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Antioxidant Protection of Photosynthesis in Two Cashew Progenies Under Salt Stress

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Abstract

The present work evaluated the indicators of photosynthetic efficiency and antioxidative protection in cashew tree seedlings subjected to salinity stress. The study was conducted with seedlings of two advanced dwarf cashew clones (CCP09 and CCP76) subjected to salt stress with increasing doses of NaCl (0, control; 25; 50; 75; 100 mM) in the nutrient solution for 30 days under greenhouse conditions. The variables of gas exchange, CO_2 assimilation (P_N), stomatal conductance (g_S), transpiration (E), intercellular CO_2 concentration (C_I), photochemical activity, potential quantum efficiency (Fv/Fm), effective quantum efficiency (ΔF/Fm') of photosystem II (PSII), photochemical quenching (qP), non-photochemical quenching (NPQ) electron transport rate (ETR) as well as the indicators of damage and oxidative protection were measured. Under these conditions, there was an intense accumulation Na^+ associated with a reduction in the K^+/Na^+ ratio in the leaves of both clones in response to salt, with higher values for this ratio in clone CCP09 than in CCP76 the highest concentration of NaCl (100 mM). Salinity reduced P_N, g_S and *E* in the two clones evaluated, with lower reductions in CCP09 than in CCP76 at the highest salt dose. Instantaneous carboxylation (P_N/C_I) and water use (P_N/E) efficiencies were strongly restricted by salinity but were less affected in CCP09 than in CCP76. Salinity stress also increased hydrogen peroxide (H_2O_2) levels in CCP09, whereas lipid peroxidation decreased in both progenies. The clones presented specific antioxidant responses due to greater enzymatic and non-enzymatic activity in CCP76, in addition to the activity of phenol peroxidase (POX) in CCP09.

Keywords: *Anacardium occidentale*, oxidative stress, photosynthesis, salinity

1. Introduction

Excess of salt in the soil solution causes metabolic disturbances in plants due to the osmotic and ionic effects of salinity, leading to reduced crop growth and productivity (Khan & Panda, 2008; Lima, Nobre, Gheyi, Soares, & Silva, 2014). The osmotic effect is immediate due to the difference in osmotic potential between the external and internal environments of the cell, whereas the ionic effect occurs later when the concentration of Na^+ and/or Cl^- reaches toxic levels in the cytosol (Shavrukov, 2013). At the time of exposure to salinity, these osmotic/ionic effects act simultaneously, affecting essential metabolic processes such as nutritional balance, water relations and photosynthesis (Shaheen, Naseer, Ashraf, & Akram, 2013; Chen, Hawighorst, Sun, & Polle, 2014).

Ionic toxicity caused by salinity stress results from increased Na^+/K^+, Na^+/Ca^{+2}, Na^+/Mg^{+2} and Cl^-/NO_3^- ratios in plant tissue, causing cellular disorders related to the physiological function of these essential nutrients (Abbaspour, Kaiser, & Tyeman, 2014; Bessa, Lacerda, Amorim, Bezerra, & Lima 2016). K^+ is a macronutrient that participates in several cellular functions, acting on osmotic potential (osmosolute function) and the functioning of metabolic pathways due to its role as an enzymatic cofactor (Wang & Wu, 2013). Thus, the

K^+/Na^+ ratio in plant tissue is considered a physiological marker of K^+ selectivity and the resulting resistance to salinity stress in some species and/or agricultural crops (Rodrigues et al., 2013; Bessa et al., 2016).

Salinity affects the absorption process by restricting K^+ accumulation in different parts of plants, a response that has been previously shown to occur in cashew trees (Ferreira-Silva, Voigt, Viégas, Paiva, & Silveira, 2009). A lower K^+/Na^+ ratio in response to salt is associated with other metabolic disturbances, such as photosynthetic limitation and photooxidative damage (Rodrigues et al., 2013). Photosynthetic limitation is among the first physiological responses exhibited by plants subjected to salinity (Shaheen et al., 2013). A reduction of this process may be associated with both stomatal limitation (Hussain, Luro, Costantino, Ollitrault, & Morillon, 2012) and non-stomatal factors (Rodrigues et al., 2013).

In addition to stomatal limitation, salinity can affect the structural components of photosystem II (PSII) and can compromise photochemical efficiency, limiting the capture and use of light energy (Silveira & Carvalho 2016). Damage to PSII can occur due to the photooxidation of structures such as pigments (chlorophylls) and proteins, particularly the D1 protein, caused by the generation of excess reactive oxygen species (ROS) at PSII (Goh, Ko, Koh, Kim, & Bae, 2012). This damage affects PSII repair systems and can lead to chronic photoinhibition, causing non-stomatal photosynthetic limitation (Hussain et al., 2012). In chloroplasts, in addition to the photochemical disturbances caused by salinity, the limitation of carbon reduction in the Calvin cycle can lead to an increase in the $NADPH/NADP^+$ ratio, stimulating the generation of ROS due to oxygen (O_2) photoreduction at photosystem I (Goh et al., 2012).

This imbalance of the photosynthetic process causes changes in the redox state that increase the content of ROS and oxidative damage (Mittler, 2002). For protection, plant cells evolved a complex system that involves enzymatic mechanisms consisting of enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and phenol peroxidase (POX) and non-enzymatic mechanisms consisting of chemical components such as ascorbate (ASC) and glutathione (GSH), which act together in cellular protection (Dinakar, Djilianov, & Bartels, 2012; Šimková, Fialová, Vaculíková & Luxová, 2016). SOD is present in different compartments of the cell and dismutates the superoxide radical ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) and water (Bhattacharjee, 2010). Peroxidases (APX and POX) remove excess generated H_2O_2 using specific electron donors (Maia et al., 2012).

Cashew (*Anacardium occidentale* L.) trees are economically important in Brazil. Cashew nut production reached 228,796 t in 2016 (IBGE, 2016). This species is cultivated under dry conditions in the Brazilian semiarid region under adverse environmental conditions. Salinity affects cashew growth, but the species is moderately salt tolerant; some clones can exclude Na^+, which is a favorable attribute for salt resistance (Ferreira-Silva et al., 2010). Genetic variability in relation to characters involved in salt resistance, such as ionic partitioning (Ponte et al., 2011) and oxidative protection (Ferreira-Silva et al., 2011), also exists.

Despite these few reports in the literature, metabolic disturbances related to photosynthetic limitation in cashew plants cultivated under salinity conditions are not characterized. In the present study, the characterization of both photosynthetic damage and metabolic disorders related to the generation of oxidative damage was carried out in two cashew tree progenies subjected to salinity stress. The effects of salinity on stomatal modulation and photochemical activity associated with the K^+/Na^+ ratio in leaf tissue are also discussed.

