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Possible Involvement of Organic Compounds and the Antioxidant Defense System in Salt Tolerance of *Medicago arborea* (L.)

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Abstract: The possible involvement organic metabolites and the antioxidative defence system in salt tolerance were investigated in *Medicago arborea* (L.). Plants were subjected to three salt treatments, 100, 200 and 300 mM NaCl for 60 days under glasshouse conditions. The plant growth, leaf water content, solutes content, H_2O_2 generation, lipid peroxidation, membranes stability index, phenols accumulation and antioxidative enzymes activities were quantified in the roots and shoots. Increasing concentrations of salinity induced decrease in plant growth in both organs parts, especially in the shoots. In addition both shoots and roots were able to accumulate a large quantity of Na^+ whereas the contents of K^+ decreased significantly. Total soluble sugars and proline content were increased by salinity but do not seem to play an important role in osmoregulation. Indeed, leaf water content was reduced to 78.9% of the control at 300 mM NaCl, explaining the lower aptitude of *M. arborea* to adjust osmotically. After stress, the accumulation of phenols and the activities of the antioxidative enzymes were changed and the extent of alteration varied between the shoots and roots. Salt stress impacts in term of H_2O_2 generation and lipid peroxidation were more pronounced in the shoots than the roots. The relative antioxidative defence capacity in the leaves of *M. arborea* may be manly attained by the increasing activities of Superoxide Dismutase (SOD) and Catalase (CAT) at lower salt stress whereas at higher salinity the antioxidative defence might be achieved essentially by Peroxidase (POD), Ascorbate Peroxidase (APX) and several metabolites such as phenols and proline. In the roots, the scavenging system might be achieved by SOD, POD and APX activities which showed to participate efficiently in restriction of oxidative damages caused by the H_2O_2 generation.

Key words: Organic metabolites, antioxidative enzymes, lipid peroxidation, salt stress, *Medicago arborea* (L.), Tunisia

INTRODUCTION

Soil salinity is the main abiotic factor limiting plant growth and productivity around the world. This problem is more severe in arid and semi-arid regions (Munns, 2002). Salinity influences plants through osmotic effects, ion specific effects and oxidative stress (Pitman and Lauchli, 2002; Munns and Tester, 2008). All these factors cause adverse effects on plant growth and development at physiological and biochemical levels (Munns, 2002; Munns *et al*., 2006) and at the molecular level (Tester and Davenport, 2003). Osmotic effects are due to salt-induced decrease in the soil water potential. Salinity results in a reduction of K^+ and Ca^{2+} content and an increased level of Na^+ and Cl^- which forms its ionic effects. Indeed, high levels of Na^+ inhibit K^+ absorption which results in a Na^+/K^+ antagonism (Tester and Davenport, 2003). Plant species adapt to high salt concentrations in soils by lowering tissue osmotic potential with the accumulation of inorganic as well as organic solutes (Samaras *et al*., 1995). Organic solutes are accumulated in the cytosol to balance the solute potential of the vacuole which is dominated by ions (Parida and Das, 2005). A large number of plant species accumulate proline in response to salinity stress and their accumulation may play a role in combating salinity stress (Ashraf and Harris, 2004; Parida and Das, 2005). However, data do not always indicate a positive correlation between the osmolyte accumulation and the adaptation to stress (Ashraf and Harris, 2004; Ashraf and Foolad, 2007). In

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addition to its known components of osmotic stress and ion toxicity, salt stress is also manifested as an oxidative stress by inducing an imbalance in cell compartment in the production of ROS and antioxidant defense (Mittler, 2002; Parvaiz and Satyawati, 2008). ROS includes superoxide (O_2^-), hydroxyl radicals (OH^{-1}), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$).

These ROS are highly reactive and can alter the normal cellular metabolism through oxidative damage to proteins and nucleic acids as well as causing peroxidation of membrane lipids (Moller *et al*., 2007). On the other hand, Malondialdehyde (MDA), a product of lipid peroxidation has been considered an indicator of salt-induced oxidative damage and tends to show greater accumulation under salt stress (Sudhakar *et al*., 2001; Meloni *et al*., 2003).

In order to prevent oxidative damages, plants have evolved a complex antioxidant system which includes both enzymatic and non-enzymatic (low-molecular mass antioxidants) components differentially found in cell compartments (Mittler, 2002; Hamed *et al*., 2007), responsible for maintaining the levels of ROS under tight control. Antioxidant compounds (non-enzymatic antioxidants) such as phenolic compounds, ascorbic acid and glutathione are secondary metabolites, their beneficial effects are related to their antioxidant activity (Heim *et al*., 2002), particularly their ability to scavenge free radicals to donate hydrogen atoms or electrons or to chelate metal cations (Sakihama *et al*., 2002; Balasundram *et al*., 2006). In plants, polyphenol synthesis and accumulation is generally stimulated in response to as salinity, suggesting that the presence of those metabolites is related to increased salt tolerance of plants (Navarro *et al*., 2006; Kiarostami *et al*., 2010).

The antioxidative enzymes are the most important components in the scavenging system of reactive oxygen species (Allen, 1995; Dat *et al*., 2000). Thus, theses enzymes such as Superoxide Dismutase (SOD), Catalase (CAT), Peroxidase (POD) and Ascorbate Peroxidase (APX) are essential components of the plant's antioxidant defence system.

Superoxide Dismutase (SOD) catalyzes the 1st step of the enzymatic defence mechanism, the conversion of superoxide radicals to yield molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). The hydrogen peroxide produced is then scavenged by catalase and a variety of peroxidases. Catalase which is apparently absent in the chloroplast, dismutates H_2O_2 into water and molecular oxygen whereas POD decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Mckersie and Leshem, 1994; Meloni *et al*., 2003). The capacity to scavenge ROS and to reduce their damaging effects on macromolecules appears to represent an important stress tolerance trait in higher plants (Xiong and Zhu, 2002; Hamed *et al*., 2007). Moreover, the ability to increase antioxidant capacity in response to salinity has been correlated with salt tolerance in a large number of plants such as *Beta maritima* (Bor *et al*., 2003), *Limonium bicolor* (Li, 2008) and *Medicago sativa* (Wang *et al*., 2009).

Medicago arborea L. is phylogenetically the oldest species in the genus Medicago and the only characterized by shrub growth habit which can adapt to extreme ecological conditions such as drought resistance. The species has potential use as an ornamental plant and is used for pasture in dry lands. The response of this species to salinity is of particular importance as it is among the important species that can be used both as a fixative soil and as fodders in dry lands of the centre and South of Tunisia. Previous studies on Medicago plants report on antioxidant system generated by stress conditions such as salinity and drought (Wang *et al*., 2009; Salah *et al*., 2010). However, the antioxidant modulation in the tissues of *M. arborea* under salt stress conditions is limited and unknown.

In this study, we examined the effects of salinity on plant growth, leaf water, ions content organic osmolytes, total phenols accumulation, level of peroxidation of lipid membranes and antioxidative enzymes activities in this fodder species with increasing salinity levels. Therefore, the main objective is to identify the tolerance mechanism developed to confront salt stress and the eventual involvement of the antioxidant defense system in this mechanism in *M. arborea* (L.).

MATERIALS AND METHODS

Plant growth conditions: *M. arborea* seeds were collected from the region of Benbla (10°79', 544°E and 35°68', 675°N) (Tunisia), sterilized for 20 sec in 3% (w/v) calcium hypochlorite and rinsed three times with deionised water. Seeds were sowed to germinate in plastic containers filled with a mixture of marketed peat and sterile sand (1:1, v/v) and irrigated with deionised water. Plants were initially grown in half-strength Hoagland (1950) solution to supply the macro- and micro-nutrients.

When seedlings were around 7 cm in height (2 months old), they were placed in plastic pots (5 L) filled with mixture of peat and perlit (2:1, v/v). Irrigation was with one-half strength Hoagland solution and with distilled water on alternated days for acclimatization during 15 days. The experiments were conducted in a greenhouse under controlled conditions with the following regimes: temperature; min/max 17/35°C; relative

humidity; min/max 30/70%; photoperiod (14/10 h day/night). At the end of the acclimatization phase, seedlings of *M. arborea* were divided into 4 groups for treatments (10 plants per treatment): 0 mM NaCl (control); 100, 200 and 300 mM NaCl. These treatments were watered with 200 mL of salt solutions every 2nd day to avoid excessive accumulation of salt due to loss of water during evaporation.

