

Salinity Effects on Organic Solutes and Antioxidative Enzymes in Two Halophytes, *Nitraria retusa* (Forssk.) and *Atriplex halimus* (L.)

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Abstract: The effect of NaCl stress on the growth, organic compounds content and antioxidant enzymes activities were investigated in two xero-halophytes *Nitraria retusa* and *Atriplex halimus*. Plants were grown in 0-800 mM NaCl for 120 days under glasshouse conditions. Both xero-halophytic species showed positive plant growth for low levels of salinity. Increasing concentrations of salinity from 400-800 mM, NaCl induced decrease in plant growth in the two species, especially in *A. halimus*. In addition, both species were able to accumulate a large quantity of Na⁺ and to maintain a higher leaf water content which was probably associated with a greater capacity for osmotic adjustment whereas the contents of K⁺ decreased significantly, resulting in an increase in the Na⁺/K⁺ ratio when NaCl concentrations increased. Organic osmotica was highly involved in osmotic adjustment in *A. halimus* leaves, especially glycinebetaine. In *N. retusa* leaves, glycinebetaine, soluble sugar and proline were increased by salinity. The relatively better salt tolerance of *N. retusa* compared to *A. halimus* plants may be related to the lower Malondialdehyde (MDA) content and hydrogen peroxide (H₂O₂) concentration and the increased activity of Catalase (CAT) and Peroxydase (POD) which participate to protect cells from ROS damage. In *A. halimus*, the higher antioxidant enzyme activities (Superoxide Dismutase, SOD; Catalase, CAT; Peroxydase, POD) play a major role in the restriction of oxidative damages caused by salt stress.

Key words: Plant growth, proline, glycinebetaine, soluble sugars, antioxidative enzymes, salt stress, *A. halimus*, *N. retusa*

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; Patel *et al.*, 2009).

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 Ca²⁺ 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 (Sampras *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 Glycinebetaine (GB) and proline in response to salinity stress and their accumulation may play a role in combating salinity stress (Ashraf and Harris, 2004; Moghaieb *et al.*, 2004). 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 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^-), 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; Jithesh *et al.*, 2006; Hamed *et al.*, 2007), responsible for maintaining the levels of ROS under tolerable level.

Antioxidative enzymes are the most important components in the scavenging system of reactive oxygen species (Allen, 1995; Dat *et al.*, 2000). Thus, these such as Superoxide Dismutase (SOD), Catalase (CAT) and Peroxidase (POD) are essential components of the plant's antioxidant defense system. Superoxide Dismutase (SOD) catalyzes, the 1st step of the enzymatic defense 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; Amor *et al.*, 2005). Moreover, the ability to increase antioxidant capacity in response to salinity has been correlated with salt tolerance in a large number of halophytes such as *Beta maritima* (Bor *et al.*, 2003), *Crithmum maritimum* (Amor *et al.*, 2005), *Limonium bicolor* (Li, 2008) and mangroves (Dasgupta *et al.*, 2010). In Tunisia, a considerable diversity of halophytic species of multiple interests including therapeutic practices and economical applications as new sources of natural antioxidants in dietary food. *Nitraria retusa* is one of the leading shrubs in steppes, deserts and saline soils belonging to the family Nitrariaceae. It is an important sand controller, its leaves and twigs are occasionally

grazed by sheep, goats and camels (Heneidy, 1996). The (Chenopodiaceae) *Atriplex halimus* is a Mediterranean xero-halophyte saltbush species, highly resistant to drought (Le Houerou, 2000) and salinity (Bajji *et al.*, 1998). *Atriplex* plays an important role as a forage crop for both wild and domestic animals in arid regions. To date, little is known about the antioxidant system response to salt stress in the two species.

In this study, researchers have examined the effects of salinity on plant growth, solutes compounds, secondary metabolites and antioxidant enzyme activity in these two fodder species with increasing salinities levels. Therefore, the main objective is to evaluate the difference in the response of *N. retusa* and *A. halimus* plants to different NaCl salinity levels and to understand the eventual involvement of organic compounds and antioxidant enzymes activities in their mechanism of salt tolerance.

MATERIALS AND METHODS

Plant growth conditions: *A. halimus* seeds were collected from Sabkha of Kalbia ($10^{\circ}59'$, $631^{\circ}E$ and $35^{\circ}49'$, $332^{\circ}N$) (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. For *N. retusa* plants were propagated by grafting from a source plant growing wild in the salt region of Sabkha of Kalbia. Polyethylene bags filled with sandy soil were employed as plant-growing containers in the plant propagation phase. Plants were initially grown in half-strength Hoagland (1950) solution to supply the macro and micronutrients. When seedlings were around 7 cm in height (2-3 months old), they were placed in plastic pots (5 L) filled with mixture of peat and perlite (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 $17/35^{\circ}C$; relative humidity: min/max 30/70%; photoperiod (14/10 h day/night).