2. Material and Methods

2.1 Plant Material and Application of Treatments

Seeds (cashew nuts) of advanced dwarf cashew (*Anacardium occidentale* L.) trees were obtained from a commercial nursery in Pacajus city, Ceará, Brazil. The experiment was carried from June to October 2015 out at the Unidade Acadêmica de Serra Talhada of the Universidade Federal Rural de Pernambuco. Nuts were subjected to superficial disinfestation with 5% (v/v) sodium hypochlorite and sown into 4.0 L pots containing a mixture of vermiculite and sand as a substrate at a ratio of 1:1 (v/v). During the germination and initial growth stages, substrate moisture was maintained near field capacity by frequent irrigation with distilled water for 20 days. Afterward, seedlings (stage of four expanded leaves) received nutrient solution described by Hoagland and Arnon (1950).

Thirty days after planting, seedlings were subjected to different salinity treatments in increasing concentrations of NaCl (0, control; 25; 50; 75; and 100 mM) dissolved in diluted nutrient solution (one-quarter strength). Treatment solutions were applied every three days for 30 days, and the photosynthetic parameters were measured at the end of the experiment. Seedlings were then collected, and leaves and stems were separated to determine their fresh mass. Part of the leaves was frozen in liquid nitrogen and stored at -80 °C to later analyze the

indicators of oxidative damage, non-enzymatic antioxidants and enzymatic activity and measure the Na^+ and K^+ contents.

The experiment was carried out in a completely randomized design in a 2 × 5 factorial arrangement: two cashew clones (CCP76 and CCP09) and five doses of NaCl (0, control; 25; 50; 75; and 100 mM). There were three replicates per treatment for a total of 30 plots, with each one represented by one pot containing one seedling. Data were subjected to the F test at 0.05% significance, and means were compared by the Tukey test at the same probability level. For the analysis, the ASSISTAT software (Statistical Assistance) was used 7.7 beta, copy updated on 10/4/2015 (Silva & Azevedo, 2016).

2.2 Fresh Mass Content, Na^+ and K^+ Contents and K^+/Na^+

The fresh mass of the above ground portion was obtained by measuring fresh leaf and stem tissue using a semi-analytical electronic balance. The extraction for the measurements of Na^+ and K^+ content from leaf tissues was performed using 50 mg of vegetable tissue and 10 mL of deionized water in a 100% water bath for 1 hour in closed thread tubes. The clear extract was obtained by filtration with cotton, and the Na^+ and K^+ contents were measured by flame photometry readings (Malavolta, Vitti, & Oliveira, 1989).

2.3 Gaseous Exchange Analysis and Chlorophyll Fluorescence

The rate of CO_2 assimilation (P_N), transpiration (E) and stomatal conductance (g_S) were measured with a portable photosynthesis system (LI-6400XT, LI-COR, USA) with saturating irradiance (1000 μmol photons m^{-2} s^{-1}) provided by an external halogen lamp to saturate PSII without damage. From these data, water use efficiency, by the P_N/E ratio (Souza, Soares, & Regina, 2001), and instantaneous carboxylation efficiency, by the P_N/C_I ratio (Ribeiro, Machado, Santos, & Oliveira, 2009), were calculated. Fluorescence measurements were subsequently performed on mature, fully expanded leaves using the saturation pulse method (Van Kooten & Snel, 1990; Schreiber, Bilger, & Neubauer et al., 1994) with a fluorometer coupled to a portable photosynthesis system (LI-6400XT, LI-COR).

From the fluorescence data, the maximum quantum efficiency of PSII based on the Fv/Fm ratio and the following parameters were calculated: PSII quantum efficiency [ΔF/F'm = (F'm – Fs)/F'm]; excitation energy capture efficiency or antenna efficiency [Fv'/Fm' = (F'm – F'o)/F'm]; apparent rate of electron transport (ETR = ΔF' - F'm × photosynthetic photon flux density × 0.5 × 0.84); non-photochemical quenching [NPQ = (Fm – F'm)/F'm]; and photochemical quenching (qP) (Rohácek, 2002). In addition, the ETR/P_N ratio was calculated to estimate excess electrons in the chloroplastic electron transport chain used in other processes not related to the rate of P_N (Ribeiro, Santos, Machado, & Oliveira, 2008).

2.4 Indicators of Oxidative Damage, Non-enzymatic and Enzymatic Antioxidants

The H_2O_2 content was determined by the method described by Cheeseman et al. (2006). Leaf samples (0.2 g of fresh tissue) were macerated in a liquid N_2 mortar, followed by extraction in 50 mM potassium phosphate buffer pH 6.0 containing 1 mM KCN. The extract was centrifuged at 12.000 × g for 30 min the 4 °C and aliquots (200 μl) of the supernatant were transferred to test tubes containing 900 μL of in reaction medium, 0,25 mM $FeSO_4$, 0.25 mM $(NH_4)_2SO_4$; 0.25 mM H_2SO_4, 124 μM xylenol orange and 99 mM sorbitol. The mixture was incubated per 30 min at 25 °C and then absorbance readings were carried out at 560 nm. The contents of H_2O_2 were obtained from standard curve and the results expressed in μmol H_2O_2 g^{-1} MF.

For the thiobarbituric acid (TBARS), ascorbate (ASC) and glutathione (GSH) reactive substance measurements reduced, leaf samples (0.1 g) were macerated in a mortar in the presence of liquid N_2, followed by extraction in solution of TCA (5%) and centrifugation at 10,000 × g per 30 min at 4 °C. Lipid peroxidation was estimated by the thiobarbituric acid reactive substance content (TBARS) according to Heath and Packer (1968). For the reaction, aliquots (0.5 mL) of the supernatant were added to 2.0 mL of 20 % TCA solution and 0.5% (m/v) TBA and heated in a 95 °C water bath in sealed tubes For 1 hour. After the reaction was stopped in an ice bath, readings were carried out at 532 and 660 nm and after subtraction of the readings, the TBARS content was estimated using the 155 mM^{-1} cm^{-1} molar extinction coefficient.

The ASC content was determined according to Kampfenkel et al. (1995). Aliquots (0,1 mL) of the supernatant were added to the reaction medium with 0.3 mL of 200 mM potassium phosphate buffer, pH 7.4; 0.1 mL of distilled water; 0.5 mL of 1% TCA; 0.4 mL of 42 % H_3PO_4; 0,4 mL of 4% bipyridyl; 0.2 mL of $FeCl_3$. The tubes were brought to the water bath at 42 ºC per 30 min and after readings were carried out at 525 nm. The content of GSH was determined according to Griffth (1980). Aliquots (0.2 mL) of the supernatant were added to the reaction medium containing 2.6 mL of 150 mM sodium phosphate buffer pH 7.4; 1 ml of 100 mM sodium phosphate buffer pH 6.8 and 0.2 ml of DTNB (5,5 'dithiobis-nitrobenzoic acid) 30 mM in 100 mM phosphate

buffer, pH 7.0. The tubes were kept in a water bath at 30 ºC for 10 min and then readings were carried out at 412 nm. The contents of ACS and GSH were estimated based on standard curve and expressed in μmol g^{-1} MF.