At the initiation of the experiment, salinity concentrations were gradually increased by 50 mM NaCl at 2nd day interval to reach maximum salinity levels. Salt solutions were completely replaced once a week to maintain salinity levels in the pots. The experiment was performed for a total period of 60 days.

Growth activity and water content: The plant material was 1st cleaned with distilled water. After the water on the plant was absorbed by tissue study, Fresh Mass (FM) was measured. The Dry Mass (DM) was measured after the fresh material was dried at 70°C for 48 h. The water content of the Leaves (LWC) was determined as follows:

$$LWC = [(FM - DM)/FM] \times 100$$

Determination of organic and inorganic solutes: Dried samples (15 mg from 4 independent plants per treatment) were ground into a fine powder for wet digestion and dry ashing. The ash was dissolved with concentrated nitric acid and then set to a volume of 20 mL with distilled water. Cations such as Na^+ and K^+ were determined with a flame photometer (Model 410, Corning, Halstead, UK). Organic solutes content were determined in leaves of four plants per treatment. Dry plant material (25 mg) was extracted with 80% ethanol at 80°C.

The solution was filtered and concentration of total soluble sugars was determined by the Anthrone colorimetric method. Proline was also determined spectrophotometrically following the Ninhydrin method described by Bates *et al.* (1973). Approximately 300 mg of dry tissue was homogenized in 10 mL of 3% aqueous sulphosalicylic acid and filtered. To 2 mL of the filtrate, 2 mL of acid ninhydrin were added followed by the addition of 2 mL glacial acetic acid and boiling for 60 min.

The mixture was extracted with toluene and the free proline was quantified spectrophotometrically at 520 nm from the organic phase using, toluene as a blank. The osmolarity was estimated by 2 (Na^++K^+)/water content and the cytoplasm organic compounds contents was achieved by reducing organic compounds content to those in the water and on the assumption that the cytoplasm is 5% of the total cell volume (Fernandez-Ballester *et al.*, 1998).

Hydrogen peroxide (H_2O_2) estimation: The H_2O_2 content was determined as described by Velikova *et al.* (2000). Fresh leaf tissue (0.5 g) was homo-genized with 5 mL of 0.1% (w/v) Trichloroacetic Acid (TCA) inapre-chilled pestle and mortar. This homogenate was then centrifuged at 12,000 g for 15 min. Total 0.5 mL of the supernatant 0.5 mL of potassium phosphate buffer (pH 7.0) and 1 mL of potassium iodide were added. The mixture was vortexed and its absorbance was read at 390 nm using a UV-visibles spectrophotometer (UV-2500, Shimadzu Corp., Kyoto, Japan) and the H_2O_2 concentration was calculated according to the standard curve.

Lipid peroxidation: Lipid peroxidation was estimated by determining the Malonyldialdehyde (MDA) content in the leaves according to the method of Cakmak and Horst (1991). Fresh leaf samples (0.5 g) were ground in 5 mL of 0.1% (w/v) Trichloroacetic Acid (TCA) at 4°C. The homogenate was centrifuged at 12,000×g for 5 min. About 1 mL aliquot of the supernatant was mixed to 4 mL of 0.5% (w/v) Thiobarbituric Acid (TBA) prepared in 20% (w/v) TCA and incubated at 90°C for 30 min. Thereafter, the reaction was stopped in ice bath. Centrifugation of the samples was performed at 10,000×g for 5 min and absorbance of the supernatant was measured at 532 nm on a spectrophotometer (UV-2500, Shimadzu Corp., Kyoto, Japan). After subtracting, the non-specific absorbance at 600 nm, the malondialdehyde content was calculated using its absorption coefficient (ε) and expressed as nmol malondialdehyde g^{-1} fresh mass following the equation:

$$MDA\ (nmoL\ g^{-1}\ FM) = [(A532 - A600) \times V \times 1000)/\varepsilon] \times W$$

Where:

ε = The specific extinction coefficient (155 mM cm^{-1})
V = The volume of crushing medium
W = The fresh weight of leaf
A600 = The absorbance at 600 nm wavelength
A532 = The absorbance at 532 nm wavelength

Membrane Stability Index (MSI): Membrane Stability Index (MSI%) was measured as described by Lutts *et al.* (1996). Samples were washed with deionized water to remove surface adhered electrolytes and cut into discs of uniform size.

Leaf and root discs were put in closed test tubes containing 10 mL of deionized water and incubated at 25°C for 24 h and sub-sequently electrical conductivity of the solution (C1) was recorded. Samples were then autoclaved at 120°C for 20 min and the final electrical

conductivity (C2) was obtained after equilibrium at 25°C. The membrane stability index was defined as:

$$MSI\ (\%) = (C1/C2)\times 100$$

Extraction and analysis of phenolic compounds: The extraction procedure was determined using the method described by Waterman and Mole (1994) with some modifications. Briefly, lyophilised leaf samples (5 g) were extracted twice with 100 mL of 70% methanol thereafter with 100 mL of 70% acetone at the temperature of 4°C. Then extraction was renewed with absolute methanol. The extracts were filtered, mixed and concentrated at 240 mbar pressure in a roto-evaporator (Heidolph Elektro GmbH and Co., WB 2000, Kelheim, Germany) at 40°C. After the elimination of organic solvents, the total aqueous excerpt was centrifuged at 15,000×g for 15 min then washed by the dichloromethane to remove the chlorophylls and the lipids traces.

The gotten total aqueous phase was evaporated until dryness and the residual was taken in the absolute methanol to constitute a total phase containing the set of polyphenols. Phenolic content was assayed using the Folin-Ciocalteu reagent following Singleton's method slightly modified (Dewanto *et al*., 2002). An aliquot (0.125 mL) of appropriately diluted sample extract was added to 0.5 mL of distilled water and 0.125 mL of the Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min before adding 1.25 mL of 7% Na_2CO_3 solution. The solution was then diluted with deionized water to a final volume of 3 mL and mixed thoroughly. After incubation for 90 min at 23°C in dark, the absorbance was measured at 765 nm by using a UV-Vis spectrophotometer (UV-2500, Shimadzu Corp., Kyoto, Japan). Total phenolic content of leaves (3 replicates treatment^{-1}) was expressed as mg Gallic Acid Equivalents (GAE) per gram of dry weight (mg GAE/g DM) through the calibration curve with gallic acid. The calibration curve range was 0-400 mg mL^{-1}.

Determination of enzymatic activities: For the enzyme assays, 0.3 g leaves were ground with 2 mL ice-cold 25 mM HEPES buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM ascorbate and 2% Polyvinyl Polypyrrolidone (PVPP). The homogenates were centrifuged at 4°C for 20 min at 12,000×g and the resulting supernatants were used for the determination of enzymatic activity and protein content assays (Zhu *et al*., 2000). All steps in the preparation of the enzyme extract were carried out at 4°C. All spectrophotometric analyses were conducted on a Shimadzu UV-2500 spectrophotometer. Each measurement of antioxidant enzymes was made four replicates in four plants. Protein concentration was determined using a Coomassie brilliant blue with bovine serum albumin as the standard (Bradford, 1976). The Superoxide Dismutase (SOD) activity was estimated by measuring its ability to inhibit the photochemical reduction of Nitroblue Tetrazolium (NBT) (Rao and Sresty, 2000). The reaction mixture (3 mL) contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT nitroblue tetrazolium, 2 μM riboflavin, 0.1 mM EDTA and 0.05 mL of enzyme extract. The reaction was started by adding 2 μM riboflavin and placing the tubes under 15 W fluorescent lamps for 15 min.