At the end of the acclimatization phase, seedlings of *N. retusa* and *A. halimus* were divided into 5 groups for treatments (10 plants treatment⁻¹): 0 mM NaCl (control); 100, 200, 400 and 800 mM NaCl. These treatments were watered with 200 mL of salt solutions every 2 days 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 100 mM NaCl in both halophytic species 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 120 days.

Growth activity and water content: Four plants for each treatment were sampled to determine leaf number and plant height. The Leaf Area (LA) was measured by using leaf area meter DT-scan (Delta-scan Version 2.03, Delta-T devices, Ltd., England). The plant material was 1st cleaned with distilled water. After the water on the plant was absorbed by tissue paper, 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:

$$LWC = \left[\frac{(FM - DM)}{FM} \right] \times 100$$

Determination of organic and inorganic solutes: Dried samples (15 mg from four 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 4 plants treatment⁻¹. 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. Glycinebetaine was extracted by stirring finely ground-dried samples with demineralised water at 100°C for 1 h. Glycinebetaine content was determined spectrophotometrically after reaction with KI-I₂ at 365 nm (Grieve and Grattan, 1983). 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. About 2 mL of the filtrate, 2 mL of acid ninhydrin were added followed by the addition of 2 mL of 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.

Hydrogen peroxide (H₂O₂) estimation: The H₂O₂ content was determined as described by Velikova. Fresh leaf tissue (0.5 g) was homo-genized with 5 mL of 0.1% (w/v) Trichloroacetic Acid (TCA) in a pre-chilled pestle and mortar. This homogenate was then centrifuged at

12,000 g for 15 min in room temperature. About 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-visible spectrophotometer (UV-2500, Shimadzu Corp., Kyoto, Japan) and the H₂O₂ 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. Total 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 formula:

$$MDA \text{ (nmol g}^{-1} \text{ FM)} = \left[\frac{(A532 - A600) \times V \times 1000}{\epsilon} \right] \times W$$

Where:

- ε = Specific extinction coefficient (= 155 mM cm⁻¹)
- V = Volume of crushing medium
- W = Fresh weight of leaf
- A600 = Absorbance at 600 nm wavelength
- A532 = Absorbance at 532 nm wavelength

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 one 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 nmol/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 H₂O₂ 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 as 1 mol g⁻¹ FM min⁻¹. Catalase (CAT) activity was estimated according to Chakraborty and Tongden (2005) which measures the initial rate of disappearance of H₂O₂ at 240 nm.

The CAT reaction mixture contained 50 mM of Na-phosphate buffer pH 7.0, 15 mM of H₂O₂ 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 as 1 mol of H₂O₂ consumed at 240 nm g⁻¹ FM min⁻¹.

Statistical analysis: A one-way ANOVA was achieved to compare the mean values using the SPSS (13) statistical

package (p<0.05). In case of significant differences, Duncan post hoc tests were performed to compare the means.

RESULTS

Effect of salinity on plant growth and relative leaf water content:

Sodium chloride treatments modified growth of both species in a concentration-dependent indicated by a several parameters such as shoot dry weight, root dry weight, average plant height, average number of leaves per plant and Leaf Area (LA). In the two halophytic species, salt stress induced modifications in Dry Mass (DM) were more pronounced in the shoots than in the roots of the plants (Table 1). The dry mass gain of plants was stimulated up to 200 mM NaCl and then reduced significantly in the highest NaCl treatment. Thus, moderate salinity (100-200 mM NaCl) appeared to be optimal for the growth of these two halophytic species. For example, shoots dry mass at 100 mM NaCl was about 15.5-29.7% higher for *N. retusa* and *A. halimus* plants, respectively. As compared with the control, shoot dry mass decreased at 800 mM NaCl by 51.5 and 69% in *N. retusa* and *A. halimus*, respectively. A one-way ANOVA indicated that salinity caused a significant (p<0.05) reduction on plant height, leaf average number and leaf area only at levels exceeding 200 mM NaCl concentration in *A. halimus* and *N. retusa*. At low level of salinity (100 mM NaCl) plant height and leaf number increased by 14.8-28% and 10.4-9.7%, respectively in *A. halimus* and *N. retusa* as compared with those non-saline controls (Table 1).

In addition, Leaf Area (LA) was significantly enhanced at moderate (100 mM NaCl) salt levels (ca. 115% and 120% of the control) in *N. retusa* and *A. halimus*, respectively. The increase of salt concentration induced promotion of the relative water content in the both species especially in *N. retusa*. This parameter was maximal in 200-800 mM NaCl in *N. retusa* and at 100-200 mM NaCl in *A. halimus* (Fig. 1).