Protein extraction was performed as described by Zimmermann et al. (2006). The activity of the enzyme superoxide dismutase (SOD; EC: 1.15.1.1) was determined according to the methodology described by Giannopolitis and Ries (1977). 0.1 ml aliquots of the protein extract were transferred to light-protected test, tubes containing 50 mM potassium phosphate buffer, pH 7.8; Containing 0.1 mM EDTA; 13 mM L-methionine and 750 μM nitroblue tetrazolium (NBT). The reaction was initiated by the addition of 2 mM riboflavin and rapid transfer of the tubes, without light protection, to a 30-watt lamp-illuminated chamber (30 μmol of photons m^{-2} s^{-1}) for 7 minutes. The reaction was interrupted by the shutdown of light and readings were carried out at 560 nm. The activity of the enzyme was estimated on the basis of inhibition of NBT and one unit of activity was considered as the amount of enzyme required to inhibit 50% of its reduction by Beauchamp and Fridovich (1971) and expressed in UA g^{-1} MF min^{-1}.

The activity of ascorbate peroxidase (APX; EC: 1.11.1.1) was determined according to the method described by Nakano and Asada (1981). Aliquots of 0.1 mL of protein extract were added to the reaction medium composed of 2.7 mL of 50 mM potassium phosphate buffer pH 6.0 containing 0.5 mM ascorbic acid. The reaction was started by adding 0.2 mL H_2O_2 30 mM and accompanied by the decline in absorbance at 290 nm in a spectrophotometer for 120 seconds, with readings at intervals of 30 sec. APX activity was estimated using the molar extinction coefficient of 2.8 mM^{-1} cm^{-1} for ascorbate at 290 nm and expressed as μmol ASC g^{-1} MF min^{-1}.

The activity of phenol peroxidase (POX; EC 1.11.1.7) was determined according to the reaction principle of the method described by Kar and Mishra (1976). 100 μL aliquots of the protein extract were transferred to assay tubes, then added 4.9 mL of 25 mM potassium phosphate buffer, pH 6.8; Containing 20 mM of pyrogallic acid and 20 mM of H_2O_2. The mixture was incubated at room temperature (25 ºC) for 1 min and the reaction Interrupted by the addition of 0.5 mL of 0.5% (v/v) H_2SO_4. Absorbance readings were taken at 40 nm. The POX activity was expressed in nmol purpurogalin g^{-1} MS min^{-1}.

3. Results and Discussion

3.1 Growth and Partition of Ions in Cashew Tree Seedlings Subjected to Salinity Stress

For all parameters evaluated, the clones presented similar responses, with the exception of K^+, in which CCP76 maintained a stable K^+ content but CCP09 tended to increase proportionally with increasing in salinity. From the 50 mM NaCl dose there was a tendency for fresh weight of shoot to decrease in response to increased salinity (Figure 1A). Torres et al. (2014) suggested that the absolute and relative growth rates of height, stem diameter, leaf number, leaf area and root length as well as fresh phytomass of cashew trees are negatively influenced by the salinity of irrigation water. These authors affirmed that salinity stress decreases plant growth due to the energy consumption required for synthesis of osmotically active organic compounds, which are associated with osmotic adjustment processes and maintenance of water relations in plants.

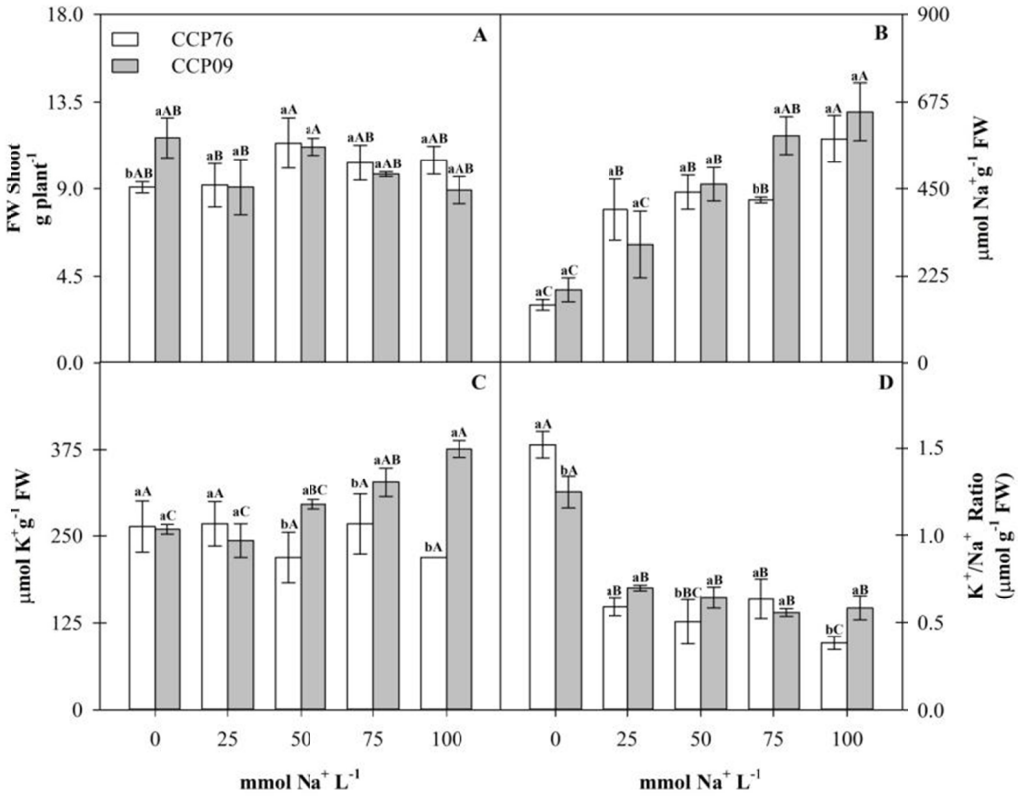

Figure 1. Fresh weight of leaves (A) as well as the Na^+ content (B), K^+ content (C) and K^+/Na^+ (D) in the leaves of early dwarf cashew clones (CCP76 and CCP09) cultivated under control conditions with distilled water and treated with 0; 25; 50; 75 and 100 mM NaCl for 30 days. The letters on the bars indicate significant differences according to the Tukey test ($p \leq 0.05$). Lowercase letters are associated with the clones; and uppercase letters with doses salinas

The increase in NaCl in nutrient solution resulted in proportional accumulations of Na^+ (Figure 1B) of 66.66% and 67.11% in clones CCP76 and CCP09, respectively, at 100 mM NaCl, compared with those of the controls. These results were similar to those of Freitas, Marques, Bezerra, Prisco & Gomes-Filho (2013), who reported an increase in Na^+ content in the leaves of cashew tree seedlings subjected to salinity stress. On the other hand, the K^+ content in the leaves increased only in clone CCP09 starting at 50 mM in response to salinity stress (Figure 1C), which may have been caused by the increment of Na^+, indicating the occurrence of ionic toxicity associated with a possible nutritional imbalance (Ferreira-Silva, Silveira, Voigt, Soares, & Viégas, 2008). Marques, Freitas, Bezerra, Prisco, and Gomes-Filho (2011) studied the effects of salinity on cashew seedlings and reported that the K^+ contents are not altered during the growth stages corresponding to germination and seedling emergence but decrease at the onset of established seedling development, suggesting that the negative effects of excess Na^+ on absorption and content of K^+ under saline conditions depend on the growth stage and duration of stress.

