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Selection of sunflower genotypes for salt stress and mechanisms of salt tolerance in contrasting genotypes

Seleção de genótipos de girassol ao estresse salino e mecanismos de tolerância ao sal em genótipos contrastantes

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ABSTRACT

Salinity is one of the main limiting factors for crop growth. The metabolic responses to salt stress are variable and depend on species characteristics. This study aimed to select sunflower genotypes tolerant to salt stress and evaluate some mechanisms of salt tolerance in two contrasting (salt-tolerant and salt-sensitive) genotypes. In the first assay, the biomass production and the accumulation of Na^+ and K^+ in 26 sunflower genotypes were evaluated. Genotypes AG963, AG967, AG972, BRS321, BRS324, H251, H360 and H863 showed lower biomass production and were characterized as salt-sensitive and the genotypes BRS323, Catisol, EXP11-26, EXP44-49, EXP60050, EXP887, HLA860HO and Olisun 5 showed higher biomass production and were considered salt-tolerant. The high K^+ content and the low Na^+ content in the leaves were the ion traits related to salt tolerance and can be used in sunflower breeding programs for this purpose. In the second assay, the plants of salt-tolerant BRS323 had lower Na^+ and Cl^- contents and higher levels of K^+ than plants of salt-sensitive AG967. A better homeostasis in the mechanisms of transport, distribution and accumulation of inorganic solutes in conjunction with a more efficient osmoregulation mechanism through the synthesis of organic solutes may, at least in part, explain the greater salt-tolerance of BRS323 genotype in comparison to AG967.

Index terms: *Helianthus annuus* L.; genotypic variation; ion distribution; osmotic tolerance; abiotic stress.

RESUMO

A salinidade é um dos principais fatores limitantes para o crescimento das culturas. As respostas metabólicas ao estresse salino são variáveis dependendo das características da espécie. O presente estudo teve como objetivo selecionar genótipos de girassol tolerantes ao estresse salino e avaliar alguns mecanismos de tolerância ao sal em dois genótipos contrastantes (tolerantes e sensíveis ao sal). No primeiro ensaio, foram avaliados a produção de biomassa e o acúmulo de Na^+ e K^+ em 26 genótipos de girassol. Os genótipos AG963, AG967, AG972, BRS321, BRS324, H251, H360 e H863 apresentaram menor produção de biomassa e foram caracterizados como sensíveis ao sal e os genótipos BRS323, Catisol, EXP11-26, EXP44-49, EXP60050, EXP887, HLA860HO e Olisun 5 apresentaram maior produção de biomassa e foram considerados tolerantes ao sal. O alto teor de K^+ e o baixo teor de Na^+ nas folhas foram as características iônicas relacionados à tolerância ao sal e podem ser utilizados em programas de melhoramento de girassol para esse fim. No segundo ensaio, as plantas de BRS323 tolerantes ao sal apresentaram menor teor de Na^+ e Cl^- e maiores níveis de K^+ do que as plantas de AG967 sensível ao sal. Uma melhor homeostase nos mecanismos de transporte, distribuição e acumulação de solutos inorgânicos em conjunto com um mecanismo de osmorregulação mais eficiente através da síntese de solutos orgânicos pode, ao menos em parte, explicar a maior tolerância ao sal do genótipo BRS323 em comparação com o AG967.

Termos para indexação: *Helianthus annuus* L.; variação genotípica; distribuição iônica; tolerância osmótica; estresse abiótico.

INTRODUCTION

In the Northeast region of Brazil, soil salinity is one of the main problems for the crop growth. Commonly, in arid and semi-arid regions, brackish water is used in irrigation, resulting in salinization of soils and reduced crop production (Melo et al., 2018).

The high concentration of salts in soils reduces the water uptake, inducing water stress, and inhibits the growth and development of plants (Isayenkov; Maathuis, 2019). The negative role of salt stress is caused mainly by osmotic and ionic effects. The osmotic effect is associated with the reduction in the free energy of water due to reduced

osmotic potential outside the roots, impairing shoot growth (Rahnama et al., 2011; Shahzad et al., 2015). The ionic effect is characterized by a gradual accumulation of toxic ions (mainly Na^+ and Cl^-) in plant tissue. This effect occurs slowly and can cause chlorosis, necrosis, and drying of old leaves (Rahnama et al., 2011; Hura et al., 2017).

Some mechanisms of salt-tolerance in plants can decrease Na^+ transport to young leaves, retaining toxic ions in lower tissues and old leaves. For example, ion sequestration by roots, partitioning of Na^+ in stems and petioles, compartmentalization of toxic ions in the vacuole, Na^+ exclusion from shoots, and the maintenance of K^+ in growing tissues include some mechanisms of salt-tolerance (Rahnama et al., 2011; Gerona et al., 2019).

For satisfactory crop production in salt-affected soils, an alternative approach is necessary, such as the choice of salt-tolerant crop varieties. The extensive genetic variability in plant species allows developing a good breeding program of new stress-tolerant genotypes (Sakina et al., 2016). Breeding of crops for salt tolerance has been studied for a long time and different methodologies have been used for this purpose based on the physiological responses of plants (Shahzad et al., 2015; Sakina et al., 2016; Cova et al., 2020).

There is a large genotypic variation related to Na^+ and K^+ uptake and accumulation in some species, which can strongly modulate the biomass production (Rahnama et al., 2011; Gerona et al., 2019). Tolerant genotypes are more effective in reducing the concentration of toxic ions in photosynthetically active leaves than sensitive ones (Gerona et al., 2019). In wheat, studies verified that the reduction of Na^+ transport from root to shoot, Na^+ sequestration in leaf sheath, lesser Na^+ accumulation and higher K^+/Na^+ ratio in plant tissues under saline conditions are associated with the genotypic characteristic of salt-tolerance (Rahnama et al., 2011). These parameters have been used for screening salt-tolerant plants, mainly through multivariate analysis, as principal components analysis (PCA) and hierarchical cluster analysis (HCA), and the identified genotypes may be considered for inclusion in the breeding program and future genetic studies for salt-tolerance (Sarabi et al., 2016).

Loss of water from the cells is another recurrent problem for plants cultivated under salinity. To maintain turgor and cell volume under this condition, plants perform the osmotic adjustment, which involves the accumulation of organic solutes of low molecular mass, such as soluble carbohydrates, proline, amino acids, and quaternary ammonium compounds (Cova et al., 2020; van Zelm; Zhang; Testerink, 2020). The accumulation of organic solutes, in addition to osmotic adjustment, also contributes to the protection of macromolecule and membranes from the deleterious effects of salinity (Silva; Azevedo Neto; Gheyi, 2019). Plant species and varieties differ in the accumulation of compatible solutes, which promotes changes in the relative contribution during osmotic adjustment (Rhodes; Nadolska-Orczyk; Rich, 2002). In addition, the allocation of organic and inorganic solutes in different organs and the distribution of the ions in leaf tissues of different developmental stages can be a new approach for selection of genotypes to salt tolerance.