A complete reaction mixture without enzyme which gave the maximal color served as control. The reaction was stopped by switching off the light and then the tubes were covered with a black cloth. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm and 1 Unit enzyme activity (U) was defined as the quantity of SOD required to produce a 50% inhibition of reduction of Nitroblue Tetrazolium (NBT) and the specific enzyme activity was expressed as unit/mg protein. POD activity was measured by monitoring the increase in absorbance at 470 nm in 50 mM of phosphate buffer (pH 5.5) containing 1 mM of guaiacol, 0.5 mM of H_2O_2 and 0.1 mL of enzyme extract. One unit of POD activity is defined by the increase in absorbance at 470 nm for 1 min due to guaiacol oxidation. Catalase (CAT) activity was estimated according to Chakraborty and Tongden (2005) which measures the initial rate of disappearance of H_2O_2 at 240 nm. The CAT reaction mixture contained 50 mM of Na-phosphate buffer pH 7.0, 15 mM of H_2O_2 and 0.1 mL of enzyme extract. Changes in the absorbance of the reaction solution at 240 nm were recorded after every 20 sec. One unit of CAT activity is defined by the decrease at 240 nm for 1 min due to H_2O_2 consumption. The activity of APX was assayed according to the method described by Nakano and Asada (1981) using ascorbic acid as a substrate. The oxidation of ascorbate was initiated by H_2O_2 and the decrease at 290 nm was monitored for 1.5 min. One unit of APX was defined as the amount of enzyme required to oxidize 1 mM of ascorbate.

Statistical analysis: A one way ANOVA was achieved to compare the mean values using the SPSS statistical package ($p<0.05$). In case of significant differences, Duncan post-hoc tests were performed to compare the means.

RESULTS AND DISCUSSION

Plant growth leaf water content and ions contents: The presence of NaCl in culture medium inhibited the growth of *M. arborea* by decreasing significantly ($p<0.05$) shoot

and root dry mass (Fig. 1a). Indeed after 2 months of treatment with 100 mM NaCl, the dry mass of shoots and roots were reduced by 23.8 and 20.1%, respectively as compared to the control. This reduction increased with increasing salinity and reached 69.3 and 51.6% at 300 mM NaCl in the shoots and roots of the plants, respectively. *M. arborea* plants accumulated Na^+ with increasing NaCl concentration and the highest levels was observed in their shoots. Indeed at 100 mM NaCl salt stress, treatment Na^+ content of shoots and roots was 7.1 and 6.7 times than that in the control (Table 1). At 300 mM NaCl level, the values of Na^+ content reached 19.6 and 23.6 times than that in control in the shoots and roots of *M. arborea*, respectively. In contrast, K^+ content decreased with increasing salt supply in both plants parts, especially in the shoots. At 100 mM NaCl level, the percentage inhibition of K^+ content as compared with control non-treated plants was 64% in the shoots and 54.6% in the roots (Table 1). For higher salinity (300 mM), K^+ content decreased down to a minimum values which represents a 85.6 and 81.6% of reduction as compared with the control in the shoots and roots of *M. arborea*, respectively. The data shows that the Na^+/K^+ ratio increases progressively with increasing salt concentration. In plants grown at 300 mM NaCl treatment, this ratio was 137 times greater in the shoots and 139 times greater as compared with the control in the roots. Leaf water content was unchanged at low salinity (100 mM NaCl) whereas, it reduced et higher salinities representing 86.4 and 78.9% of the controls in the plants subjected to 200 and 300 mM NaCl, respectively (Fig. 1b).

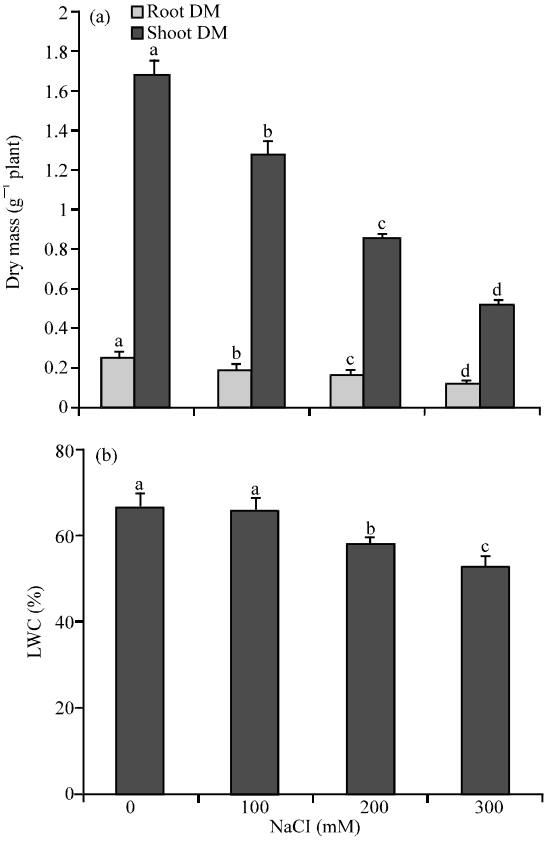

Fig. 1: Effects of NaCl treatments (0, 100, 200 and 300 mM) on; a) shoot and root dry mass and b) leaf water content in *M. arborea* plants. Bars followed by the same letter are not statistically different at $p<0.05$ (Duncan's multiple range test). Averages of four repetitions are presented with bars indicating SE

Oxidative stress evaluation: Results in Table 2 showed that lipid peroxidation (MDA content), H_2O_2 generation and Membrane Stability Index (MSI) changed with increasing salinity in *M. arborea* shoots and roots. As compared with control, the MDA amount and H_2O_2 content showed significantly increase as salinity raise and the greatest increase was observed in the shoots as compared with roots. Thus, *M. arborea* plants treated

Table 1: Effect of different NaCl concentrations on the content of sodium and potassium and Na^+/K^+ ratio in the shoots and roots of *M. arborea* plants

	Na^+ (μmol g^{-1} DM)		K^+ (μmol g^{-1} DM)		Na^+/K^+	
NaCl (mM)	Shoot	Root	Shoot	Root	Shoot	Root
0	198±14^d	112±7^d	2156±72^a	2593±55^a	0.09±0.003^d	0.04±0.003^d
100	1423±75^c	760±45^c	775±40^b	1176±16^b	1.84±0.16^c	0.64±0.04^c
200	3160±111^b	1861±105^b	483±22^c	739±31^c	6.56±0.50^b	2.53±0.13^b
300	3881±113^a	2650±110^a	310±32^d	476±31^d	12.35±1.24^a	5.58±0.31^a

Data are means values±SE of four measurements. Values in each column with the same letter are not significantly different (p = 0.05) as described by Duncan's test

Table 2: Effects of NaCl treatments (0, 100, 200 and 300 mM) on the concentration of hydrogen peroxide (H_2O_2), Malondialdehyde (MDA), Membrane Stability Index (MSI) and total phenols in *M. arborea* plants

	H_2O_2 (μmol g^{-1} FM)		MDA (nmol g^{-1} FM)		MSI (%)		Total phenols (mg GAE g^{-1} DM)
NaCl (mM)	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
0	4.6±0.4^d	4.5±0.4^b	15.4±0.8^d	14.2±0.4^d	90.5±3.8^a	90.6±0.4^a	0.48±0.12^c
100	6.2±0.3^c	4.6±0.4^b	21.7±1.2^c	15.5±0.4^c	80.2±2.9^b	88.5±0.4^a	1.27±0.21^b
200	7.5±0.5^b	5.1±0.4^a	43.2±3.5^b	18.4±0.4^b	59.7±1.7^c	74.7±0.4^b	1.45±0.18^b
300	9.7±0.6^a	5.3±0.4^a	77.5±4.6^a	25.1±0.4^a	33.2±1.5^d	61.8±0.4^c	3.07±0.24^a

Data are means values±SE of four measurements. Values in each column with the same letter are not significantly different (p = 0.05) as described by Duncan's test

with 100 and 300 mM NaCl showed large increases in lipid peroxidation, revealed by MDA concentrations, representing 1.4 to 5 fold, the control in shoots and only 1.1-1.7 fold the control in roots, respectively at 100 and 300 mM NaCl (Table 2). In this range of salinities, the increase in H_2O_2 content reached 1.3 to 2.1-fold increases in shoots and only 1.01 to 1.16-fold increases due to salt stress in the roots. The Membrane Stability Index (MSI), estimated as electrolyte leakage decreased significantly under salinity stress, especially in the shoots. Indeed after 2 months of treatment with 300 mM NaCl the membrane stability index were reduced by 63.3% in the shoots and only by 31.7% in roots as compared to the control (Table 2 and Fig. 2a). There was a positive linear correlation ($r = 0.96$, $F = 160.2$, $p<0.0001$ in the shoots; $r = 0.84$, $F = 34.6$, $p<0.0001$ in the roots; Fig. 3a) between the MDA accumulation and H_2O_2 content in shoots and roots of *M. arborea*. Whereas a negative relationship was observed between the Membrane Stability Index (MSI) and H_2O_2 generation ($r = -0.97$, $F = 262.4$, $p<0.0001$ in the shoots; $r = -0.92$, $F = 74.1$, $p<0.0001$ in the roots; Fig. 3b). On the other hand, a highly negative relationship was observed between MDA accumulation and biomass production ($r = -0.94$, $F = 111.6$, $p<0.0001$ in the shoots; $r = -0.91$, $F = 65.1$, $p<0.0001$ in the roots; Fig. 4a).

