (NIT) only differed ($p \leq 0.05$) in the roots of the sorghum grain plant, with an average value of 0.04 µmol kg^{-1} in the dry matter (DM) of the control (without application of saline concentrations) and with a 50% increase in relation to the saline concentrations of

1.5 to 2.0 M. In leaves, the NIT quantity ranges from 0.04 to 0.06 μmol kg^{-1} nitrate in the DM, but with no distinction among concentrations (Table 1). Sorghum plants treated with ion concentrations (NaCl) caused the highest nitrate accumulation in the stem tissue, which is an important storage organ for accumulation of these ions and their subsequent use in the leaves (Lobo et al., 2011). The decrease in NIT concentration in plants grown under salinity (NaCl) can be related to competition from the chloride ions (Cl^-) for the nitrate conveyors (NO^{3-}) (Ogawa et al., 2000). Aragon et al. (2011) observed that in saline conditions via the addition of NaCl at a concentration of 100 mM (-0.45 MPa) solution, the nitrate content in leaves was significantly low only on the 8th and 10th day of the experiment, whereas in the roots saline stress caused significant difference throughout the experiment.

Regarding the availability of ammonium (AMO), the highest values were observed in the roots, especially at the applied saline concentration of 2.0 M (24.09 μmol ammonium kg^{-1}) and with increases of 26.85% in relation to the control treatment (17.62 μmol ammonium kg). In the leaf there was no distinction among concentrations, the average ranging from 5.96 to 7.80 μmol ammonium kg^{-1} (Table 1). Although the accumulation of AMO was not observed in the leaves in the study results, Wolf et al. (2011), in a similar study, found the opposite: under saline concentration conditions there was ammonium storage both in sorghum leaves as well as in the forage sorghum stalk.

The nitrate reductase activity (NRA) had higher average values in the leaves and roots in the control treatment, with 0.11 and 0.04 μmol NO_{2-} g^{-1} h^{-1}, respectively; demonstrating that the NRA decreases as the saline concentration increases (Table 1). The same behavior can be observed for the availability of amino acid (AMINOA), protein (PRT) and starch (AMID), in both the root and leaves of sorghum plants (Table 1). Decreases in the NRA under saline stress conditions were also obtained by Aragon et al. (2011) and Lobato et al. (2009), with reductions in soluble protein concentrations compared to the root and leaf control treatments. Furthermore, an increase in AMINOA content in the leaves and roots was verified, but with no distinction between treatments (Aragão et al., 2011).

The concentration of proline (PROL), carbohydrates (CAR), sucrose (SAC) and glycine betaine (GLIC) had higher average values under high saline concentrations (Table 1). The SAC showed increases of 16.69 and 23.02%, in leaves and roots, respectively, with 2.0 M concentration compared to the control treatment (Table 1). Higher increases were reached in the PROL available in leaves with 47.57 and 59.39% in the control treatment at concentrations of 1.5 and 2.0 M respectively. Increases in the PROL content in the aerial part of plants subjected to saline stress were also found by Wolf et al. (2011). These increases are proportional to the salt content in the culture medium, the concentration of PROL in the sorghum leaves and also CAR, PROT and AMINOA concentrations (Oliveira et al., 2006). Under hydric or saline stress conditions there was initially loss of cell turgor, resulting in PROL accumulation to help reduce the osmotic potential in plant tissue (Munns and Tester, 2008). Furthermore, the addition of such an osmoregulator in plant tissue is associated with the plant osmotic stress behavior (Parida and Das, 2005) and to different sorghum genotypes (IPA SF-25, IPA 02-03-01, IPA 42-70-02, CFS-4, CSF-5, CSF-6, CSF-7, CSF-8, CSF-9 and CSF-10) (Oliveira et al., 2006).

Biochemical compounds in the leaf and root of sorghum plants analyzed by multivariate analysis

An alternative to better represent the relationship of inter and intradependence of qualitative and quantitative variables is the multivariate analysis because it allows simultaneous analysis of multiple measures that facilitate the characterization and principally the visualization of certain behaviors that would hardly be identified using only basic statistics (Hair et al., 2005). In the multivariate analysis (hierarchical method), there were distinct and sub-groups in the sorghum plant treatments. Group 1 consisted of the root part and within this group there was the formation of two subgroups: Subgroup 1 (saline concentrations 1.5 to 2.0) and subgroup 2 (0 saline concentration). The leaf part was concentrated in group 2, however without formation of subgroups, suggesting a different pattern by the nature of these formations (Figure 1). The distinction between leaf and root is related to the difference in the accumulation of ions (Na^+ and Cl^-) in the plant. In the leaf blades the amount of water absorbed during stress application is higher (Larcher, 2000), and also the distinction of the exclusion and retention capacity of ions from these tissues (Boursier and Lauchli, 1989).