In the present study, both clones subjected to the NaCl treatments (25, 50, 75 and 100 mM) presented a 65% reduction in the K^+/Na^+ ratio in the aerial portions of the plants (Figure 1D). When these values are lower than 1, ionic toxicity due to the excessive accumulation of Na^+ in relation to K^+ is evident (Rodrigues et al., 2013).

3.2 Photosynthetic Changes Induced by Salinity in Cashew Tree Seedlings

In the analyzed variables of photosynthetic metabolism, a significant difference was observed between treatments, in which clones displayed similar behavior among themselves. This behavior differed only with respect to water use efficiency (P_N/E) and qP. Salinity stress dramatically affected P_N (Figure 2A). In the CCP76 clone, P_N decreased by 89.25% in response to the dose of 100 mM and in CCP09 by 79.51% in response to the dose of 75 mM compared to control. In cowpea plants, salinity stress promotes a lower rate of P_N, which is caused by a reduction in g_S (Souza, Machado, Silveira, & Ribeiro, 2011). This effect was also observed in cashew seedlings under salinity stress.

There was also a significant effect on intercellular CO_2 concentration (C_I) due to salinity (Figure 2B). There was an increase in C_I in the CCP76 clone of 45.26% from 100 mM NaCl but only 7.14% in CCP09 at the same concentration, when compared to control. The results are in agreement with those reported in other studies (Warren, 2008; Sousa et al., 2012) in several plant species, such as Cocos nucifera, Swietenia macrophylla, Zea mays and Arachis hypogaea subjected to salinity stress and/or different water regimes**.** As reported in these studies, the increase in C_I may be caused by the direct interference of salinity stress in absorption of water and nutrients; therefore, the amount of water available influences stomatal opening and closure. High concentrations of Na^+ reduce the turgidity potential of foliar tissues, interfering in the absorption of water. At the same time, stomatal closure and reduction in C_I occur. The reduction in g_S shown in Figure 2C likely impaired the stability of gas exchange, reducing the P_N rate of cashew clones and becoming correlated with the lower influx of CO_2 in the sub-stomatal cavity for photosynthesis (Romero-Aranda et al., 1998; Chaves, Flexas, & Pinheiro, 2009).

In cashew seedlings subjected to salinity stress, drastic reductions in the instantaneous carboxylation efficiency (P_N/C_I) of approximately 92.85% for CCP76 and 67% for CCP09 were observed compared with those of plants in the absence of NaCl (Figure 2D). The P_N/C_I ratio decreased with increasing NaCl content in leaf tissue. This decrease can be explained in part by the toxic effect of increasing concentrations of Na^+ and Cl^- in leaves, as reported in other species such as cowpea (Souza et al., 2011) and soybean (Shi, Meng-Zhaolai, Wang, Xu, & Xu, 2015). It is probable that the reduction in the P_N/C_I ratio is associated with a decrease in P_N (Figure 2A) and therefore lower availability of CO_2 as a substrate for photosynthesis.

Increased NaCl concentration significantly reduced the g_S of clones CCP76 (60%) and CCP09 (45.45%) subjected to 100 mM salinity concentrations (Figure 2D). Among the clones, a significant difference was observed in response to doses of 75 and 100 mM, with the g_S of CCP76 overlapping with that of CCP09 at 75 mM and the g_S of CCP09 with that CCP76 at 100 mM NaCl.

