BIOLOGIA PLANTARUM 54 (1): 159-163, 2010

BRIEF COMMUNICATION

Antioxidative enzymatic protection in leaves of two contrasting cowpea cultivars under salinity

J.M. MAIA*, C.E. COSTA de MACEDO**, E.L. VOIGT*, J.B.S. FREITAS* and J.A.G. SILVEIRA*[1]

*Departamento de Bioquímica e Biologia Molecular, Instituto Nacional de Ciência e Tecnologia em Salinidade, Universidade Federal do Ceará, CP 6033, CEP 60451-970, Fortaleza, Ceará, Brazil**
*Universidade Federal do Rio Grande do Norte, Natal, CP 1648, CEP 59078-670, Rio Grande do Norte, Brazil***

Abstract

The aim of this work was to investigate the role of the antioxidant enzymes in salt tolerance comparing the salt-sensitive (Pérola) and a salt-tolerant (Pitiúba) cultivar of cowpea [*Vigna unguiculata* (L.) Walp.]. Salt stress (100 mM NaCl for 8 d) reduced the leaf growth rate more in the sensitive cultivar. The salt-induced decrease in the relative water content, Na^+ accumulation and increase in leaf electrolyte leakage was similar in both cultivars. Salt stress induced a higher increase in the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and phenol peroxidase (POX) in the tolerant cultivar than in sensitive one.

Additional key words: ascorbate peroxidase, oxidative damage, peroxidase, salt stress, salt tolerance, superoxide dismutase, *Vigna unguiculata.*

Oxidative stress is a common response to environmental adverse conditions including high soil salinity (Apel and Hirt 2004). Salt-induced water deficit and ionic toxicity may cause several metabolic disturbances that trigger the over-production of reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen (Asada 1992, Möller *et al.* 2007, Procházková and Wilhelmová 2007). ROS can act as signaling molecules or disrupt the cell metabolism, oxidizing lipids, proteins and nucleic acids (Alscher *et al.* 2002, Imlay 2003). The plant cells display nonenzymatic and enzymatic antioxidant systems to mitigate these oxidative damages caused by ROS (Apel and Hirt 2004). The antioxidant enzymes include superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.1), catalase (CAT; EC 1.11.1.6), phenol peroxidase (POX; EC 1.11.1.7) and other enzymes of the ascorbate-glutathione cycle (Hernández *et al.* 2001).

In the leaves, oxidative stress may be generated as salt-induced unbalance between the light-driven NADPH production and consumption in the CO_2 assimilation (Foyer and Noctor 2003). The superoxide anions generated at photosystem 2 may be dismutated by the Cu/Zn-SOD isoforms located inside the chloroplasts (Edreva 2005, Möller *et al.* 2007). Stromal and thylakoid membrane-bound APX scavenge the H_2O_2 released as a product of the SOD catalysis (Shigeoka *et al.* 2002). However, the exceeding H_2O_2 that diffuses to other cell compartments may be detoxified by other APX isoenzymes as the H_2O_2 generated by photorespiration is mainly eliminated by CAT inside the peroxisomes (Foyer and Noctor 2003). Many POX isoforms may also contribute to the H_2O_2 removal at different cell compartments, including the cell wall POX that utilizes apoplastic H_2O_2 in lignification (Passardi *et al.* 2004, Baťková *et al.* 2008).

Salt-tolerance has been generally attributed, at least in part, to either high constitutive or upregulated activities

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Received 23 June 2008, *accepted* 22 December 2008.

Abbreviations: APX - ascorbate peroxidase, CAT - catalase, DAE - days after emergence, DM - dry matter, EDTA - ethylene diamine tetraacetic acid, MDA - malondialdehyde, NBT - *p*-nitroblue tetrazolium chloride, POX - phenol peroxidase, RGR - relative growth rate, ROS - reactive oxygen species, RWC - relative water content, SOD - superoxide dismutase, TBA - thiobarbituric acid, TBARS - thiobarbituric acid reactive substances, TCA - trichloroacetic acid.

