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NADP-Dependent Malic Enzyme Protects *Vigna unguiculata* against Reactive Oxygen Species under Osmotic Stress

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ABSTRACT

Analysis of the effect of a water stress (41 mM of polyethyleneglycol, or PEG) and a salt stress (150 mM NaCl) on enzymes implicated in the antioxidant system of two cultivars (Vita 3 and Vita 5) of *Vigna unguiculata* showed modifications in the SOD-APX-GR cycle. Catalase (EC 1.11.1.6) activity was strongly increased in both cultivars, under either PEG or NaCl treatment; superoxide-dismutase (EC 1.15.1.1) activity increased only for Vita 3 during water stress. On the other hand, ascorbate peroxidase (EC 1.11.1.11) activity increased only for Vita 5 following PEG treatment. Glutathione reductase (EC 1.6.4.2) and NADP-dependent malic enzyme (EC 1.1.1.40) activities increased significantly for Vita 3 under PEG treatment. Besides, the malondialdehyde (MDA) content increased more in Vita 5. The modification in the SOD-APX-GR cycle demonstrated that both treatments triggered an oxidative stress in both cultivars. Except for the results obtained for APX, Vita 3 had more efficient mechanisms to counteract reactive oxygen species than Vita 5. An increase in NADP-ME activity could maintain a more reductive environment (more NADPH). Therefore, we conclude that NADP-ME is also involved in the detoxification process in C_3 plants.

Keywords: antioxidants, drought stress, NADP-ME, oxidative stress, Vita 3 and Vita 5 cultivars

Abbreviations: APX, ascorbate peroxidase; **DTN**, 5,5'-dithio-nitrobenzoic acid; **DTNB**, 5,5'-dithiobis(2-nitrobenzoic acid); **DTT**, dithiotreitol; **EGTA**, ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid; **GR**, glutathione reductase; **GSH**, reduced glutathione; **GSSH**, oxidized glutathione; **NADP-ME**, NADP-dependent malic enzyme; **NBT**, nitroblue tetrazollium; **OD**, optics density; **PEG**, polyethylene glycol; **PMS**, phenazine methosulfate; **PVP**, polyvinylpyrolidone; **PVPP**, polyvinylpolypyrrolidone; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase

INTRODUCTION

Salinity is a great problem for agriculture in the world today. The main causes of soil salinity are: low pluvial precipitation or flooding and mismanagement of irrigation procedures. In arid and semi-arid areas, as is the case of Northeast Brazil, the salinity levels are high because the amount of rain is not enough to drain the accumulated salts in the soil. In addition, being in the vicinity of the equator increases the evaporation and transpiration levels, which aggravate the salinity of the soil, rendering it virtually unusable for agriculture.

The bean is the principal food source for the poor people from Northeast Brazil. Cowpea (*Vigna unguiculata*) possesses several cultivars that differ from each other with regards to their degree of tolerance to salt stress (Guimarães 1988). Hence, an understanding of the factors that determine the sensitivity of beans to water and salt stress is particularly important.

The metabolic activities of plants are profoundly changed under osmotic stress. Oscillation in the malate levels and changes in the activities of enzymes implicated in malate metabolism under stress conditions suggests a role of malate in plant defence (Lance and Rustin 1984; Aragão *et al*. 1997). Malic enzymes (MEs) catalyze the oxidative decarboxylation of l-malate, producing pyruvate, CO_2 and NAD(P)H in the presence of a divalent cation (Chang and Tong 2003). Different isoforms from NADP-dependent malic enzyme (NADP-ME) have been found in bundle sheath chloroplasts of some C_4 plants and the cytosol of some Crassulacean acid metabolism (CAM) plants, where they take part in photosyntetic metabolism, as well as in the cytosol and plastids of photosyntetic and nonphotosyntetic tissues of C_3, C_4, and CAM plants playing different postulated nonphotosyntetic roles (Drincovch *et al*. 2001; Lai *et al*. 2002a, 2002b).

Actually, NADP-MEs from plants can be classified into four groups: group I corresponding to cytosolic isoform from dicot, while group II includes plastidic isoform from dicot; group III comprehends an isoform found into monocot and the novel group IV comprises an isoform found into both monocot and dicot. Recently, Wheeler et al (2005) found four NADP-ME isoforms from *Arabidopsis thaliana*, a dicot plant, being named AtNADP-ME1 to AtNADP-ME4. AtNADP-ME2 and NADP-ME3 belonging to group I, while AtNADP-ME4 is included in the group II and AtNADP-ME1 is incorporated the group IV. Neither of the AtNADP-ME isoforms belongs to group III.