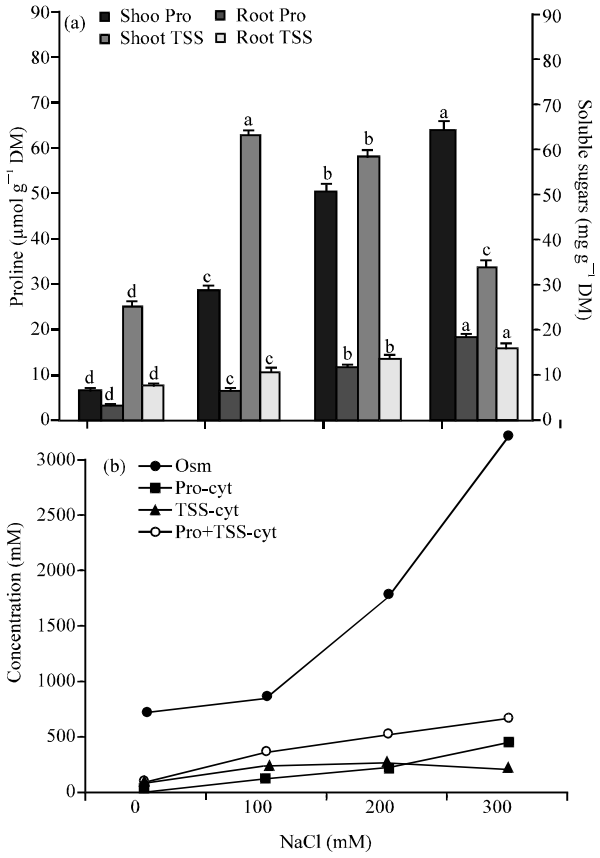

Fig. 2: Effects of NaCl treatments (0, 100, 200 and 300 mM) on; a) Proline (Pro) and Total Soluble Sugars (TSS) content in the shoots and roots of *M. arborea*; b) the comparison between the osmolarity (Osm) and cytoplasmic concentration of Proline (Pro cyt) and soluble sugars (TSS-cyt) of *M. arborea* shoots. Bars followed by the same letter are not statistically different at $p<0.05$ (Duncan's multiple range test). Averages of 4 repetitions are presented with bars indicating SE

Proline, soluble sugar and total phenols accumulation: The study showed that proline content increased linearly with NaCl concentration and the higher accumulation was occurred in the shoots reaching 9.3-fold higher at 300 mM NaCl than that in control and only 5.9-fold higher in the

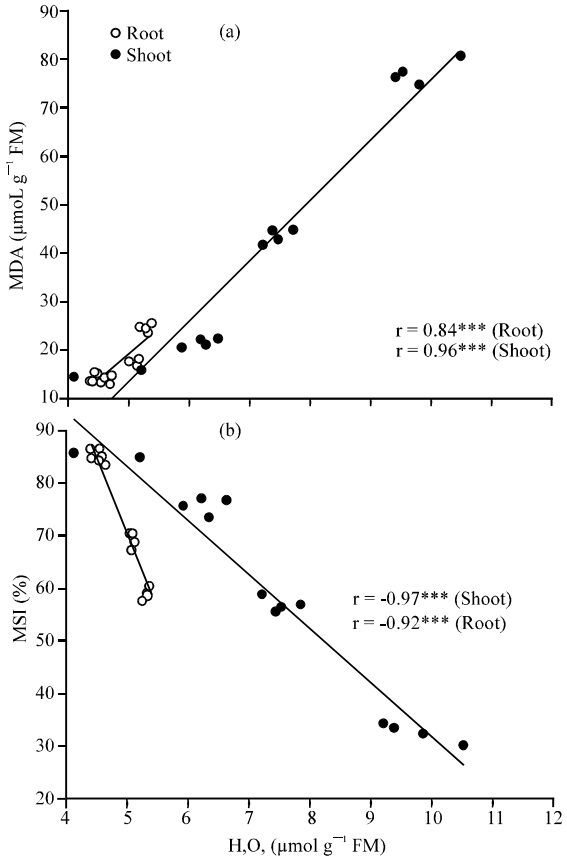

Fig. 3: Relationships between a) Hydrogen peroxide (H_2O_2) contents and Malondialdehyde (MDA) accumulation and b) Membrane Stability Index (MSI) in the shoots and roots of *M. arborea* cultivated under salt stress. An average of 5 repetitions and confidence interval was calculated at the threshold of 95%

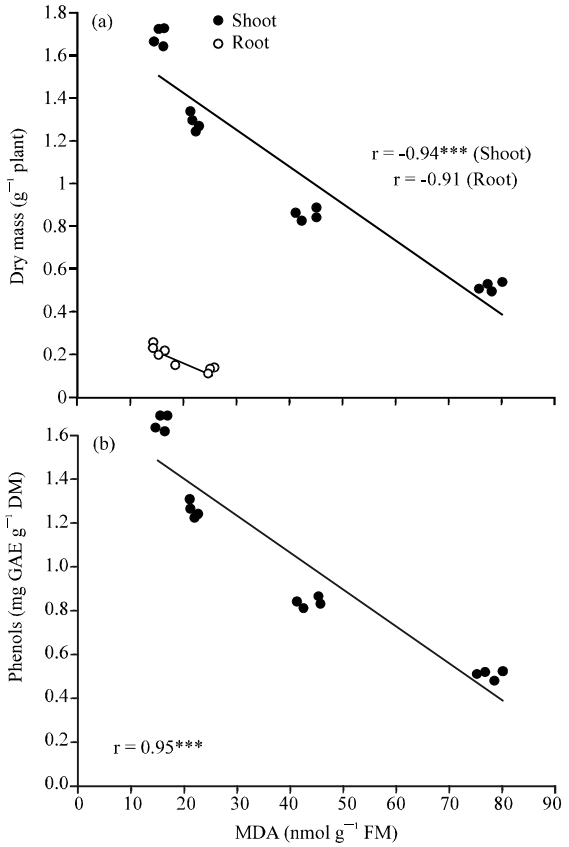

Fig. 4: Correlation between; a) Malondialdehyde (MDA) and biomass accumulation and b) total phenols amount at *M. arborea* cultivated under salt stress. An average of 5 repetitions and confidence interval was calculated at the threshold of 95%

roots (Fig. 2a). Shoot total soluble sugars content was promoted at 100-200 mM NaCl treatments and reached 248% of the control at 100 mM. However, the amount of soluble sugars decreased at 300 mM but still higher than the amount accumulated in control plants (Fig. 2a).

The roots total soluble sugars amount increased linearly with NaCl concentration reaching 2.1-fold higher at 300 mM NaCl than the control. The amount of total phenols showed a significant raise over salt stress (Table 2).

At 100 mM NaCl, this parameter was increased by 264% as compared to the control. Higher salinity (300 mM NaCl) was accompanied by large increases in total phenols, representing 6.4-fold the control. On the other hand, a positive correlation between phenols accumulation and lipid peroxidation (MDA content) was observed in *M. arborea* ($r = 0.94$, $F = 139.7$, $p<0.0001$; Fig. 4b).