Table 1: Effect of NaCl concentrations on dry mass, plant height, leaf number and Leaf Area (LA) in *N. retusa* and *A. halimus* plants

Species	NaCl (mM)	Dry mass (g plant ⁻¹)			Plant height (cm)	Leaf number	LA (cm ²)
		Shoot	Root	Shoot root ⁻¹			
<i>N. retusa</i>	0	5.65±0.08 ^c	1.15±0.04 ^a	4.89±0.22 ^{ab}	45.0±0.7 ^b	393±21.5 ^b	313.2±6.7 ^b
	100	6.53±0.17 ^a	1.24±0.07 ^a	5.27±0.35 ^a	57.6±8.2 ^a	432±12.6 ^a	362.2±5.0 ^a
	200	6.18±0.24 ^b	1.14±0.14 ^a	5.50±0.81 ^a	53.9±1.7 ^a	425±6.2 ^a	360.6±6.3 ^a
	400	4.13±0.12 ^d	0.95±0.08 ^b	4.33±0.35 ^b	34.8±2.5 ^c	395±10.6 ^b	267.4±5.2 ^c
<i>A. halimus</i>	0	2.74±0.15 ^e	0.76±0.04 ^c	3.59±0.17 ^c	16.3±2.5 ^d	257±10.4 ^c	151.6±8.2 ^d
	100	8.44±0.31 ^c	1.30±0.08 ^b	6.47±0.38 ^a	96.4±3.4 ^b	167±8.9 ^b	283.3±12.5 ^b
	200	10.95±0.21 ^a	1.63±0.15 ^a	6.75±0.63 ^a	110.7±3.8 ^a	185±10.2 ^a	340.5±7.0 ^a
	400	9.01±0.27 ^b	1.37±0.07 ^b	6.56±0.15 ^a	101.7±8.1 ^b	164±5.6 ^b	287.8±9.7 ^b
	800	4.16±0.19 ^d	0.88±0.07 ^c	4.75±0.55 ^b	70.7±6.2 ^c	107±6.8 ^c	140.6±5.4 ^c
	800	2.61±0.11 ^e	0.66±0.04 ^d	3.94±0.34 ^c	46.8±2.8 ^d	68±4.8 ^d	63.8±5.7 ^d

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

Inorganic ion content: Both species accumulated Na⁺ with increasing NaCl concentration and the highest levels was observed in their shoots. Indeed at 200 mM NaCl salt stress treatment, Na⁺ content of shoots and roots was (8.9 ; 3.9) and (3.8 ; 3.2) times than that in the control in *N. retusa* and *A. halimus*, respectively (Table 2). At 800 mM NaCl level the values of *N. retusa* shoots Na⁺ content reached 10.6 times than that in control and 5.4 times than that in control in *A. halimus*. In contrast, K⁺

content of shoots decreased with increasing salt supply in the two plant species. At 200 mM NaCl level, the percentage inhibition of shoots K⁺ content as compared with control non-treated plants was 37.1% in *A. halimus* and 30.4% in *N. retusa* (Table 2).

The rooted potassium content decreased down to a minimum values in *N. retusa* and *A. halimus* plants grown in the presence of 200 mM NaCl which represents a 31.5 and 42.8% of reduction as compared with the control, non-treated plants.

For higher salinities levels (400-800 mM) roots K⁺ content was insensitive in *N. retusa* or decreased slightly in *A. halimus*. The data shows that the Na⁺/K⁺ ratio increases progressively with increasing salt concentration. In shoots grown at 200 mM NaCl treatment, this ratio was 12.8 times greater in *N. retusa* and only 6.1 times greater as compared with the control for *A. halimus*.

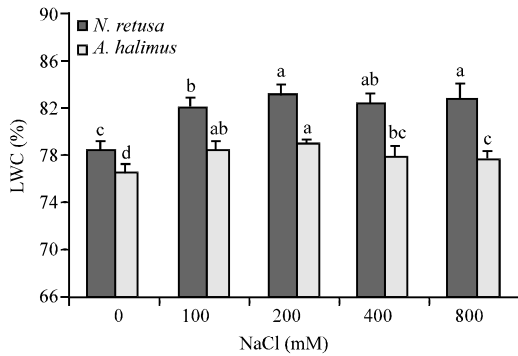


Fig. 1: Effect of NaCl treatments on relative Leaf Water Content (LWC) in *N. retusa* and *A. halimus* leaves. Bars followed by the same letter are not statistically different at $p < 0.05$ (Duncan's multiple range test). Averages of four repetitions are presented with bars indicating SE