In a previous paper we demonstrated an increase of malic enzymes (NAD-ME and NADP-ME) activities in *Eucalyptus citriodora* submitted to a salt treatment (Aragão *et al*. 1997). It was suggested that an increase in NADP-ME activity was necessary by providing reducing power (NADPH) to supply the detoxification system responsible for elimination of reactive oxygen species (ROS).

ROS are byproducts from reactions involved in normal metabolism, such as photosynthesis and respiration. Furthermore, ROS act as signals for the activation of stress-

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response and defense pathways and processes such as programmed cell death (PCD) and pathogen defense (for review, see Mittler 2002, Bailley-Serres and Mittler 2006). Thus, ROS can be viewed as cellular indicators of stress and as secondary messengers involved in the stress-response signal transduction. Nevertheless, the production of ROS in cells is low under normal growth conditions, whereas, under stress conditions an imbalance occurs between ROS productions and antioxidant content. Therefore, plants need a reduced environment for their protection against the deleterious effects of ROS.

Resistance to oxidative stress is accompanied by a more active ascorbate-glutathione cycle and implicated in cross-tolerance to a variety of stresses (Dalton *et al*. 1996). Therefore, the study of the effect of salt and water stresses on antioxidant enzymes could provide a way to understand the different responses of *V. unguiculata* cultivars to stress. Due to the important role of NADPH in defense reactions of plant to ROS, it was necessary to confer possible roles of the supplement of NADPH by NADP-ME.

We previously verified modifications on RUBISCO (ribulose-1,5-bisphosphate carboxylase:oxygenase) contents from two cultivars of *V. unguiculata*: Vita 3, considered being more resistant and Vita 5, considered more susceptible to salt stress (Aragão *et al*. 2005). Furthermore, we demonstrated that both cultivars showed a significant reduction in shoot length, leaf area and dry mass. These reductions were more accentuated for cv. Vita 5, although this cultivar showed an increase in RUBISCO activity as well as RUBISCO content.

In the present study, we report the action of salt and water stresses on NADP-ME from cvs. Vita 3 and Vita 5, as well as some enzymes that participate in the defense system of the plant against oxidative stress, more specifically superoxide dismutase (SOD), glutathione reductase (GR), ascorbate peroxidase (APX) and catalase (CAT). The analyze also include measurements of osmotic potential, stomatal conductance and relative water content (RWC), which are indicators of salt and water stress, as well as malondialdehyde (MDA) content, the major product from lipid peroxidation by ROS.

MATERIALS AND METHODS

Plants

Seeds of two cultivars from *Vigna unguiculata* (Vita 3 and Vita 5) were obtained of seed's bank from *Universidade Federal do Ceará*. The seeds were treated with 0.4% (w/v) sodium hypochlorite and germinated in the dark for 4 days, in perlite soaked in distilled water. The plants were then transported to a greenhouse and watered daily with a nutritive solution contained: 5.6 mM $Ca(NO_3)_2$, 2 mM KNO_3, 2 mM KH_2PO_4, 2 mM $MgSO_4$, 0.1 mM FeEDTA, 40.4 µM H_3BO_3, 7.5 µM $MnCl_2$, 0.2 µM $CuCl_2$, 0.05 µM MoO_4 and 0.6 µM $ZnSO_4$ made up in deionized H_2O and a pH around 6.5. Ten days after germination, three groups of plants were separated and the following treatments were applied: one group was watered with 150 mM NaCl into nutritive solution (salt stress); the second group was watered with a solution nutritive enriched with 41 mM PEG (water stress) and a control group still being watered with nutritive solution only. Concentrations were calculated for a water potential equal to -0.37 MPa in both treatments, since NaCl concentrations higher 150 mM inhibited drastically growth of cultivar seedlings (results not shown). On the 10th day after treatment application, the plants were collected and used for different experimental purposes.

Stress parameters

Determination of relative water content (RWC) was measured in leaves from plants cultured in control conditions and plants grown under stresses (150 mM NaCl and 41 mM PEG). Therefore, leaves were detached, weighted and dried in stuff during 72 hours at 60° C, then weighted again. RWC was calculated according to formula: RWC = (FW-DW)x100/(TW-DW) where FW means fresh weight, DW signifies dry weights and TW corresponds to turgid weight, i.e. leaves were left overnight in deionized water and weighed.