Effect of salt stress on antioxidant enzymes activities: The activity of SOD was raised significantly with the increase of salinity in *M. arborea*, especially in the leaves thus, at 300 mM NaCl there was observed a 318.9% increase in leaf SOD activity and only a 174.3% increase in roots.

The leaves treated with 100 and 200 mM NaCl showed a 170.3 and 310.3% increase in SOD activity, respectively compared with control plants at the end of the experimental period (Fig. 5a). However, the activity of SOD remained significantly unchanged with increasing salinity for 200-300 mM NaCl.

The activity of POD increased with the increase of the concentration of NaCl in both organs parts (Fig. 5b). *M. arborea* plants treated with 100-300 mM NaCl showed a 167.8-373.9% increase in leaf POD activity and only a 106.6-194.5% increase in root POD activity, respectively compared with control plants (Fig. 5b). Concerning the activity of CAT, it remained unaffected by salinity in the roots.

In the leaves, CAT activity was significantly increased under 100-200 mM NaCl and reached 156.9 and 142.2% compared with control plants under 100 and 200 mM, respectively. However, it decreased significantly and reached 70.4% compared with control plants (Fig. 5c). The APX activity in the leaves and roots of *M. arborea* treated with NaCl stress was significantly higher than that of control plants (Fig. 5d). Thus, the treatment of the plants by 100-300 mM NaCl caused 290.7-755.8% increase in leaf APX activity and 341.1-1210.6% increase in root APX activity in comparison to the control plants.

Growth inhibition is a common response to salinity (Parida and Das, 2 005). In agreement with this idea, *M. arborea* respond to salt stress by decreasing plant growth progressively with the increase of medium salinity. Many studies have shown that *Medicago* sp. could survive under low saline conditions up to 200 mM NaCl (Sibole *et al*., 2003; Rabhi *et al*., 2007). The inhibitory effects of salt stress on plant growth were also reported in other researches using various plants (Soussi *et al*., 1998; Trajkova *et al*., 2006). The depressive action of salt on growth appeared by a significant reduction of the aerial organ growth activity suggesting that the shoot was more sensitive to salinity than the root. The decline in plant growth due to salinity is associated with a number of physiological and biochemical processes (nutrient imbalance, accumulation of compatible solutes, antioxidants and enzyme activities) governing plant growth (Munns and Tester, 2008). Deleterious effects of salinity are thought to result from osmotic effects, ion

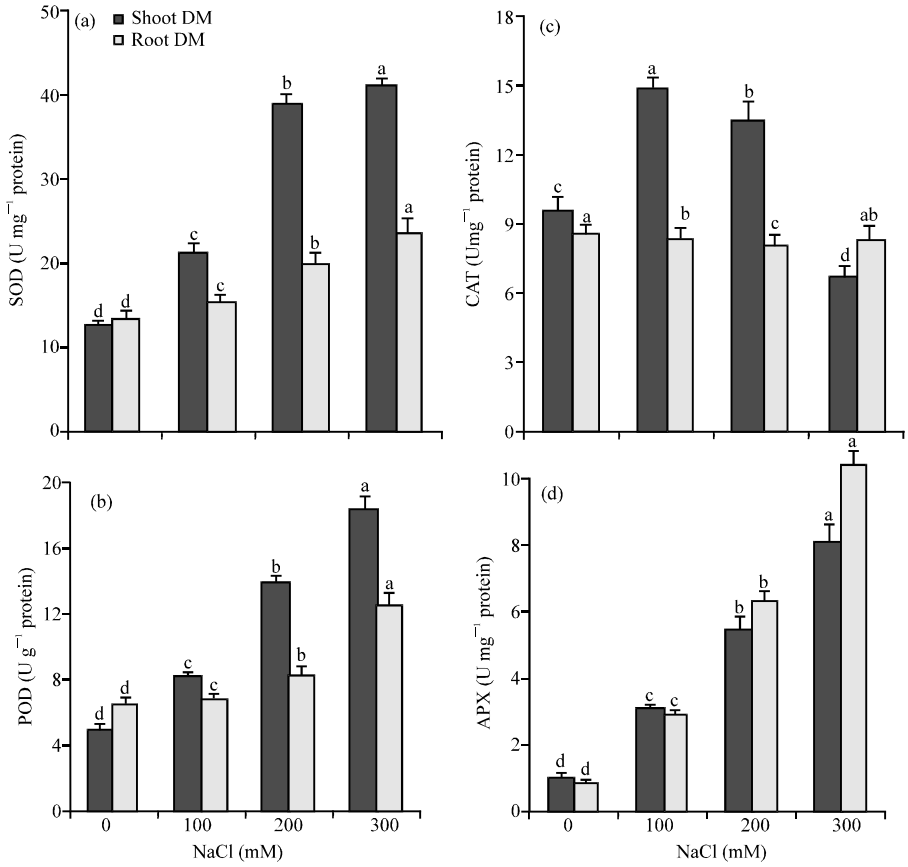

Fig. 5: Effect of NaCl treatments on the contents of: a) Superoxide Dismutase (SOD); b) Peroxidase (POD); c) Peroxidase (POD) and d) Ascorbate Peroxidase (APX) in the shoots and roots of *M. arborea*. Bars followed by the same letter are not statistically different at $p<0.05$ (Duncan's multiple range test). Averages of 4 repetitions are presented with bars indicating SE

toxicities and nutrient deficiencies (Munns, 2002; Munns *et al*., 2006). In this research, the negative relationship between the aerial biomass production of *M. arborea* and its Na^+ content suggest that the growth decrease was due to the ionic toxicity. From the results of the present study, it is obvious that increasing supply of NaCl caused a marked accumulation of Na^+ in the shoots. In contrast, increasing the external NaCl concentration was accompanied by a concomitant decrease in K^+ content and increase Na^+/K^+ ratio. A high concentration of Na^+ can interfere with K^+ uptake, resulting in deficiency and stunted growth (Meloni *et al*., 2008). Thus, the interaction between relative K^+ and Na^+ concentration has been considered a key factor in determining salt tolerance in plants. The results showed that the accumulation of Na^+ in photosynthetic organs was associated with an increase of water content, suggesting that the decrease in plants growth under high salinity can be explained by reduction in cell turgor (Fig. 1b). Because the reduction in leaf RWC is a general response when plants are under osmotic stress conditions (Bertamini *et al*., 2006), reduced leaf RWC seems to suppress the growth of *M. arborea* plants after long-term exposure to salt stress. This higher negative relationship between water and Na^+ contents suggests that *M. arborea* species may be deprived of efficient systems for Na^+ vacuolar compartmentation and a probable apoplastic accumulation of Na^+ in leaves. This decline in leaf ion compartmentation capacity can caused decrease in photosynthetic ability in this species (Boughalleb *et al*., 2009). In some species, salt tolerance is associated with accumulation of compatible compound in the cytoplasm thus balancing the osmotic pressure of ions in the vacuoles to maintain water economy and preserving enzyme activity in the presence of toxin ions (Hasegawa *et al*., 2000; Lee *et al*., 2008). Generally, salt stress induces proline accumulation in many crops (Bor *et al*., 2003; Parida and Das, 2005; De Abreu *et al*.,

2008). In addition, several reasearches showed that soluble sugar was involved in alleviating salt stress in plants (Shen and Chen, 2001; Song *et al*., 2006). The data (Fig. 2a) showed a progressive increase in proline with increasing salinity in both plant organs parts. Soluble sugar showed slightly increase with salinity in the roots while it was remarkably accumulated in shoots at 100-200 mM NaCl and reduced at higher salt treatment.

