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Alteration of biochemical and antioxidant mechanisms in rice plants under salt stress

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The aim of this study was to examine possible alterations in the metabolism of rice plants (*Oryza sativa* L.) that were exposed to high salt concentrations. BRS Ligeirinho seeds were sown, and the plants were grown in a greenhouse. At 14 days following sowing, the plants were alternately irrigated with a nutrient solution and water containing 0, 150 and 300 mM NaCl. After 30 days, the leaves and roots were collected, and the levels of proline, photosynthetic pigments, total protein, hydrogen peroxide, lipid peroxidation and the activities of the antioxidant enzymes superoxide dismutase, catalase and ascorbate peroxidase were analysed. There was a gradual increase in the levels of proline, total protein, hydrogen peroxide and lipid peroxidation with increasing salt concentrations in the irrigation water. The synthesis of photosynthetic pigments increased until the NaCl concentration reached 150 mM, after which the activities of the antioxidant enzymes decreased. These results suggest that proline may have protective effects against protein degradation and that carotenoids may aid in the protection of chlorophyll. Moreover, although, antioxidant enzymes were shown to possess low levels of activity, a large proportion of the hydrogen peroxide that is produced is preferentially directed towards lipid peroxidation.

Key words: *Oryza sativa* L., salinity, oxidative stress, reactive oxygen species, lipid peroxidation, chlorophyll.

INTRODUCTION

Salt stress, which is primarily caused by the accumulation of Sodium Chloride (NaCl), affects agriculture worldwide and is more severe in arid and semiarid regions where high rates of evapotranspiration and low levels of rainfall combined with inadequate soil and water management have contributed to an increase in salinized soils (Zhu, 2001). According to data from the Food and Agriculture Organization of the United Nations (FAO, 2008), approximately 20% of cultivated land worldwide is encountering problems with salinisation. The ionic imbalances that are caused by the accumulation of toxic ions, such as Na^+ and Cl^-, and the depletion of ions, such as K^+ and Ca^{2+}, are also directly affected by salinity (Sumithra et al., 2006). Other consequences of salt stress include disturbances in membrane integrity, changes in the levels of growth regulators, alterations in metabolic activities, including photosynthesis, and the increased production of reactive oxygen species (ROS) (Zhu, 2001; Panda and Khan, 2009). Additional biochemical changes that have been observed in plants grown under salt stress include altered concentrations of total soluble carbohydrates, total phenols, glycinebetaine,

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proline (Lacerda et al., 2001; Ashraf and Foolad, 2007), chlorophyll (Netono et al., 2004) and total proteins (Lunde et al., 2007).

Under osmotic stress, proline acts as an osmoregulator and osmoprotectant, aiding in the redox balance within the cell, in addition to being an excellent source of carbon and nitrogen. It has been proposed that proline may also contribute to the stabilities of protein structures, control ROS and act as an indicator of adaptive responses (Maggio et al., 2002). In recent years, special attention has been given to the cellular damage caused by the accumulation of ROS under conditions of stress. These radicals, when produced in excess, may be destructive to cells by reacting with the unsaturated fatty acids of phospholipid membranes, altering their functionalities and promoting lipid peroxidation. Some of the antioxidant enzymes that are involved in the elimination of ROS in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), catalase (CAT), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Scandalios, 2005). In addition to other physiological mechanisms, the efficiency of the antioxidant system increases the tolerance capacity of the plant by diminishing the effects that are caused by ROS (Giannakoula et al., 2010).

Thus, the objective of this study was to investigate the responses of rice plants (*Oryza sativa* subspecies *indica*) to high concentrations of NaCl, examining alterations in the levels of proline, total proteins and photosynthetic pigments as well as the antioxidant mechanism.

MATERIALS AND METHODS

Plant materials and cultivation conditions

Rice seeds from the cultivar BRS Ligeirinho (subspecies *indica*) were sown in 5-L plastic pots that had been perforated at the bases to guarantee sufficient levels of water percolation. Sand that had been previously washed with water and 1% hydrochloric acid was used as the substrate. The plants were grown under greenhouse conditions at 70% relative humidity temperatures of 25 ± 2°C and were watered daily, alternating between pure water and nutrient solutions that were developed by Hoagland and Arnon (1938). At 14 days after sowing, the plants were alternately watered with a nutrient solution and water containing NaCl at concentrations of 0, 150 and 300 mM. In total, 100 ml of saline solution was used to water each pot. Leaves and roots were collected for analysis 30 days after the initiation of the treatments.