Measurements of osmotic potential were realized in a Vapor Pressure Osmometer (VAPRO model 5520, WESCOR). Fluids from leaves were obtained from control and stressed plants by pressure with syringes according to the manufacturer's instructions. Ten µl of different samples were placed into the osmometer calibrated with different concentrations of NaCl and osmotic potentials were calculated automatically.

Stomatal conductance was determined daily during the treatment period by measuring the flux of air traversing a cell containing the leaf sample utilizing the Li-Cor 6200 photosynthesis system (Li-Cor, Inc, Lincoln, NE, USA). All measurements were made at 22 ± 2°C and 70 ± 5% relative humidity.

Protein extraction

Crude extracts were obtained from the leaves of both cultivars, in either control conditions (watered just with nutritive solution), or water stress (solution nutritive more 41 mM PEG) or salt stress (solution nutritive more 150 mM NaCl salt) treatments. All utilized reagents were purchased from Sigma except to Sephadex G-25 columns which were Pharmacia. The leaves were ground in a homogenizer (Ultra-turrax Jauke et Kunkel) containing extraction buffer (50 mM Tris/HCl, 5 mM EGTA, 5 mM $MgCl_2$, 0.15% (w/v) PVP 25,000, 0.20% (w/v) PEG 20,000, 5 mM ascorbate, 0.04% (w/v) triton, 50% (w/v) PVPP, pH 7.8) and centrifuged (20,000 × *g* for 30 min, ultra-centrifuge Kontron). The homogenate was desalted by molecular exclusion chromatography on a Sephadex G-25 column, balanced with the wash buffer (50 mM Tris, 5 mM EGTA, 5 mM $MgCl_2$, 5 mM ascorbate). Protein contents of the crude extracts obtained were determined according to Bradford (1972).

Enzyme activities

Enzyme activities were determined spectrophotometrically in the crude extracts obtained from the leaves of both cultivars according to the following protocols:

NADP-ME activity was measured according to Aragão *et al*. (1997) following an increase in the absorbance of NADPH at 340 nm. The unit employed was the nanokatal, defined as the amount (in nmol) of NADPH produced *per* second, *per* mg of protein.

GR activity was measured following the formation of DTN from DTNB at 418 nm (Smith *et al.* 1988). NADPH reduces GSSH and DTNB is reduced by GSH formed from NADPH. The unit employed was the nanokatal, defined as the amount (in nmol) of consumed NADPH with DTN formation, *per* second, *per* mg of protein.

SOD activity was measured using the system of adrenochrome formation at 480 nm, due to the oxidation of adrenaline by superoxide ions (Misra and Fridovich 1972; Polle *et al.* 1989). SOD inhibits adrenochrome formation by diminishing superoxide ions concentration. The data were plotted and the activity was calculated by the method of Asada *et al*. (1974). A unit of SOD (U) was defined as the amount of protein capable of inhibiting 50% of adrenaline oxidation.

CAT activity was measured following the decrease in absorbance of H_2O_2 at 240 nm (Chance and Maehly 1955). The unit (U) was defined as the amount (in µmol) of consumed H_2O_2, *per* minute, *per* mg of protein.

APX activity was measured following the decrease in absorbance of ascorbate at 298 nm due dehydroascorbate formation (Nakano and Asada 1981). The unit (U) was defined as the amount (in µmol) of consumed ascorbate, *per* minute, *per* mg of protein.

Malondialdehyde content

Malondialdehyde (MDA) content of crude extracts obtained from leaves of the different cultivars grown either control or stress conditions (water and salt) were measured according to Heath and Packer (1968) with some modifications. One volume equals 0.5% thiobarbituric acid dissolved in 20% trichloroacetic; samples were incubated at 90°C for 30 min. After centrifugation (20,000 × *g* for 30 min, ultra-centrifuge Kontron), the amount of MDA was calcu-

lated according to the following formula:

$$\text{MDA (nmol (mg protein)}^{-1}) = \frac{(OD_{562} - OD_{600}) \times 1000}{\varepsilon \times b \times \text{protein } (\mu g)}$$

[ε: (extinction coefficient) = 155 liter mmol^{-1} cm^{-1}. B: (light distance) = 1 cm].

