

*Full Length Research Paper*

# Enhanced accumulation of root hydrogen peroxide is associated with reduced antioxidant enzymes under isoosmotic NaCl and Na<sub>2</sub>SO<sub>4</sub> salinities

Mahmoudi Hela<sup>1\*§</sup>, Baatour Olfa<sup>1§</sup>, Ben Salah Imen<sup>2</sup>, Nasri Nawel<sup>1</sup>, Wissal Abidi<sup>1</sup>, Huang Jun<sup>3</sup>, Zargouni Hanen<sup>1</sup>, Hannoufa Abdelali<sup>4</sup>, Lachaal Mokhtar<sup>1</sup> and Ouerghi Zeineb<sup>1</sup>

<sup>1</sup>Unité de Physiologie et Biochimie de la Tolérance au Sel des Plantes, Département des Sciences Biologiques, FST, Campus Universitaire, 2092 Tunis, Tunisie.

<sup>2</sup>Laboratoire d'Adaptation des Plantes aux Stress Abiotiques, CBBC, BP901, 2050 Hammam-Lif, Tunisia.

<sup>3</sup>Molecular Genetics, Agriculture and Agri-Food Canada, 107 Science Place, Saskatoon, Saskatchewan, Canada S7N 0X2.

<sup>4</sup>Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, 1391 Sandford Street, London, Ontario, Canada, N5V 4T3.

Accepted 8 February, 2012

The inhibitory effect of salt stress on lettuce is one of the main reasons for the reduction of plant growth and crop productivity. In the present study, the response of two lettuce varieties Verte and Romaine to isoosmotic NaCl and Na<sub>2</sub>SO<sub>4</sub> treatments were examined. Both varieties were grown in pots containing nutrient Hoagland solution with or without 100 mM NaCl or 77 mM Na<sub>2</sub>SO<sub>4</sub>. Relative growth rate (RGR), hydraulic parameters, root ion content, proline and several antioxidant activities in roots were measured after 12 days of treatment. After prolonged exposure to salt stress, relative growth rate and water content of lettuce significantly decreased. Roots accumulated high level of Na<sup>+</sup> under both salts, whereas the accumulation of K<sup>+</sup> and Ca<sup>2+</sup> decreased. High level of Na<sup>+</sup> inside the cells inhibited the K<sup>+</sup> uptake and resulted in increased K<sup>+</sup>/Na<sup>+</sup> ratio. In addition, salt stress also caused an increase in the accumulation of proline. This result suggests that proline may play a crucial role in protecting lettuce under salt stress especially in response to Na<sub>2</sub>SO<sub>4</sub> treatment. Membrane damage estimated by electrolyte leakage (EL) increased especially in response to Na<sub>2</sub>SO<sub>4</sub> treatment in both varieties, but Verte had significantly lower EL relative to Romaine under 100 mM NaCl. A reduction in the activities of CAT in both varieties under 100 mM, and GPX activity in Verte under Na<sub>2</sub>SO<sub>4</sub> treatment coincided with an increase in H<sub>2</sub>O<sub>2</sub> level, indicative of cellular damage and a general depression of the antioxidant enzymatic system in lettuce roots.

**Key words:** Lettuce, NaCl, Na<sub>2</sub>SO<sub>4</sub>, RGR, mineral nutrition, antioxidant activities, proline.

## INTRODUCTION

Salinity affects plant growth by the osmotic stress induced by salt around the roots as well as the toxicity effects caused by excessive accumulation of salt in

leaves. Growth response to salinity has two phases. The first phase is characterized by a large decrease in growth rate caused by the salt outside the roots, that is, an 'osmotic' response, whereas the second phase typically involves an additional decline in growth caused by salt having built up to toxic levels within plants, that is a 'salt-specific' response (Munns, 2005). As a consequence of these primary effects, secondary stresses such as oxidative damage and nutritional imbalance often occur (Zhu, 2001), also affecting plant growth. It has been

\*Corresponding author. E-mail: mahmoudihela@yahoo.fr.

#These two authors contributed equally to this work.

shown that reduction of relative growth rate (RGR) is greater during the time immediately after salt treatment, and there is a partial difference in RGR between control and salt treatments during the long term (Cramer et al., 1994).

Membrane damage, as measured by electrolyte leakage (EL) has been documented for salt-treated plants and can be alleviated by adding Ca (Leopold and Willing, 1984), although the exact mechanism of membrane injury has not been determined. Excess salt concentrations cause enhanced generation of reactive oxygen species (ROS) in plants (Muscolo et al., 2003; Kim et al., 2005). These ROS can cause serious oxidative damages by disrupting lipids, proteins and nucleic acids (Ghorbanli et al., 2004).