Sunflower is an oilseed crop used for the production of quality biofuel and for human and animal food (Machekposhti et al., 2017; Birck et al., 2017). In Brazil, in 2019 the cultivated area under this crop was 80,818 hectares with a production of 131,173 Mg achenes (Instituto Brasileiro de Geografia e Estatística – IBGE, 2019). This crop, is an alternative for crop rotation and succession in several producing regions because it adapts to different latitude, longitude and photoperiod (Birck et al., 2017). However, its tolerance to salt stress varies according to the genetic material. Li et al. (2020) observed that out of 552 sunflower genotypes, only 30 were considered to be highly tolerant and 53 tolerant to salt in the germination phase under 300 mM NaCl. For the genotype *H. annuus* L. cv. Azargol the threshold soil salinity was 1.6 dS m^{-1} for oil production (Machekposhti et al., 2017). In case of cultivar Embrapa 122/V-2000, Nobre et al. (2011) observed linear decreases in the leaf area, dry matter of the aerial parts and roots, production of achenes and the harvest index when electrical conductivity of irrigation water (ECw) exceeded 0.5 dS m^{-1}. For the same genotype, productivity decreased by 119.93 kg ha^{-1} with per unit increment of ECw (Santos et al., 2016). Therefore, this study aimed to select sunflower genotypes tolerant to salt stress and to evaluate the mechanisms of salt tolerance in two contrasting (salt-tolerant and salt-sensitive) genotypes, through the accumulation and distribution of inorganic and organic solutes in the distinct organs and pairs of leaves of different age.

MATERIAL AND METHODS

The study was carried out in a greenhouse with mean values of air temperature, relative air humidity, and photosynthetic active radiation (at noon) of 34 ºC, 65%, and 1200 µmol m^{-2} s^{-1}, respectively. Two experiments were carried out: the first one in a completely randomized design, a 26 (genotypes) × 2 (salt levels - 0 and 100 mM NaCl) factorial with four replicates, and the second one in

a completely randomized design, a 2 (genotypes) × 2 (salt levels - 0 and 100 mM NaCl) factorial with five replicates.

Assay 1: Selection of sunflower genotypes differing in salt tolerance

Seeds of 26 sunflower genotypes were obtained from six different companies: Ceapar (AG862, AG963, AG967, AG972, AG975); Embrapa Soja (BRS321, BRS322, BRS323, BRS324, BRS-G27), Coordenadoria de Assistência Técnica Integral (Catissol), Helianthus do Brasil (EXP11-26, EXP44-49, EXP44-63, EXP60050, EXP887, H250, H251, H358, H360, H863, HLA860HO, TC8122), Instituto Agronômico de Campinas (IAC-Uruguai), and Atlântica sementes (Olisum 3, Olisum 5).

Seeds were surface-sterilized with 2% sodium hypochlorite for five minutes and then washed three times with distilled water for three minutes each. Sterilized seeds were sown in 200-mL plastic recipients containing washed sand and irrigated with half-strength Hoagland's nutrient solution (Hoagland; Arnon, 1950). Seven days after germination, the seedlings were transferred to plastic containers with 12-L of aerated full-strength Hoagland's nutrient solution in a Floating hydroponic system. The nutrient solutions were renewed weekly and daily volume was completed with distilled water. The pH was daily monitored and adjusted to between 6.0 and 6.5 with 1.0 M hydrochloric acid (HCl) or 1.0 M sodium hydroxide (NaOH).

After eight days under these conditions, the seedlings received their respective salt-treatments (Control (C) - nutrient solution without NaCl or salt stress (S) - nutrient solution with 100 mM NaCl). NaCl was gradually added (25 mM day^{-1}) to avoid osmotic shock. Plants were harvested at ten days after the end of salt additions.

At the harvest, the plants of all treatments were carefully removed from the nutrient solution. The roots were washed with distilled water and plants were divided into leaves, stems, and roots. After drying at 65 °C in an oven for 72 h, the dry masses of the leaf (LDM), stem (SDM), and root (RDM) were determined and the total dry mass (TDM) was calculated by summation. Data of dry mass yield in different plant organs were expressed as a percentage relative to control, using the formula: % of control = 100 - (DM_S × 100) / DM_C, where:
DM_C = dry mass yield of the leaves, stems or roots in the control treatment;
DM_S = dry mass yield of the leaves, stems or roots in the salt stress treatment.

For the determination of Na^+ and K^+ contents in leaves, stems, and roots, the extracts were prepared as described by Gondim et al. (2011). In test tubes, 0.1 g dried powdered plant material and 10 mL deionized water were added. The tubes were heated to 95 °C in a water bath for one hour and then centrifuged at 5.000 × g for five minutes. The supernatants were filtered in quantitative filter paper, collected and stored at -20 °C for further analysis. The Na^+ and K^+ contents were determined by flame photometry (Jones Jr., 2001).

Assay 2: Inorganic and organic solutes in salt-tolerant and salt-sensitive sunflower genotypes

This assay was carried out using seeds of two contrasting genotypes (BRS323 and AG967) selected from the first assay and respectively classified as salt-tolerant and salt-sensitive. The treatment of seeds, sowing, production of seedlings, cultivation system, salt-treatments and the management of nutrient solutions were the same as that of Assay 1. Plants remained under salt stress for 20 days, and after this period, were harvested and divided into leaves, stems, and roots for the determination of organic and inorganic solutes content.

Samples (about 1 to 2 g) of the first pair of fully expanded leaves and the younger third of the root system were collected, immediately frozen in liquid N_2 and lyophilized for analysis of organic solutes (soluble carbohydrates, free amino acids, soluble proteins, and free proline) content. Extract preparation and determination of organic solutes were carried out as described by Sacramento et al. (2014). The soluble carbohydrates were determined at 490 nm, by sulfuric acid-phenol method. Free amino acids were determined at 570 nm, by ninhydrin method, and the proline was determined at 520 nm, by acid-ninhydrin method. Soluble proteins were determined at 595 nm by protein-dye binding method.

Samples (about 1 to 2 g) of the 6^{th}, 7^{th}, 8^{th}, and 9^{th} pairs of fully expanded leaves, counted from of base of the stem, were collected separately for analysis of Na^+, K^+, and Cl^- content (inorganic solutes) in leaves of different age. The remaining plant material was oven-dried at 65 °C for 72 h and ground to a powder for analysis of the concentrations of Na^+, K^+, and Cl^- in different organs (leaves, stems and roots). The extracts for determination of Na^+, K^+, and Cl^- contents in leaves were prepared as described in Assay 1. Na^+ and K^+ were determined by flame photometry, and Cl^- content by spectrophotometry (Jones Jr., 2001).

Statistical analysis

In Assay 1, the results were subjected to analysis of variance (F test) and the means were compared by the

Scott-Knott's test at 0.05 probability, using the Sisvar 5.6 statistical software (Ferreira, 2019).

Principal components analysis (PCA) for traits of this assay (TDM, Na^+ and K^+) was performed using the R Statistical Software (R Statistical Software - R Core Team, 2020), following the use of the 'cluster' and 'dendextend' packages (Galili, 2015; Maechler et al., 2019) for application of hierarchical cluster analysis (HCA) by the 'Ward' method. For the visualization of the obtained results, the 'factoextra' R package was used (Kassambara; Mundt, 2020).

In Assay 2, the results were subjected to analysis of variance (F-test) and the means were compared by Tukey's test at 0.05 probability, using the Sisvar 5.6 statistical software (Ferreira, 2019).

RESULTS AND DISCUSSION

In general, the biomass of all sunflower genotypes studied was reduced by salt stress, and Figure 1 shows the relative biomass production of salt-stressed genotypes in comparison to their respective control. The lowest values of LDM (60 to 75%) were observed in the genotypes: AG862, AG963, AG967, AG972, BRS321, BRS324, BRS-G27, EXP44-63, H251, H360, H863, TC 8122 and Olisum 3 under salinity (Figure 1A). In contrast, the highest values of LDM (77 to 95%) were observed in AG975, BRS322, BRS323, Catisol, EXP11-26, EXP44-49, EXP60050, EXP887, H250, H358, HLA860HO, IAC-Uruguai and Olisum 5.

Also, the lowest relative productions of SDM (41 to 66%) were found in the genotypes: AG862, AG963, AG967, AG972, BRS321, BRS324, BRS-G27, Catisol, EXP44-49, EXP44-63, EXP887, H250, H251, H360, H863, TC 8122 and Olisun 3 under salinity, while the genotypes AG975, BRS323, EXP11-26 and Olisum 5 showed the highest relative production of SDM (83 to 93%) in comparison to control conditions (Figure 1B).