Experimental design

The experiment was conducted using a completely randomised design with 5 replicates/treatments for the photosynthetic pigments and proline analyses and 3 replicates for the remaining analyses. Each replicate was represented by a pot containing 10 plants. The data were subjected to analyses of variance to test for the sources of variation (2 tissues versus 3 NaCl concentrations) and their possible interactions. The results were considered significant when $P \leq 0.05$, and the mean values were compared using Tukey's test with a 5% probability.

Quantification of levels of proline, photosynthetic pigments, H_2O_2 and lipid peroxidation

A modified version of the method described by Bates et al. (1973) was used to determine the proline levels. Approximately, 1.5 g of leaf tissue was homogenised in porcelain mortar with 10 ml of 3% aqueous sulphosalicylic acid (w/v) and then centrifuged at 12,000 x g for 20 min. Next, 1 ml of the supernatant and 1 ml of ninhydrin acid were added to the tubes, which were kept in a boiling water bath for 60 min. They were then cooled in an ice bath, mixed with 4 ml of toluene and vigorously vortexed. Upon reaching room temperature, readings were obtained at 520 nm using a spectrophotometer with toluene as the blank. The absorbances were compared with the standard curve of proline, and the results were expressed as mmol proline g^{-1} FW (fresh weight). The photosynthetic pigments were extracted from 200 mg of macerated leaves, completely homogenised in 80% acetone and quantified using a spectrophotometer as described by Lichtenthaler (1987). The levels of chlorophyll *a,* chlorophyll *b,* total chlorophyll and total carotenoids were expressed as µg mg^{-1} FW. The chlorophyll *a* and *b* values were used to calculate the chlorophyll *a*/*b* ratio. The hydrogen peroxide concentrations were measured according to Sinha et al. (2005). Briefly, 200 mg of leaf and root tissues were macerated separately in 0.1% (w/v) trichloroacetic acid (TCA). The homogenates were then centrifuged at 12,000 x g for 15 min at 4°C. Subsequently, 0.5 ml of the supernatants were collected, and 0.5 ml of buffer consisting of 10 mM potassium phosphate (pH 7.0) and 1 ml of 1 M potassium iodide (KI) were added.

Absorbance readings at 390 nm were obtained using a spectrophotometer, and the H_2O_2 concentrations were calculated by comparing measurements using a H_2O_2 standard curve; the results were expressed in µmol H_2O_2 g^{-1} FW. Lipid peroxidation was determined by quantifying thiobarbituric acid reactive species (TBARS) as described by Buege and Aust (1978). Approximately, 200 mg of plant tissue (leaf and root) were macerated in liquid N_2 plus 20% polyvinylpolypyrrolidone (PVPP) and homogenized in 0.1% trichloroacetic acid (TCA) (w/v). The samples were centrifuged at 10,000 x g for 10 min. A 250 µL aliquot of supernatant was added to 1 ml of the reaction medium containing 0.5% (w/v) thiobarbituric acid (TBA) and 10% (w/v) TCA. The mixture was incubated at 95°C for 30 min. Subsequently, the reaction was stopped by incubation on ice, and spectrophotometric readings were performed at 535 and 600 nm. TBA forms complexes with malondialdehyde (MDA), which is a secondary product of the peroxidation process. The concentration of the MDA/TBA complex was calculated, and peroxidation was expressed as nmol MDA g^{-1} FW.

Enzymatic analyses

The leaf and root samples (200 mg) were macerated in liquid N_2 and 50% PVPP and homogenized in 1.5 ml of extraction buffer containing 100 mM potassium phosphate (pH 7.8), 0.1 mM ethylenediaminetetra-acetic acid (EDTA) and 10 mM ascorbic acid. The homogenate was centrifuged at 13,000 x g for 10 min at 4°C, and the supernatant was then collected to determine the SOD, CAT and APX activities. The total protein was eluted with the same buffer and quantified using the Bradford method (1976). The total soluble protein content was expressed as mg protein g^{-1} FW. The SOD activity (EC 1.15.1.1) was assessed by the ability of this

enzyme to inhibit the photoreduction of nitroblue tetrazolium (NBT) in a reaction medium that included 100 mM potassium phosphate (pH 7.8), 14 mM methionine, 0.1 µM EDTA, 75 µM NBT and 2 µM riboflavin (Giannopolitis and Reis, 1977). Tubes containing the reaction medium and the samples were illuminated for 7 min with a 20 W fluorescent lamp. As a control, the same reaction medium without a sample was illuminated under the same conditions, whereas the blank was kept in the dark. Readings at 560 nm were obtained using a spectrophotometer, and one unit of SOD activity was considered to be the amount of enzyme that was capable of inhibiting 50% of the photoreduction of NBT under these experimental conditions. SOD activity was expressed as U mg^{-1} protein.

