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BRIEF COMMUNICATION

Partial oxidative protection by enzymatic and non-enzymatic components in cashew leaves under high salinity

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Abstract

The work evaluated the role of enzymatic and non-enzymatic antioxidants in cashew (*Anacardium occidentale*) leaves under 0, 50, 100, 150 and 200 mM NaCl. Salt stress increased protein oxidation and decreased the lipid peroxidation, indicating that lipids are less susceptible to oxidative damage. The superoxide dismutase (SOD) activity was not changed, ascorbate peroxidase (APX) activity steadily decreased while the catalase (CAT) activity strongly increased with the increasing NaCl concentration. High salinity also induced alterations in the ascorbate (AsA) and glutathione (GSH) redox state. The salt resistance in cashew may be associated with maintaining of SOD activity and upregulation of CAT activity in concert with the AsA and GSH antioxidants.

Additional key words: *Anacardium occidentale*, antioxidative enzymes, ascorbate peroxidase, catalase, NaCl, oxidative stress, superoxide dismutase.

Plants acclimate to changes in their environment by achieving a new state of cellular homeostasis. This acclimation requires a delicate balance among multiple pathways and has a direct impact on metabolic cell efficiency (Miller *et al.* 2010). Plants exposed to saline soils exhibit reduced growth and low productivity due to osmotic and ionic stresses (Munns 2005, Munns and Tester 2008). Stomatal closure induced by the salinity decreases the CO_2/O_2 ratio in chloroplasts, restricting the CO_2 assimilation, decreasing the $NADP^+$/NADPH ratio and leading to the production of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) (Foyer and Noctor 2003). To avoid the oxidative damages caused by excessive production of ROS, the plant cells employ enzymatic and non-enzymatic antioxidants (Møller *et al.* 2007). Superoxide dismutase (SOD) catalyzes the $O_2^{\bullet-}$ dismutation to H_2O_2 and O_2 and is in the first line of cell defense against ROS (Alscher *et al.* 2002). In the ascorbate-glutathione cycle, ascorbate peroxidase (APX) is an H_2O_2-scavenging enzyme that acts in synchrony with dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHR) and glutathione reductase (GR) (Shigeoka *et al.* 2002). Catalase (CAT) is essential for the removal of H_2O_2 produced in the peroxisomes by photorespiration (Foyer *et al*. 2009, Silva *et al*. 2010). Ascorbate (AsA) and glutathione (GSH) are the main non-enzymatic antioxidants (Shalata and Neumann 2001, Kocsy *et al.* 2002, Kranner *et al.* 2002, Tarchoune *et al.* 2010). Salt tolerance has been associated with the enhanced expression and/or activity of antioxidative enzymes (Lin and Pu 2010, Zhong *et al*. 2010).

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Abbreviations: APX - ascorbate peroxidase; AsA - ascorbic acid; CAT - catalase; DHA - dehydroascorbate; DHAR - dehydroascorbate reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; LPO - lipid hydroperoxides; MDHR - monodehydroascorbate reductase; PUFA - polyunsaturated fatty acid; ROS - reactive oxygen species; SOD - superoxide dismutase; TBARS - thiobarbituric acid-reactive substances.
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Nevertheless, the precise interaction between the enzymatic and non-enzymatic components in oxidative protection under salinity remains poorly understood. In the present work, we evaluated the role of the SOD, APX and CAT enzymes and of AsA and GSH antioxidants in the oxidative protection of cashew leaves subjected to salinity. Cashew was utilized as the plant model because it is a species widely cultivated in semi-arid regions often exposed to salinity (Silveira *et al*. 2003, Ferreira-Silva *et al*. 2008).

