

Full Length Research Paper

Changes in physiological and biochemical indicators associated with salt tolerance in cotton, sorghum and cowpea

Valdinéia Soares Freitas¹, Nara Lídia Mendes Alencar¹, Claudivan Feitosa de Lacerda², José Tarquinio Prisco¹ and Enéas Gomes-Filho^{1*}

¹Department of Biochemistry and Molecular Biology, National Institute of Science and Technology in Salinity (INCTSal), Federal University of Ceará, Fortaleza-CE, Brazil.

²Departament of Agronomy Engineering, Center of Agricultural Sciences, Federal University of Ceará, Fortaleza-CE, Brazil.

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The aim of this study was to evaluate the interaction between salinity, growth, and enzymatic antioxidants in cotton, cowpea and sorghum. Salt stress significantly reduced plant growth, especially in cowpea. Na⁺ and Cl⁻ concentrations increased in leaves and roots of these three species, especially for cotton at 4.0 and 8.0 dS/m, in relation to control. Salinity significantly increased lipid peroxidation levels in cowpea, whereas in cotton these levels were reduced. Superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) enzyme activities in leaves were not changed by saline treatments at 4.0 and 8.0 dS/m. However, reductions were observed in the SOD activity in cotton at 8.0 dS/m NaCl and in CAT activity in sorghum at 4.0 and 8.0 dS/m. The growth results obtained confirmed the highest cotton tolerance and the highest cowpea susceptibility to salinity, while antioxidant enzyme activities changes suggest that cotton constitutive enzyme system seems to be more efficient than the others.

Key words: Salt stress, antioxidant enzymes, inorganic solutes, *Gossypium hirsutum*, *Vigna unguiculata*, *Sorghum bicolor*.

INTRODUCTION

Abiotic stress may be caused by numerous factors such as drought (Simova-Stoilova et al., 2009), cold (Van Heerden et al., 2003), high temperature (Reynolds-Henne et al., 2010), salinity (Meloni et al., 2003), heavy metals (Smeets et al., 2009) and ultraviolet radiation (Gao and Zhang, 2008). Among the various abiotic stresses to which plants are constantly exposed, salt stress is the one that most affects growth and productivity of plants around the world (Vaidyanathan et al., 2003; Veeranagamallaiah et al., 2007), reaching more than 800 million land hectares around the globe (FAO, 2005).

In general, salt stress consists of two components:

osmotic and ionic. The first is a result of the high concentration of salts in the root environment that leads to decreased in soil water potential and reduces the availability of water for the plant. The ionic component arises from the accumulation of certain ions (usually Na⁺ and Cl⁻) and can cause nutritional imbalance, toxicity or both (Greenway and Munns, 1980; Munns, 2002; Munns and Tester, 2008). In addition to these effects, salt stress also causes an imbalance in redox status of cells, generating an oxidative stress through overproduction of reactive oxygen species (ROS) such as superoxide (O₂⁻) and hydroxyl (HO•) radicals and hydrogen peroxide (H₂O₂) (Vaidyanathan et al., 2003). These ROS are highly reactive and can alter normal cellular metabolism through the oxidation of important biomolecules such as proteins and nucleic acids (McKersie and Leshem, 1994; Mittler, 2002).

*Corresponding author. E-mail: egomesf@ufc.br. Tel: (85) 3366.9405. Fax: (85) 3366.9829.

To minimize oxidative damage caused by ROS, plants have developed a complex antioxidant system that includes both non-enzymatic antioxidants of low molecular weight (mainly ascorbate and glutathione) and ROS scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (McKersie and Leshem, 1994). The superoxide radical is eliminated mainly by SOD, resulting in the formation of H_2O_2 (Alscher et al., 2002). The H_2O_2 produced is then removed by CAT and peroxidases activities (McKersie and Leshem, 1994). The generation of ROS and increased activity of many antioxidant enzymes during salt stress have been reported in cotton (Meloni et al., 2003), sorghum (Costa et al., 2005), tomato (Mittova et al., 2002) and mangrove (Parida et al., 2004). However, until date, biochemical and physiological studies involving plants with different responses to salinity in the same experimental conditions are scarce.