The sorghum plant has the ability to retain Na^+ in the stem, resulting from stress, and remains constant for 20 days under saline stress conditions. While, there is minimal increase in toxic ion content in the leaf blades. The sorghum plant has the capacity to export the Na^+ present in the stalk to the leaves (Trindade et al., 2006). This behavior can be characterized as a mechanism for tolerating salinity stress conditions. When in the same group, it is considered that these groups have similarities. In addition, between one group and another group (G1 and G2, for example), there is dissimilarity, being that both measurements take into consideration various characteristics simultaneously. Within this context, Figure 1 represents the natural grouping structure of variables analyzed in this study. It is noted that although Group 1 presents a similarity that distinguishes it from Group 2, this subdivides by the saline concentrations evaluated, and it can be inferred that when 1.5 and 2.0 M concentrations were applied at the root of the sorghum plant they present similar behavior, but are dissimilar in

Table 1. Descriptive statistics of biochemical compounds in the shoot and root of sorghum plants grown in saline concentrations.

SC (M)	Leaf			Root		
	Average	SD	CV	Average	SD	CV
Nitrate (µmol kg^{-1})						
0	0.06[a]*	0.01	15.38	0.04[a]	0.001	2.50
1.5	0.06[a]	0.02	34.48	0.02[b]	0.001	5.00
2.0	0.04[a]	0.02	47.62	0.02[b]	0.001	5.00
Ammonium (µmol kg^{-1})						
0	7.80[a]	0.97	12.44	17.62[b]	3.14	17.82
1.5	5.96[a]	2.22	37.25	21.72[ab]	2.31	10.64
2.0	5.99[a]	2.44	40.73	24.09[a]	2.11	8.76
Nitrate reductase activity (µmol g^{-1})						
0	0.11[a]	0.03	27.27	0.04[a]	0.001	2.50
1.5	0.05[b]	0.02	40.00	0.02[b]	0.001	5.00
2.0	0.04[b]	0.02	50.00	0.01[b]	0.001	10.00
Amino acid (µmol g^{-1})						
0	63.59[a]	26.85	42.22	40.27[a]	7.15	17.76
1.5	43.51[a]	18.73	43.05	35.65[a]	7.22	20.25
2.0	36.74[a]	13.98	38.05	29.76[a]	4.96	16.67
Protein (mg g^{-1})						
0	3.89[a]	1.06	27.25	1.57[a]	0.41	26.11
1.5	2.89[ab]	0.61	21.11	1.11[ab]	0.45	40.54
2.0	2.42[b]	0.52	21.49	0.73[b]	0.21	28.77
Carbohydrate (mmol g^{-1})						
0	1.03[b]	0.20	19.42	0.84[b]	0.27	32.14
1.5	1.57[a]	0.18	11.46	1.21[ab]	0.26	21.49
2.0	1.84[a]	0.19	10.33	1.45[a]	0.34	23.45
Starch (mmol g^{-1})						
0	0.34[a]	0.02	5.88	0.06[a]	0.001	1.67
1.5	0.25[b]	0.02	8.00	0.04[b]	0.001	2.50
2.0	0.21[c]	0.01	4.76	0.04[b]	0.001	2.50
Sucrose (mmol g^{-1})						
0	59.73[b]	1.61	2.70	54.95[c]	2.62	4.77
1.5	68.75[a]	1.61	2.34	63.96[b]	2.14	3.35
2.0	71.70[a]	2.55	3.56	71.39[a]	2.88	4.03
Glycine betaine (µmol g^{-1})						
0	9.77[a]	0.70	7.16	5.56[a]	0.50	8.99
1.5	12.10[a]	4.57	37.77	11.44[a]	5.22	45.63
2.0	12.15[a]	6.11	50.29	12.05[a]	7.03	58.34
Proline (mmol kg^{-1})						
0	4.32[b]	0.24	5.56	0.77[c]	0.05	6.49
1.5	8.24[a]	1.01	12.26	2.62[b]	0.30	11.45
2. 0	10.64[a]	2.46	23.12	3.61[a]	0.27	7.48

N = 05; SC = saline concentration in molar concentration (M); SD = standard deviation of the mean; CV = coefficient of variation. * Means followed by the same letter in the column do not differ by Tukey's test at 0.5% probability.

relation to non-application (Concentration 0). On the other hand, in sorghum leaves the concentrations (0, 1.5 and 2.0 M) are similar, and therefore have the same behavioral pattern.