A modified methodology described by Azevedo et al. (1998) was used to determine CAT activity (EC 1.11.1.6), which was monitored based on the degradation of hydrogen peroxide (H_2O_2) using a spectrophotometer at 240 nm for 2 min in a reaction medium containing 100 mM potassium phosphate buffer (pH 7.0), 12.5 mM H_2O_2 and 50 ml of plant extract at 28°C. The same reaction medium without plant extract was used as the blank. CAT enzyme activity was expressed as µmol H_2O_2 min^{-1} mg^{-1} protein. APX activity (EC 1.1.11.1) was determined according to Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm. A reaction medium containing 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid and 0.1 mM H_2O_2 was incubated at 28°C. Decreases in absorbance were monitored for 2 min from the beginning of the reaction, and APX activity was expressed as mmol ASA min^{-1} mg^{-1} protein.

RESULTS

Changes in the levels of photosynthetic pigments, proline, total proteins, H_2O_2 and MDA

The analysis of variance for the photosynthetic pigments showed that the average of the chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoid concentrations were significantly different *(P* ≤ 0.05) between the NaCl concentrations that were tested, whereas the chlorophyll *a/b* ratio did not vary as a function of the presence of salt in the irrigation water. At a salt concentration of 150 mM, the synthesis of chlorophyll a, b and total chlorophyll increases of the 46, 45 and 46%, respectively, compared with the control. Chlorophyll synthesis was lower at 300 mM than at 150 mM. However, the mean values were also higher than those that were observed in the control treatments, but no significant differences were present (Figure 1A, B and C). The levels of total carotenoids as a function of the NaCl concentrations showed a similar pattern compared with the chlorophylls; increased synthesis occurred following treatment with 150 mM NaCl, which increased by 26%, compared with the control. At concentrations of 300 mM, the levels of total carotenoids decreased but were higher than those that were observed in the control treatment (Figure 1D). In general, the salinity levels significantly affected those of proline, which significantly differed for all treatments *(P* ≤ 0.05) (Figure 2A). The plants that were irrigated with water containing NaCl showed higher accumulations of proline in their leaves compared with the control plants; gradual increases of 4.3 and 12.3 fold were observed for the treatments with 150 and 300 mM NaCl, respectively, compared with the unstressed plants.

The total protein levels gradually increased in concordance with increases in salinity in both the leaves and roots. For this variable/response in the analysis of variance, there was a significant interaction (*P* ≤ 0.05) between the sources of variation (tissues versus NaCl concentrations). In the leaves, the total protein levels significantly increased, and differences were observed between the 150 and 300 mM NaCl concentrations compared with the control. However, the differences between the treatments for the roots were not significant. At a concentration of 300 mM, there were higher levels of total proteins in the leaves and roots ,13.75 and 2.55 mg protein g^{-1} FW, corresponding with 54 and 33% increases, respectively, compared with the control plants (Figure 2B). The analysis of variance detected significant effects for the interactions tested between factors for the quantification of H_2O_2. Although, the results showed that both the leaves and roots produced higher amounts of H_2O_2 under conditions of salt stress, these changes were significant only in the leaves, in which the H_2O_2 concentration increased in parallel with the addition of salt to the irrigation water and was 21 and 29% higher at NaCl concentrations of 150 and 300 mM, respectively, compared with the control after 30 days of exposure (Figure 2C).

In the present study, the damage to cellular membranes due to salinity was evident by the increase in cellular MDA, which is a secondary product of the process of peroxidation. In the analysis of variance, there was a significant interaction between the factors that were tested. The leaves of the plants that were treated with 150 and 300 mM salt showed significant increases of 54 and 69%, respectively, in the MDA concentrations compared with the control plants. In the roots, the peroxidation values did not change, as shown by the H_2O_2 levels, indicating that, for the cultivars studied, the effects of lipid peroxidation were more severe in the leaves (Figure 2D).