Acknowledgements: The authors appreciated financial support by Fundação Cearense de Apoio à Pesquisa (FUNCAP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). J.A.G. Silveira is a CNPq fellowship honored researcher and J.M. Maia and E.L. Voigt are CNPq fellowship granted students.

[1] Corresponding author; fax: (+55) 85 3366 9821, e-mail: silveira@ufc.br

of different antioxidant enzymes (Attia *et al.* 2008). The activities of SOD, APX, CAT and POX are reported to increase under salinity and salt-tolerant cultivars may show higher activities as compared to salt-sensitives ones as was reported in millet (Sreenivasulu *et al.* 2000), rice (Vaidyanathan *et al.* 2003), cotton (Meloni *et al.* 2003), wheat (Sairam *et al.* 2005) and maize (Azevedo-Neto *et al.* 2006). However, rice and cotton salt-tolerant cultivars show enhanced CAT activity under salinity (Gosset *et al.* 1994, Vaidyanathan *et al.* 2003), whereas those of wheat and cucumber demonstrated its salt-induced down-regulation (Foyer and Noctor 2000, Shim *et al.* 2003).

Cowpea [*Vigna unguiculata* (L.) Walp.] is a species widely cultivated in the semi-arid regions of Africa and Brazil and some cultivars are adapted to cope with salinity (Cavalcanti *et al.* 2007). In previous works with this species, the SOD activity remains unaltered, the CAT activity is strongly decreased and only the POX activity is prominently increased in the tolerant Pitiúba cultivar under salt stress (Cavalcanti *et al.* 2004, 2007). The aim of the present work is to investigate the role of antioxidative enzymes in salt tolerance comparing the oxidative responses displayed by the leaves of a salt-sensitive (Pérola) and a salt-tolerant (Pitiúba) cultivar of cowpea.

Seeds of cowpea [*Vigna unguiculata* (L.) Walp.] cvs. Pitiúba (salt-tolerant) and Pérola (salt-sensitive) (Freitas 2006) were obtained from the Departamento de Fitotecnia of the Universidade Federal do Ceará (UFC). The seeds were surface-sterilized in 70 % (v/v) ethanol for 3 min and 0.2 % (m/v) sodium hypochlorite for 1 min, thoroughly rinsed with distilled water and germinated in plastic pots containing sand and vermiculite (1:1, v/v). The seedlings were grown in a greenhouse under natural conditions and watered daily with distilled water until fourth day after emergence (DAE). From fifth DAE, the seedlings were watered daily with tenth-strength Hoagland's nutrient solution. All the waterings were accomplished with nutrient solution up to 70 % of the substrate field capacity. After the thirteenth DAE, cowpea plants showing the first trifoliate fully expanded leaf were irrigated daily with 25 mmol dm^{-3} NaCl in 1/4-strength Hoagland's nutrient solution up to reaching 100 mM NaCl around the roots. The controls were maintained without salt addition. The salt treatment was carried out for 8 d. Then, control and salt-treated plants were transferred to a controlled growth room (27 ± 2 °C, 72 % RH, 230 μmol m^{-2} s^{-1} of photon flux density) and after 2 h of acclimation the plants with mature trifoliate leaves were harvested and samples for biochemical determinations were immediately frozen in liquid N_2 and stored to -80 °C until the assays.

Leaf relative growth rate (RGR) was calculated as $[(DM_2 - DM_1)/(t_2 - t_1) \times 1/DM_1]$, where $DM_2 - DM_1$ represents the difference between two leaf dry mass measurements and $t_2 - t_1$ represent the difference between two times assessed. Leaf relative water content (RWC) was determined according to Cairo (1995) and 30 leaf discs (1.0 cm in diameter) with known fresh mass (FM) were saturated with distilled water for 6 h at 25 °C to obtain SM. The RWC was calculated using the equation RWC = (FM - DM)/(SM - DM) × 100.