Plants have developed various protective mechanisms to eliminate or reduce ROS, which are effective at different levels of stress-induced deterioration (Beak and Skinner, 2003). The enzymatic antioxidant system is one of these protective mechanisms, and includes superoxide dismutase (SOD: EC 1.15.1.1), which can be found in various cell compartments and catalyses the disproportion of two  $O_2^-$  radicals to  $H_2O_2$  and  $O_2$  (Scandalios, 1993).  $H_2O_2$  is eliminated by various antioxidant enzymes such as catalases (CAT: EC 1.11.1.6) (Scandalios, 1993) and peroxidases (POX: EC 1.11.1.7) (Gara et al., 2003) which convert  $H_2O_2$  to water. Moreover, increased POX enzyme activity in responses to salinity was reported by Neto et al. (2006).

Several reports have suggested that the extent of oxidative cellular damage in plants exposed to abiotic stress is controlled by the capacity of their anti-oxidant systems (Silvana et al., 2003). We previously analyzed seedlings of two lettuce varieties (Verte and Romaine) for their physiological and biochemical responses to equivalent osmotic levels of two types of salinity (NaCl and  $Na_2SO_4$ ) (Mahmoudi et al., 2010). In the present study, we aimed to study the implications of  $Na^+$  accumulation in the roots, and the limitations of essential nutrients acquisition. We also evaluated the effects of NaCl and  $Na_2SO_4$  salts on antioxidant enzymes.

## MATERIALS AND METHODS

### Plant material and culture condition

The experiments were carried out in a greenhouse under the following conditions: 16 h light/8 h dark photoperiod and day/night temperature cycle of 20°C/17°C. Seeds were germinated in Petri dishes at room temperature in the dark. Seven-day old seedlings were irrigated with distilled water during the first week. The tested plants (Verte and Romaine) were grown in pots containing Hoagland nutrient solution (Hoagland and Arnon, 1950) diluted 8-fold as the control. After 7 days of acclimation, salt treatment was initiated by adding increments of 20 mM  $Na_2SO_4$  and 25 mM NaCl daily until the desired concentrations of 77 mM  $Na_2SO_4$  and 100 mM NaCl in order to avoid osmotic shock. Individual plants were grown in the control (without salt), control plus 100 mM NaCl, or control plus 77 mM  $Na_2SO_4$  for 12 days prior to harvest. Fresh

weight (FW), dry weight (DW) of roots and rosette leaves of six plants from each treatment were separately recorded. Tissue water content was expressed as a percentage of (fresh weight-dry weight)/fresh weight. Tissues were harvested and stored at -80°C until biochemical analysis.

Relative growth rate (RGR) was calculated from the increase in the dry weight of plant at initial and final sampling. Plant harvests were taken of six replicate plants for each genotype and each treatment at two sampling times 0, and 12 days after final salt concentrations (100 mM NaCl and 77 mM  $Na_2SO_4$ ). Plant DW was determined after drying fresh tissues at 60°C for 48 h. Relative growth rate was calculated for each period as per the equation:

$$RGR = (\ln W_2 - \ln W_1) / (t_2 - t_1) \quad (1)$$

Where, W is the plant dry weight and t is time in days at the start and finish of each period.

### Nutrition parameters

Ions were extracted with 0.5%  $HNO_3$ ;  $K^+$ ,  $Na^+$  and  $Ca^{2+}$  concentrations were assayed by flame photometry (Eppendorf apparatus) and  $Cl^-$  by coulometry (Butcher Cotlove apparatus). The ability of the plants to maintain tissue  $K^+$  or  $Ca^{2+}$  concentration in saline conditions is indicated as  $K^+$  selectivity. It is defined as the ratio of  $K^+/(K^++Na^+)$  in the tissue divided by the ratio of  $K^+/(K^++Na^+)$  in the external medium (Ashraf and McNeilly, 1990).

### Proline content

Proline in root and leaf tissues was extracted and analysed according to Bates et al. (1973). Ten milligram (10 mg) of powder of dry plant material was mixed with 1.5 ml aqueous sulfosalicylic acid (3%, w/v). The homogenate was centrifuged at 14,000 x g for 10 min, and the supernatant was transferred to a fresh 1.5 ml tube. The extracted solution was reacted with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 g ninhydrin in 30 ml glacial acetic and 20 ml 6 M  $H_3PO_4$ ) and incubated at 100°C for 1 h. The reaction was terminated by placing the tube in an ice bath. The reaction mixture was vigorously mixed with 2 ml of toluene. After warming at 25°C, the chromophore was measured at 520 nm. L-proline was used as a standard.

### Electrolyte leakage (Relative ion leakage ratio)

EL was measured to assess membrane permeability according to the procedure of Lutts et al. (1996) using an Electrical Conductivity Meter (EC). Leaf samples were taken and cut into 1 cm sections. The samples were then placed in individual stoppered vials containing 10 ml of distilled water after three washes with distilled water to remove surface contamination and incubated at room temperature (Ca 25°C) on a shaker (100 rpm) for 24 h. Electrical conductivity (EC) of bathing solution ( $EC_1$ ) was recorded after incubation. The same samples were then placed in an autoclave at 120°C for 20 min and a second reading ( $EC_2$ ) was taken after cooling the solution to room temperature. The electrolyte leakage was calculated as  $EC_1/EC_2$  and expressed as a percentage.