In this study, we examined the interactions between salinity, growth, lipid peroxidation and antioxidant enzyme activities in three species with contrasting tolerance to salt (cotton, sorghum and cowpea) and aiming a better understanding of these salt tolerance indicators in these species.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of cotton [*Gossypium hirsutum* (L.) Mast.], cultivar BRS 113 7MH, cowpea [*Vigna unguiculata* (L.) Walp.], cultivar Epace 10, and sorghum [*Sorghum bicolor* (L.) Moench.], genotype CSF 20, were sown in plastic cups containing vermiculite moistened with half-strength Hoagland's nutrient solution ($\frac{1}{2}$ HNS) and irrigated daily with distilled water. Fifteen day old seedlings were transferred to hydroponic media in plastic bowls (12 L) containing $\frac{1}{2}$ HNS and after six days they were transferred to plastic buckets (5 L) and subjected to three saline treatments with electrical conductivity (EC) of 0.9, 4.0 and 8.0 dS/m. The lowest EC treatments consisted of plants in $\frac{1}{2}$ HNS (low salinity - control), while 4.0 (moderate salinity) and 8.0 dS/m (high salinity) treatments consisted of plants in $\frac{1}{2}$ HNS with the addition of NaCl, which resulted in these conductivities. They were kept aerated and their pH was checked daily and adjusted between 5.5 to 6.5 with 0.1 N NaOH or 0.1 N HCl when necessary. Every seven days, the nutrient solution was exchanged for a new one. The experiment was carried out under greenhouse conditions. Temperature and relative humidity mean values were 28.5°C and 65.5%, respectively.

Growth measurements

Plants were harvested 25 days after the commencement of the addition of salt, when leaves, culms and roots were separated. Leaf area was determined using a Li-Cor area meter LI-3000 (Li-Cor., Inc., Lincoln, Nebraska, USA). The first and second fully expanded leaves from the base and apex roots (one third of the apical extremity) were frozen in liquid nitrogen and kept in a freezer (-80°C) for further analyses. The rest of the plant material was weighed and after drying in a forced air circulation oven at 60°C for

48 h for dry mass (DM) measurement.

Ion determination

Second leaf and root fresh matter were macerated in a mortar and pestle, thereafter pressed and filtered in a disposable syringe, using a muslin cloth. The juice extracted from the plant material was centrifuged at 12,000 g for 10 min. The supernatant (cell juice) was used for determining Na^+ and K^+ concentration using flame photometry and Cl^- concentration was determined according to the method of Gaines et al. (1984). The inorganic ion concentrations were expressed in mM.

Extract preparation

First leaf (1 g) and root (1 g) were homogenized in a mortar and pestle with 4 mL of ice-cold extraction buffer (100 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 2 mM ascorbate). The homogenate was filtered through muslin cloth and centrifuged at 12,000 g for 15 min. The supernatant fraction was used as a crude extract for enzyme activity assays. All operations were carried out at 4°C.

Enzyme assays

SOD (EC 1.15.1.1) activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT), as described by Giannopolitis and Ries (1977). The SOD activity results were expressed as U/g fresh mass (FM), being one unity of SOD activity (U) defined as the enzyme amount required to cause 50% inhibition of the NBT photoreduction rate. CAT (EC 1.11.1.6) activity was measured according to Havar and McHale (1987), by H_2O_2 decrease that was monitored by reading the absorbance at 240 nm. The difference in absorbance was divided by the H_2O_2 molar extinction coefficient (36/M.cm) and enzyme activity expressed as μmol of $H_2O_2/\text{min.g}$ FM. APX (EC 1.11.1.1) activity was performed according to Nakano and Asada (1981), being the ascorbate oxidation monitored by reading at 290 nm absorbance. The absorbance difference was divided by the ascorbate molar extinction coefficient (2.8/mM.cm) and enzyme activity expressed as μmol of $H_2O_2/\text{min.g}$ FM, considering that 2.0 mol of ascorbate is required for reducing 1.0 mol of H_2O_2 (McKersie and Leshem, 1994).

Lipid peroxidation in leaves

Lipid peroxidation was performed using the thiobarbituric acid according to Cakmak and Horst (1991) method. The malondialdehyde (MDA) concentration was calculated using a 155/mM.cm extinction coefficient and results expressed as nmol/g FM.

Experimental design and statistical analysis

The experimental design was completely randomized, corresponding to factorial three (species) \times three (salt levels), with five replicates of two plants each. Enzyme activities, MDA concentration and ion determination were assayed in duplicate. Data were analyzed using a two-way analysis of variance (ANOVA). The values were compared using Tukey's test ($p < 0.05$).