Cashew nuts (*Anacardium occidentale* L.) of the CCP 06 genotype were provided by *EMBRAPA-CNPAT*, Fortaleza, Brazil. The nuts were sown in plastic pots with *Vermiculite* and grown in greenhouse. 35-d-old plants were irrigated with nutrient solution (Hoagland and Arnon 1950) supplied with increasing NaCl concentrations (0, 50, 100, 150, and 200 mM) for 15 d. The salt treatments were gradually applied by increasing the NaCl concentration by 50 mM per day. Plant harvest was performed when transpiration rate in the 200 mM NaCl treatment was below 80 % of that of control. Leaf discs were harvested to determine the relative water content (RWC) and membrane damage. The relative growth rate (RGR) was calculated using the equation $RGR = [(\ln DM_2 - \ln DM_1)/(T_2 - T_1)]$, where T = time [d], DM_1 = dry mass at T_1 and DM_2 = dry mass at T_2. The Na^+ content $[Na^+]$ was measured by flame photometry, and its relative uptake rate (RUR) was estimated using the relation $RUR = (\ln [Na^+]_2 - \ln [Na^+]_1)/(T_2 - T_1)$. The leaf RWC was measured by the successive weighing of leaf discs (fresh, fully saturated and dry) and membrane damage (MD) was estimated by leaf K^+ leakage (Cavalcanti *et al.* 2004). Measurement of transpiration rate was performed daily using a portable porometer (*LI-COR 1600*, Lincoln, USA). Hydrogen peroxide content was determined according to Cheeseman *et al.* (2006). Lipid peroxidation was determined by measuring the thiobarbituric acid-reactive substances (TBARS) and lipid hydroperoxides (LPO) content. TBARS was determined as described by Heath and Packer (1968) and the LPO content was measurement using an assay kit according to the manufacturer's instructions (*Cayman Chemical*, USA). The carbonyl group (C=O) content was determined after the derivatization of proteins with dinitrophenylhydrazine (DNPH) as described by Levine *et al.* (1994). The contents of reduced ascorbate (AsA) and total ascorbate (AsA + DHA) were determined according to the method of Kampfenkel *et al.* (1995). The content of reduced (GSH) and total glutathione (GSH + GSSG) was determined according to (Griffth 1980). The oxidized fractions of glutathione (GSSG) and ascorbate (DHA) were calculated by subtracting the reduced fraction from the total content. The activities of superoxide dismutase (SOD; EC: 1.15.1.1), ascorbate peroxidase (APX; EC: 1.11.1.1) and catalase (CAT; EC: 1.11.1.6) were determined as previously described (Maia *et al*. 2010). Soluble protein content of the enzymatic extracts was measured according to Bradford (1976). The experiment was carried out with a completely randomized design comprising five salinity treatments and four replicates. The data were analyzed by an *F*-test with significance set at $P < 0.05$, and the means were compared using Tukey's test ($P < 0.05$).

The root and leaf RGR of cashew plants subjected to increasing NaCl concentration progressively decreased and such reduction was associated with increase in the Na^+ uptake rate (Table 1). The leaf relative water content was not changed by salt stress, but the transpiration was severely dropped. The cashew plants subjected to salinity exhibited atypical response in terms of changes in the oxidative stress indicators. The content of TBARS and LPO in leaves decreased as the NaCl levels increased. The leaf H_2O_2 content was not changed, but the protein carbonylation increased in parallel to membrane damage (Table 1). H_2O_2 accumulation in the plant tissues is likely to occur under salt stress and indicates ROS overproduction by metabolic disturbances (Foyer and Noctor 2003, Daneshmand *et al*. 2010, Hernandez *et al*. 2010). Salt-induced oxidative stress also is usually associated with increase in lipid peroxidation (Møller *et al.* 2007). In the membrane lipids, the double bonds of PUFA moieties are targets of ROS-mediated peroxidation, which damage the membrane structure. The increase in lipid peroxidation has been considered an indicator of oxidative damage in leaves of several plant species subjected to salt stress such as cowpea (Cavalcanti *et al.* 2004, 2007), rice (Demiral and Türkan 2005), maize (Azevedo-Neto *et al.* 2006) and sorghum (Chai *et al*. 2010). Surprisingly, however, the lipid peroxidation decreased in salt-stressed cashew leaves as indicated by reduction in the TBARS and LPO contents. These results suggest that cashew plants under salinity could trigger an efficient ROS-scavenging system associated with an intense turnover in the membrane lipids which could be favorable to oxidative protection. Similar results were obtained in roots of maize (Azevedo-Neto *et al.* 2006) and cowpea (Cavalcanti *et al.* 2007). In halophyte species subjected to salt stress, the decreased TBARS content is strictly related to the enhanced linoleate/linolenate ratio, which limits the MDA formation caused by lipid peroxidation (Hamed *et al*. 2005). In spite of the unaltered H_2O_2 content and reduction in lipid peroxidation in the leaves of cashew plants under salinity, a mild salt-induced oxidative stress could have occurred as indicated by increase in protein carbonylation and membrane damage (K^+ leakage). The increase in protein carbonylation has been utilized as an indicator of oxidative damage in plants exposed to several abiotic stresses (Moran *et al.* 1994, Gomez *et al.* 1999, Boscolo *et al*. 2003, Miller *et al.* 2007). The increase (25 %) in protein carbonylation suggests the occurrence of a mild oxidative damage caused by the attack of ROS on leaf proteins.