Compared to the control, the genotypes: AG862, AG963, AG967, AG972, AG975, BRS321, BRS322, H250, H251, H358, H360, H863, IAC-Uruguai and Olisun 3 had the lowest RDM values (62 to 79%) (Figure 1C). On the other hand, the relative productions of RDM in EXP44-49, EXP887, and HLA860HO genotypes were not affected by salt stress.

Also, the genotypes BRS323, Catisol, EXP11-26, EXP44-49, EXP60050, EXP887, HLA860HO and Olisun 5 showed the highest relative productions of TDM (83 to 93%) under salt stress in comparison to control conditions (Figure 1D). The most salt-affected genotypes were AG963, AG967, AG972, BRS321, BRS324, H251, H360, and H863, which showed relative productions of TDM ranging from 55 to 68%.

The variation in salt tolerance among genotypes of the same species is commonly reported in the literature (Shtereva; Vassilevska-Ivanova; Karceva, 2015), and the use of salt-tolerant cultivars is more economically viable than the techniques for reclamation of the salt-affected areas (Liang et al., 2018). According to Munns (2002), the relative production of biomass is a valid indicator of salt tolerance in plants, so the criterion used to classify the genotypes as salt-tolerant or salt-sensitive was the relative dry mass yield.

Salinity strongly increased Na^+ content in all plant parts (Table 1). In leaves of salt-stressed plants, Na^+ content varied among genotypes. The lowest values were observed in IAC-Uruguai, BRS322, EXP60050, AG975, Catisol, EXP44-49, H250 and BRS323, and the highest Na^+ content in AG967. A significant variation in Na^+ content among the genotypes under salt stress was also observed in stem (3.30 to 4.07 mmol g^{-1} DM) and roots (4.14 to 5.20 mmol g^{-1} DM). In roots, the lowest values were found in the genotypes IAC-Uruguai, Olisun 3, EXP60050, H358, AG963, EXP11-26, HLA860HO, H251 and H250, and the higher values in H360, BRS323, AG972, BRS324, and BRS-G27.

In the salt stress treatment, a significant variation between the Na^+ and K^+ levels in the plant organs of the genotypes was observed. However, it is noteworthy that in the leaves of the genotypes classified as salt-sensitive the highest Na^+ and the lowest K^+ levels were observed, in contrast to the leaves of tolerant ones, which showed the lowest Na^+ and the highest K^+ contents. However, in the stem and roots, no relationship was observed between the accumulation of these ions and the degree of salt tolerance.

Principal component analysis (PCA) showed that PC1 and PC2 together explained 94.17% of the variance (Figure 2A). PC1 explained the largest variance observed in the data (79.29%), while PC2 accounted for 14.88% of the total variance. In this figure, it is possible to see the dispersion of the variables according to the score and the correlation between them. Considering that the cosine of the angle between any two vectors representing variables indicates the coefficient of correlation between those variables (Jolliffe; Cadima, 2016), the leaf K^+ content and TDM correlated positively, in contrast to the leaf Na^+ content, which showed a negative correlation with the dry mass yield.

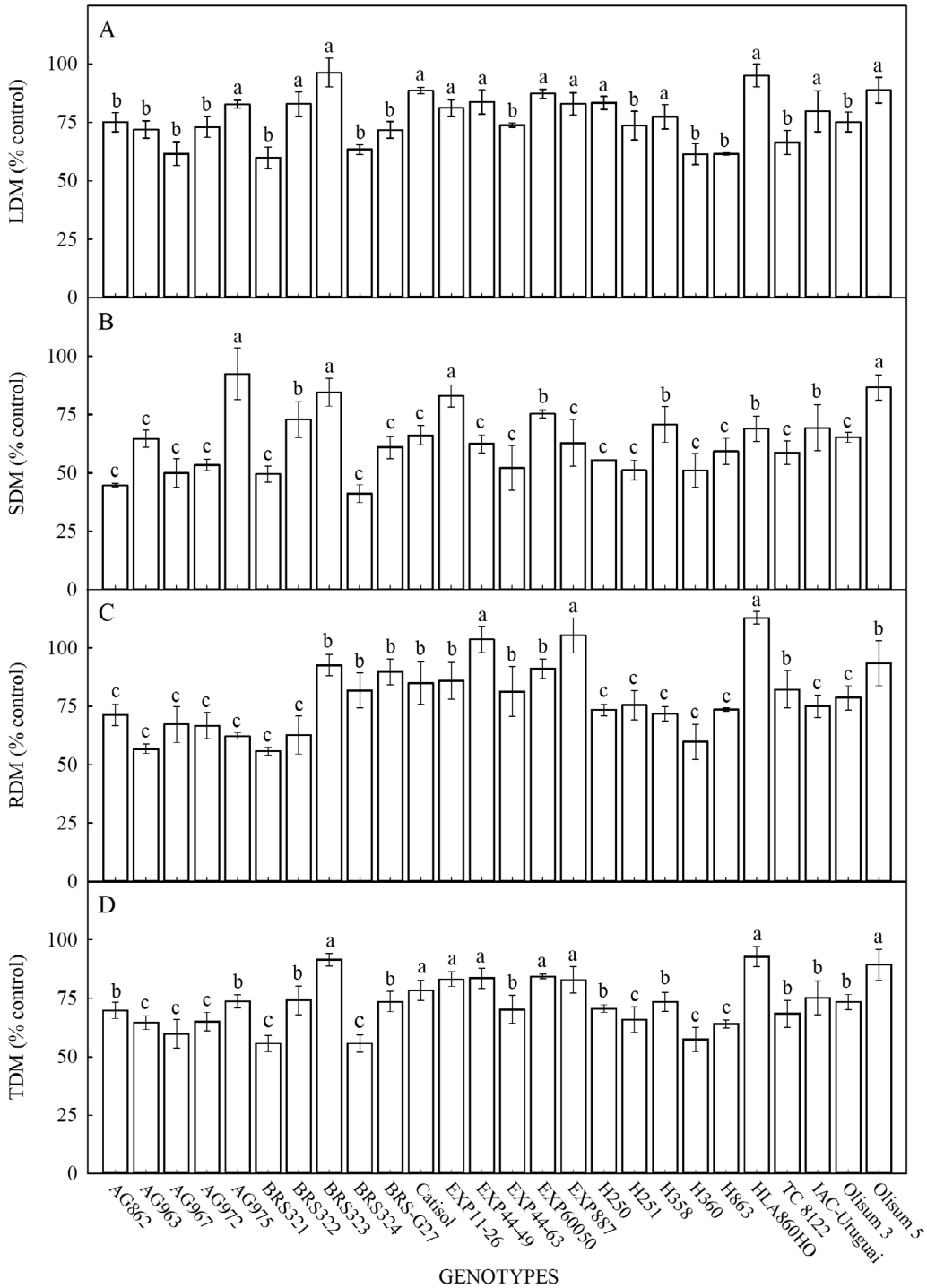

Figure 1: Dry mass yield, expressed as percentage of the control, of leaf - LDM (A), stem - SDM (B), root - RDM (C) and total - TDM (D) of 26 sunflower genotypes grown under greenhouse conditions for ten days in nutrient solution containing 100 mM of NaCl.

Means followed by the same letters do not significantly differ by the Scott-Knott's test at $p \leq 0.05$. Vertical bars represent the standard errors (n=4).

Table 1: Content of sodium and potassium (mmol g^{-1} DM) in leaves, stems and roots of 26 sunflower genotypes grown under greenhouse conditions for ten days with nutrient solution (control - C) or nutrient solution containing 100 mM NaCl (salt stress - S).