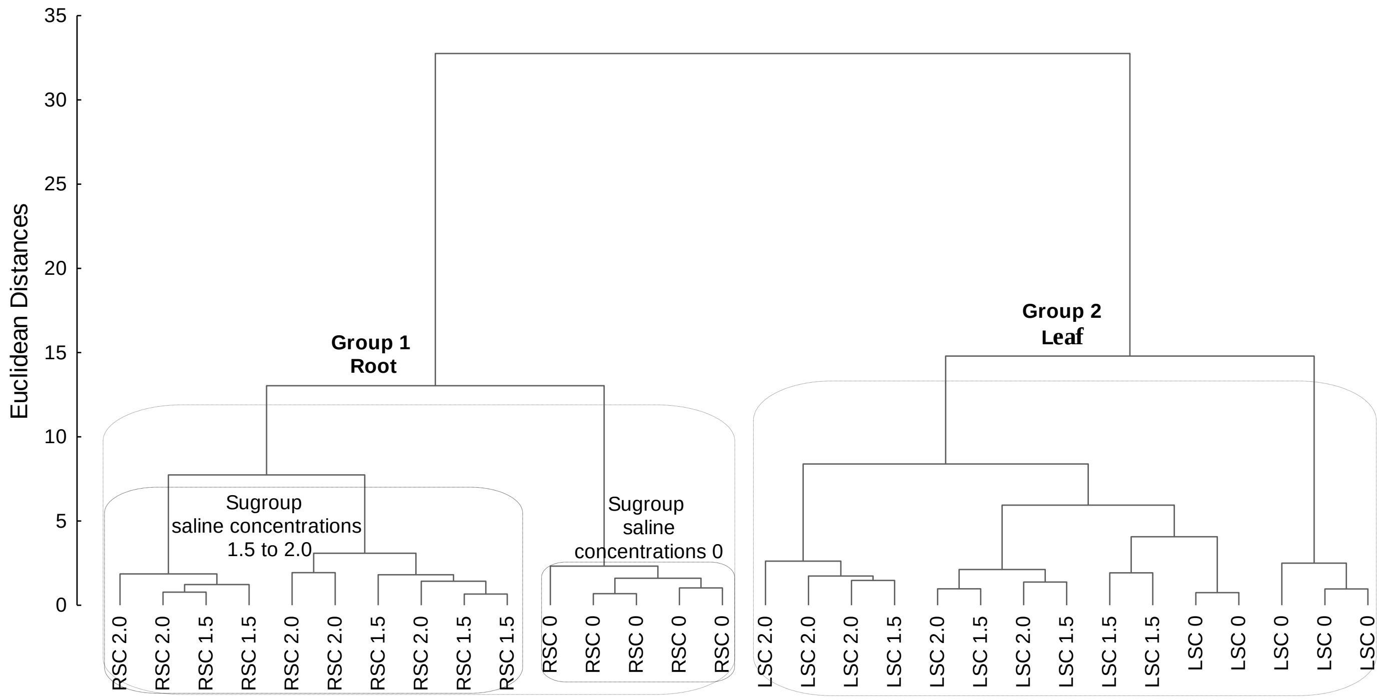

Figure 1. Dendrogram showing the hierarchy of groups in the aerial part of the leaf (L) and root (R) of sorghum plants subjected to saline concentrations (SC 0, SC 1.5 and SC 2.0 M) resulting from the cluster analysis by the hierarchical method.

Biochemical behavior in the leaf

The results obtained with the cluster analysis (Figure 1) confirm the results obtained in the principal component analysis (PCA), since in this analysis it was also possible to distinguish the leaf (Figure 2) and root areas (Figure 3). Through the PCA, it was possible to identify which biochemical compounds are characteristic, that is, are more expressive or not in a specific saline concentration when assessing both the sorghum root as well as the plant leaf. The two-dimensional plane generated with the first two principal components (PC) corresponds to 86.26% of the information contained in the original data: 61.79% in Principal Component 1 (PC1) and 24.47% in principal component 2 (PC2) when analyzing the sorghum plant leaf (Figure 2). These results are consistent with the criteria established by Sneath and Sokal (1973), wherein the number of PCs used in the interpretation must be such that they explain at least 70% of the total variance.