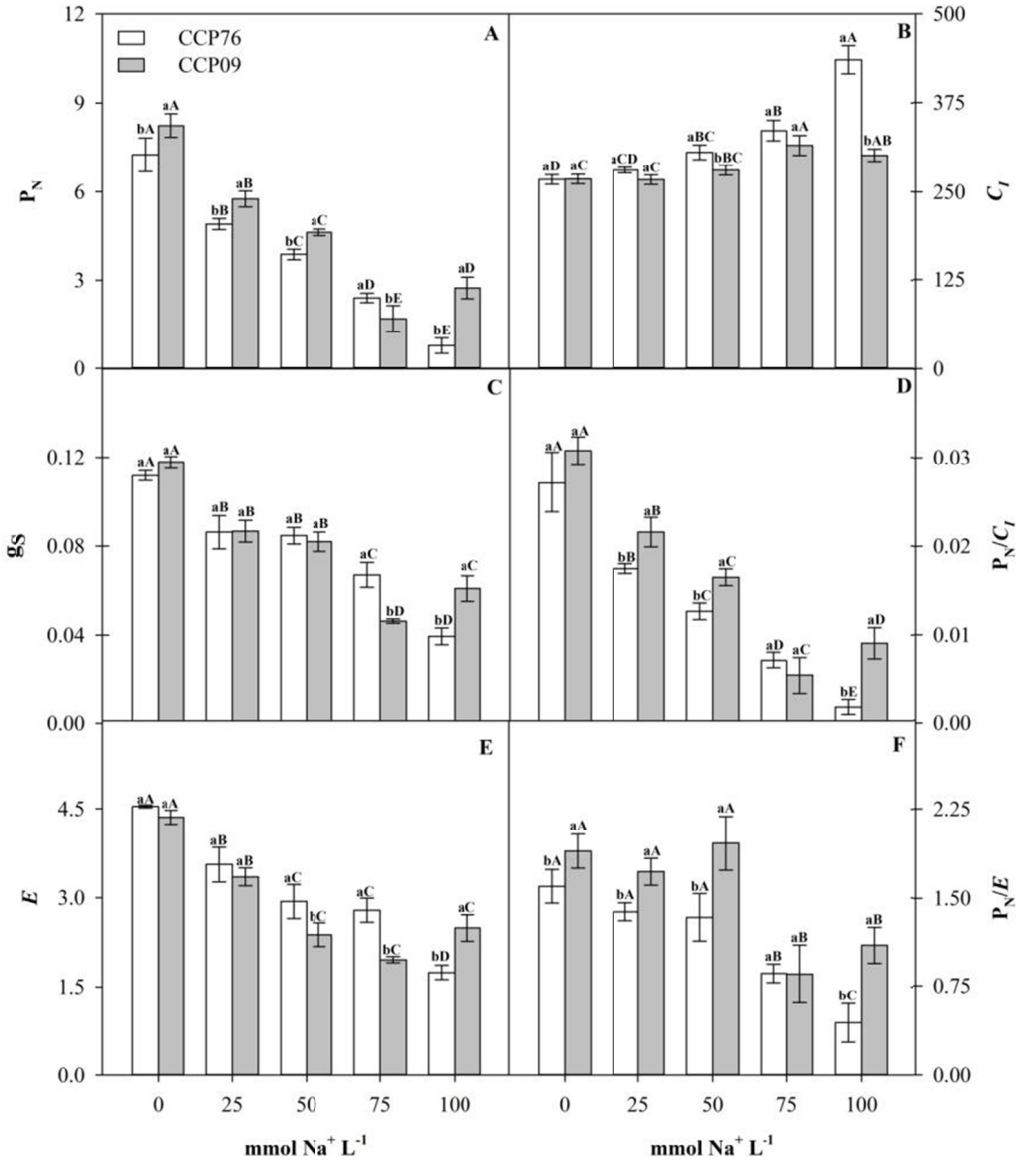

Figure 2. CO_2 assimilation rate (PN) (A), intercellular CO_2 concentration (CI) (B), maximum carboxylation efficiency (P_N/C_I) (C), stomatal conductance (gS) (D), transpiration rate (E) and water use efficiency (PN/E) (F) in the leaves of early dwarf cashew clones (CCP76 and CCP09) grown under controlled conditions with distilled water and treated with 0; 25; 50; 75 and 100 mM NaCl for 30 days. The letters on the bars indicate significant differences according to the Tukey test ($p \leq 0.05$). Lowercase letters are associated with clones; and uppercase letters with doses salinas

Hussain et al. (2012) reported that the accumulation of salts in leaf affects stomatal closure due to the dehydration of guard cells. Similar results were observed in cashew seedlings exposed to both combined and isolated factors under high temperature and salinity (Ferreira-Silva et al., 2011). The same behavior was also reported in other species of plants exposed to salinity, such as *Ipomoea pes-caprae* (Suárez, 2011). However, Amorim, Gomes Filho, Bezerra, Prisco, and Lacerda (2010) reported varying results in clones CCP76 and CCP06 but no significant changes in g_S when plants were exposed to salt stress with different electrical conductivities (0.5; 4.0; 8.0 and 12.0 dS m^{-1}).

The rate of *E* decreased with increasing salt dose (Figure 2E), with mean reductions of 62.22% for clone CCP76 and 41.86% for CCP09 in the treatment with 100 mM NaCl compared to control. The decrease in the *E* rate may be related to the reduction in g_S. A reduction in g_S reduces water consumption through *E* and contributes to water retention in leaves, which may be a key factor for avoiding drastic effects of drought on growth and

photosynthesis of cashew seedlings. According to Carneiro et al. (2012), an increase in salinity causes a reduction in the transpiration rate of cashew clone CCP76 plants due to the osmotic effect of salinity stress, affecting mass flow to root zone.

A progressive reduction in P_N/E in cashew leaves exposed to different concentrations of Na^+ (Figure 2F) was also observed. Clones were stable through the 50 mM NaCl dose, but there was a 60.16% reduction in P_N/E for the CCP76 clone and a 69% reduction for the CCP09 clone at concentrations of 100 mM NaCl. However, CCP09 stood out more than CCP76 did in all salinity concentrations. This decrease in P_N/E can be explained by the reduction in P_N (Figure 2A), demonstrating the ability of plants to absorb and assimilate CO_2 at the expense of water loss through evapotranspiration (Silva et al., 2014).

The apparent ETR was also influenced by NaCl. In the CCP09 clone, the ETR remained constant through the dose of 50 mM but decreased by 21.42% with dose of 100 mM compared with to control (Figure 3A). In the CCP76 clone, there was an increase with the 25 mM dose followed by a drastic reduction of 41.09% with the dose of 100 mM NaCl compared to control. It is possible that the salinity directly affected PSII due to photoinhibitory processes, enabling the reduction in the electron flux necessary for the formation of ATP and NADPH (Ghannoum et al., 2003). Similar results have been reported in bean, corn, sorghum and *Brachiaria* (Dias & Brüggemann, 2010; Santos et al., 2014) under water and salinity stresses.

Salinity stress also provided excess electrons for photosynthesis, as indicated by the increase in ETR/P_N. There was an increase in both clones, with increases of 81.81% in CCP76 at a dose of 100 mM and of 78% in clone CCP09 through the dose of 75 mM NaCl compared with that of the controls (Figure 3B). The increase in the ETR/P_N ratio indicated the occurrence of excess electrons for the carbon reduction process, a potential condition for the formation of excessive ROS.

The increase in the ETR/P_N ratio represents an imbalance between electron flux and P_N during photosynthesis, which is often associated with an increase in the electron flux to other physiological processes rather than to reactions for P_N (Baker, Harbinson, & Kramer, 2007; Ribeiro et al., 2009). The increase in the ETR/P_N ratio is associated with a reduction in the P_N/C_I ratio, which may indicate a loss in photosynthetic efficiency in cashew seedlings caused by salinity stress.

The maximum Fv/Fm was reduced by 3% (not significant) in both cashew clones (Figure 3C) from the treatment of 75 mM NaCl. Jamil, Lee, J. M. Kim, H. S. Kim, and Rha (2007), and Silveira, S. L. Silva, E. N. Silva, and Viégas (2010) reported that salinity stress causes stomatal closure, which reduces photosynthesis and disturbs PSII functioning, causing reductions in maximum Fv/Fm values in cashew tree seedlings, especially at high salinity concentrations (14.1 dS m^{-1}). This reduction was also confirmed in studies of changes in photochemical reactions caused by salinity stress in glycophytes, reported by Cha-Um and Kirdmanee (2011), when comparing the results of potential Fv/Fm in yellow passion fruit plants irrigated with saline water (Freire, Dias, Cavalcante, Fernandes, & Lima-Neto, 2014).

The quantum efficiency of PSII (ΔF/Fm') decreased as salt levels increased, corresponding to a 37.5% reduction for the clone CCP76 and a 20% reduction for the clone CCP09 exposed to 100 mM NaCl compared with that of the controls. However, an increase in ΔF/Fm' under the 25 mM treatment was noted, although a drastic reduction in Fv/Fm was observed under the higher salt regimes (Figure 3D). Similar results were observed in eucalyptus irrigated with saline water (Mendonça, Carneiro, Freitas, & Barroso, 2010) and in cowpea under conditions of salinity stress (Souza et al., 2011), in which reductions in ΔF/Fm' were observed.