SOD, CAT and APX activities

For SOD activity, there was a significant interaction (*P* ≤ 0.05) between the sources of variation tested; reduced activities were observed as NaCl concentrations increased. In the leaves, a 36% reduction was observed, and the difference between the 0 and 300 mM concentrations was significant. In the roots, a decrease of 24% was observed; however, the mean values between the salt concentrations did not differ significantly (Figure 3A). In the leaves, CAT activity decreased by 22 and 33% at concentrations of 150 and 300 mM, respectively,

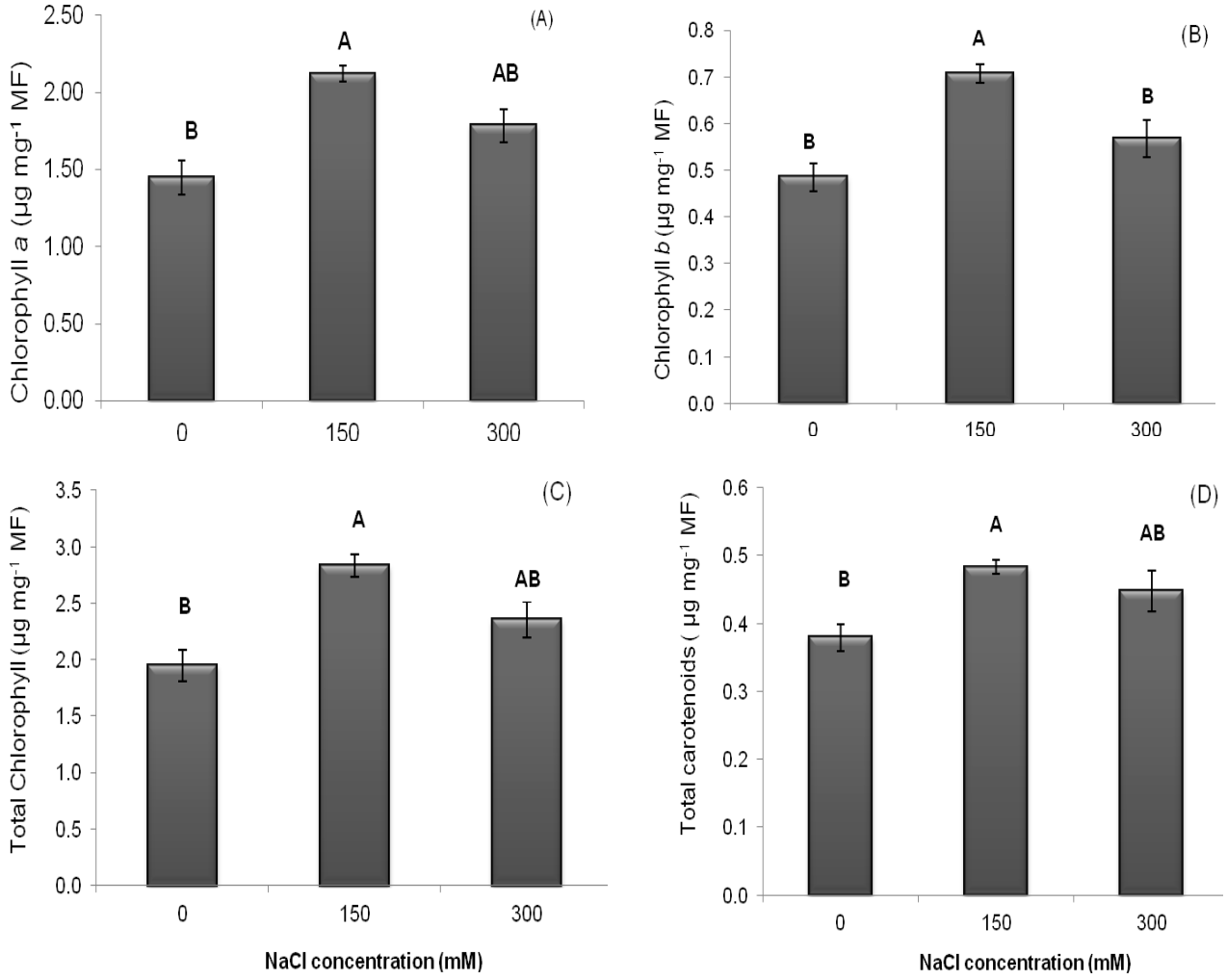

Figure 1. Contents of chlorophyll *a* (A), *b* (B), total chlorophyll (C) and total carotenoids (D), in plants of *O. sativa* L., from the cultivar BRS Ligeirinho, subjected to different concentration of NaCl for 30 days. Means followed by the same letter were not significantly different based on mean comparison by Tukey's test at $P \leq 0.05$. Vertical bars indicate mean ± SE.

compared with the control treatment. This variation between the concentrations that were tested was significant. Conversely, in the root system, the same enzyme showed only a slight decrease in activity of approximately 6% in plants that were treated with 150 mM NaCl compared with the control plants, whereas at the highest concentration (300 mM), the activity increased to levels comparable with that of the control (Figure 3B). There was also decreased APX activity in the leaves. The plants that were subjected to NaCl concentrations of 150 and 300 mM showed decreases of 2 and 15% in APX activity compared with the control plants (0 mM).

The 0 and 150 mM NaCl treatments were significantly different than the 300 mM NaCl treatment. In the root system, APX activity only tended to show a slight decrease; however, these differences were not significant (Figure 3C).

DISCUSSION

Chlorophylls are the most abundant natural pigments in plants and are common in all photosynthetic cells. The pigments involved in photosynthesis include chlorophylls *a* and *b* and the carotenoids. In this study, the plants retained their capacities for synthesising chlorophyll under salt stress, increasing their chlorophyll *a*, chlorophyll *b* and total chlorophyll concentrations. Considering the fact that at the intermediate NaCl