The significance of organic compounds accumulation and their role in osmotic adjustment and salt tolerance has been questioned. The great difference between the accumulation of these osmolytes and the osmolarity detected in *M. arborea* show that the contribution of proline and soluble sugar was not sufficient to provide osmotic adjustment and to maintain water balance of *M. arborea* plants under salinity (Fig. 2b). On the other hand, the relative maintenance of water content observed at 100 mM NaCl can be explained at last in part by the enhanced level of soluble sugar occurred in this salt treatment. These results are consistent with several other researches showing that proline does not seem to play an important role in the mechanism of salt tolerance (Ashraf and Foolad, 2007). Furthermore, Wang and Han (2009) found in study of *Medicago sativa* salt tolerance that the salt-sensitive cultivar had the higher increased proline accumulation indicating that proline accumulation was a contributing factor to the inhibited growth of plants under salt stress. Besides their role in osmoregulation, proline may be act as radical scavenger and protects cells against salt induced oxidative stress (Hong *et al*., 2000). The excessive accumulation of Reactive Oxygen Species (ROS) in plants is one of the major damage induced by salinity. The product of lipid peroxidation (content of MDA) and the generation of hydrogen peroxide have been considered as indicators of oxidative damage (Meloni *et al*., 2003).

In the present study, the H_2O_2 accumulation resulted in a marked increase in MDA content and a decrease in MSI. The high positive correlation between H_2O_2 generation and MDA amount (r = 0.96*** in shoots; r = 92*** in roots; Fig. 3a) which was negatively associated with the decrease of MSI (r = -0.97*** in shoots; r = 0.84*** in roots; Fig. 3b) confirmed the hypothesis that H_2O_2 brings about lipid peroxidation leading to membrane damages (Hichem *et al*., 2009). Furthermore, a negative correlation was observed between biomass production and MDA contents (r = -0.94*** in shoots; r = 0.91*** in roots; Fig. 4a) indicating that higher lipid peroxidation resulted in reduced biomass production. This increase in lipid peroxidation may be due to the incapability of antioxidants to scavenger reactive oxygen species results from salt stress. Similar results were observed on other glycophytes such as cotton (Meloni *et al*., 2003), sesame (Koca *et al*., 2007) and tomato (Li, 2009). They reported that growth reduction under salt stress in different cultivars is closely associated with increased lipid peroxidation levels. On the other hand, the research showed that roots had a lower membrane permeability, MDA content and H_2O_2 amount than shoots under increasing salt concentrations. This suggests that the antioxidative defense system would not be as effective in leaves as in roots.

To cope with oxidative damage induced by salt stress, plants have the ability to detoxify ROS by up-regulating antioxidant enzymes as well as some non-enzymatic antioxidant metabolites. The phenolic compounds produced through the phenyl propanoid pathway has been considered pertinent in oxidative stress tolerance (Moyer *et al*., 2002; Sgherri *et al*., 2004). Their accumulation can be changed by salt stress but this is critically dependent on the salt sensitivity of plants (Kim *et al*., 2008). In the present study, the total phenols content was significantly increased with salt stress *M. arborea* leaves which is similar to that in sugarcane and radish reported by Wahid and Ghazanfar (2006) and Kim *et al*. (2006), respectively. Furthermore, the significant negative correlation (r = -0.95***; Fig. 4b) between leaf MDA and phenolic contents under salt stress, lest to conclude that higher shoot biomass production may be due to low production of MDA content. This reduction in MDA contents may be due to reduced production of ROS hence decreased oxidative stress. The phenolic compounds can play an important role in neutralizing ROS, alleviating ion-induced oxidative damage (Zheng and Wang, 2001; Moyer *et al*., 2002) and protect cytoplasmic structures and chloroplasts from adverse effects of salinity. In addition to the antioxidants metabolites, plants have evolved ROS-scavenging enzymes such as SOD, CAT, POD and APX to protect cellular membranes and organelles from damaging effects of ROS (Gomez *et al*., 2004). Salt tolerance is often correlated with a more efficient antioxidative system (Bor *et al*., 2003).

In the present study, a higher SOD activity was observed in the shoots, compared with the roots which suggests that this enzyme may function as a ROS scavenger by converting O_2^- to H_2O_2 and the higher capacity for this conversion was occurred in the shoots. Similar increases in the activities of SOD have been reported in pea (Hernandez and Almansa, 2002), tomato (Koca *et al*., 2006) and *Limonium bicolour* (Li, 2008). The H_2O_2 produced by SOD to prevent cellular damage, must be eliminated by conversion to H_2O in sub-sequent

reactions involving POD, CAT and APX which regulate H_2O_2 accumulation in plants. POD is a key enzyme involved in the detoxification of toxic compounds such as H_2O_2 which are produced in chloroplasts as a result of oxidative stress (Chaparzadeh *et al*., 2004). The data showed enhanced activity of POD, especially in shoots (Fig. 5b). This result is consistent with those found in mulberry (Harinasut *et al*., 2003) indicating that POD play an important role in eliminating ROS under salt stress. Higher activity of CAT decrease H_2O_2 level in cell by breaking it down directly to form H_2O and O_2 and increase the stability of membranes and CO_2 fixation because several enzymes of the Calvin cycle within chloroplasts are extremely sensitive to H_2O_2. A high level of H_2O_2 directly inhibits CO_2 fixation (Yamazaki *et al*., 2003). The data demonstrated that CAT activity was significantly higher in leaves of *M. arborea* treated with 100-200 mM NaCl but significantly lower at 300 mM NaCl than the control. This deactivation of CAT activity observed at higher salinity can be explained by the great accumulation of singlet oxygen and peroxyl radicals (Escobar *et al*., 1996). In addition, higher concentration of NaCl causing higher hydrogen peroxide accumulation, this may be one of the factors that results in the inactivation of CAT (Velikova *et al*., 2000). A similar result was also reported in mulberry (Harinasut *et al*., 2003) and *Medicago sativa* (Wang *et al*., 2009). Conversely, no salt-induced alterations were observed on root CAT activity (Fig. 5c) showing a salt-independent root CAT activity under salt stress. In agreement with the finding of Cavalcanti *et al*. (2007) and De Abreu *et al*. (2008) in cowpea and dwarf-cashew roots exposed to NaCl stress. APX is an important antioxidant enzymes involved in ascorbate-glutathione cycle which plays a key role in destroying the H_2O_2 (Foyer and Noctor, 2005).

In the present study, salt stress led to a significant increase in APX activity and this increase was higher in roots than in shoots. It suggested that *M. arborea* may mainly employ this enzyme for detoxification of H_2O_2 in roots rather in shoots under salt stress. Salt led to an increase in APX activity in pea (Hernandez *et al*., 2000), sugar beet (Bor *et al*., 2003) and alfalfa (Wang *et al*., 2009).

On the other hand, the enhanced conversion of H_2O_2 produced by SOD to H_2O and O_2 was essentially played by CAT activity at low level of salinity (100 mM NaCl) however, at higher salinities *M. arborea* plants may mainly employ POD and APX for the detoxification of H_2O_2 in their shoots. Similar, result was occurred in *Solanum tuberosum* by Benavides *et al*. (2000) suggesting that APX activity was likely to be more important than CAT in the detoxification. In the roots, characterised by lower levels of H_2O_2 and lipid peroxidation than in shoots, this detoxification was realised by POD and essentially by APX activity which can protect the plants from lipid peroxidation of membrane systems and oxidative damages under salt. The lack of increase in SOD activity occurred in the shoots of *M. arborea* under higher salinity (300 mM) suggests a probable overproduction of ROS creating a risk for serious cellular damage and that *M. arborea* leaves may mainly employ POD and APX to scavenging H_2O_2 than SOD to defend against salinity-induced oxidative stress. Furthermore, it appeared that non-enzymatic ways like phenols and proline might have been involved in the scavenging of superoxide radicals in *M. arborea* leaves treated with higher salinity.

Table 3: Correlations coefficients (r) of the shoot dry mass, total phenols content and antioxidant enzyme activity (Superoxide Dismutase, SOD; Peroxidise, POD; Catalase, CAT; Ascorbate Peroxidise APX) with Malondialdehyde (MDA) accumulation in *M arborea* leaves

Parameters	MDA content
Shoot dry mas	-0.94***
Total phenols	0.95***
SOD content	0.87***
POD content	0.96***
CAT content	-0.59**
APX content	0.96***

Significant at *p<0.05, **p<0.01 and ***p<0.001

As the membrane permeability index and MDA content increased significantly under higher salinity (Table 3), it is possible that the scavenging of the dangerous radical was not done perfectly. Thus, the increased accumulation of metabolites and antioxidant enzymes might have been not enough to prevent peroxidation of lipid membranes caused by high concentration of NaCl. Consequently, this radical attacks vital biomolecules and damage to membranes happens.