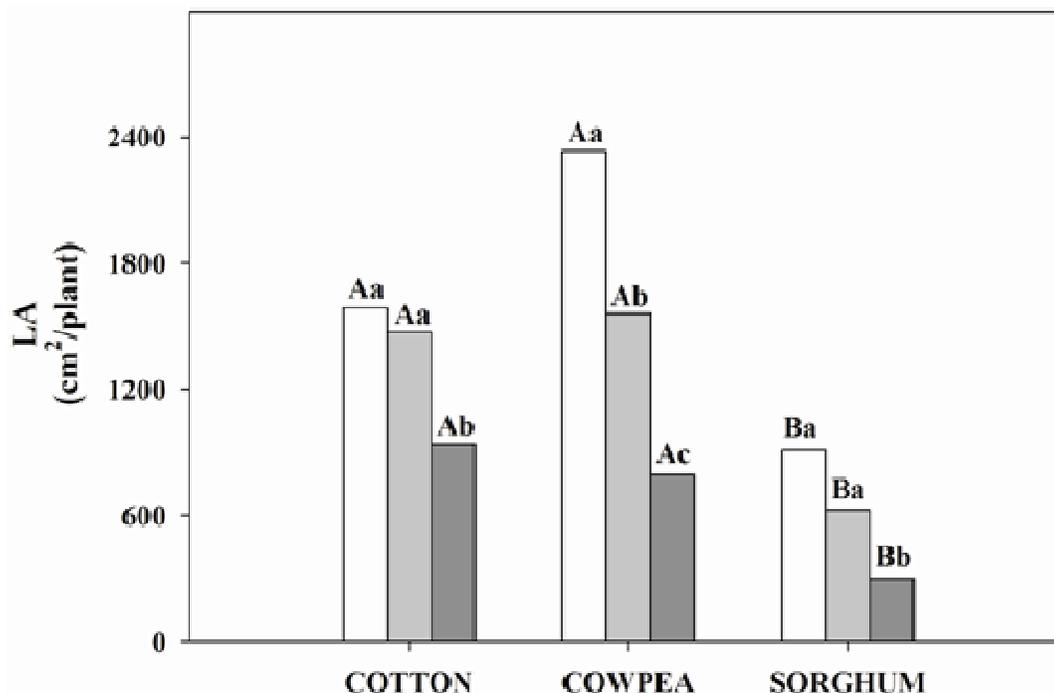


Figure 1. Leaf area (LA) of cotton, cowpea and sorghum plants subjected to salinity (low- □, medium - ▒ and high - ■ levels). Columns followed by the same capital letters within each treatment and with the same lowercase letters in the same species are not significantly different ($p \leq 0.05$) by Tukey test.

RESULTS

Plant growth

The leaf area (LA) of these three species was affected by salt stress, but this reduction was more pronounced in cowpea (Figure 1). In cowpea, at the moderate (4.0 dS/m) and high salinity (8.0 dS/m) levels the reduction of LA reached values of 33 and 66%, respectively, in relation to control, whereas for sorghum and cotton plants LA reductions were observed at 8.0 dS/m, corresponding to 67 and 41%, respectively, compared to the lowest level.

In cotton and sorghum plants exposed to a moderate level (4.0 dS/m), there was no significant reduction in shoot dry mass (SDM) compared with the lowest salinity level (0.9 dS/m) (Figure 2a). However, reductions were observed at 41 and 58% for the two species, respectively, when exposed to the highest salinity level (8.0 dS/m). In cowpea, this parameter was significantly affected by the salinity moderate level, which remained unchanged even with higher salinity, showing average reductions of 50%, in these two salinity levels, in relation to plants subjected to 0.9 dS/m (Figure 2a). Sorghum showed a significant reduction in root dry mass (RDM) at 8.0 dS/m when compared to the low level of salinity, while cotton and cowpea did not suffer significant changes in this parameter (Figure 2b). In cotton and sorghum plants, the SDM/RDM ratio did not differ

statistically among the three treatments; however, this relationship was significantly reduced in cowpea, about 39% at moderate and high salinity treatments in relation to the low level of salinity (Figure 2c).