Native electrophoresis

The desalted crude extracts from leaves of both cultivars – either control condition or under salt and water stress – were submitted to native electrophoresis in a 7 or 12% polyacrylamide gel. Electrophoresis were performed according to the discontinuous system developed by Davis (1964), at 4°C using a mini-protean system (BIO-RAD) and the polyacrylamide gel was run for 40 mA and at an initial voltage equal to 150 V. Each sample was applied to the gel in a fixed amount (20 µg). Gels were stained for SOD activity according to Droillard *et al*. (1989) and treated with inhibitors (KCN and H_2O_2) according to Beauchamps and Fridovich (1971). Gels analyzed for NADP-ME activity were first incubated for 10 min in a solution of 50 mM Tris-HCl (pH 8.0), then incubated in the same buffer enriched with 60 mM malate, 8 mM NADP, 4 mM $MnCl_2$, 0.16 mM PMS and 0.35 mM NBT (Aragão *et al*. 1977). The procedure for developing colour was carried out in the dark until the appearance of colored bands. Another gel was used for testing for APX according to Dalton *et al*. (1987). Attempts to detect GR and CAT were unsuccessful.

Statistic analysis

All experiments were repeated three times or more and the data were subjected to one-way analysis of variance (ANOVA). Where significant effects were found, the least significant difference at the 0.05 level of probability was calculated according to Scheffe F Test and used to compare the mean values.

RESULTS AND DISCUSSION

Osmotic potential (**Table 1**) from leaves' fluids of both cultivars was not modified by water stress. However, salt stress significantly decreased the osmotic potential in Vita 5. Recently, we demonstrated that both cultivars showed a significant reduction in several parameters grown, as shoot length, leaf area and dry mass when developed in presence of 100 mM NaCl and the reductions were more accentuated for Vita 5 cultivar (Aragão *et al*. 2005). In this work, we confirmed a higher susceptibility of Vita 5 to salt stress, since lower osmotic potential indicates an elevation in NaCl molarity; therefore, it was less effective than Vita 3 to impede the exacerbated rise of salt towards photosynthetic tissues.

Evolution of the stomatal conductance in Vita 3 and Vita 5 cultivars was measured daily during the application of both treatments (**Figs. 1A, 1B**). The results demonstrated an accentuated decrease of this evolution in Vita 5, indicating that Vita 5 is, indeed, more susceptible to salt and water stress, since a decrease of the stomatal conductance promotes a decrease in the absorption of CO_2. These results explain a diminution of biomass for this cultivar under salt stress in spite of a higher expression of RUBISCO as we have previously demonstrated (Aragão *et al*. 2005).

RWC measures of plant water status in terms of the physiological consequence of cellular water deficit. It estimates the current water content of the sampled leaf tissues relative the maximal water content it can hold at full turgidity. RWC decreased in cv. Vita 3 when submitted to water stress. However, there was no significant difference for Vita 5 in either salt or PEG treatment (**Figs. 2A, 2B**).

Table 1 Osmotic potential of leaves' fluids from Vita 3 and Vita 5 cultivars grown under control conditions or under water and salt stresses. The unit used was MPa.

Cultivar	Control	41 mM PEG	150 mM NaCl
Vita 3	-0.336 ± 0.077	-0.321 ± 0.036	-0.349 ± 0.036
Vita 5	-0.307 ± 0.048	-0.292 ± 0.046	-0.433 ± 0.070*

(*) Differences from control values were significant at P < 0.05 according to the Scheffe F test.