	Leaves		Stem		Roots	
Genotypes	C	S	C	S	C	S
	Na^+ (mmol g^{-1} DM)					
AG862	0.01Ab (0.000)	2.19Ea (0.143)	0.25Ab (0.028)	3.76Aa (0.144)	1.24Ab (0.043)	4.67Ca (0.157)
AG963	0.02Ab (0.002)	2.72Ca (0.149)	0.23Ab (0.028)	3.47Ba (0.203)	1.16Ab (0.084)	4.42Da (0.164)
AG967	0.01Ab (0.001)	3.23Aa (0.070)	0.21Ab (0.023)	3.63Aa (0.031)	1.23Ab (0.037)	4.52Ca (0.141)
AG972	0.01Ab (0.000)	2.15Ea (0.153)	0.17Ab (0.014)	3.85Aa (0.171)	1.36Ab (0.046)	5.14Aa (0.223)
AG975	0.01Ab (0.000)	2.02Ea (0.135)	0.20Ab (0.009)	3.33Ba (0.132)	1.59Ab (0.063)	4.75Ba (0.228)
BRS321	0.01Ab (0.000)	2.61Ca (0.126)	0.16Ab (0.016)	3.43Ba (0.066)	1.02Bb (0.080)	4.87Ba (0.222)
BRS322	0.01Ab (0.000)	1.73Fa (0.157)	0.13Ab (0.016)	3.38Ba (0.063)	0.91Bb (0.042)	4.62Ca (0.124)
BRS323	0.01Ab (0.001)	1.60Fa (0.082)	0.15Ab (0.010)	3.86Aa (0.182)	1.35Ab (0.116)	5.01Aa (0.109)
BRS324	0.01Ab (0.000)	2.41Da (0.084)	0.16Ab (0.016)	4.07Aa (0.264)	1.11Bb (0.082)	5.16Aa (0.183)
BRS-G27	0.01Ab (0.001)	2.33Da (0.060)	0.19Ab (0.010)	3.71Aa (0.187)	1.20Ab (0.087)	5.20Aa (0.121)
Catissol	0.01Ab (0.000)	1.60Fa (0.100)	0.17Ab (0.017)	3.68Aa (0.196)	1.22Ab (0.085)	4.85Ba (0.075)
EXP11-26	0.01Ab (0.001)	1.89Ea (0.067)	0.18Ab (0.013)	3.74Aa (0.166)	1.19Ab (0.089)	4.16Da (0.219)
EXP44-49	0.01Ab (0.001)	1.67Fa (0.080)	0.18Ab (0.012)	3.75Aa (0.301)	1.02Bb (0.031)	4.54Ca (0.060)
EXP44-63	0.01Ab (0.001)	2.09Ea (0.132)	0.15Ab (0.019)	3.60Ba (0.298)	0.90Bb (0.078)	4.63Ca (0.300)
EXP60050	0.01Ab (0.001)	1.79Fa (0.167)	0.22Ab (0.011)	3.75Aa (0.092)	1.28Ab (0.075)	4.41Da (0.140)
EXP887	0.01Ab (0.001)	2.03Ea (0.165)	0.19Ab (0.015)	3.87Aa (0.114)	1.09Bb (0.061)	4.82Ba (0.180)
H250	0.02Ab (0.002)	1.83Fa (0.091)	0.15Ab (0.018)	4.00Aa (0.095)	1.30Ab (0.095)	4.14Da (0.244)
H251	0.02Ab (0.002)	2.62Ca (0.132)	0.16Ab (0.013)	3.65Aa (0.273)	1.61Ab (0.043)	4.20Da (0.089)
H358	0.01Ab (0.001)	2.07Ea (0.181)	0.20Ab (0.017)	3.61Ba (0.051)	1.32Ab (0.018)	4.23Da (0.205)
H360	0.02Ab (0.000)	2.70Ca (0.102)	0.36Ab (0.022)	3.59Ba (0.313)	1.03Bb (0.116)	5.10Aa (0.124)
H863	0.02Ab (0.001)	2.91Ba (0.061)	0.16Ab (0.016)	3.41Ba (0.082)	1.22Ab (0.061)	4.82Ba (0.108)
HLA860HO	0.02Ab (0.000)	2.05Ea (0.175)	0.25Ab (0.008)	3.54Ba (0.172)	1.04Bb (0.075)	4.39Da (0.148)
TC 8122	0.01Ab (0.001)	2.45Da (0.116)	0.12Ab (0.005)	3.32Ba (0.034)	0.82Bb (0.031)	4.65Ca (0.089)
IAC-Uruguai	0.02Ab (0.002)	1.74Fa (0.122)	0.20Ab (0.001)	3.50Ba (0.263)	1.01Bb (0.104)	4.40Da (0.232)
Olisum 3	0.01Ab (0.000)	2.37Da (0.134)	0.13Ab (0.015)	3.36Ba (0.089)	1.15Ab (0.093)	4.31Da (0.132)
Olisum 5	0.01Ab (0.000)	1.93Ea (0.195)	0.11Ab (0.010)	3.30Ba (0.219)	0.77Bb (0.073)	4.66Ca (0.121)
	K^+ (mmol g^{-1} DM)					
AG862	0.60Aa (0.008)	0.55Ba (0.040)	0.52Da (0.032)	0.59Ba (0.017)	0.49Cb (0.012)	0.58Ba (0.032)
AG963	0.64Aa (0.023)	0.56Ba (0.025)	0.49Da (0.021)	0.43Ca (0.036)	0.50Ca (0.036)	0.58Ba (0.018)
AG967	0.61Aa (0.022)	0.53Ba (0.016)	0.75Ba (0.055)	0.81Aa (0.023)	0.49Cb (0.010)	0.63Ba (0.028)
AG972	0.60Aa (0.015)	0.58Ba (0.043)	0.60Ca (0.030)	0.59Ba (0.061)	0.46Ca (0.026)	0.52Ca (0.037)
AG975	0.60Aa (0.018)	0.57Ba (0.046)	0.69Ba (0.043)	0.74Aa (0.056)	0.54Cb (0.038)	0.63Ba (0.047)
BRS321	0.60Aa (0.055)	0.52Ba (0.027)	0.82Aa (0.031)	0.75Aa (0.027)	0.65Ab (0.017)	0.77Aa (0.061)
BRS322	0.58Aa (0.021)	0.53Ba (0.038)	0.45Da (0.035)	0.53Ca (0.021)	0.45Ca (0.028)	0.50Ca (0.011)
BRS323	0.61Aa (0.024)	0.68Aa (0.043)	0.72Ba (0.034)	0.70Aa (0.048)	0.45Ca (0.019)	0.40Da (0.010)
BRS324	0.66Aa (0.024)	0.60Ba (0.041)	0.62Ca (0.049)	0.64Ba (0.025)	0.48Ca (0.027)	0.51Ca (0.017)

Continue...

Table 1: Continuation.