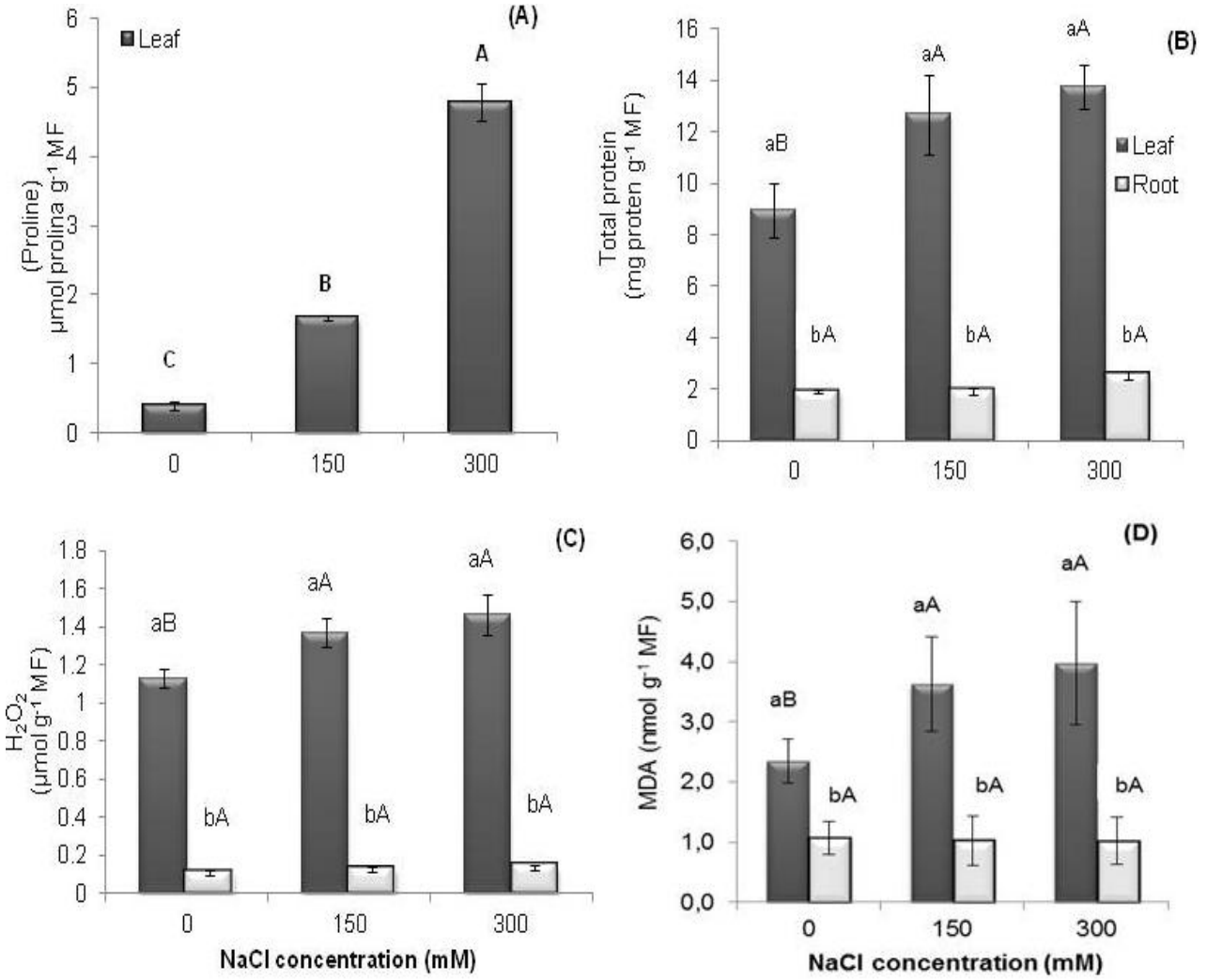

Figure 2. Level of proline (A), total protein (B), H_2O_2 (C) and lipid peroxidation – MDA (D), in plants of *O. sativa* L., from the cultivar BRS Ligeirinho, subjected to different concentration of NaCl for 30 days. Means followed by the same letter were not significantly different based on mean comparison by Tukey's test at $P \leq 0.05$. Lowercase letters compare the different tissues within each concentration and capital letters compare the same tissues in different concentrations. Vertical bars indicate mean ± SE.

concentration that was tested (150 mM), higher mean values were observed for the synthesis of these pigments. This response may be partially explained by Santos (2004), who noted that the enzyme chlorophyllase may be stimulated to synthesise chlorophyll under conditions of moderate stress. However, such activity may be inhibited by high salt concentrations. Abiotic stress has been strongly linked with the decreased capacity to synthesise chlorophyll or with increased chlorophyll degradation in various types of plants, including aquatic plants (*Spirodela polyrhiza*) (Chang et al., 2011), legumes (*Medicago sativa*), cereals (*Avena sativa*) and grasses (*Lolium multiflorum*) (Hernandez-Pinero et al., 2002). However, the results of the current study indicate that the role of pigments in combating salt stress is still divergent and varies according to species. Conversely, in the present study, the maintenance of the synthesis of total carotenoids was observed together with increased levels of chlorophyll. This response is possibly related to the important role that carotenoids play as photoprotectants of chloroplast membranes, as suggested by Bartley and Scolnik (1995). According to Sharma and Hall (1991), carotenoids are accessory pigments that absorb and transfer radiant energy and protect chlorophyll from photooxidation. Therefore, the maintenance of carotenoid synthesis may involve the protection of chlorophyll, indicating that, under stress, rice plants appear to use carotenoids to avoid problems with