Membrane damage was estimated by electrolyte leakage as previously described by Blum and Ebercon (1981). Twenty leaf discs (1.0 cm in diameter) were placed in test tubes containing 10 cm^3 deionized water. Flasks were incubated in a shaking water bath at 25 °C for 6 h and the electric conductivity in the medium (L_1) was measured. After that, the discs were boiled at 100 °C for 60 min, cooled to 25 °C and the electric conductivity (L_2) was measured again. The relative electrolyte leakage (percentage of membrane damage, MD) was estimated using the formula: MD [%] = $L_1/L_2 \times 100$. The Na^+ content of the leaf tissues were determined by flame photometry according to Silveira *et al.* (2001). Dry leaves were finely powdered and samples of 50 mg were extracted with 10 cm^3 of deionized water at 100 °C for 60 min in hermetically closed tubes. After cooling, the extract was filtered through cotton cloth and the determinations were performed by a flame photometer (*Mod 462*, *Micronal*, Brazil).

The lipid peroxidation was determined in terms of thiobarbituric acid-reactive substances (TBARS) content, as described by Heath and Packer (1968) with minor modifications. Leaf tissue (25 mg) was homogenized in 3 cm^3 1.0 % (m/v) trichloroacetic acid (TCA) at 4 °C. The homogenate was centrifuged at 20 000 *g* for 15 min at 4 °C and 0.5 cm^3 of the supernatant obtained was added to 3 cm^3 0.5 % (v/v) thiobarbituric acid (TBA) in 20 % TCA. The mixture was incubated at 95 °C in a shaking water bath for 50 min, and the reaction was stopped by cooling the tubes in an ice bath. Then, samples were centrifuged at 9 000 *g* for 10 min, and the absorbance of the supernatant was read at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using the absorption coefficient (155 mM^{-1} cm^{-1}).

For enzyme assays, samples of 1.0 g of fresh leaves were homogenized with a mortar and pestle in 3 cm^3 of ice-cold 50 mM potassium phosphate buffer, pH 7.0 containing 0.1 mM EDTA and 1 mM L-ascorbic acid. After filtration through cheesecloth, the homogenate was centrifuged at 13 000 *g* for 15 min and the supernatant was used as the source of enzymes. The activity of SOD was determined by adding 0.05 cm^3 of the enzymatic extract to a solution containing 13 mM L-methionine, 75 μM *p*-nitroblue tetrazolium chloride (NBT), 100 μM EDTA and 2 μM riboflavin in a 50 mM potassium phosphate buffer pH 7.8. The reaction was started by turning the 30 W fluorescent lamp on, and stopped 5 min later by turning it off (Van Rossum *et al.* 1997). The blue formazane produced by NBT photoreduction was measured at 560 nm. One SOD unit was defined as the amount of enzyme required to inhibit 50 % of the NBT photoreduction. Ascorbate peroxidase (APX) activity was measured spectrophotometrically by monitoring the ascorbic acid dependent reduction of H_2O_2 at 290 nm using coefficient of absorbance 2.8 mM^{-1} cm^{-1} (Koshiba 1993). Catalase (CAT) activity was determined by adding 0.05 cm^3 enzymatic extract to 3 cm^3 of a solution containing 50 mM potassium phosphate buffer (pH 7.0) and 20 mM H_2O_2 and measuring the decrease in absorbance at 240 nm and 30 °C (Havir and McHale 1987). The activity of phenol peroxidase (POX) was determined by adding 0.025 cm^3 of the crude enzyme preparation to 2 cm^3 of a solution containing 50 mM potassium phosphate buffer pH 6.8, 20 mM pyrogallic acid and 20 mM H_2O_2. After incubation at 30 °C for 10 min, the reaction was stopped by adding 0.5 cm^3 5 % (v/v) H_2SO_4 and the absorbance was read at 480 nm (Urbanek *et al.* 1991).

The experiments were carried out in a completely randomized design with two treatments and two cultivars. Six replicates were performed per treatment and replicate was submitted to *ANOVA* and the differences were assessed according to the Tukey's test at 5 % of significance.