	----------Leaves----------		----------Stem----------		----------Roots----------	
Genotypes	C	S	C	S	C	S
BRS-G27	0.66Aa (0.020)	0.64Aa (0.033)	0.55Da (0.042)	0.63Ba (0.051)	0.44Ca (0.012)	0.49Ca (0.017)
Catissol	0.63Aa (0.015)	0.65Aa (0.099)	0.51Da (0.010)	0.50Ca (0.042)	0.47Cb (0.010)	0.56Ca (0.056)
EXP11-26	0.63Aa (0.031)	0.65Aa (0.077)	0.63Ca (0.035)	0.56Ba (0.020)	0.47Ca (0.031)	0.50Ca (0.025)
EXP44-49	0.64Aa (0.008)	0.70Aa (0.066)	0.62Ca (0.026)	0.47Cb (0.040)	0.64Aa (0.016)	0.70Aa (0.029)
EXP44-63	0.65Aa (0.021)	0.62Aa (0.038)	0.78Aa (0.045)	0.82Aa (0.060)	0.66Aa (0.004)	0.64Ba (0.041)
EXP60050	0.64Aa (0.022)	0.69Aa (0.025)	0.72Ba (0.037)	0.76Aa (0.071)	0.58Ba (0.023)	0.64Ba (0.017)
EXP887	0.60Ab (0.003)	0.74Aa (0.011)	0.88Aa (0.042)	0.82Aa (0.073)	0.68Aa (0.025)	0.63Ba (0.025)
H250	0.61Aa (0.014)	0.57Ba (0.041)	0.48Da (0.039)	0.46Ca (0.014)	0.45Ca (0.038)	0.44Da (0.013)
H251	0.62Aa (0.008)	0.57Ba (0.081)	0.55Da (0.051)	0.53Ca (0.049)	0.46Ca (0.029)	0.47Ca (0.022)
H358	0.58Aa (0.014)	0.52Ba (0.016)	0.53Da (0.016)	0.48Ca (0.034)	0.64Aa (0.008)	0.69Aa (0.023)
H360	0.61Aa (0.027)	0.57Ba (0.032)	0.53Da (0.040)	0.58Ba (0.032)	0.54Cb (0.034)	0.63Ba (0.021)
H863	0.63Aa (0.023)	0.55Ba (0.040)	0.74Ba (0.024)	0.63Ba (0.037)	0.57Bb (0.026)	0.67Ba (0.009)
HLA860HO	0.63Aa (0.012)	0.71Aa (0.055)	0.70Ba (0.025)	0.52Cb (0.033)	0.50Ca (0.017)	0.54Ca (0.035)
TC 8122	0.66Aa (0.056)	0.62Aa (0.045)	0.55Da (0.038)	0.52Ca (0.040)	0.50Ca (0.030)	0.55Ca (0.026)
IAC-Uruguai	0.64Aa (0.019)	0.64Aa (0.070)	0.58Ca (0.041)	0.49Ca (0.105)	0.47Ca (0.043)	0.52Ca (0.008)
Olisum 3	0.60Aa (0.029)	0.60Ba (0.010)	0.48Da (0.019)	0.51Ca (0.019)	0.38Ca (0.019)	0.39Da (0.038)
Olisum 5	0.63Aa (0.051)	0.67Aa (0.040)	0.50Da (0.030)	0.40Ca (0.013)	0.49Ca (0.018)	0.55Ca (0.022)

In each plant organ, means followed by the same capital letters in a column and same lowercase letters in a row do not significantly differ by the Scott-Knott's test ($p \leq 0.05$). Numbers between parentheses represent standard errors (n=4).

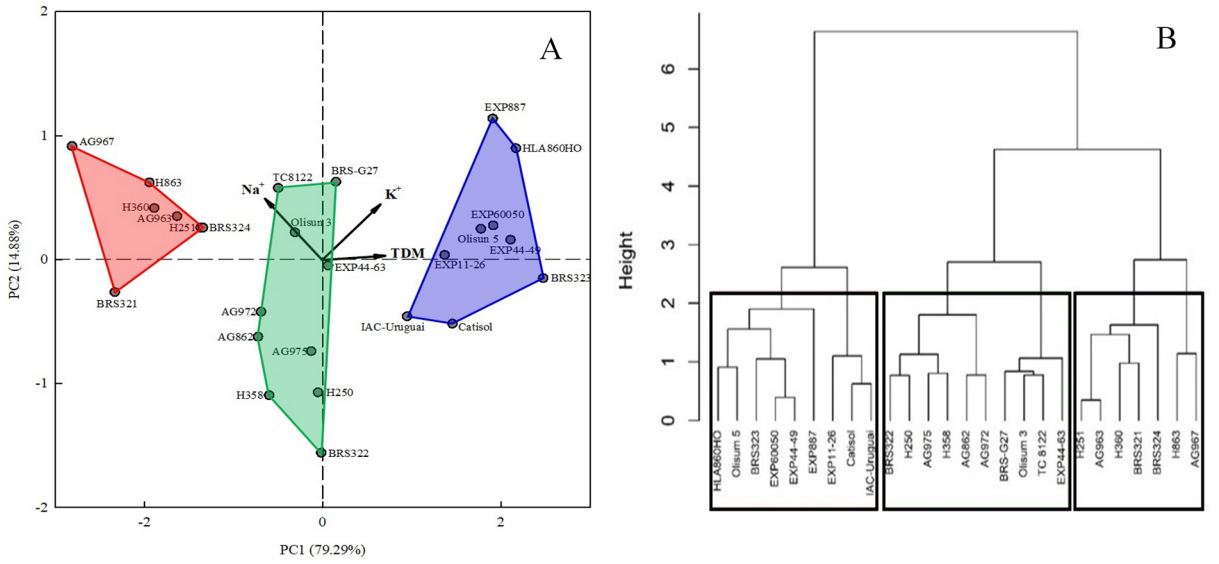

Figure 2: (A) Principal components analysis (PCA) using the leaf contents of sodium (Na^+) and potassium (K^+) and relative production of total dry mass (TDM) of 26 sunflower genotypes grown under greenhouse conditions for ten days in nutrient solution containing 100 mM NaCl; and (B) dendrogram of hierarchical cluster analysis (HCA) by the 'Ward' method of the studied variables (Na^+, K^+ and TDM).

Hierarchical cluster analysis (HCA) divided the genotypes into three groups, estimated by 'Ward' method based on Euclidian distance (Figure 2B). The first cluster (on the left) included all genotypes with the highest TDM (Figure 1D), plus the genotype IAC-Uruguai. On the opposite side (on the right), the third cluster included the genotypes with the lowest TDM, except the AG972.

In agreement with HCA, the correlations of the PC1 axis discriminate against the groups of genotypes with higher and lower dry mass production. The right side of the PC1 gathered nine sunflower genotypes (BRS323, Catisol, EXP11-26, EXP44-49, EXP60050, EXP887, HLA860HO, IAC-Uruguai, and Olisun 5), which expressed high TDM, high leaf K^+ content and low leaf Na^+ content. By contrast, the left side of the PC1 associated seven genotypes (AG963, AG967, BRS321, BRS324, H251, H360, and H863) with opposite traits. It can also be seen in Figure 2A that BRS323 and AG967 genotypes occupy the most extreme positions on the PC1 axis.

The combinations of the HCA and PCA have been used to distinguish salt-tolerant cultivars from salt-sensitive ones (Sarabi et al., 2016). These analyses were also used to evaluate the drought tolerance level of 49 switchgrass genotypes (Liu et al., 2015). In our study, PC1 was the main salt-related component, so the salt-tolerant genotypes (high TDM) are located on the right side of Figure 2A and the salt-sensitive ones on the left side (low TDM). Therefore, HCA and PCA contributed to explaining that the degree of salt-tolerance in sunflower is associated with the concentrations of Na^+ and K^+ in the leaves. The genotypes located on the extreme sides of the PC1 axis, BRS323 and AG967, were used in assay 2 as the most salt-tolerant and the most salt-sensitive, respectively.

Figure 3 shows the dry mass yield of two sunflower genotypes (BRS323 and AG967) that were selected in assay 1 as most salt-tolerant and most salt-sensitive, respectively. In assay 2, salinity decreased LDM (43%), SDM (56%), RDM (52%), and TDM (50%) of AG967 in comparison to control conditions. However, in BRS323, salinity induced a smaller reduction in RDM (26%) and TDM (21%) and did not affect the LDM and SDM, in contrast to results of assay 1. It can also be seen in Figure 3 that the dry mass yields of both genotypes were similar under control conditions. However, under salt stress, the LDM, SDM, RDM, and TDM of the BRS323 genotype were, respectively, 37, 87, 67, and 61% higher than those of AG967.