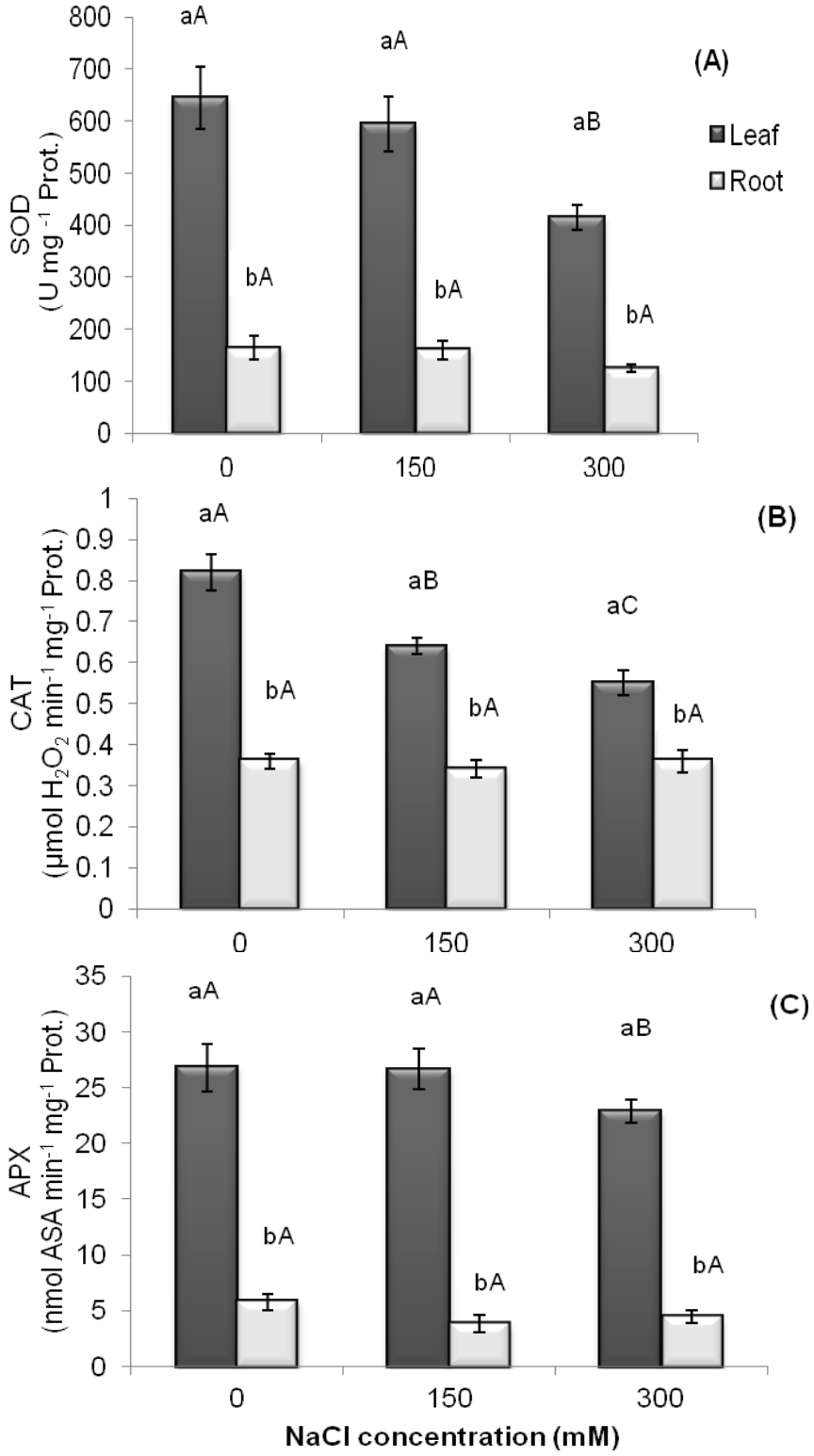

Figure 3. SOD (A), CAT (B) and APX (C) activity in plants of *O. sativa* L., from the cultivar BRS Ligeirinho, subjected to different concentration of NaCl for 30 days. Means followed by the same letter were not significantly different based on mean comparison by Tukey's test at $P \leq 0.05$. Lowercase letters compare the different tissues within each concentration and capital letters compare the same tissues in different concentrations. Vertical bars indicate mean ± SE.

photoinhibition and photooxidation.

The increased production of osmolytes, such as proline, is one of the biochemical changes that are caused by salt stress (Sripinyowanich et al., 2010). It has long been considered that proline is merely an osmolyte that protects subcellular structures and macromolecules under osmotic stress (Banu et al., 2009). However, proline accumulation is related to protective mechanisms against stress in several ways; for example, it has been shown to act as a molecular chaperone that is capable of protecting protein integrity and the activities of various enzymes (Szabados and Sauvoré, 2009). The gradual increase in free proline concentrations that were observed in the rice plants during this study indicate that salinity markedly affects them to produce a rapid osmotic adjustment, suggesting that it is a protective mechanism for salt stress in rice plants of the studied cultivar. These results are consistent with the findings of studies that have been performed using other cereals. For example, Goudarzi and Pakniyat (2009) observed a 2.6-fold increase in the proline concentrations of wheat that was irrigated with salt water over a four-week period. Under salt stress, there is typically a reduction in protein concentrations that is caused either by reduced protein synthesis or increased proteolysis (Parida and Das, 2005). However, according to Tester and Davenport (2003), there may also be an increase in the synthesis of a wide variety of proteins in response to salt stress, which primarily act to stabilise cellular membranes.