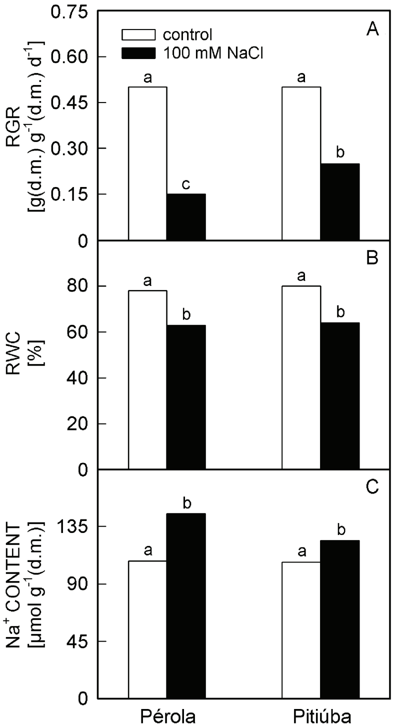

Fig. 1. Leaf relative growth rate, RGR (*A*), leaf relative water content, RWC (*B*) and leaf Na^+ content (*C*) of salt-stressed and control plants of cvs. Pérola and Pitiúba. The significant differences according to Tukey's test ($\alpha = 0.05$) were indicated by different letters.

The leaf RGR was reduced by 70 % in salt-sensitive Pérola and only by 50 % in salt-tolerant Pitiúba at 100 mM NaCl (Fig. 1*A*). Also, the leaf relative water content (RWC) decreased significantly after 8 d of salt treatment, although this reduction was not as drastic as that observed for the leaf growth (Fig. 1*B*). As expected, the Na^+ content increased in the leaf tissues of both cultivars, but it increased about 26 % in the Pérola and 14 % in the Pitiúba under 100 mM NaCl (Fig. 1*C*). It has been suggested that cowpea plants show efficient mechanisms to prevent Na^+ accumulation in the leaves, avoiding its toxic effects on the photosynthetic tissues (Silveira *et al.* 2001). Thus it is probable that the decreased leaf RGR reflected the water deficit induced by the salt treatment.

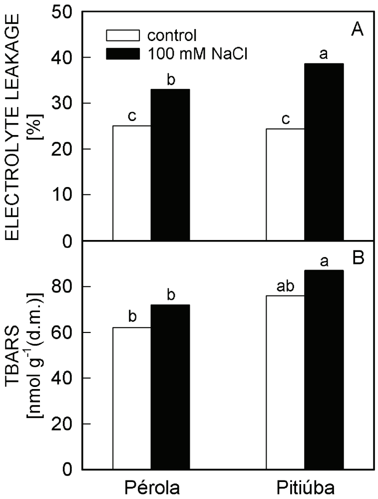

Fig. 2. Electrolyte leakage (*A*) and lipid peroxidation, TBARS (*B*) in leaves of salt-stress and control plants of cvs. Pérola and Pitiúba. The significant differences were indicated by different letters.

Cell membrane stability is frequently related to salt tolerance in plants (Dionisio-Sese and Tobita 1998) and electrolyte leakage is usually used as an indicator of membrane injuries in salt-treated plants (Shanker *et al.* 2004, Mandhania *et al.* 2006). Electrolyte leakage, was increased at 100 mM NaCl by 37 and 28 % in Pitiúba and Pérola, respectively (Fig. 2*A*). Conversely, lipid peroxidation estimated by the TBARS content was not significantly changed in both cultivars (Fig. 2*B*) and so membrane lipid turnover may play a role in the maintenance of the membrane structure under salt stress.

Oxidative stress generated by salinity may be overcome by the upregulation of antioxidative enzymes (Ashraf and Ali 2008). In the Pitiúba cultivar submitted to salt stress, it was previously demonstrated that the SOD activity was unchanged, the CAT activity was strongly decreased and the POX activity was enhanced (Cavalcanti *et al.* 2004, 2007). In the present experiments, the activity of SOD, APX and POX was increased in the leaves of Pérola and Pitiúba plants when exposed to the salt treatment. In fact, the SOD activity increased by 32 and 12 % in the Pitiúba and Pérola, respectively (Fig. 3*A*). Likewise, the APX activity was enhanced by about 33 % in the Pitiúba and by only 14 % in the Pérola (Fig. 3*B*). A remarkable increase of 93 % was verified in the POX activity for the Pitiúba, while it was stimulated by only 50 % in the Pérola (Fig. 3*C*). Conversely, the CAT activity decreased more than 65 %