Osmotic solute accumulation: The study showed that proline concentration in *A. halimus* leaves was unchanged up to 100 mM NaCl but increased rapidly at higher salinities (Table 3). For *N. retusa* proline concentration, it was 1.6 fold higher at 100 mM and 3.3 fold higher at 800 mM NaCl than that in control while it was maintained at constant value in the range 100-200 mM NaCl. The glycinebetaine content increased

Table 2: Effect of NaCl concentrations on the content of sodium and potassium and Na/K ratio in the shoots and roots of *N. retusa* and *A. halimus* plants

Species	NaCl (mM)	Na ⁺ (μmol g ⁻¹ DM)		K ⁺ (μmol g ⁻¹ DM)		Na ⁺ /K ⁺	
		Shoot	Root	Shoot	Root	Shoot	Root
<i>N. retusa</i>	0	573±52 ^e	597±53 ^e	1104±83 ^a	1164±59 ^a	0.52±0.09 ^d	0.51±0.07 ^d
	100	3087±214 ^d	1961±68 ^d	791±31 ^b	875±78 ^b	3.90±0.27 ^e	2.25±0.19 ^e
	200	5104±86 ^c	2377±136 ^c	768±54 ^b	797±48 ^b	6.66±0.44 ^b	2.99±0.32 ^b
	400	7574±172 ^a	2815±128 ^a	834±33 ^b	895±19 ^b	9.08±0.56 ^a	3.14±0.17 ^b
	800	6089±613 ^b	3376±227 ^a	687±25 ^c	738±26 ^c	8.86±0.85 ^a	4.57±0.35 ^a
<i>A. halimus</i>	0	1058±57 ^d	259±11 ^d	1484±51 ^a	605±44 ^a	0.71±0.14 ^e	0.42±0.05 ^d
	100	2527±78 ^c	954±31 ^b	1041±74 ^b	395±24 ^b	2.43±0.23 ^d	2.42±0.23 ^c
	200	4059±230 ^b	835±60 ^c	933±32 ^c	346±17 ^b	4.35±0.26 ^c	2.41±0.21 ^c
	400	5578±232 ^a	1545±70 ^a	712±33 ^d	315±6 ^c	7.84±0.61 ^b	4.90±0.28 ^b
	800	5734±191 ^a	1472±49 ^a	592±28 ^e	263±11 ^d	9.70±0.71 ^a	5.58±0.16 ^a

Table 3: Effects of NaCl treatments (0, 100, 200, 400 and 800 mM) on the concentration of Hydrogen peroxide (H₂O₂), Malondialdehyde (MDA), proline, glycinebetaine and soluble sugars of *N. retusa* and *A. halimus* shoots

Species	NaCl (mM)	H ₂ O ₂	MDA	Proline	Glycinebetaine	Soluble sugars
		(μmol g ⁻¹ FM)	(nmol g ⁻¹ FM)	(μmol g ⁻¹ DM)	(mg g ⁻¹ DM)	(mg g ⁻¹ DM)
<i>N. retusa</i>	0	12.3±0.8 ^e	6.5±0.5 ^e	40.1±2.7 ^d	6.5±0.6 ^d	26.1±0.9 ^e
	100	11.2±0.7 ^d	4.9±0.4 ^d	58.2±3.1 ^c	8.6±0.9 ^d	18.4±1.4 ^f
	200	10.8±0.8 ^d	5.8±0.5 ^e	61.1±3.3 ^{bc}	15.1±2.3 ^c	23.6±1.2 ^f
	400	15.4±1.1 ^b	7.6±0.6 ^b	67.7±3.7 ^b	51.4±4.2 ^a	34.1±1.8 ^e
	800	21.8±1.5 ^a	8.7±0.6 ^b	121.6±5.4 ^a	47.1±3.1 ^b	46.5±1.4 ^e
<i>A. halimus</i>	0	14.2±1.2 ^{cd}	7.2±0.5 ^e	31.5±1.9 ^d	28.4±1.5 ^e	17.1±1.6 ^f
	100	13.8±1.1 ^d	6.9±0.6 ^e	33.1±1.8 ^d	30.7±1.3 ^d	21.4±2.6 ^f
	200	15.1±1.1 ^c	7.3±0.6 ^e	51.3±3.4 ^c	68.7±5.1 ^c	27.4±1.5 ^e
	400	19.2±1.4 ^b	10.7±0.8 ^b	142.2±5.8 ^b	224.5±5.8 ^b	58.4±3.8 ^e
	800	34.2±2.3 ^a	13.8±1.0 ^a	151.2±6.7 ^a	296.6±7.2 ^a	110.2±8.7 ^e

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

consistently with increasing external salt. *A. halimus* plants accumulated higher levels of glycinebetaine in their leaves under salt conditions than *N. retusa* plants. Soluble sugar content decreased in *N. retusa* with moderate levels (100-200 mM NaCl) but increased with higher salinities. In contrast, a clear increase in soluble sugar concentration was detected in the leaves of *A. halimus* plants treated with NaCl concentrations of 100 mM or higher. The variation of cytoplasm organic compounds contents (Fig. 2) induced by increasing salinity present the same tendency.