Inorganic solutes

In leaves, Na^+ concentration did not show differences between the species at the lowest salinity level (Figure 3a). In cotton, a significant increase ($p < 0.05$) was verified in Na^+ concentration, at 4.0 and 8.0 dS/m. Plants exposure to the highest level of salinity, when compared to the lowest, resulted in leaf, Na^+ concentration increases for cotton, cowpea and sorghum, corresponding respectively to 32.7, 4.4 and 20.6 times (Figure 3a). In roots, high salinity (8.0 dS/m) showed Na^+ concentration increase of 46.4, 24.1 and 20.6 times in cotton, cowpea and sorghum, respectively, when compared to the lowest salinity level (Figure 3b). In comparison with 0.9 dS/m treatment, K^+ concentration only increased in cotton and cowpea leaves at the highest salinity level (Figure 3c). In contrast, in sorghum plants, K^+ concentration in leaves was progressively reduced by salt stress (Figure 3c). In roots, K^+ concentration decreased significantly for cotton (16.9%) and cowpea (20.5%) with NaCl increase from 0.9 to 8.0 dS/m (Figure 3d). In sorghum roots subjected at 4.0 dS/m treatment, K^+ concentration increased 17.8%, when

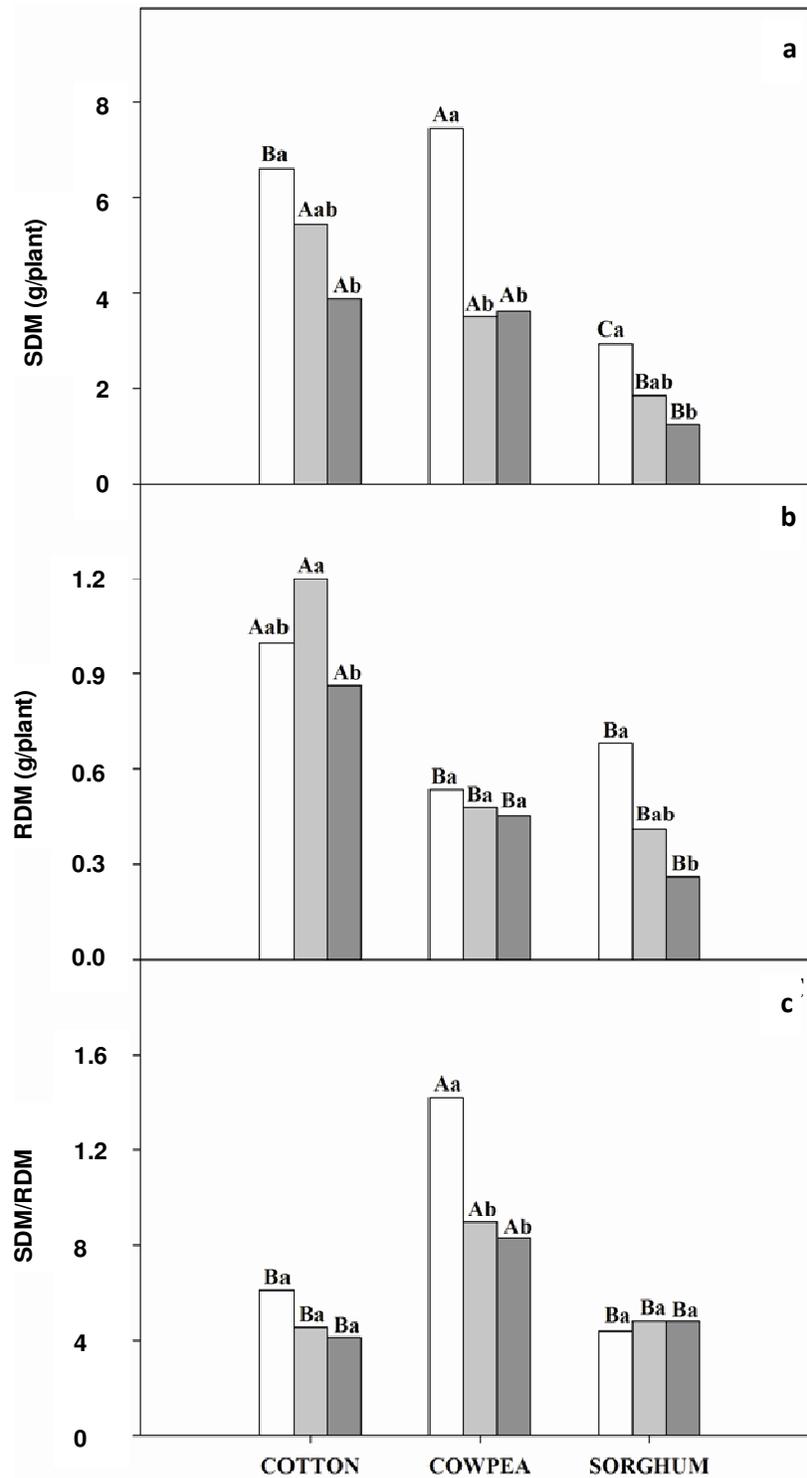


Figure 2. Shoot dry mass (SDM, a), root dry mass (RDM, b) and SDM/RDM ratio (c) of cotton, cowpea and sorghum plants subjected to salinity (low - □, medium - ■ and high - ■ levels). Columns followed by the same capital letters within each treatment and with the same lowercase letters in the same species are not significantly different ($p \leq 0.05$) by Tukey test.

compared with 0.9 dS/m treatment (Figure 3d). Leaf Cl^- concentration increased with the salinity increment in the

three species, however in cotton and cowpea the Cl^- accumulation was more pronounced than sorghum