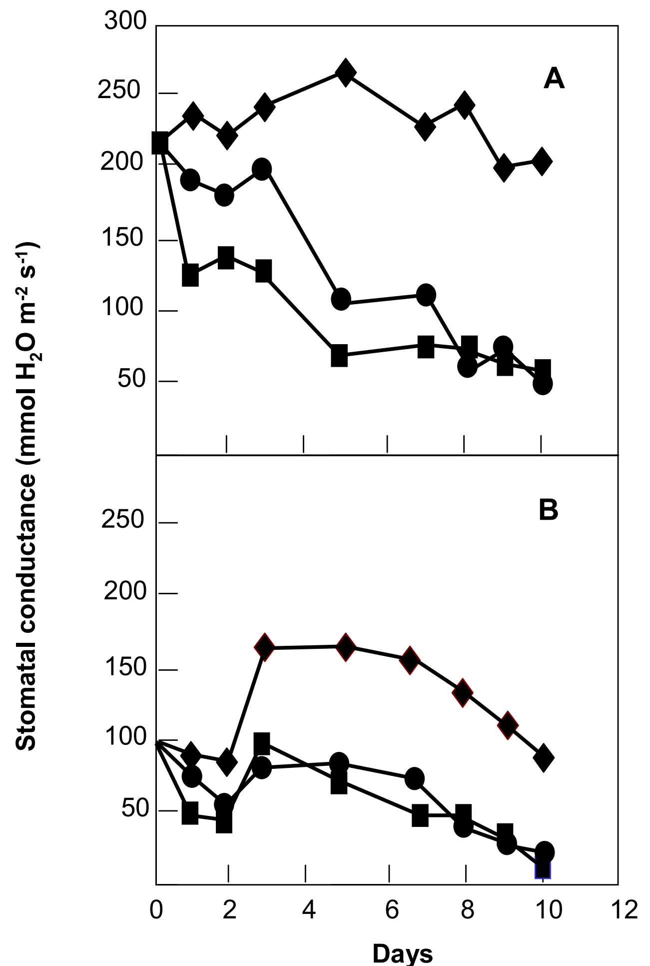

Fig. 1 Evolution of stomatal conductance from Vita 3 (A) and Vita 5 (B) cultivars of *Vigna unguiculata* grown under control conditions (♦), in a water (41 mM PEG) (■) and in salt (150 mM NaCl) (●) stress on the stomatal conductance. Results represent the average of 3 different samples.

Early papers analyzed the susceptibility of Vita 5 to salt treatment by evaluation of important enzyme from energetic metabolism. Thus, Melo *et al.* (1994) demonstrated that mitochondrial ATPase was inhibited in Vita 5 cultivar under a salt treatment. Otoch *et al*. (2001) showed that 2-day-old seedlings from Vita 5 submitted to treatment with 100 mM NaCl decreased the proton transport and hydrolytic activities of both tonoplast V-ATPase and the H^+ -PPase. Therefore, in the present study we examined others important enzymes implicated in the metabolism as well as those responsible in defense against oxidative metabolites. Thus, comparative studies in enzyme activities were carried out in crude extracts of control and of stressed plants (water and salt stresses) from both cultivars and these spectrophotometric analysis revealed significative differences between control and stressed plants as well as among different cultivars from cowpea under two types of stress (**Figs. 3, 4**). NADP-ME activity increased significantly when Vita 3 was subjected to water stress (**Fig. 3**). But, this activity did not change in cv. Vita 5 under both stress treatments. This increase in NADP-ME activity in Vita 3 was accompanied by an increase in GR activity, which was also not verified for Vita 5 (**Fig. 3**). SOD activity was not modified by salt stress in both cultivars, although there were increase in cv. Vita 3 subjected to water stress (**Fig. 4**).

Native electrophoresis revealed four isoforms from

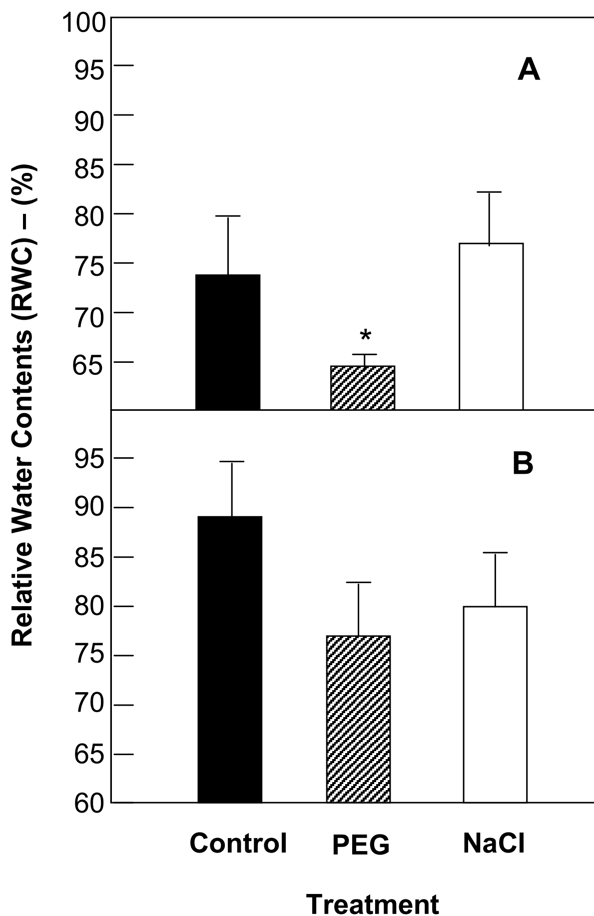

Fig. 2 Effect of water (41 mM PEG) and salt (150 mM) stress on the relative water contents (RWC) from Vita 3 (A) and Vita 5 (B) cultivars of *Vigna unguiculata*. Differences from control values were significant at P < 0.05 (*) according to the Scheffe F test.