Oxidative stress evaluation: Results shows in Table 3 that in both species, lipid peroxidation (MDA content) changed with increasing salinity. As compared with control, the leaf MDA content showed significantly increase only at higher salinities (400-800 mM NaCl). At moderate salinity, the MDA content was unaffected in the

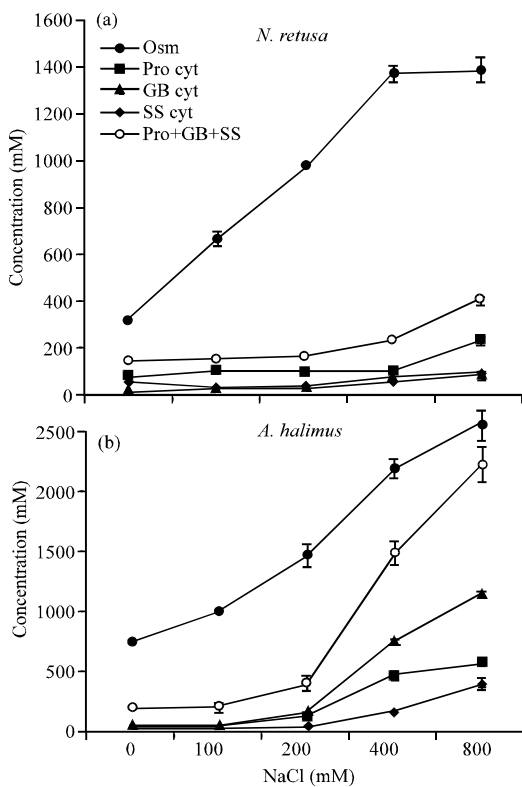


Fig. 2: Comparison between the Osmolarity (Osm) and cytoplasmic concentration of proline (Pro cyt), glycinebetaine (GB cyt) and Soluble Sugars (SS cyt) of *N. retusa*; a) and *A. halimus*; b) shoots. Bars followed by the same letter are not statistically different at $p < 0.05$ (Duncan's multiple range test). Averages of four repetitions are presented with bars indicating SE

leaves of *A. halimus* moreover, this parameter decreased significantly and reached 74.7% compared with control plants at 100 mM NaCl in *N. retusa*.

On the other hand, the MDA accumulation was greater in *A. halimus* than in *N. retusa* thus at 800 mM NaCl level, lipid peroxidation increased by 33.7 and 92% in *N. retusa* and *A. halimus*, respectively (in relation to control). For the H_2O_2 generation, it has been noted that in both species, this parameter as well as the MDA accumulation was correlated with growth inhibition, it increased in all treatments which reduced growth and it was always significantly higher in *A. halimus* as compared to that in *N. retusa* (Table 3).

In *N. retusa*, the H_2O_2 content decreased significantly between 9 and 12.2% at 100-200 mM NaCl and increased significantly at 400 and 800 mM NaCl to 125 and 177% of the control, respectively. In *A. halimus*, the H_2O_2 content decreased slightly by 2.3% at 100 mM NaCl. At higher salinities, the H_2O_2 generation was significantly higher in *A. halimus* than that *N. retusa*. In comparison with the control plants, this parameter increased to 135 and 241% under 400 and 800 mM NaCl, respectively (Table 3). There was a positive linear correlation ($r = 0.87$, $F = 54.2$, $p < 0.0001$ in *N. retusa*; $r = 0.94$, $F = 145.3$, $p < 0.0001$ in *A. halimus*; Fig. 3a) between the MDA accumulation and H_2O_2 content in both species. On the other hand, a highly negative relationship was observed between MDA accumulation and biomass production ($r = -0.89$, $F = 67.9$, $p < 0.0001$ in *N. retusa*; $r = -0.93$, $F = 123.1$, $p < 0.0001$ in *A. halimus*; Fig. 3b).