in the salt-treated plants of both cultivars (Fig. 3*D*). It may be an example of the CAT-APX-POX crosstalk, considering that the decreased CAT activity may be compensated by the activities of APX and POX. In fact, several works have reported similar responses in other plant species (*e.g.* Shigeoka *et al.* 2002, Vaidyanathan *et al.* 2003). Additionally, the decreased CAT activity in the leaves of cowpea plants under salt stress may reflect unbalances between its synthesis and degradation, as well as irreversible damages in the CAT protein structure (Cavalcanti *et al.* 2004, Cavalcanti *et al.* 2007).

It is noteworthy that POX activity is more stimulated by NaCl in the tolerant cultivar. Considering that the leaf RGR is strongly impaired by salinity in both cultivars, it is possible that this increased POX activity is simply related to H_2O_2 scavenge and/or it is involved in H_2O_2-dependent cell wall lignification, reducing leaf growth. Indeed, many POX isoforms are located in different cell compartments, utilizing diverse phenolic compounds as substrates to eliminate H_2O_2 (Azevedo-Neto *et al.* 2005, 2006, Radic *et al.* 2006, Ashraf and Ali 2008, Bogdanović *et al.* 2008). Besides this protective role, other results point out that the cell wall POX is strictly involved in mechanisms underlying growth regulation, utilizing apoplastic H_2O_2 to cell wall strengthening through lignin synthesis (Bacon *et al.* 1997, Lin and Kao 2002, Passardi *et al.* 2004). Thus, the enhanced POX activity suggests the involvement of this antioxidant enzyme in the redox homeostasis during salt acclimation in the leaves of cowpea plants. However, an important question remains unsolved: whether the cell wall lignification mediated by POX is a consequence of salt-induced disturbances in the oxidative metabolism or it is a biochemical strategy to mitigate oxidative damages under tight regulation.

In conclusion, the differential responses of the SOD-APX-POX system in the leaves of the Pitiúba and Pérola reinforce the contrasts between these cultivars in terms of salt tolerance.

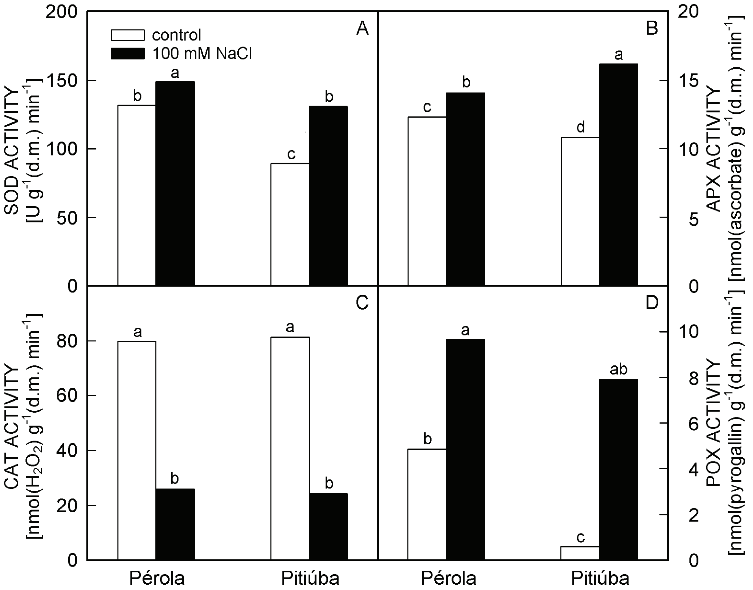

Fig. 3. Activity of superoxide dismutase, SOD (*A*), ascorbate peroxidase, APX (*B*), catalase, CAT (*C*) and phenol peroxidase, POX (*D*) in leaves of salt-stressed and control plants of cvs. Pérola and Pitiúba. The significant differences were indicated by different letters.

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