CONCLUSION

In this study, the results indicate that *M. arborea* grown in the presence of NaCl could accumulate large amounts of ions in its leaves. The very limited ability of salt-treated plants to prevent the entry of Na^+ to their photosynthetic organs could explain the marked decline of their growth. These accumulated ions were evidently incorrectly compartmentalized because their accumulation leads to tissue dehydration and the leaves remained affected by oxidative damage. The relative salt tolerance of this species may be a consequence of the accumulation of compatible compounds, especially soluble sugar to make osmotic adjustments and improved resistance to oxidative stress by enhancing their antioxidative capacity via increasing the activities of SOD, POD, CAT and APX

in the shoots of *M. arborea* whereas in the roots, the scavenging system might be achieved by SOD, POD and APX activities. In addition to the antioxidants enzymes, several metabolites were involved in the antioxidant defence system such as phenols and proline which they increased with salinity and it appeared that their role was essentially occurred in the shoots at higher salinity.

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REFERENCES

Allen, R.D., 1995. Dissection of oxidative stress tolerance using transgenic plants. Plant Physiol., 107: 1049-1054.

Ashraf, M. and P.J.C. Harris, 2004. Potential biochemical indicators of salinity tolerance in plants. Plant Sci., 166: 3-16.

Ashraf, M. and M.R. Foolad, 2007. Roles of glycinebetaine and proline in improving plant abiotic stress resistance. Environ. Exp. Bot., 59: 206-216.

Balasundram, N., K. Sundram and S. Samman, 2006. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence and potential uses. Food Chem., 99: 191-203.

Bates, L.S., R.P. Waldren and I.D. Teare, 1973. Rapid determination of free proline for water-stress studies. Plant Soil, 39: 205-207.

Benavides, M.P., P.L. Marconey, S.M. Gallego, M.E. Comba and M.I. Tamaro, 2000. Relationship between antioxidant defence systems and salt tolerance in *Solano tobarasum*. Aust. J. Plant Physiol., 27: 273-278.

Bertamini, M., L. Zulini, K. Muthuchelian and N. Nedunchezhian, 2006. Effect of water deficit on photosynthetic and other physiological responses in grapevine (*Vitis vinifera* L. cv Riesling) plants. Photosynthetica, 44: 151-154.

Bor, M., F. Ozdemir and I. Turkan, 2003. The effect of salt stress on lipid peroxidation and antioxidants in leaves of suger beet *Beta vulgaris* L. and wild beet *Beta maritime* L. Plant Sci., 164: 77-84.

Boughalleb, F., M. Denden and B.B. Tiba, 2009. Anatomical changes induced by increasing NaCl salinity in three fodder shrubs, *Nitraria retusa*, *Atriplex halimus* and *Medicago arborea*. Acta Physiol. Plant, 31: 947-960.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Cakmak, I. and W.J. Horst, 1991. Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase and peroxidase activities in root tip of soybean (*Glycine max*). Physiol. Plant., 83: 463-468.

Cavalcanti, F.R., J.P.M.S. Lima, S.L. Ferreira-Silva, R.A. Viegas and J.A. Silveira, 2007. Roots and leaves display contrasting oxidative response during salt stress and recovery in cowpea. J. Plant Physiol., 164: 591-600.

Chakraborty, U. and C. Tongden, 2005. Evaluation of heat acclimation and salicylic acid treatments as potent inducers of thermotolerance in *Cicer arietinum* L. Curr. Sci., 89: 384-389.

Chaparzadeh, N., M.L. D'Amico, R.A. Khavari-Nejad, R. Izzo and F. Navari-Izzo, 2004. Antioxidative responses of *Calendula officinalis* under salinity conditions. Plant Physiol. Biochem., 42: 695-701.

Dat, J., S. Vandenbeele, E. Vranova, M. van Montagu, D. Inze and F. van Breusegem, 2000. Dual action of the active oxygen species during plant stress responses. Cell. Mol. Life Sci., 57: 779-795.

De Abreu, C.E.B., J.T. Prisco, A.R.C. Nogueira, M.A. Bezerra, C.F. de Lacerda and F. Gomes-Filho, 2008. Physiological and biochemical changes occurring in dwarf-cashew seedlings subjected to salt stress. Braz. J. Plant Physiol., 20: 105-118.

Dewanto, V., X. Wu, K.K. Adom and R.H. Liu, 2002. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agric. Food Chem., 50: 3010-3014.

Escobar, J.A., M.A. Rubio and E.A. Lissi, 1996. Sod and catalase inactivation by singlet oxygen and peroxyl radicals. Free Radic. Biol. Med., 20: 285-290.

Fernandez-Ballester, G., V. Martinez, D. Ruiz and A. Cerda, 1998. Changes in inorganic solutes in citrus growing under saline stresses. J. Plant Nutr., 21: 2497-2514.

Foyer, C.H. and G. Noctor, 2005. Oxidant and antioxidant signalling in plants: A re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ., 28: 1056-1071.

Gomez, J.M., A. Jimenez, E. Olmos and F. Sevilla, 2004. Location and effects of long-term NaCl stress on superoxide dismutase and ascorbate peroxidase isoenzymes of pea (*Pisum sativum* cv. Puget) chloroplasts. J. Exp. Bot., 55: 119-130.

Hamed, K.B., A. Castagna, E. Salem, A. Ranieri and C. Abdelly, 2007. Sea fennel (*Crithmum maritimum* L.) under salinity conditions: A comparison of leaf and root antioxidant responses. Plant Growth Regul., 53: 185-194.

Harinasut, P., D. Poonsopa, K. Roengmongkol and R. Charoensataporn, 2003. Salinity effects on antioxidant enzymes in mulberry cultivar. Sci. Asia, 29: 109-113.

Hasegawa, P.M., R.A. Bressan, J.K. Zhu and H.J. Bohnert, 2000. Plant cellular and molecular responses to high salinity. Ann. Rev. Plant Physiol. Plant Mol. Biol., 51: 463-499.

Heim, K.E., A.R. Tagliaferro and D.J. Bobilya, 2002. Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. J. Nutr. Biochem., 13: 572-584.

Hernandez, J.A., A. Jimenez, P. Mullineaux and F. Sevilla, 2000. Tolerance of pea (*Pisum sativum* L.) to long term salt stress is associated with induction of antioxidant defences. Plant Cell Environ., 23: 853-862.

Hernandez, J.A. and M.S. Almansa, 2002. Short-term effects of salt stress on antioxidant systems and leaf water relations of pea leaves. Physiol. Plant., 115: 251-257.

Hichem, H., D. Mounir and E. Naceur, 2009. Differential responses of two maize (*Zea mays* L.) varieties to salt stress: Changes on polyphenols composition of foliage and oxidative damages. Ind. Crop Prod., 30: 144-151.

Hong, Z., K. Lakkineni, Z. Zhang and D.P.S. Verma, 2000. Removal of feedback inhibition of Ä^{1}-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol., 122: 1129-1136.

Kiarostami, K., R. Mohseni and A. Saboora, 2010. Biochemical changes of *Rosmarinus officinalis* under salt stress. J. Stress Physiol. Biochem., 6: 114-122.

Kim, H.J., F. Chen, X. Wang and J.H. Choi, 2006. Effect of methyl jasmonate on phenolics, isothiocyanate and metabolic enzymes in radish sprout (*Raphanus sativus* L.). J. Agric. Food Chem., 54: 7263-7269.

Kim, H.J., J.M. Fonseca, J.H. Choi, C. Kubota and D.Y. Kwon, 2008. Salt in irrigation water affects the nutritional and visual properties of romaine lettuce (*Lactuca sativa* L.). J. Agric. Food Chem., 56: 3772-3776.

Koca, H., F. Ozdemir and I. Turkan, 2006. Effect of salt stress on lipid peroxidation and superoxide dismutase and peroxidase activities of *Lycopersicon esculentum* and *L. pennellii*. Biol. Plant., 50: 745-748.

Koca, H., M. Bor, F. Ozdemir and I. Turkan, 2007. The effect of salt stress on lipid peroxidation, antioxidative enzymes and proline content of sesame cultivars. Environ. Exp. Bot., 60: 344-351.