Effect of salt stress on antioxidant enzymes activities:

The activity of SOD was raised significantly with the increase of salinity in *A. halimus*. The leaves treated with 100, 400 and 800 mM NaCl showed a 29.8, 57.1 and 66.3% increase in SOD activity, respectively compared with control plants at the end of the experimental period (Fig. 4a). However in *N. retusa*, the activity of SOD remained slightly changed with increasing salinity thus, there was observed a slight increase (102.4-104.7% of the control) only at moderate salinities (100-200 mM NaCl). The activity of POD increased with the increase of the concentration of NaCl in the both species (Fig. 4b). The more pronounced increase of POD concentration, particularly at high salinity was observed in *A. halimus*. For example in leaves treated with 800 mM NaCl, the activity of POD reached 418% of control in *A. halimus* and only 180% of the control in *N. retusa*. Concerning the activity of CAT, it remained statistically unchanged with moderate salinities (100-200 mM) in *A. halimus* and even we observed a slightly decreases in the activity of catalase in *N. retusa* plants treated with the same range of

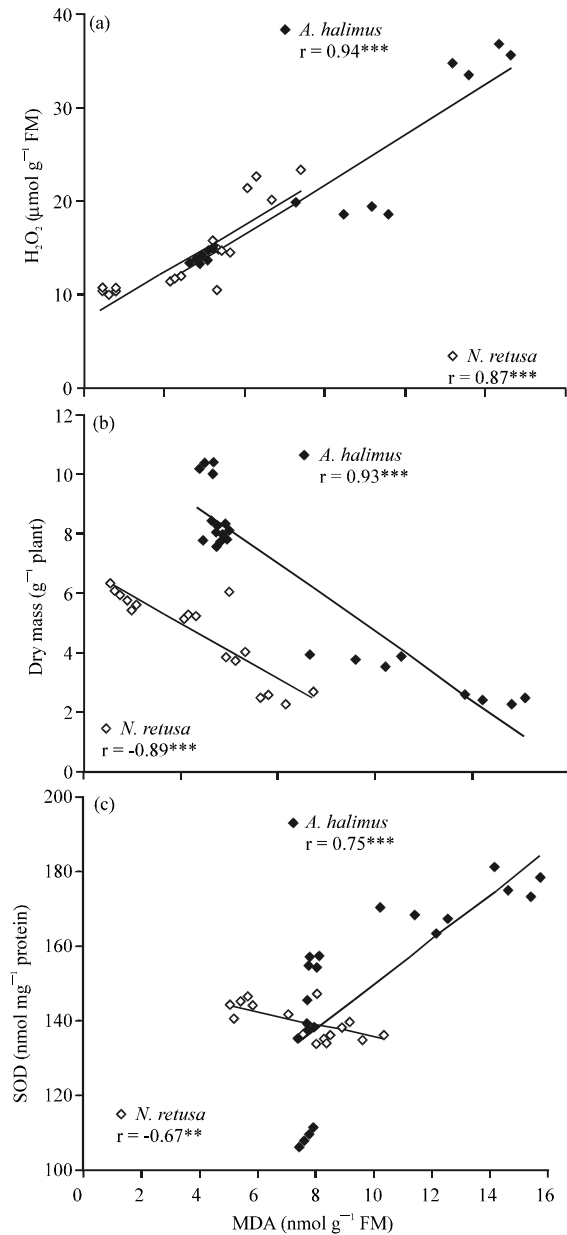


Fig. 3: Effect of NaCl treatments on the contents of; a) Superoxide Dismutase (SOD); b) Peroxidase (POD) and c) Peroxidase (POD) in *N. retusa* and *A. halimus* leaves. Bars followed by the same letter are not statistically different at p<0.05 (Duncan's multiple range test). Averages of four repetitions are presented with bars indicating SE

salt stress and the greater accretion was measured in *A. halimus*. Thus, plants treated with 800 mM NaCl showed a 216.8 and 348% increase in CAT activity in salinity (Fig. 4c). At higher salinities, the amount of CAT increased significantly and progressively with increasing