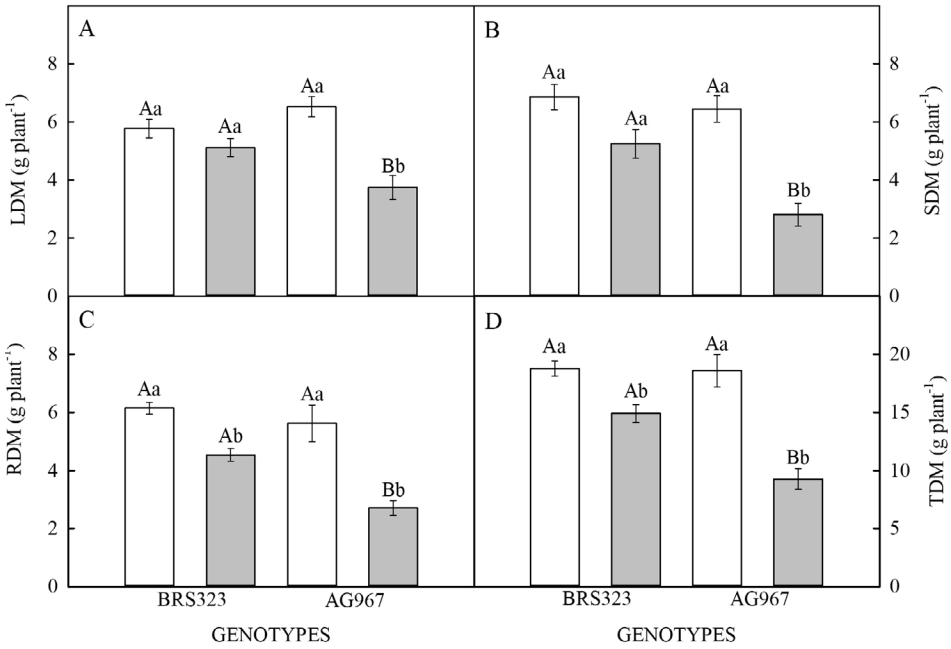

Figure 3: Leaf - LDM (A), stem - SDM (B), root - RDM (C) and total - TDM (D) dry mass yield of two sunflower genotypes grown under greenhouse conditions for 20 days in nutrient solution - control (□) or nutrient solution containing 100 mM NaCl - salt stress (■).

Means followed by the same letters do not significantly differ by the Tukey's test at $p \leq 0.05$. Uppercase letters compare genotypes at same salt levels and lowercase letters compare salt levels in same genotype. Vertical bars represent the standard errors (n=4).

These results support the findings that BRS323 is a salt-tolerant genotype and that AG967 is a salt-sensitive one. The most widely accepted explanations for the salt-induced biomass reduction are associated with a combination of factors such as water stress due to a reduction of osmotic potential, accumulation of Na^+ and Cl^- toxic ions in plant tissues and imbalance of nutritional status (Shtereva; Vassilevska-Ivanova; Karceva, 2015).

Salinity increased the contents of Na^+ and Cl^- in leaves, stems and roots of both genotypes (Figure 4). In salt-stressed leaves, the Na^+ content in the AG967 genotype was 52% higher than in BRS323 (Figure 4A). On the other hand, in the stem and roots, the Na^+ contents in BRS323 were, respectively, 73 and 20% higher than in AG967. Regarding Cl^- in the salt stress treatment, the levels in the leaves, stem and roots of AG967 genotype were 33, 16, and 9% higher than in BRS323 (Figure 4C).

Salinity did not affect the concentrations of K^+ in the leaves and roots of BRS323 and increased by 89% the content in the stem (Figure 4B). In AG967, salt stress decreased (33%) the K^+ concentration in the leaves and increased by 91 and 101%, respectively, the content of this nutrient in the stem and roots.

Figure 5 shows the concentrations of Na^+, Cl^- and K^+ in the 6th, 7th, 8th and 9th leaf pairs, counted from base of the stem, that is, from the oldest to the youngest fully expanded leaves. Salt stress increased Na^+ and Cl^- contents in all pairs of leaves of both sunflower genotypes in comparison to control conditions. However, these increases were more pronounced in AG967 genotype (Figure 5A and C).

The BRS323 genotype showed similar Na^+ contents, regardless of the leaf age considered (Figure 5A). In contrast, the Na^+ content in the AG967 genotype increased progressively with leaf age (Figure 5A). Thus, the Na^+ content in the 6th, 7th, 8th and 9th pair of leaves of AG967 were respectively, 76, 52, 56 and 17% higher than in the same pairs of leaves of the BRS323 genotype.

In the BRS323 genotype, the levels of K^+ were similar, regardless of treatment or pair of leaves evaluated (Figure 5B). In AG967, salt stress reduced the K^+ content in the 6th and 7th pairs of leaves by respectively, 77 and 54%, compared to the same pairs under control conditions (Figure 5B). In contrast with Na^+, the K^+ content in salt stress treatment was higher in the younger leaves.

Salinity changed the levels of Na^+ and K^+ in all organs of both genotypes, but they differ in accumulation of ions in the tissue. The leaves of salt-sensitive AG967 accumulated more Na^+ and less K^+ than salt-tolerant BRS323. In the stem and roots, the genotype AG967 concentrated more K^+ and less Na^+ than BRS323. Additionally, AG967 accumulated more Na^+ and less K^+ in older leaves, in contrast to BRS323, whose contents of these ions did not differ between leaves.

Our results suggest that the retention of Na^+ in the stem and roots of BRS323 mitigated the harmful effects of this ion on the leaves (Figure 4), and indicate that this genotype has an important mechanism related to salt tolerance (Wu et al., 2019). Additionally, the ability of BRS323 to maintain the leaf K^+ content with a very low level of Na^+ gives this genotype a relatively better physiological state under salt stress. This response is also considered a mechanism of salt tolerance as it is a key factor in mitigating the deleterious effects of NaCl-induced stress on plants (Abid et al., 2020).

The Cl^- content in AG967 genotype was higher (23%) in younger leaves (8th and 9th pairs) than in the older ones (6th and 7th pairs) (Figure 5C), in contrast with BRS323, in which Cl^- content was 33% lower when compared the same pairs of leaves mentioned (Figure 5C).

The Cl^- content in all organs of the AG967 genotype was higher than that of BRS323. The AG967 also had a remarkable high Cl^- content in younger leaves, as opposed to that observed in BRS323. Li, Tester and Gilliham (2017) affirm that reduction of Cl^- concentration in the xylem is a key step to reduce the Cl^- toxicity, as it prevents a large accumulation in young tissues, indicating a greater selectivity in uptake and long-distance transport of this ion in salt-tolerant plants.

From the results of inorganic solutes, it can be hypothesized that the greater tolerance of BRS323 was, at least in part, due to physiological mechanisms of Na^+ and Cl^- exclusion and reduction of K^+ efflux from leaves, reducing the disturbances in ion homeostasis and in the cell metabolic activity of this genotype.

The addition of NaCl in the nutrient solution changed the levels of organic solutes in both genotypes, however, the reductions were more expressive in AG967 and the increases were more significant in BRS323 genotype (Figure 6A-D). Thus, salinity significantly decreased leaf contents of soluble carbohydrates (32%), free amino acids (30%), soluble proteins (24%) and free proline (29%) in AG967. In the roots, salt stress also decreased soluble carbohydrates and free amino acids (45 and 27%, respectively) and increased soluble proteins by 28%. However, in the BRS323 genotype, salt stress decreased only 14% the soluble proteins and increased proline (41%) in leaves. In the roots, salinity decreased amino acids by 29%, but increased soluble carbohydrates and proline by 45% and 111%, respectively.