Lee, G., R.N. Carrow, R.R. Duncan, M.A. Eiteman and M.W. Rieger, 2008. Synthesis of organic osmolytes and salt tolerance mechanism in *Paspalum vaginatum*. Environ. Exp. Bot., 63: 19-27.

Li, Y., 2008. Kinetics of the antioxidant response to salinity in the halophyte *Limonium bicolor*. Plant Soil Environ., 54: 493-497.

Li, Y., 2009. Physiological responses of tomato seedlings (*Lycopersicon esculentum*) to salt stress. Mod. Applied Sci., 3: 171-176.

Lutts, S., J.M. Kinet and J. Bouharmont, 1996. NACl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. Ann. Bot., 78: 389-398.

Mckersie, B.D. and Y.Y. Leshem, 1994. Stress and Stress Coping in Cultivated Plants. 1st Edn., Kluwer Academic Publishers, Dordrecht, The Netherlands, pp: 55-78.

Meloni, D.A., M.A. Oliva, C.A. Martinez and J. Cambraia, 2003. Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Environ. Exp. Bot., 49: 69-76.

Meloni, D.A., M.R. Gulotta and C.A. Martinez, 2008. Salinity tolerance in *Schinopsis quebracho* colorado: Seed germination, growth, ion relations and metabolic responses. J. Arid Environ., 72: 1785-1792.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci., 7: 405-410.

Moller, I.M., P.E. Jensen and A. Hansson, 2007. Oxidative modifications to cellular components in plants. Annu. Rev. Plant Biol., 58: 459-481.

Moyer, R.A., K.E. Hummer, C.E. Finn, B. Frei and R.E. Wrolstad, 2002. Anthocyanins, phenolics and antioxidant capacity in diverse small fruits: Vaccinium, rubus and ribes. J. Agric. Food Chem., 50: 519-525.

Munns, R., 2002. Comparative physiology of salt and water stress. Plant Cell Environ., 25: 239-250.

Munns, R., R.A. James and A. Lauchli, 2006. Approaches to increasing the salt tolerance of wheat and other cereals. J. Exp. Bot., 57: 1025-1043.

Munns, R. and M. Tester, 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol., 59: 651-681.

Nakano, Y. and K. Asada, 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplast. Plant Cell Physiol., 22: 867-880.

Navarro, J.M., P. Flores, C. Garrido and V. Martinez, 2006. Changes in the contents of antioxidant compounds in pepper fruits at different ripening stages, as affected by salinity. Food Chem., 96: 66-73.

Parida, A.K. and A.B. Das, 2005. Salt tolerance and salinity effects on plants: A review. Ecotoxicol. Environ. Saf., 60: 324-349.

Parvaiz, A. and S. Satyawati, 2008. Salt stress and phyto-biochemical responses of plants-a review. Plant Soil Environ., 54: 89-99.

Pitman, M.G. and A. Lauchli, 2002. Global Impact of Salinity and Agricultural Ecosystems. In: Salinity Environment-Plants Molecules, Lauchli, A. and V. Luttge (Eds.). Kluwer Academic Press, The Netherlands, pp: 3-20.

Rabhi, M., Z. Barhoumi, R. Ksouri, C. Abdelly and M. Gharsalli, 2007. Interactive effects of salinity and iron deficiency in *Medicago ciliaris*. C. R. Biol., 30: 779-788.

Rao, K.V.M. and T.V.S. Sresty, 2000. Antioxidative parameters in the seedlings of pigeon pea (*Cajanus cajan* L. Millspaugh) in response to Zn and Ni stresses. Plant Sci., 157: 113-128.

Sakihama, Y.M., F. Cohen, S.C. Grace and H. Yamasaki, 2002. Plant phenolic antioxidant and prooxidant activities: Phenolics-induced oxidative damage mediated by metals in plants. Toxicology, 177: 67-80.

Salah, I.B., T. Slatni, A. Albacete, M. Gandour and C.M. Andujar *et al*., 2010. Salt tolerance of nitrogen fixation in *Medicago ciliaris* is related to nodule sucrose metabolism performance rather than antioxidant system. Symbiosis, 51: 187-195.

Samaras, Y., R.A. Bressan, L.N. Csonka, M.G.D. Garcia-Rios, P. Urzo and D. Rodes, 1995. Proline Accumulation During Drought and Salinity. In: Environment and Plant Metabolism, Flexibility and Acclimation, Smironoff, N. (Ed.), BIOS Scientific Publishers, Oxford UK., pp: 161-187.

Sgherri, C., B. Stevanovic and F. Navari-Izzo, 2004. Role of phenolics in the antioxidative status of the resurrection plant *Ramonda serbica* during dehydration and rehydration. Physiol. Plant, 122: 478-485.

Shen, Y.G. and S.Y. Chen, 2001. Molecular mechanism of plant response to salt stress. Hereditas, 23: 365-369.

Sibole, J.C., C. Cabot, C. Poschenrieder and J. Barcelo, 2003. Ion allocation in two different salt-tolerant Mediterranean *Medicago* species. J. Plant Physiol., 160: 1361-1365.

Song, J., G. Feng, C.Y. Tian and F.S. Zhang, 2006. Osmotic adjustment traits of *Suaeda physophora*, *Haloxylon ammodendron* and *Haloxylon persicum* in field or controlled conditions. Plant Sci., 171: 113-119.

Soussi, M., A. Ocana and C. Lluch, 1998. Effects of salt stress on growth, photosynthesis and nitrogen fixation in chick-pea (*Cicer arietinum* L.). J. Exp. Bot., 49: 1329-1337.

Sudhakar, C., A. Lakshmi and S. Giridarakumar, 2001. Changes in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. Plant Sci., 161: 613-619.

Tester, M. and R. Davenport, 2003. Na^+ tolerance and Na^+ transport in higher plants. Ann. Bot., 91: 503-527.

Trajkova, F., N. Papadantonakis and D. Savvas, 2006. Comparative effects of NaCl and $CaCl_2$ salinity on cucumber grown in a closed hydroponic system. HortScience, 41: 437-441.

Velikova, V., I. Yordavow and A. Edreva, 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: Protective role of exogenous polyamines. Plant Sci., 151: 59-66.

Wahid, A. and A. Ghazanfar, 2006. Possible involvement of some secondary metabolites in salt tolerance of sugarcane. J. Plant Physiol., 163: 723-730.

Wang, W.B., Y.H. Kim H.S. Lee, K.Y. Kim, X.P. Deng and S.S. Kwak, 2009. Analysis of antioxidant enzyme activity during germination of alfalfa under salt and drought stresses. Plant Physiol. Biochem., 47: 570-577.

Wang, X.S. and J.G. Han, 2009. Changes of proline content, activity and active isoforms of antioxidative enzymes in two alfalfa cultivars under salt stress. Agric. Sci. China, 8: 431-440.

Waterman, P.G. and S. Mole, 1994. Extraction and Chemical Quantification. In: Analysis of Phenolic Plant Metabolites, Waterman, P.G. and S. Mole (Eds.). Blackwell Scientific Publications, Oxford, UK., ISBN-13: 9780632029693, pp: 80-84.

Xiong, L. and J.K. Zhu, 2002. Molecular and genetic aspects of plant responses to osmotic stress. Plant Cell Environ., 25: 131-139.

Yamazaki, J., A. Ohashi, Y. Hashimoto, E. Negishi and S. Kumagai *et al*., 2003. Effects of high light and low temperature during harsh winter on needle photodamage of *Abies mariesii* growing at the forest limit on Mt. Norikura in Central Japan. Plant Sci., 165:257-264.

Zheng, W. and S.Y. Wang, 2001. Antioxidant activity and phenolic compounds in selected herbs. J. Agric. Food Chem., 49: 5165-5170.

Zhu, Z., J. Gerendas, R. Bendixen, K. Schinner, H. Tabrizi, B. Sattelmacher and U.P. Hansen, 2000. Different tolerance to light stress in NO_3^- and NH_4^+-grown *Phaseolus vulgaris* L. Plant Biol., 2: 558-570.