In the first principal component and in order of importance, the compounds that showed higher correlation coefficients were CAR (-0.98), AMID (0.97), SAC (-0.97), NRA (0.91), PROL (-0.84), PROT (0.83), NIT (0.77) and AMINOA (0.72). In the second principal component, we have GLIC (0.91) and AMO (0.78). The correlations are presented in Table 2 and represented by the arrows of each attribute and their projections are given in Figure 2. Compounds having charges (correlation values) with the same sign are directly correlated (positively) among themselves, while those with different signals have an inverse relationship; for example, carbohydrate (CAR), sucrose (SAC) and proline (PROL) have a direct relationship; on the other hand, they are negatively correlated to starch (AMID), nitrate reductase activity (NRA), protein (PRT), nitrate (NIT) and amino acid (AMINOA).

In PC1, AMINOA, PROT, NRA and AMID compounds located to the right of the PC1 are characteristics of sorghum leaves without the use of saline concentrations, that is, these compounds are found in greater amounts in the leaves under such conditions (Figure 1); a similar behavior is shown in Table 1; but, by the Tukey test, the AMINOA and PROT compounds do not differ in relation to the concentrations, although they present higher values in the treatment without saline concentration (Table 1). This fact reinforces the discussion on the relationships that are observed in the multivariate analysis and sometimes do not appear in basic statistics. In contrast, the compounds PROL, CAR and SAC located to the left of PC1 indicate that saline concentrations 1.5 and 2.0 favor greater quantities of these compounds in the sorghum leaves, especially at the 2.0 concentration (Table 1).

Piza et al. (2003) observed reduction in total soluble protein content in pineapple plants after 15 days of evaluation at all salinity levels (0, 0.57, 1.15 and 2.30 g L^{-1} de NaCl) studied. According to the authors, the

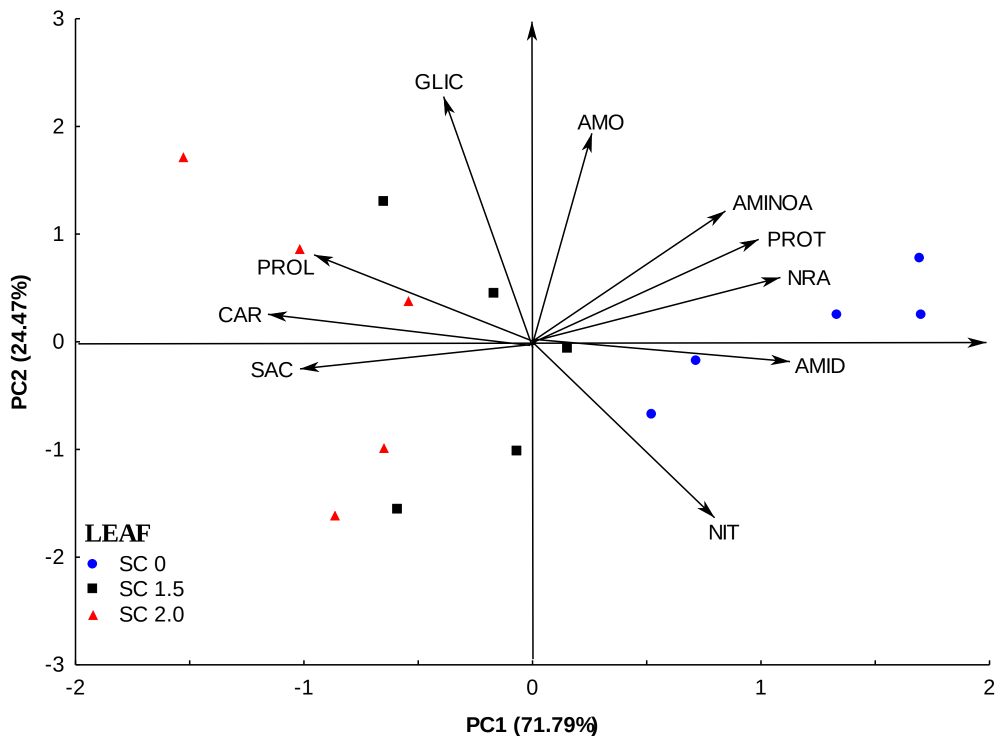

Figure 2. Biplot graphs containing the biochemical compounds in the leaf and the respective saline concentrations (SC) for the evaluated variables. NIT, nitrate; AMO, ammonium; NRA, nitrate reductase activity; AMINOA, amino acid; PROT, protein; CAR, carbohydrate; AMID, starch; SAC, sucrose; GLIC, glycine betaine; PROL, proline. In the Figure: (L) is the leaf. SC 0: is the 0 M saline concentration; SC 1.5: 1.5 M saline concentration; and SC 2.0: 2.0 M saline concentration.