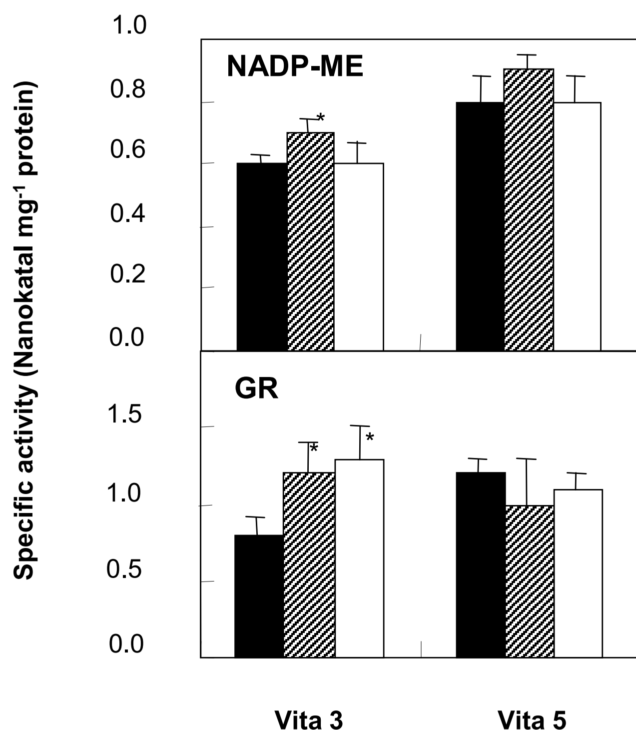

Fig. 3 Effect of water and salt stress on the specific activities of NADP-dependent malic enzyme (NADP-ME) and glutathione reducetase (GR). The plants were grown in nutritive solution (control) (■), nutritive solution enriched with 41 mM PEG (▒) or nutritive solution enriched with 150 mM NaCl (□). The leaves were collected for determining the enzymatic activities. Each bar represents the mean ± SD for three repetitions. Differences from control values were significant at P < 0.05 (*) according to the Scheffe F test.

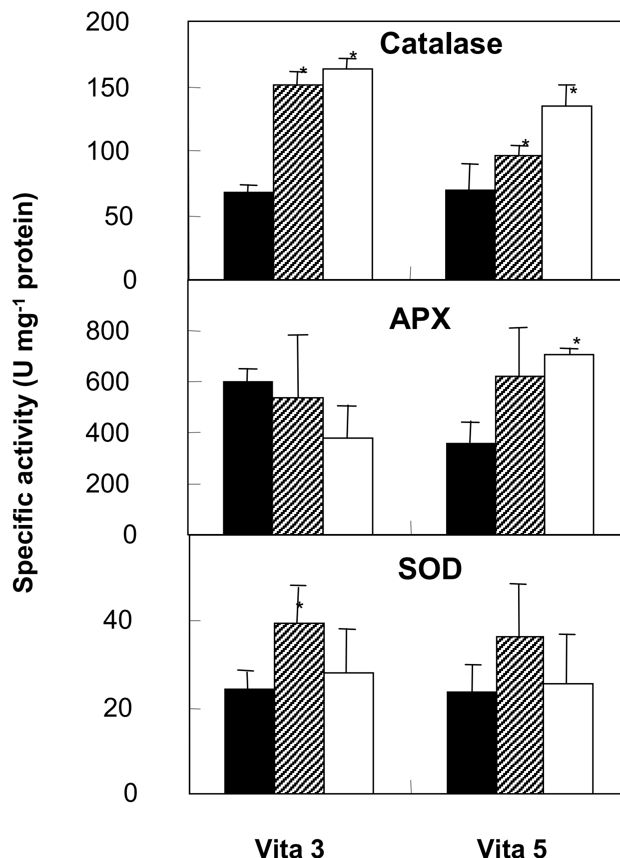

Fig. 4 Effect of water and salt stress on the specific activities of catalase, ascorbate-peroxydase (APX) and superoxide-dismutase (SOD). The plants were grown in nutritive solution (control) (■), nutritive solution enriched with 41 mM PEG (▒) or nutritive solution enriched with 150 mM NaCl (□). The leaves were collected for determination of the enzymatic activities. Each bar represents the mean ± SD for three repetitions. Differences from control values were significant at P < 0.05 (*) according to the Scheffe F test.