The SOD activity was not affected by increasing NaCl concentration, while APX activity was progressively decreased and CAT activity strongly increased (Table 1). Overall, these different responses may be associated with modulation in gene expression,

Table 1. Root and leaf relative growth rate, RGR; root and leaf Na^+ relative uptake rate, RUR; leaf relative water content, RWC; transpiration rate, E; thiobarbituric acid-reactive substances content, TBARS; lipid hydroperoxides content, LPO; hydrogen peroxide content, H_2O_2; protein carbonyl group content, C=O; membrane damage, K^+ leakage; soluble protein content; superoxide dismutase activity, SOD; ascorbate peroxidases activity, APX; catalase activity, CAT; content and redox state of AsA and GSH antioxidants in leaves of cashew plants subjected to increasing NaCl concentrations (0 - 200 mM) for 15 d. The data represent the mean of four replicates. In each row, the values followed by the same letter did not differ at 0.05 of probability according to the Tukey test.

Parameter	0 mM	50 mM	100 mM	150 mM	200 mM
Root-RGR [mg DM g^{-1}(DM) d^{-1}]	18.25a	12.75b	11.00b	6.75c	5.50c
Leaf-RGR [mg DM g^{-1}(DM) d^{-1}]	18.00a	15.00ab	13.50b	9.50c	8.75c
Root Na^+-RUR [µmol kg^{-1}(DM) d^{-1}]	8.40c	324.00b	380.00a	424.00a	416.00a
Leaf Na^+-RUR [µmol kg^{-1}(DM) d^{-1}]	7.20b	388.00a	384.00a	440.00a	444.00a
RWC [%]	91.00a	91.00a	91.00a	89.00a	91.00a
E [mmol (H_2O) m^{-2} s^{-1}]	2.02a	1.03b	0.51c	0.36cd	0.27d
TBARS [µmol g^{-1}(DM)]	112.00a	84.00b	81.00b	80.00b	60.00c
LPO [µmol g^{-1}(DM)]	5.84a	5.24a	4.20b	3.72b	3.64b
H_2O_2 [µmol g^{-1}(DM)]	19.12a	17.84a	18.61a	18.82a	18.51a
C=O [µmol mg^{-1}(protein)]	3.05b	3.08b	3.97a	4.25a	4.35a
K^+ leakage [%]	21.00b	26.00ab	30.00a	32.00a	31.00a
Soluble protein [mg g^{-1}(DM)]	48.63a	47.00a	44.00a	50.24a	48.28a
SOD [A.U. mg^{-1}(protein) min^{-1}]	2.36a	2.65a	2.81a	2.64a	2.63a
APX [µmol (AsA) mg^{-1}(protein) min^{-1}]	0.77a	0.83a	0.79b	0.47c	0.40d
CAT [µmol (H_2O_2) mg^{-1}(protein) min^{-1}]	1.62e	4.70c	10.36a	7.48b	2.38d
AsA+DHA [µmol g^{-1}(DM)]	27.36a	25.61a	30.81a	27.96a	27.36a
AsA [µmol g^{-1}(DM)]	14.41a	12.56a	12.68a	12.62a	9.24b
DHA [µmol g^{-1}(DM)]	12.72b	13.04b	12.56b	19.00a	18.72a
AsA/(AsA+DHA) [%]	53.51a	49.06a	50.24a	40.91b	33.05b
GSH + GSSG [µmol g^{-1}(DM)]	5.64a	5.56a	5.72a	5.72a	5.56a
GSH [µmol g^{-1}(DM)]	3.72c	3.64c	4.36a	4.41a	4.56a
GSSG [µmol g^{-1}(DM)]	1.84a	1.61a	1.63a	1.32b	1.07c
GSH/(GSH + GSSG) [%]	65.96c	65.47c	76.22b	76.92b	82.01a