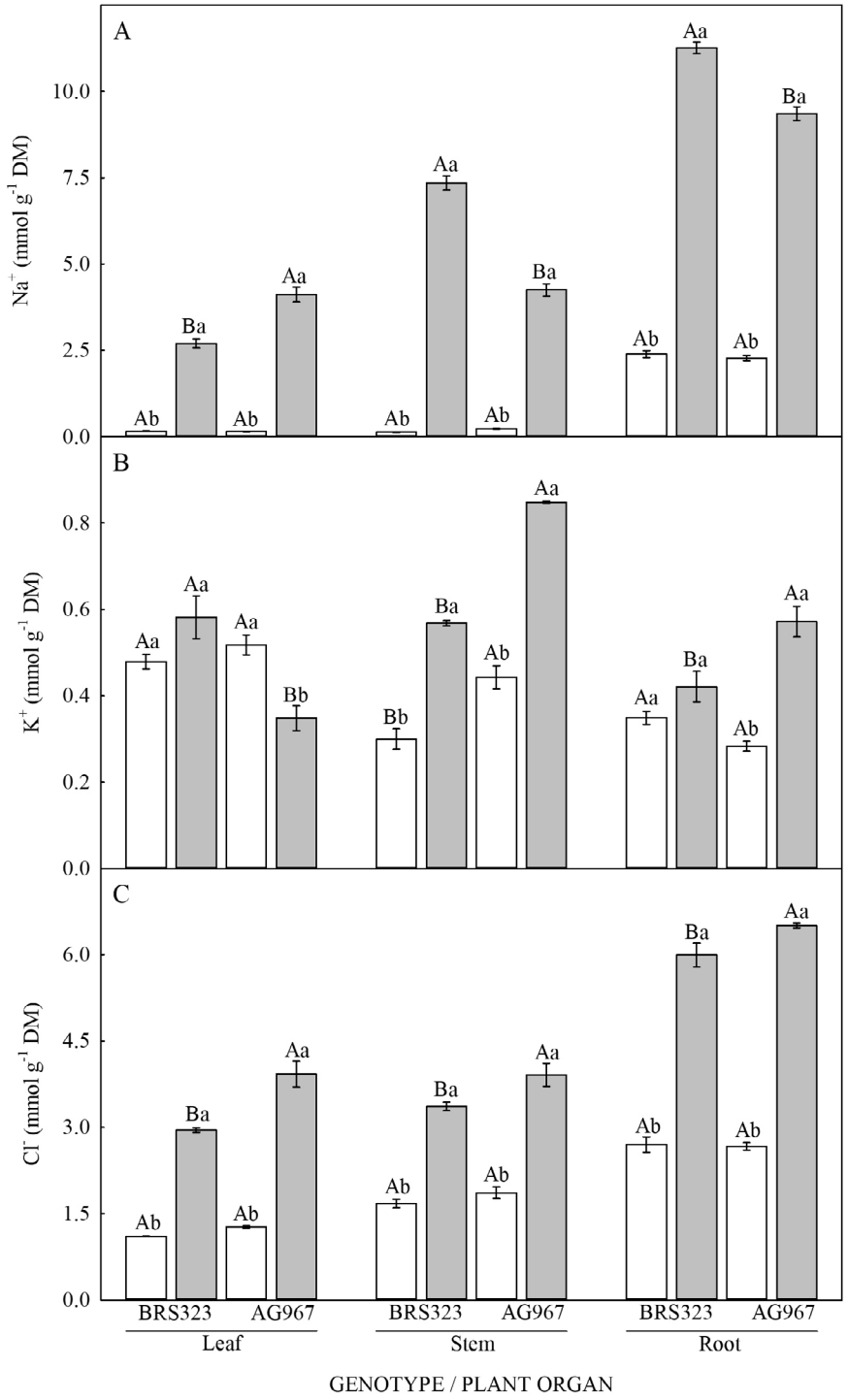

Figure 4: Sodium (A), potassium (B) and chloride (C) contents in leaves, stems and roots of two sunflower genotypes grown under greenhouse conditions for 20 days in nutrient solution - control (□) or nutrient solution containing 100 mM NaCl - salt stress (■).

Means followed by the same letters do not significantly differ by the Tukey's test at $p \leq 0.05$. Uppercase letters compare genotypes at same salt levels and lowercase letters compare salt levels in same genotype. Vertical bars represent the standard errors (n=4).

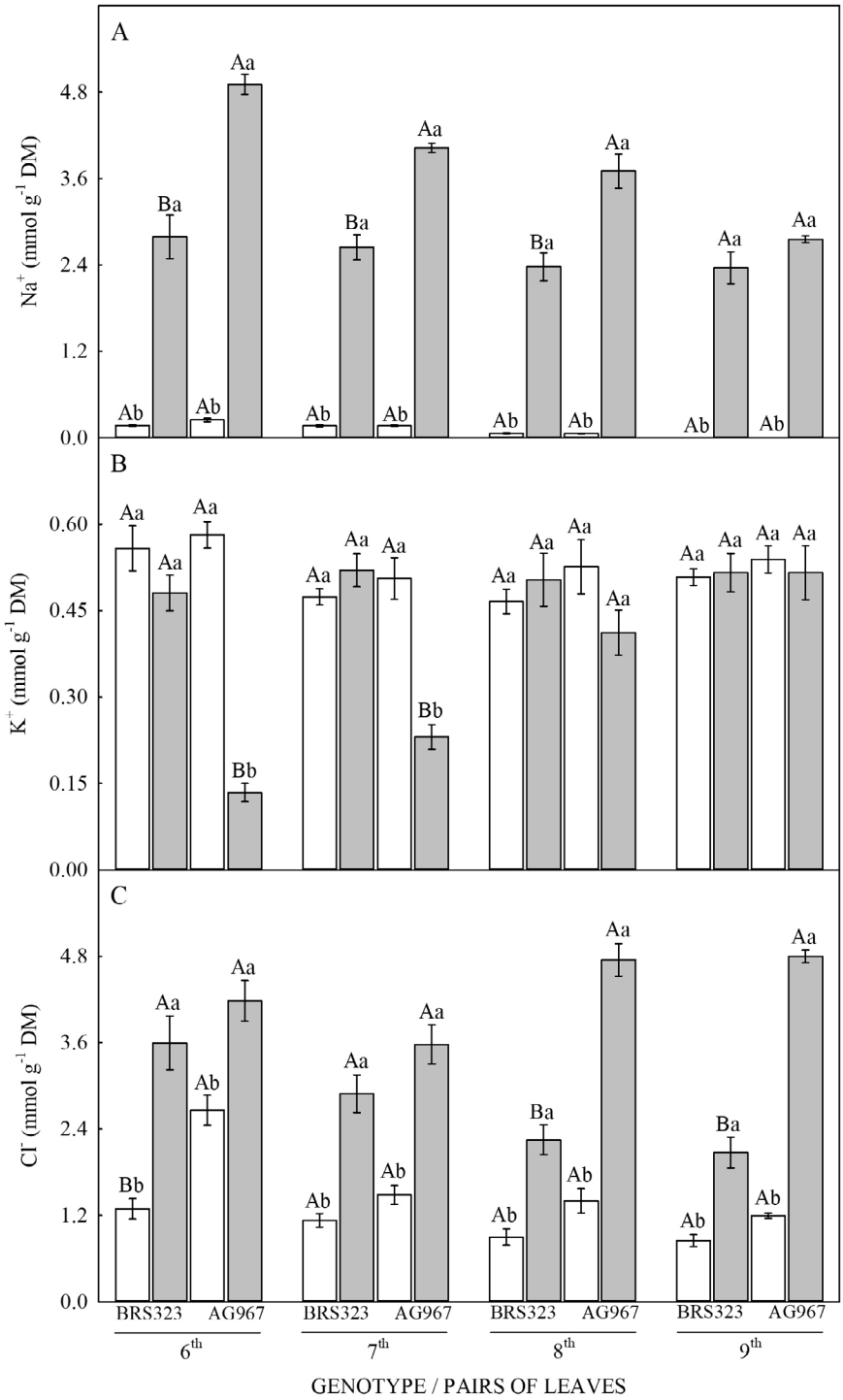

Figure 5: Sodium (A), potassium (B), and chloride (C) contents in leaves of different ages of two sunflower genotypes grown under greenhouse conditions for 20 days in nutrient solution - control (□) or nutrient solution containing 100 mM NaCl - salt stress (■).