Table 2. The correlation coefficient between the principal component scores for the variables in the shoot (leaf) of sorghum plants subjected to saline concentrations (0, 1.5 and 2.0 M).

Variable	PC1 (61.79%)*	PC2 (24.47%)*
Nitrate	**0.77**	-0.65
Ammonium	0.22	**0.78**
Nitrate reductase activity	**0.91**	0.26
Amino acid	**0.72**	0.48
Protein	**0.83**	0.40
Carbohydrate	**-0.98**	0.09
Starch	**0.97**	-0.04
Sucrose	**-0.97**	-0.06
Glycene betaine	-0.31	**0.91**
Proline	**-0.84**	0.34

*Percentage of the variability of the original data set retained by their respective principal components. Correlations in bold (> 0.70 in absolute value) were considered in the interpretation of the principal component.

reduction in protein synthesis can delay or even accelerate protein degradation, leading to an increase in the concentration of free amino acids. Jampeetong and Brix (2009) found that the proline content increased in *Salvinia natans* plants under saline conditions. The GLIC and AMO compounds retained in PC2 present a direct relationship with each other, but are not related to saline concentrations in the sorghum leaves.

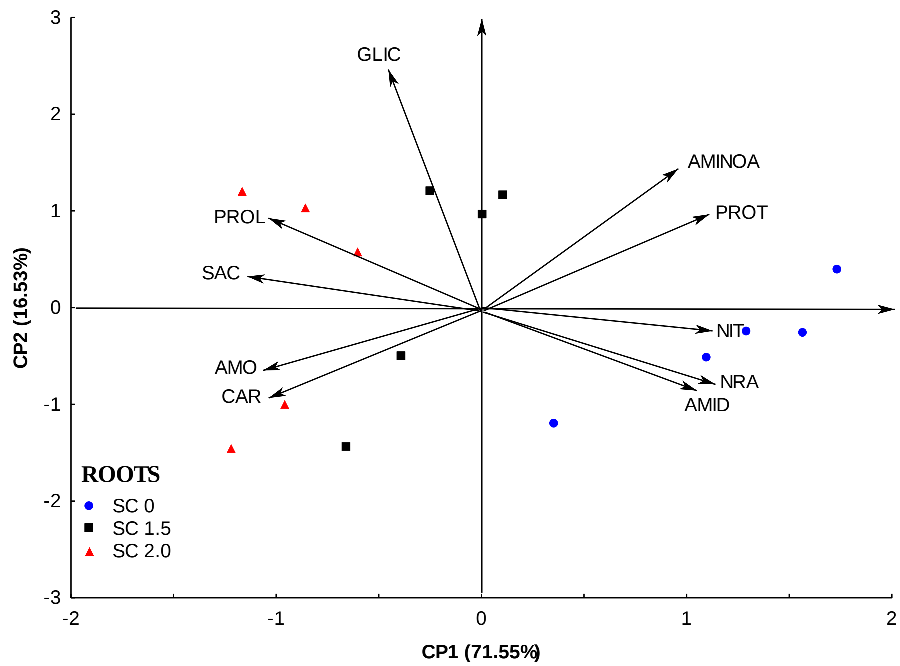

Figure 3. Biplot graph containing the biochemical compounds in the roots and their respective saline concentrations (SC) for the evaluated variables. NIT, nitrate; AMO, ammonium; NRA, nitrate reductase activity; AMINOA, amino acid; PROT, protein; CAR, carbohydrate; AMID, starch; SAC, sucrose; GLIC, glycine betaine; PROL, proline. In the figure: R represents the root. SC 0: the 0M saline concentration; SC 1.5: the 1.5 M saline concentration; and SC 2.0: the 2.0 M saline concentration.