In this study, there was a trend towards increasing protein concentrations with concurrent increasing salt concentrations in the irrigation water. These results are consistent with those that were reported by Goudarzi and Pakniyat (2009), who observed increases in total protein concentrations in both salt-tolerant and salt-sensitive wheat cultivars. In rice plants that were exposed to 200 mM NaCl, Hien et al. (2003) reported that the protein levels were stable in both the leaves and roots of the salt-tolerant and salt-sensitive rice plants. The accumulation of ROS leads to oxidative stress, which occurs when there is an imbalance between ROS production and antioxidant defence systems (Wang et al., 2005). Lee et al. (2001) showed that NaCl treatments resulted in the increased accumulation of H_2O_2 in the leaves but not the roots of rice plants. These results are consistent with those that were observed in this study with the rice cultivar BRS Ligeirinho, in which there was a significant increase in H_2O_2 levels in the leaves of plants that were treated with NaCl compared with the control plants, whereas these significant changes were not observed in the roots. Cho and Seo (2005) observed an accumulation of H_2O_2 in the leaves of *Arabidopsis thaliana* that was proportional to an increase in cadmium concentrations when the plants were subjected to this stress for 21 days. The authors admit that this was due to the reduced activities of enzymes that are directly involved in H_2O_2 elimination, such as peroxidase (POX), APX and glutathione reductase (GR). Conversely, Lin and Kao (2000) observed decreased H_2O_2 levels in the leaves of rice plants that were subjected to salt stress, indicating that this response is due to the increased activities of enzymes that are responsible for eliminating H_2O_2.

According to Scandalios (1993), the induction of lipid peroxidation is one of the most damaging effects of ROS and is an indication of ROS production. Our results show that increased levels of salinity induced oxidative stress as shown by lipid peroxidation, which was observed in the leaves of the rice plants and was highly significant at NaCl concentrations of 300 mM. This fact was evidenced by the increased formation of MDA in parallel with increased H_2O_2 levels. MDA is a product of lipid peroxidation, and thus, it may be used as an indicator of the degree of lipid peroxidation (Tartoura and Youssef, 2011). Several studies have reported increased lipid peroxidation under different types of stress, including high doses of lead in *O. sativa* L. (Verma and Dubey, 2003), cold stress in *Glycine max* (L). Merr. (Posmyk et al., 2005), cadmium excess in *Nicotiana tabacum* (Islam et al., 2009) and salt stress in *Vigna unguiculata* L. (Deuner et al., 2011). In the present study, SOD, CAT and APX showed reduced activities in the leaves of rice plants as a function of salinity. However, in the roots, although, the activities were also lower, the reduction was less pronounced. Decreased activities of antioxidant enzymes after long periods of stress exposure have been reported by other authors. Lee et al. (2001) quantified SOD, CAT, APX and GR activities in rice plants that were grown with 150 mM NaCl and observed increased SOD and APX activities during the first three days of salt exposure, which decreased thereafter. Deuner et al. (2011) studied the effects of NaCl on the development of four genotypes of *V. unguiculata* L. seedlings and observed increased SOD activity up to a concentration of 150 mM and increased APX and CAT activities only up to a concentration of 100 mM. However, the activities of these enzymes under 200 mM NaCl stress were reduced compared with those that were observed in the control treatment.

According to Carmak and Horst (1991), reductions in the activities of some enzymes, such as CAT, indicates that in some plants that are maintained under stress conditions, the H_2O_2 that is produced is more rapidly consumed by oxidative processes, such as lipid peroxidation, than eliminated via metabolism by the actions of antioxidant enzymes. This may explain the low enzymatic activities and high lipid peroxidation levels that were observed in this study.

Conclusions

Under salt stress, rice plants, specifically the cultivar BRS

Ligeirinho, maintain chlorophyll and protein synthesis at levels approaching or exceeding those that are found in plants grown without NaCl. This response may possibly be related to increased proline levels. Conversely, after 30 days of salt exposure, the rice plants showed decreased antioxidant enzymatic activity, and the majority of the H_2O_2 that was produced was preferentially consumed by lipid peroxidation. Finally, the changes that were caused by salinity were more pronounced in the leaves. However, further studies are necessary to understand the manner in which these mechanisms are activated and triggered within the plant cell.

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