Regarding qP, there was a 50% decrease in the CCP76 clone from the 100 mM dose compared with that of the control seedlings (Figure 3E). There was also a significant increase in qP in the CCP09 clone and a gradual reduction in qP under the 25 mM dose, mainly in CCP76. At higher concentrations, NaCl led to a reduction in qP due to the accumulation of Na^+ and/or Cl^- in the chloroplasts, negatively affecting the biochemical and photochemical processes involved in photosynthesis (Munns & Tester 2008). An analysis of qP indicates the percentage of energy directed to photosynthesis, demonstrating PSII capacity to use light energy to reduce $NADP^+$ (Ribeiro et al., 2009). In this work, the reduction of qP indicated that PSII and ETR is reduced too (Dias & Brüggemann, 2010). The same effect has been observed in bean, corn, sorghum and *Brachiaria* under water stress and saline conditions (Souza et al., 2011; Santos et al., 2014).

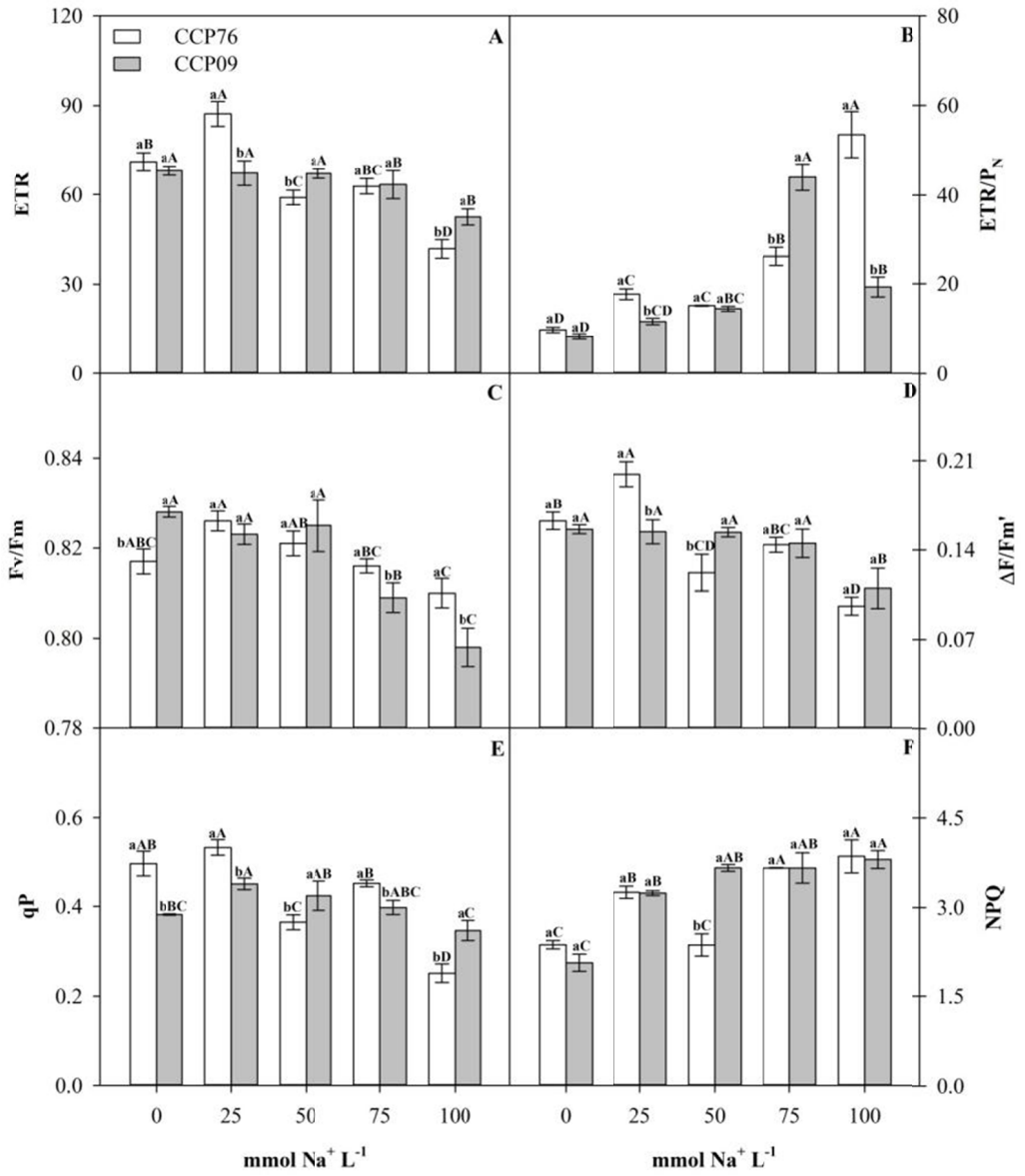

Figure 3. Apparent rate of electron transport (ETR) (A), excess electrons for photosynthesis (ETR/P_N) (B), quantum efficiency of photosystem II (Fv/Fm) (E) and non-photochemical quenching (NPQ) (F) in the leaves of early dwarf cashew clones (CCP76 and CCP09) grown under control conditions with distilled water and treated with 0; 25; 50; 75 and 100 mM NaCl for 30 days. The letters on the bars indicate significant differences according to the Tukey test ($p \leq 0.05$). Lowercase letters are associated with the clones; and uppercase letters with doses salinas

The NPQ increased with increasing salinity, with the greatest influence from the dose of 100 mM NaCl, reaching 33.33% in clone CCP76 and 43.24% in clone CCP09 compared with that of the respective controls (Figure 3F). The increase in NPQ is associated with non-photochemical energy dissipation mechanisms caused by reductions in both the effective Fv/Fm of PSII and qP, which is expected due to the reduction of P_N by salinity stress. After the exposure of cashew clones to salinity stress, the photochemical process became activated, providing energy transfer via NPQ proportional to the increase in salt dose.

3.3. Oxidative Protection in Response to Salinity in Cashew Plants

The cashew clones presented different H_2O_2 contents. In CCP76, treatment with 25 mM NaCl increased the content of H_2O_2 in the leaves by 30.76% compared with that of the controls, whereas under the other treatments, the H_2O_2 content remained similar to that of the control (Figure 4A). On the other hand, the CCP09 clone

maintained a constant H_2O_2 content throughout the treatments, differing only from the 100 mM dose, in which the H_2O_2 increased by 36%. The excessive accumulation of H_2O_2 in plant tissues is indicative of the overproduction of ROS originating from metabolic disturbances caused by salinity stress (Daneshmand, Arvin, & Kalantari, 2010; Hernandez, Fernandez-Garcia, Diaz-Vivancos, & Olmos, 2010). The results, however, differ from those of Ferreira-Silva et al. (2012), in which the contents of H_2O_2 in cashew tree leaves subjected to different concentrations of NaCl (0; 50; 150 and 200 mM) presented no significant effects.