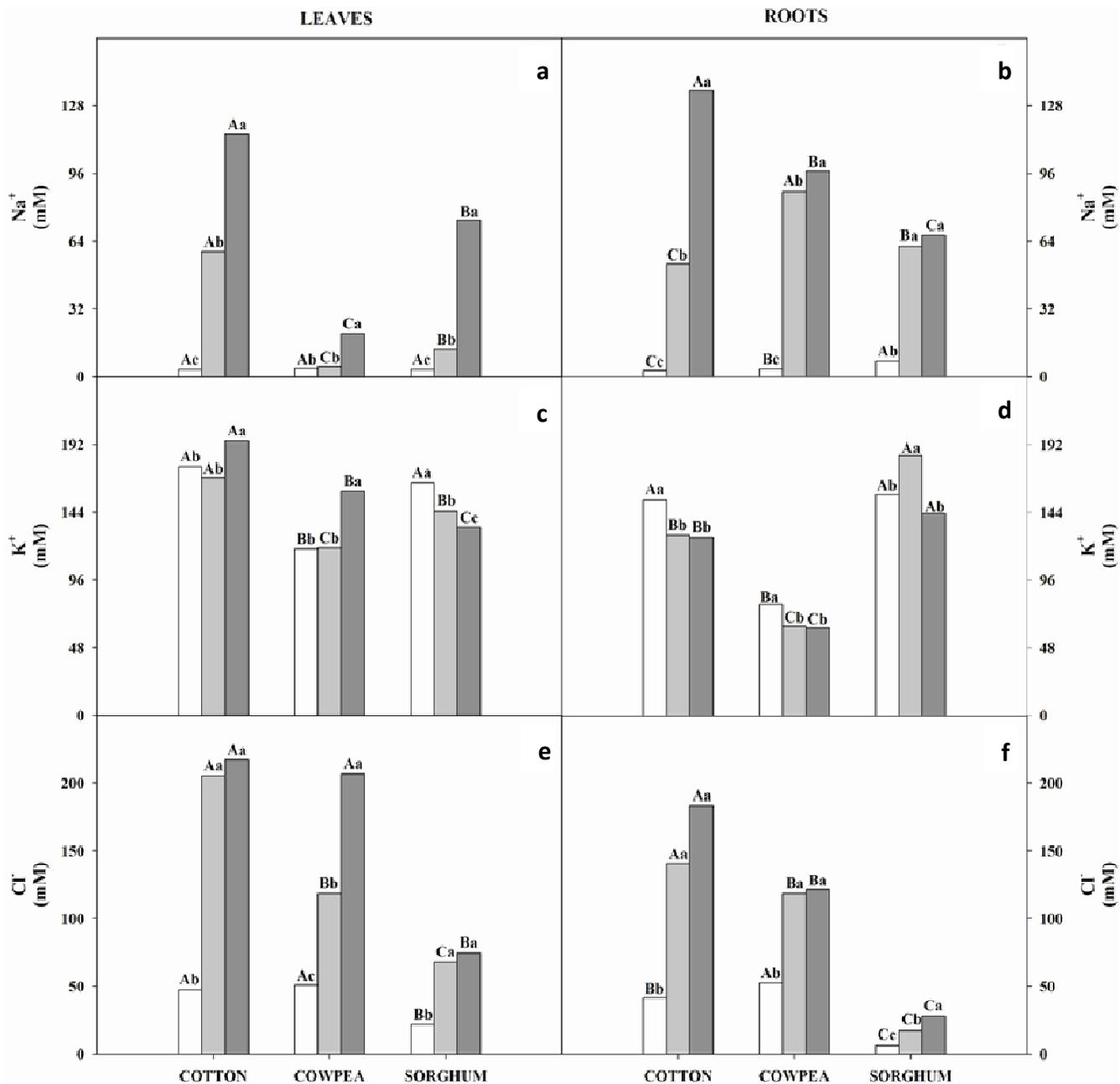


Figure 3. Na⁺, K⁺ and Cl⁻ concentrations in leaves and roots of cotton, cowpea and sorghum plants subjected to salinity (low - □, medium - ◻ and high - ◼ levels). Columns followed by the same capital letters within each treatment and with the same lowercase letters in the same species are not significantly different ($p \leq 0.05$) by Tukey test.

(Figure 3e). Root Cl⁻ concentration increased significantly in these species, being higher in cotton (Figure 3f).

SOD, CAT and APX activities

Constitutive SOD activity in leaves was higher in sorghum followed by cotton and cowpea (Figure 4a). In general, 4.0 and 8.0 dS/m treatments compared to 0.9 dS/m did not strongly affect SOD activity in leaves and

roots (Figures 4a and b), except by reductions of SOD activities in cotton leaves (Figure 4a). Constitutive CAT activity was markedly higher in cotton and sorghum leaves than in cowpea, whereas in roots the constitutive CAT activity was higher in cotton than in cowpea and sorghum (Figure 4d). In leaves and roots of cotton and cowpea, CAT activity remained practically constant under all NaCl levels (Figures 4c and d). However, CAT activity in sorghum leaves decreased about 37% with the salinity increase. APX activity was not significantly affected by