post-transcriptional mechanisms, inactivation/activation of proteins and/or protein denaturation triggered directly or indirectly by salt stress. In the ROS-scavenging enzymatic system, SOD is critical for the removal of the $O_2^{\bullet-}$ generated mainly in the chloroplasts (Alscher *et al.* 2002). H_2O_2 produced by SOD activity is then eliminated by APX within different cell compartments (Shigeoka *et al.* 2002). Moreover, CAT activity is fundamental for the removal of H_2O_2 generated by the photorespiration in the peroxisomes (Mittler 2002, Foyer *et al.* 2009). In the present study, SOD activity was not affected by salinity and electrophoretic analysis revealed two Fe-SOD isoforms and three Cu/Zn-SOD isoenzymes (data not shown). The decrease in APX activity and increase in CAT suggest a possible ROS-scavenging compensatory mechanism in cashew leaves under salt stress. Recent research highlight that APX is the main cytosolic H_2O_2-scavenging enzyme (Mittler 2002, Shigeoka *et al*. 2002) and that a crosstalk between APX and CAT plays a central role in the control of intracellular H_2O_2 level (Palatnik *et al.* 2002). The maintenance of adequate H_2O_2 content as well as the protection against lipid peroxidation may be explained, at least in part, by the positive modulation of CAT activity. Likewise, membrane stability in a salt-tolerant rice cultivar is attributed mainly to H_2O_2 scavenging by CAT (Vaidynathan *et al.* 2003). Moreover, the reduction in AsA/(AsA+DHA) redox state associated with decreased APX activity under high NaCl concentration may indicate a direct utilization of AsA in H_2O_2-scavenging by a chemical reaction as proposed by Del Rio *et al.* (2006).

The reduced ascorbate (AsA) decreased while the oxidized ascorbate (DHA) increased and, as a consequence, the AsA/(AsA+DHA) redox state decreased. The reduced glutathione (GSH) content increased and the oxidized glutathione (GSSG) decreased allowing the increase in the GSH/(GSH+GSSG) redox state. The increase in the glutathione redox state can confer better antioxidative protection and would be considered an acclimation response to salt stress (Tauz *et al*. 2004). In this study, the GSH/(GSH+GSSG) redox state in leaves of cashew plants increased in response to salt stress. Similar results were obtained in salt-tolerant tomato (Mittova *et al*. 2003) and rice (Vaidyanathan *et al*. 2003) cultivars when compared to salt-sensitive cultivars. Thus, the reduction in ascorbate redox state associated with increase in glutathione redox state may represent an efficient interactive mechanism between enzymatic and non-enzymatic protection to avoid oxidative damage in cashew leaves under high salinity.

The obtained data indicate that cashew plants subjected to salinity exhibited a specific oxidative response. Soluble proteins were apparently more sensitive to ROS attack than lipids but the protein oxidation was

not sufficient to induce important oxidative damage. In the literature there are rare reports about the differential sensitivity of the main targets of ROS as well as the damage threshold level for each of these indicators in plant cells (Foyer and Noctor 2005, Cavalcanti *et al*. 2007). Further studies employing non-model plants, such as cashew, are needed since the antioxidant responses involve redundant metabolic networks which could vary among plants species under different stressful conditions.

In summary, cashew leaves have an efficient antioxidative mechanism under salt stress, which is apparently more effective in preventing lipid peroxidation and H_2O_2 accumulation than in preventing protein oxidation. Moreover, this oxidative protection might be attributed to the maintaining of SOD activity in concert with stimulation of CAT associated with favorable modulation in the glutathione and ascorbate redox states.

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