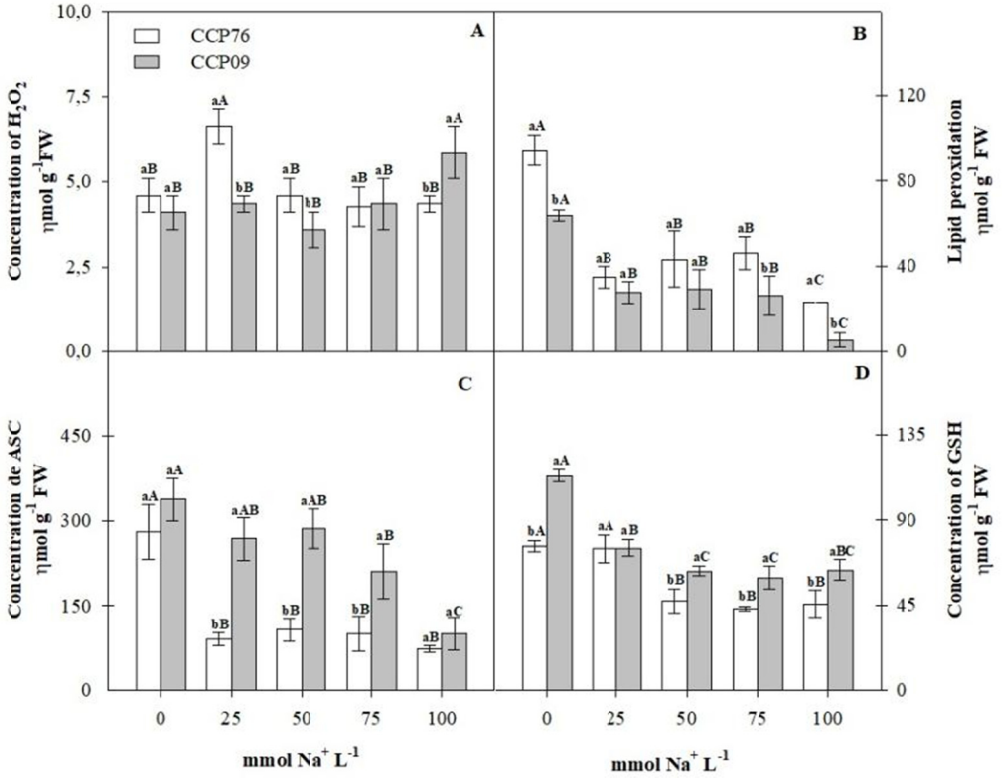

Figure 4. H_2O_2 concentration (A), lipid peroxidation (thiobarbituric acid [TBARS]) (B), ascorbate concentration (ASC) (C) and glutathione (GSH) reductase concentration (D) in the leaves of early dwarf cashew clones (CCP76 and CCP09) grown under control conditions with distilled water and treated with 0; 25; 50; 75 and 100 mM NaCl for 30 days. The letters on the bars indicate significant differences according to the Tukey test ($p \leq 0.05$). Lowercase letters are associated with the clones; and uppercase letters with doses salinas

It was noted that in seedlings subjected to salinity there was an intense reduction in TBARS content (Figures 4B). The TBARS content was reduced by 68.42% in CCP76 and 84.61% in CCP09 compared to control. Similar results were reported by Ferreira-Silva et al. (2012) in which the TBARS content in cashew tree leaves decreased proportionally to increasing salt dose. These results suggest that cashew tree seedlings under salinity stress may have efficient mechanisms to restore damaged membrane lipids and/or to remove produced ROS, which is favorable for oxidative protection. Similar results were obtained in the roots of cowpea beans (Maia et al., 2012) and in cashew tree leaves (Ferreira-Silva et al., 2012).

Analogous to lipid peroxidation, contents of non-enzymatic antioxidants, ASC and GSH, experienced drastic changes in response to salinity stress (Figures 4C and 4D). The NaCl treatments reduced the ASC content in cashew tree leaves, with drastic decreases of 71.42% for CCP76 and 73.52% for CCP09 at the concentration of 100 mM. However, CCP09 had higher concentrations of reduced ASC at doses of 25; 50 and 75 mM (67%, on average) relative to those of CCP76 (Figures 4C and 4D).

According to Noctor and Foyer (1998), ASC is considered the main antioxidant substrate for H_2O_2 reduction. Since APX uses two reduced ASC molecules as electron-specific donors to catalyze the reduction of a molecule of H_2O_2 to H_2O and O_2, ASC is an essential metabolite involved in the oxidative metabolism and maintenance of vital cellular functions (Nakano & Asada, 1987; Gratão, Polle, Lea, & Azevedo, 2005). In the present study, the

ASC content in CCP76 was inversely proportional to the APX activity, supporting the hypothesis of the efficient interaction of the enzymatic and non-enzymatic antioxidant mechanisms in the oxidative protection of cashew plants under saline conditions.

In the CCP09 clone, the high amounts of reduced ASC content associated with low APX activity indicated ASC consumption decreased, suggesting a role of ASC in the oxidative protection of this clone. Similar results were observed in cashew trees (Ferreira-Silva et al., 2012) and corn plants (Shan, Liu, Zhao, & Wang, 2014), in which the reduction of the ASC redox state, which is associated with a decrease in APX activity under high NaCl concentrations, may indicate the direct use of ASC in H_2O_2 oxidation.

The content of GSH in the leaf tissue decreased with increasing salinity, mainly from 50 mM NaCl (Figure 4D). In both clones, there was a drastic reduction in GSH content (38.66% for CCP76 and 42.60% for CCP09) under 100 mM NaCl stress compared to control. However, CCP09 was more responsive than was CCP76 and exhibited 34.78% more GSH than did CCP76 compared to control and an average decrease of 30% in GSH concentration under treatments of 50, 75 and 100 mM NaCl (Figure 4D). GSH is considered the main thiol compound that promotes protection against ROS (Foyer & Noctor, 2003; Tausz, Šircelj, & Grill, 2004; Delaplace, Fauconnier, & DuJardin, 2011). In this study, the reduction in ASC and GSH was directly proportional to the reduction in TBARS, corroborating the hypothesis that these molecules are important for antioxidant protection in plants. This effect was also observed in *Vigna radiata* (Shan & Zhao, 2014) and *Zea mays* (Shan, Liu, Zhao, & Wang, 2014).