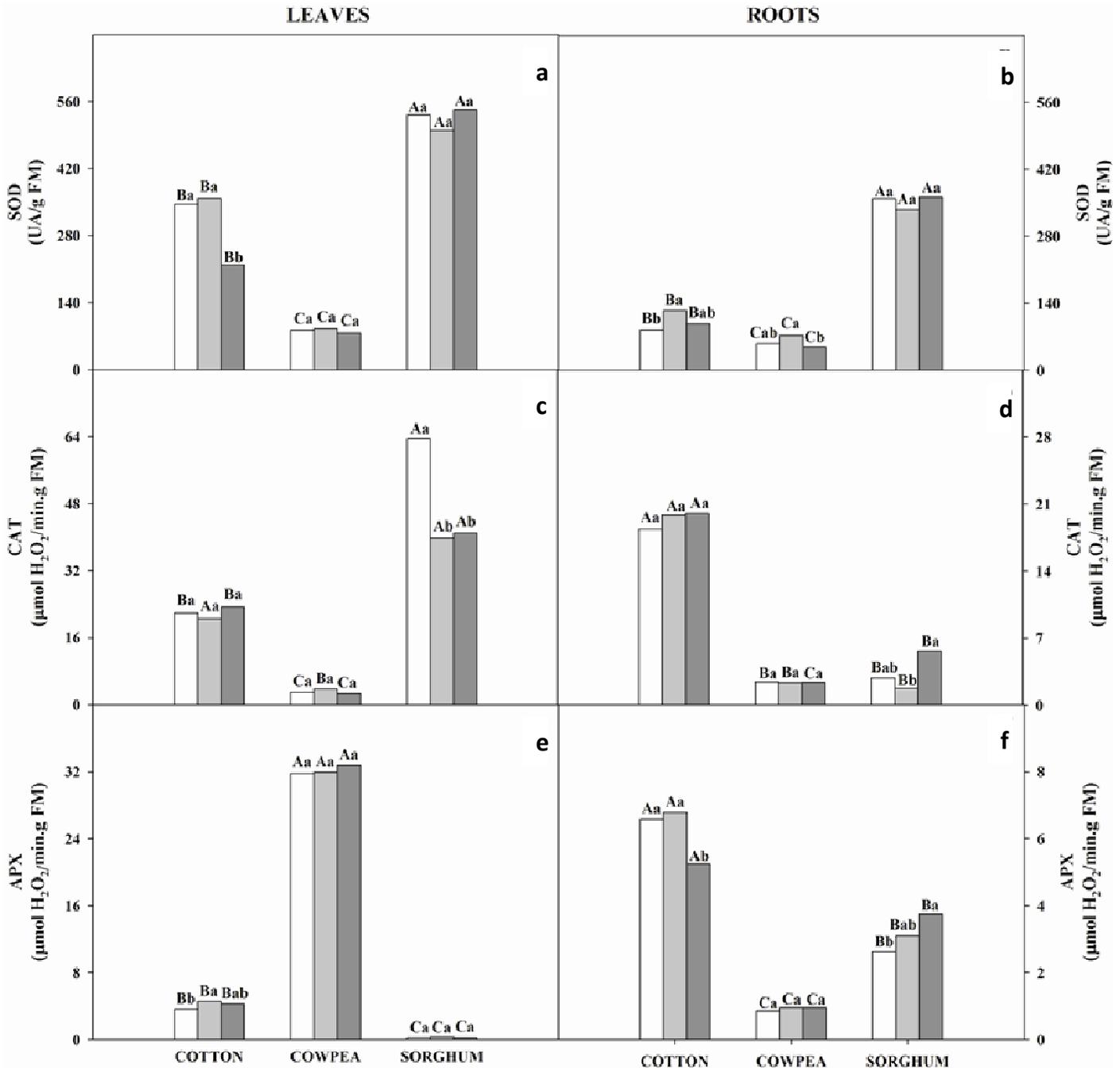


Figure 4. Superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities in leaves and roots of cotton, cowpea and sorghum plants subjected to salinity (low - □, medium - ◻ and high - ◼ levels). Columns followed by the same capital letters within each treatment and with the same lowercase letters in the same species are not significantly different ($p \leq 0.05$) by Tukey test.

salt treatments in cowpea leaves and roots and in sorghum leaves (Figures 4e and f). However, APX activity in sorghum roots increased 42.6% at 8.0 dS/m treatment. In cotton leaves, APX activity increased in the treatment with moderate salinity, when compared to that with low salinity (Figure 4e), whereas in roots, the 8.0 dS/m treatment promoted a 20.4% decrease in total APX activity in comparison to 0.9 dS/m treatment (Figure 4f).

Lipid peroxidation in leaves

The salt stress (4.0 and 8.0 dS/m) caused a marked increase in MDA content in leaves only in cowpea (Figure 5), showing a medium increase of about 39.6% as compared to the lowest level of salinity. In sorghum, there were no significant differences in MDA content between the treatments, whereas in cotton, there were significant