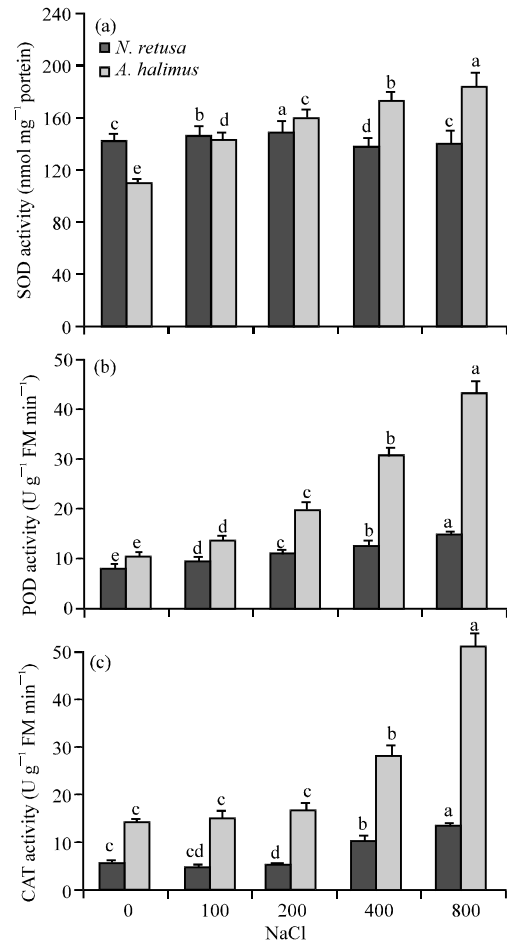


Fig. 4: Relationships between hydrogen peroxide (H₂O₂) contents; a) shoots biomass accumulation; b) and Superoxide Dismutase (SOD) contents; c) with malondialdehyde (MDA) accumulation at *N. retusa* and *A. halimus* plants cultivated under salt stress. An average of five repetitions and confidence interval was calculated at the threshold of 95%

N. retusa and *A. halimus*, respectively compared with control plants (Fig. 4c). A highly positive correlation was observed between SOD activity and MDA content in *A. halimus* (r = 0.75, F = 22.3, p<0.0001; Fig. 3c) whereas this relationship was significantly negative in *N. retusa* (r = -0.67, F = 15.2, p<0.016; Fig. 3c).

DISCUSSION

Both *N. retusa* and *A. halimus* responded to low salinity by increased biomass production, plant height, leaf number and leaf surface area indicating that *N. retusa* and *A. halimus* are two typical halophyte species. But

higher salinities levels (400-800 mM NaCl) were inhibitory to plant growth especially in *A. halimus*. The stimulation of plant growth at moderate salinity is consistent with previous studies on salt requiring halophytes (Moghaieb *et al.*, 2004; Silveira *et al.*, 2009). 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 (Gonzalez *et al.*, 2002; Abogadallah, 2010). The decline in plant growth due to high salinity is associated with a number of physiological and biochemical processes (nutrient imbalance, accumulation of compatible solutes and antioxidants enzyme activities) governing plant growth.

The present study reveals that increasing supply of NaCl caused a marked accumulation of Na⁺ in the plant organs. Ion distributional patterns in halophytic plants were typical for those of salt tolerant species where the major portion of the absorbed ions is translocated to the shoots (Munns, 1993). 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.

These results are consistent with those reported in other halophytes (Pagter *et al.*, 2009; Patel *et al.*, 2009). The present findings (Fig. 1) showed that the accumulation of Na⁺ in photosynthetic organs was associated with an improvement of water content for all levels of salinity in the both species, suggesting that the decrease in plants growth under high salinity can not be explained by reduction in cell turgor. The ability of these species to keep leaf LWC at a level above the control, despite high external salinity makes them less vulnerable to the osmotic and ionic stress caused by salinity (Silveira *et al.*, 2009). The positive relationship between water and Na⁺ contents suggests that *N. retusa* and *A. halimus* plants are able to accumulate Na⁺ in their leaves and to maintain turgor and osmotic adjustment. Thus, high salinity increased leaf and mesophyll parenchyma thickness of *A. halimus* and *N. retusa* and caused lower leaf stomatal density (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 and preserving enzyme activity in the presence of toxic ions (Hasegawa *et al.*, 2000; Lee *et al.*, 2008). The data (Table 3) showed a progressive increase in proline

and glycinebetaine with increasing salinity gradient. The glycinebetaine content in *N. retusa* leaves was 4-6 fold lower than that in *A. halimus* indicating that GB can play an important role in osmotic adjustment of *A. halimus* plants and that *N. retusa* is not considered among the hyperaccumulator of GB. Similar results have been reported for *A. griffithii* and *A. nummularia* (Khan *et al.*, 2000; Silveira *et al.*, 2009).

Soluble sugar involved in alleviating salt stress in many halophytes (Shen and Chen, 2001; Song *et al.*, 2006) in agreement with this view, present data showed that soluble sugar increased as a result of salinity increase for the two species and the most progressive sugar increments were observed in *A. halimus*. The significance of organic compounds accumulation and their role in osmoregulation and salt tolerance has been questioned. To see if these organic compounds play a role in the osmotic adjustment between the cytoplasm and the vacuole, we compared the osmolarity (estimated by $2(\text{Na}^+ + \text{K}^+)/\text{water content}$) with the cytoplasm organic compounds contents. The latter is 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). Figure 2 shows a relative weedy contribution of proline and soluble sugar in maintaining osmotic balance between the vacuole and the cytoplasm in both halophytic species studied while the role of GB was more important especially in *A. halimus*. On the other hand, the sum of the organic compounds concentrations was similar to the osmolarity in *A. halimus* suggesting that these osmolytes are the major compounds involved in maintaining osmotic balance between the vacuole and the cytoplasm. In contrast, the great difference between the accumulation of these osmolytes and the osmolarity detected in *N. retusa*, shows that the osmotic adjustment in this species was provided mainly by inorganic solutes (Na⁺ and Cl⁻) than by compatible compounds. These results are consistent with several other works indicating that GB and proline contributed only a few per cent to osmotic adjustment (Martinez *et al.*, 2005; Geissler *et al.*, 2009).