Means followed by the same letters do not significantly differ by the Tukey's test at $p \leq 0.05$. Uppercase letters compare genotypes at same salt levels and lowercase letters compare salt levels in same genotype. Vertical bars represent the standard errors (n=4).

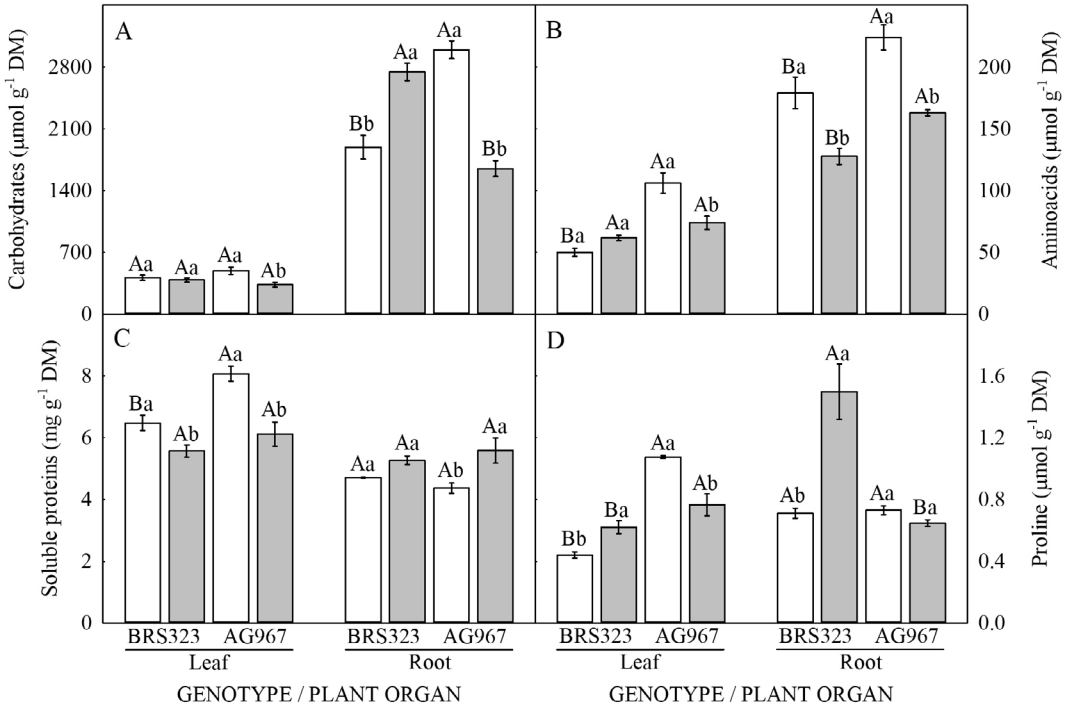

Figure 6: Carbohydrates (A), free amino acids (B), soluble proteins (C) and free proline (D) contents in leaves and roots of two sunflower genotypes grown under greenhouse conditions for 20 days in nutrient solution - control (□) or nutrient solution containing 100 mM NaCl - salt stress (■). Additional details as in Figure 3.

Means followed by the same letters do not significantly differ by the Tukey's test at $p \leq 0.05$. Uppercase letters compare genotypes at same salt levels and lowercase letters compare salt levels in same genotype. Vertical bars represent the standard errors (n=4).

By comparing the genotypes in the stress treatment, substantial differences were not found between the leaf contents of each organic solute. However, in the stressed roots of BRS323, the levels of soluble carbohydrates and proline were 66 and 132% higher than those of AG967, respectively.

For physiological level, the synthesis and accumulation of organic solutes (also known as compatible solutes or compatible osmolytes) in plants grown under salinity is an acclimation mechanism that enables the maintenance of turgor (Singh et al., 2015). In this study, salinity reduced the content of all organic solutes in leaves and the carbohydrates and amino acids in roots of salt-sensitive AG967 genotype. On the other hand, in BRS323, salinity increased the leaf and root proline contents and the root carbohydrates content. Furthermore, the levels of these solutes in the stressed roots of the BRS323, were substantially higher than those of AG967. Therefore, our results indicate that the leaves and roots of the BRS323 genotype had a much more efficient osmoregulation mechanism to cope with salt stress than that observed in salt-sensitive AG967.

In higher plants, carbohydrate metabolism is co-regulated with amino acid metabolism and protein synthesis and involves reciprocal regulation. Amino acid biosynthesis uses carbohydrate backbones, while degradation of all amino acids produces carbohydrate backbones that can be converted into citric acid cycle intermediates and used as an energy source (Pratelli; Pilot, 2014). So, the decrease in photosynthesis was probably the critical factor for reducing the carbohydrates and amino acids contents in both leaves and roots of the AG967 (Abdul Qados, 2011), and can be an additional support to explain the salt-sensitivity of this genotype when compared to BRS323.

Regarding proline, the marked increase in proline content in both leaves and roots of BRS323 can be considered a biochemical trait related to tolerance to salt stress (Reddy et al., 2017). This compatible solute is reported to accumulate in response to several environmental stresses, but the role of proline in the osmotic adjustment is still controversial. In our study, the proline content in the stressed leaves (0.71 μmol g^{-1} DM) and roots (1.5

μmol g^{-1} DM) of BRS323 represented, respectively, 0.04 and 0.05% of the carbohydrates content (1,892 and 2,744 μmol g^{-1} DM), indicating that the contribution of proline to sunflower osmoregulation is negligible when compared to that of carbohydrates. These findings are in agreement with those obtained by other authors (Sacramento et al., 2014; Silva; Azevedo Neto; Gheyi, 2019; Cova et al., 2020).

In addition to its role in osmotic adjustment, a number of other functions are related to proline in plant acclimation to salt stress (Azevedo Neto; Silva, 2015). Thus, despite the minor importance for osmotic adjustment, our results suggest that the proline accumulation in leaves and roots does not exclude its beneficial role in the acclimation of sunflower plants to salt stress.

CONCLUSIONS

Genotypes AG963, AG967, BRS321, BRS324, H251, H360 and H863 were characterized as salt-sensitive, especially AG967 genotype, while BRS323, Catisol, IAC-Uruguai, EXP11-26, EXP44-49, EXP60050, EXP887, HLA860HO and Olisun 5 were characterized as salt-tolerant, especially BRS323 genotype. The high K^+ content and the low Na^+ content in the leaves were the ion traits related to salt tolerance in sunflower and can be used in breeding programs for this purpose. The better homeostasis in the mechanisms of transport, distribution and accumulation of inorganic solutes in conjunction with a more efficient osmoregulation mechanism through the synthesis of organic solutes may, at least in part, explain the greater salt-tolerance of BRS323 genotype in comparison to AG967.

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