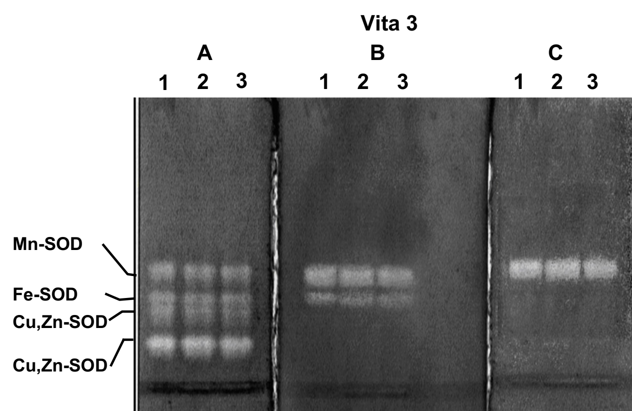

Fig. 5 Detection of SOD activity extracted of leaves from Vita 3 cultivar grown under control conditions (lane 1), under water (lane 2) or salt (lane 3) stresses. Gel B was subjected to KCN and C was subjected to H_2O_2. 40 μg of soluble proteins were loaded into each lane.

SOD into two cultivars: one Mn-SOD, one Fe-SOD and two Cu, Zn-SOD (**Figs. 5, 6**). Three isoforms were detected for APX (**Fig. 7**). Concerning to NADP-ME, only one isoform was detected in both cultivars (**Fig. 8**).

According to Wheeler *et al*. (2005) NADP-ME2 is responsible for the major part of NADP-ME activity in mature tissues of Arabidopsis. They demonstrated that NADP-ME2 and NADP-ME4 are constitutively expressed, while the expression of NADP-ME1 and NADP-ME3 is restricted to developmental stages of the plant rather than being involved in primary metabolism. Only one isoform of NADP-

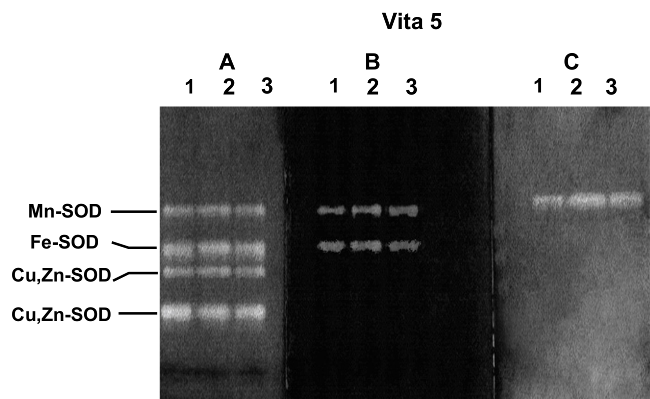

Fig. 6 Detection of SOD activity extracted of leaves from Vita 5 cultivar grown under control conditions (lane 1), under water (lane 2) or salt (lane 3) stresses. Gel B was subjected to KCN and C was subjected to H_2O_2. 40 µg of soluble proteins were loaded on each lane.

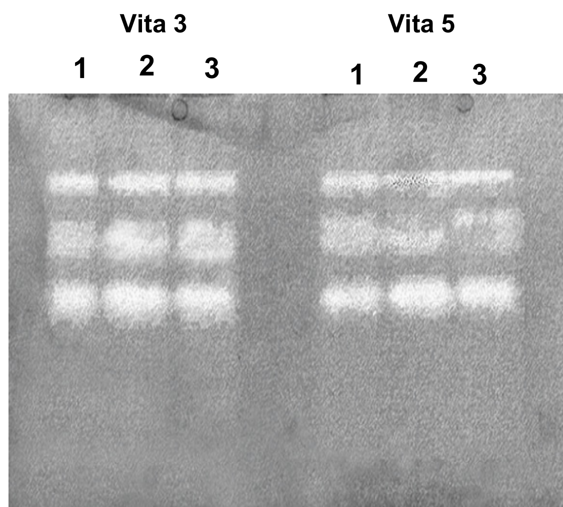

Fig. 7 Detection of APX activity extracted of leaves from Vita 3 and Vita 5 cultivars grown under control conditions (lane 1), under water (lane 2) or salt (lane 3) stresses. 40 µg of soluble proteins were loaded into each lane.