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 both species, the leaf H₂O₂ and MDA accumulation was increased significantly only at higher NaCl concentration (400-800 mM NaCl). This accumulation was greater in *A. halimus* than in *N. retusa* (Table 3). The high positive correlation between H₂O₂ generation and MDA amount ($r = 0.87^{***}$ in

N. retusa; $r = 0.94^{***}$ in *A. halimus*, Fig. 3a) 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 shoot biomass production and leaf MDA contents ($r = -0.89^{***}$ in *N. retusa*; $r = -0.93^{***}$ in *A. halimus*, Fig. 3b) in both species under salt stress indicating that low lipid peroxidation resulted in increased biomass production which may be attributed to the highly tolerance of these species to moderate salinities and their effective detoxification mechanisms.

However, the increase of MDA and H_2O_2 contents coupled with reduced plant growth at higher salinities indicating that membrane stability had been destroyed and lipid peroxidation had occurred. The 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 halophyte such as *Cakile maritima* and *Limonium bicolor* (Amor *et al.*, 2005; Li, 2008).

Plants have evolved ROS-scavenging enzymes such as SOD, CAT and POD to protect cellular membranes and organelles from damaging effects of ROS (Gómez *et al.*, 2004). Salt tolerance is often correlated with a more efficient antioxidative system (Bor *et al.*, 2003). In the study, the activities of POD and CAT in both species increased significantly with increased salinity gradient in *A. halimus*. The SOD activity increased sharply in *A. halimus* whereas there was only a minor increase in this enzyme activity at moderate salinities (100-200 mM NaCl) in *N. retusa*. This result observed in *A. halimus* is in good agreement with Suaeda salsa (Qiu-Fang *et al.*, 2005) and *Catharanthus roseus* (Elkahoui *et al.*, 2005) and *Limonium bicolor* (Li, 2008) indicating that SOD, POD and CAT play an important role in eliminating ROS under salt stress.

The significant increase observed in SOD activity in *A. halimus* suggested that the enzyme may function as a ROS scavenger by converting O_2^- to H_2O_2 (Alscher *et al.*, 2002; Costa *et al.*, 2005). The CAT and POD destroy the H_2O_2 produced by SOD and other reactions (Foyer *et al.*, 1994). POD is thought to be involved in the oxidation of phenolics (Lagrimini, 1991) and the detoxification of toxic compounds such as H_2O_2 which are produced as a result of oxidative stress (Chaparzadeh *et al.*, 2004). Higher activity of CAT decrease H_2O_2 level in cell 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). In *N. retusa* plant, the result indicated that there was a negative relationship between SOD activity and MDA

content ($r = -0.67^{**}$, Fig. 3c). But this does not imply that *N. retusa* have the higher oxidative stress in contrast the lipid peroxidation and MDA contents considered as the symptom of oxidative damage are lower in comparison with *A. halimus*.

Similar results were found by Sairam *et al.* (1998) who showed that H_2O_2 scavenging systems by APX and CAT are more important than SOD in drought tolerance of wheat. On the other hand, the decrease in the activity of SOD observed at 800 mM NaCl suggests that SOD does not seem to be the only source of H_2O_2 . In agreement with the findings of Tsaia *et al.* (2005) suggested that source of H_2O_2 in NaCl-treated leaves of rice can be NADPH oxidase.

CONCLUSION

In this study, results indicate that both the halophytic studies species *N. retusa* and *A. halimus* showed increase in growth at moderate salinities. The ability to survive at higher salinities in *N. retusa* plants may due to the inclusion of great amount of Na^+ in relation to their advanced leaf succulence and to improved resistance to oxidative stress via increased the activities of POD and CAT. In *A. halimus*, salt tolerance may be due to its ability to use Na^+ in the shoots for osmotic adjustment and specially the synthesis of compatible solutes (glycinebetaine, proline and soluble sugar). In this species, it appears that the antioxidant enzymes (SOD, POD and CAT) have a major role to prevent oxidative damages induced by salinity.

The minor inhibition of the growth and increase of the hydrogen peroxide generation and lipid peroxidation at higher salinities (400-800 mM NaCl) in *N. retusa*, suggest the better tolerance to salt stress of this species as compared to *A. halimus*.

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