However, enzymatic responses were visually clone specific, with CCP76 presenting higher activities of SOD and APX but lower POX activity; for CCP09, there was a significant increase in POX activity (Figures 5A, 5B, and 5C). Salt treatment caused changes in leaf SOD activity in both clones. SOD activity increased significantly only in the CCP76 clone, with a 24.39% increase at the dose of 75 mM NaCl compared to control (Figure 5A). In addition, regarding the stability of H_2O_2 and the decrease in TBARS, it is suggested that these plants did not undergo oxidative stress and that the maintenance of ROS content is due to the action of antioxidants other than SOD. Similar results have been reported in species such as *Zea mays* (Kholova, Sairam, & Meena, 2010) and *Saccharum officinarum* (Satbhai & Naik, 2014).

APX activity was significantly distinct between clones. The APX content significantly decreased only in CCP76, with a decrease of 85.71% with the dose of 100 mm NaCl compared to control (Figure 5B). However, clones differed by 81% on average from the doses of 0; 25; 50 and 75 mm NaCl. The effects of salt on the decrease in APX activity in plants has been previously reported (Ferreira-Silva et al., 2012; Maia et al., 2012). Ferreira-Silva et al. (2012) suggested that this decrease in APX activity of cashew trees is caused by an association to eliminate ROS.

POX activity gradually increased in both clones (Figure 5C). In particular, in the CCP76 clone, the POX activity increased by 32.14% from the 25 mM dose, and the CCP09 clone increased by 21% from the treatment of 75 mM, both compared to control. However, the activity of this enzyme in the shoots was 35% higher on average in CCP09 than in CCP76 at all doses. POX has been associated with antioxidant protection against diverse abiotic stresses (Abdelgawad, Khalafaallah, & Abdallah, 2014; Laxman et al., 2014) and the control of growth under various conditions of stress (Maia et al., 2012). In the present work, the significant increase in POX activity may be related, at least in part, to the decrease in accumulation of biomass in cashew tree shoots, given the early maturation of tissues caused by increased POX activity. This effect has also been observed in *Arabidopsis* (Mhamdi, Noctor, & Baker, 2012) and cowpea (Maia et al., 2012).

Taken together, the increased activity of these enzymes indicates antioxidant protective function against salinity stress (Harter, Harter, Deuner, Meneghello, & Villela, 2014). However, in the present work, POX apparently represents low relevance in the process of maintaining the ROS pool in cashew trees, due to its low responsiveness to increased salt dose and subtle relationship with the peroxidation of lipids.

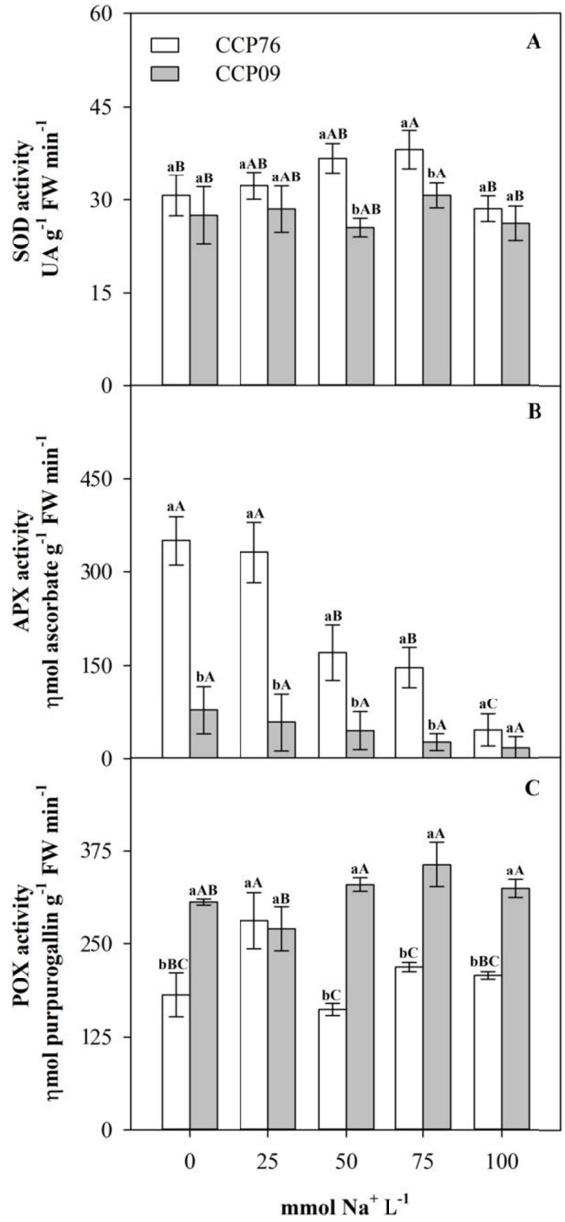

Figure 5. Superoxide dismutase (SOD) (A), ascorbate peroxidase (APX) (B) and phenol peroxidase (POX) (C) activity in the leaves of early dwarf cashew clones (CCP76 and CCP09) grown under control conditions with distilled water and treated with 0; 25; 50; 75 and 100 mM NaCl for 30 days. The letters on the bars indicate significant differences according to the Tukey test ($p \leq 0.05$). Lowercase letters are associated with the clones; and uppercase letters with doses Salinas

In cashew plants, high doses of NaCl did not drastically affect the fresh mass of above ground portions, despite showing ionic toxicity, as evaluated by the K^+/Na^+ ratio. The photosynthetic efficiency of cashew plants was affected by salinity, mainly by dramatic reductions in P_N, P_N/C_I and g_S. The last one is possibly related to the lower efficiency regarding the mobilization of K^+. Additionally, mechanisms of antioxidant protection are clone specific, as there was a tendency of enzymatic responses in clone CCP76 and non-enzymatic responses, besides the high activity of POX, in CCP09. Taken together, results suggest that the best photosynthetic performance was in the CCP09 clone. CCP09 exhibited greater efficiency of P_N and water use in its tissues than did CCP76 due to the protective effect of K^+ on leaves and to relevant non-enzymatic antioxidant protection promoted by ASC and GSH, in addition to the enzymatic activity of POX.

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Abbreviations

APX: Ascorbate peroxidase; ASC: Ascorbate; C_I: Intercellular CO_2 concentration; E: Sweating rate; ETR: Apparent rate of transport and electrons; ETR/P_N: Excess electrons for photosynthesis; Fv/Fm: Maximus quantum efficiency of photosystem II; GSH: Glutathione reductase; H_2O_2: Hydrogen peroxide; NBT: Nitroblue tetrazolium; NPQ: Non-photochemical quenching; P_N: CO_2 assimilation rate; P_N/C_I: Maximum carboxylation efficiency; P_N/E: Efficiency of water use; POX: Phenol Peroxidase; PS: Photosystem; qP: Photochemical Quenching; ROS: Oxigen-reactive species; SOD: Superoxide dismutase; TBARS: Lipid peroxidation; TCA: Trichloroacetic acid; ΔF/Fm': Quantum efficiency of photosystem II.

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