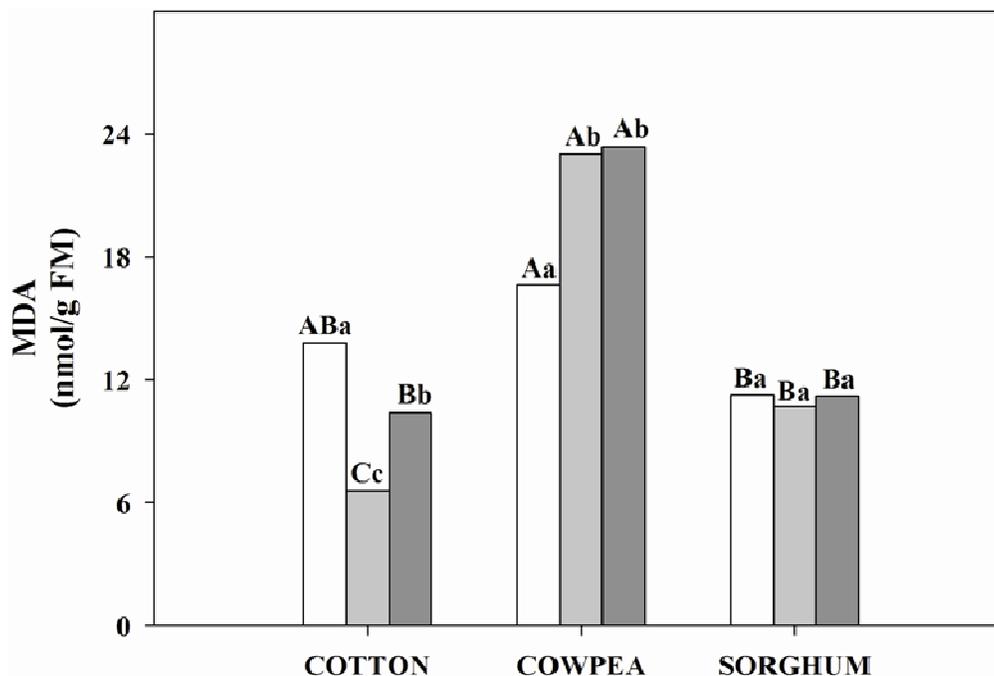


Figure 5. Malondialdehyde (MDA) content in leaves of cotton, cowpea and sorghum plants subjected to salinity (low - □, medium - ▒ and high - ■ levels). Columns followed by the same capital letters within each treatment and with the same lowercase letters in the same species are not significantly different ($p \leq 0.05$) by Tukey test.

decreased at 4.0 and 8.0 dS/m treatments (Figure 5).

DISCUSSION

Salt stress can have many effects on plant growth being a complex syndrome that involves osmotic stress, ion toxicity, mineral deficiencies and formation of ROS such as $O_2^{\bullet-}$, H_2O_2 and HO^{\bullet} (Hasegawa et al., 2000; Munns, 2002; Vaidyanathan et al., 2003; Munns and Tester, 2008). In this study, cotton, cowpea and sorghum varied markedly in salt tolerance. The cowpea was the one that showed severe reductions in LA and SDM since at moderate salinity level (4.0 dS/m), whereas sorghum and cotton showed more severe reductions in these parameters only at the highest salinity level (8.0 dS/m), which clearly demonstrates cowpea's greater susceptibility to salinity compared with other species tested (Figures 1 and 2). This severe reduction in cowpea biomass may be due to the biggest sensitive to Na^+ and Cl^- accumulation in leaves and roots (Figure 3). Cotton seems to be a species that does not use mechanisms of exclusion of toxic ions (especially Na^+ and Cl^-) of photosynthetic tissues, as with other species of glycophytes (Lacerda et al., 2003). Therefore, depending on the specie, salt tolerance could be associated with the rate of ion transport to the shoot and/or plant capacity to compartmentalize toxic ions in different tissues or cells (Lacerda et al., 2001).

The biggest values for SOD and CAT activities in

cotton and sorghum leaves, suggest that their constitutive antioxidant enzyme system is more efficient than in the cowpea (Figure 4). The SOD and CAT low activities in cowpea can at least be partially responsible for its bigger susceptibility to salt stress. This idea is supported by other studies that suggest greater tolerance to salinity in species that have