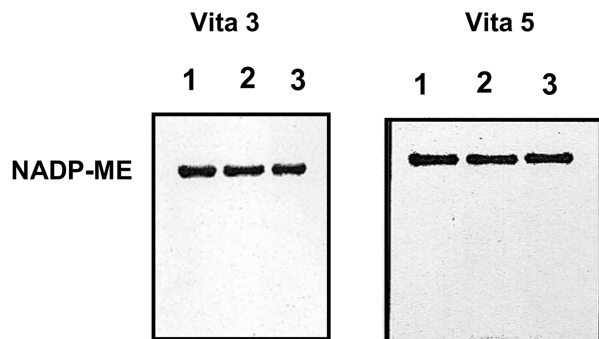

Fig. 8 Detection of NADP-ME activity extracted of leaves from Vita 3 and Vita 5 cultivars grown under control conditions (lane 1), under water (lane 2) or salt (lane 3) stresses. 40 µg of soluble proteins were loaded into each lane.

ME was detected by native electrophoresis and it showed a profile similar to citosolic isoforms (NADP-ME1, NADP-ME2 and NADP-ME3). Therefore, we suggest that the detected isoform refers to NADP-ME2.

The different gels were scanned and the volume of blots (OD x mm x mm) were quantified with a ScnImage software (Scion Corporation, USA). The results for NADP-ME were compatible with that shown in activities measure by spectrophotemetric analysis.

Enzyme activities, in general, were affected by salt and water stresses. The modifications verified in the enzymes of the SOD-APX-GR cycle (**Fig. 4**) suggested that both water and salt stress induced oxidative stress in both cowpea cultivars. Cv. Vita 5 was less efficient than Vita 3 in overcome the deleterious effects of ROS. Exception was seen for the results obtained for APX that showed an accentuated enhancement for Vita 5 in relation to Vita 3 cultivar (**Fig. 4**). D'arcy-Lameta *et al.* (2006) also showed an elevation of APX activity in another *V. unguiculata* cultivar named '1183' (more susceptible to salt stress) in relation to 'EPACE 1' cultivar, a more tolerant cultivar. Furthermore, the same team found an augmentation transcript of glutathione reductase from 'Epace 1' more accentuated when compared with the same transcript by '1183' cultivar submitted to drought stress (Contour-Ansel *et al.* 2006).

MDA content increased more in the less tolerant cultivar (Vita 5) (**Table 2**), which corroborate with the results obtained with the enzymatic activities that indicate a higher susceptibility for Vita 5 to osmotic stresses.

NADP-ME displays an important role in C_4 and CAM plants by production of CO_2 to photosyntetic process. Thus, a constitutive isoform chlroplastic in C_4 plants and a cytosolic isoform in plant CAM were extensively characterized (Bailley-Serres and Mittler 2006). It is known that an inducible cytosolic isoform from NADP-ME is express in plants as *Flaveria* sp. that display different C_3 and C_4 or CAM photosynthetic pathways (Lai *et al.* 2002a, 2002b). Nevertheless, an increase in expression level of non-photosynthetic NADP-ME mRNA under high-salt or water conditions was reported in the facultative halophyte and CAM plant, *Mesembryanthemum crystallium* demonstrating the importance of malic enzymes in display others roles in process of plant defenses (Cushman 1992). Similar results were found in *Aloe vera*, an obligate monocot CAM when exposed to salt stress conditions (Sun *et al.* 2003). Furthermore, *E. citriodora* (a C_3 plant) increased its NADP-ME activity when submitted to salt stress (Aragão *et al.* 1997). Therefore, these results suggest important biological roles of NADP-MEs in constraint situations, apart from being involved in C_4 and CAM photosynthesis.

Therefore, NADP-ME plays a role in the detoxification process by supplying reducing power (NADPH) for the SOD-APX-GR cycle. Hereby, this result can also explain the greater susceptibility of cv. Vita 5 to stress conditions. This cultivar inefficiently maintained an appropriate redox state inside its biosynthetic machinery to face with the ROS.

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