Biochemical behavior in the root

The two-dimensional plane generated in the biochemical behavior analysis of the sorghum plant roots due to saline concentrations accounts for 88.08% of the variability of the original data: 71.55% retained in PC1 and 16.53% in PC2 (Figure 3). Most of the biochemical compounds were retained in PC1, with the exception of glycine betaine (GLIC) in PC2 (Table 3). In PC1, the compounds AMO, CAR, SAC and PROL (same negative sign) are directly correlated and are found in greater amounts in the roots of sorghum plants that received saline concentrations of 1.5 and 2.0 (Figure 3 and Table 3). In the root, the compounds, AMO, CAR, SAC and PROL, are responsible for characterizing the sorghum plants that received saline concentrations (1.5 and 2.0 M), and the control treatment (Figure 1). While in sorghum leaves these compounds are CAR, SAC and PROL (Figure 2).

The effect of proline accumulation in plants on both root and leaf subjected to stress has been studied for over 45 years (Kavi Kishor et al., 2005), and is currently considered as an indicator of acquired tolerance of the plants (Bellinger et al., 1991). The relative availability of K^+/Na^+ ions (Maathuis and Amtmann, 1999) and peroxidase phenols are also considered indicators of stress in plants (Lee et al., 2001). NIT, NRA, AMINOA, PROT and AMID (same positive sign) compounds were located to the right in PC1 (Figure 3), as occurred in sorghum leaves (Figure 2), and have higher availability in the roots of plants of the control treatment. Salinity conditions promote plant stress contributing to reduction in the NIT (Aragão et al., 2011; Lobato et al., 2009) and AMINOA activity (Elshintinawy and Elshoubagy, 2001). With reduced availability of AMINOA, the PROT amount decreases. The variance of glycine betaine (GLIC) was retained and isolated from other variables in PC2 (Figure 3). The amount of GLIC was higher in sorghum roots subjected under 1.5 M saline concentration. According to Khan et al. (2000), the GLIC levels increase in the leaves and roots of plants under saline stress, confirming the observations in the present study.

The changes in the biochemical behavior of the sorghum plant leaves and roots occur because the stimulus to saline stress causes excess Na^+ and Cl^- in the protoplasm. This causes physiological disturbances that affect photophosphorylation, respiratory chain, nitrogen assimilation and protein metabolism (Trindade et al., 2006). In addition, excess Na^+ inhibits many enzymes requiring potassium (Greenway and Munns, 1980), since there is a competition between Na^+ and K^+ for the active site. Consequently, the alterations observed in the sorghum plants under saline stress cause low productivity, decrease in the stand of the plants and, in

Table 3. The correlation coefficient among the principal component scores for the variables in the root part (root) of sorghum plants subjected to saline concentrations (0, 1.5 and 2.0 M).

Variables	PC1 (71.55%)*	PC2 (16.53%)*
Nitrate	**0.86**	-0.02
Ammonium	**-0.87**	-0.28
Nitrate reductase activity	**0.88**	-0.28
Amino acid	**0.75**	0.51
Protein	**0.88**	0.34
Carbohydrate	**-0.85**	-0.39
Starch	**0.94**	-0.26
Sucrose	**-0.96**	0.09
Glycene betaine	-0.40	**0.89**
Proline	**-0.91**	0.33

*Percentage of the variability of the original set of data retained by their respective principal components. Correlations in bold (> 0.70 in absolute value) were considered in the interpretation of the principal component.

severe cases, death of seedling (Silva and Pruski, 1997). A similar situation occurs in other crops, such as rice - *Oryza sativa spp. japonnica* (Carvalho et al., 2011), bean - *Vigna unguiculata (L.) Walp*, corn - *Zea mays* (Willadino et al., 1999; Azevedo Neto and Tabosa, 2000) and soy bean - *Glycine max L*. (Carvalho et al., 2012).

Conclusion

Under salinity condition, there occurs a distinction in the biochemical behavior of sorghum plants between the position of the leaf and root, with the formation of two dissimilar groups in the cluster analysis. The salinity increase causes an adverse effect on the biochemical behavior in the sorghum roots and leaves, a reduction of nitrate reductase activity, amino acids, proteins and starches, while there is an increase in the amount of proline, carbohydrates, sucrose and glycine. In the multivariate analysis of principal components, the direct correlation of carbohydrates, sucrose and proline in the leaves and roots of sorghum plants is verified, while there is an inverse correlation with the availability of amino acids, nitrates, protein and nitrate reductase activity.

Conflict of Interests

The authors have not declared any conflict of interests.

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