a more efficient constitutive antioxidant enzyme system (Bandeoglu et al., 2004; Stepien and Klobus, 2005; Demiral and Türkan, 2005). Furthermore, the highest values of MDA (Figure 5) in any treatment were observed in cowpea leaves, indicating that, among the species, it has higher lipid peroxidation, thus corroborating the suggestion of being the species with the least effective constituent antioxidant system. APX is an important antioxidant enzyme, which acts by reducing H_2O_2 to water using ascorbate as a reducing agent (Asada, 1992). In this study, the constitutive activity of APX in sorghum roots was higher than that found in leaves, whereas with cowpea plants, the constitutive activity was higher in leaves than in roots (Figures 4e and f). Bandeoglu et al. (2004), while studying lentil under saline conditions found that APX activity was significantly increased in the leaves, while no changes were observed in roots and that their oxidative protection was due to the enzyme's high constitutive activity coupled with SOD increases.

Malondialdehyde (MDA) is a decomposition product of polyunsaturated fatty acids and their increases in plants have been widely used as an indicator of lipid

peroxidation in salt stress, often serving to discriminate between species that are sensitive and tolerant to salinity (Meloni et al., 2003; Stepien and Klobus, 2005). In the present study, lipid peroxidation levels were significantly increased in medium and high salinity treatments in the most susceptible species (cowpea), while in the most tolerant (cotton) for the same treatments, reductions were observed (Figure 5). These results suggest that salinity does not induce lipid peroxidation in cotton plants, or even that these plants have high oxidative protection under saline conditions.

Conclusion

The growth results obtained confirmed the highest cotton tolerance and the highest susceptibility of the cowpea to salt stress, which can be related to the higher efficiency of cotton's constitutive antioxidant enzyme system, in reducing oxidative damage caused by salinity, than cowpea.

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