### Hydrogen peroxide assay

The hydrogen peroxide ( $H_2O_2$ ) content was determined according to Kotchoni et al. (2006) and as described by Mahmoudi et al. (2011). Briefly, fresh tissue (100 mg) was homogenized with 1 ml of 25 mM  $H_2SO_4$  and then centrifuged at 14000 rpm for 10 min at 4°C. Twenty

microliter (20  $\mu$ l) of the supernatant was added to 200  $\mu$ l of working reagent (WR) using PeroXoquant Quantitative Peroxide Assay Kit (Cat no. 23285, Thermo Fisher Scientific, Ottawa, ON, Canada). The absorbance of the supernatant was measured at 560 nm in a spectrophotometer.  $H_2O_2$  was used as a standard calibration curve.

### Antioxidant activities

Fresh root samples were submersed for 5 min in liquid nitrogen. The frozen roots were kept at  $-80^\circ\text{C}$  for further analyses. Enzymes were extracted from 0.5 g leaf tissue using a mortar and pestle with 5 ml extraction buffer containing 50 mM potassium phosphate buffer, pH 7.6 and 0.1 mM Na-EDTA. The homogenate was centrifuged at 15,000 g for 15 min and the supernatant fraction was used to assay for the various enzymes. All steps in the preparation of enzyme extracts were performed at  $4^\circ\text{C}$ . Gaiacol peroxidase activity (GPX) according to Srinivas et al. (1999) was assayed using guaiacol as an electron donor with a reaction mixture containing 20 mM phosphate buffer (pH 7.0), and 30 mM  $H_2O_2$ . The increase of absorbance due to tetraguaiacol formation was recorded at 470 nm. One unit of peroxidase activity catalyzes the oxidation of 1  $\mu$ mol of guaiacol. APX activity was determined by measuring the consumption of ascorbate following the absorbance at 290 nm. One unit of APX activity was defined as the amount of enzyme required to consume 1  $\mu$ mole ascorbate  $\text{min}^{-1}$  (Cakmak and Marschner, 1992). SOD activity was estimated by recording the decrease in absorbance of superoxidenitro blue tetrazolium complex by the enzyme (Sairam and Srivastava, 2002). About 3 ml of reaction mixture, containing 0.1 ml of 200 mM methionine, 0.1 ml of 2.25 mM nitro-blue tetrazolium (NBT), 0.1 ml of 3 mM EDTA, 1.5 ml of 100 mM potassium phosphate buffer, 1ml distilled water and 0.05 ml of enzyme extraction were taken in test tubes in duplicates from each enzyme sample. Two tubes without enzyme extract were taken as the control. The reaction was started by adding 0.1 ml riboflavin (60  $\mu$ M) and placing the tubes below a light source of two 15 W fluorescent lamps for 15 min. Reaction was stopped by switching off the light and covering the tubes with a black cloth. Tubes without enzyme developed maximal colour. A non-irradiated complete reaction mixture which did not develop colour served as blank. Absorbance was recorded at 560 nm and one unit of enzyme activity was taken as the quantity of enzyme which reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes. CAT activity was measured according to Aebi (1984). About 3 ml reaction mixture containing 1.5 ml of 100 mM potassium phosphate buffer (pH=7), 0.5 ml of 75 mM  $H_2O_2$ , 0.05 ml enzyme extraction and distilled water to make up the volume to 3 ml. Reaction was started by adding  $H_2O_2$  and the decrease in absorbance was recorded at 240 nm for 1 min. Enzyme activity was computed by calculating the amount of decomposed  $H_2O_2$ .

### Data analysis

The values of the parameters were subjected to two-way analysis of variance (ANOVA) and the mean differences were compared by Duncan Test. Each data point was the mean of indicated replicates and comparisons with P-values  $<0.05$  were considered significantly different.

## RESULTS

### Effect of salts on growth

Some growth related characteristics such as plant biomass, relative growth rate (RGR), rosette diameter,

leaf number, leaf area and root length of both lettuce varieties were studied to distinguish the effect of sodium sulfate on lettuce plants. In the control condition, relative growth rate was higher in Verte than in Romaine. Salinity significantly affected RGR in both lettuce varieties (Figure 1). However, a significant difference in RGR between genotypes and treatments was found but results showed that RGR reduced severely with  $Na_2SO_4$ . Verte showed higher relative growth rate at 100 mM NaCl than Romaine. Thus, Verte was more tolerant to NaCl than Romaine. Furthermore, RGR reduced more by  $Na_2SO_4$  than by NaCl in both varieties suggesting that the toxic effect is more important with  $Na_2SO_4$  than with NaCl.

Dry weights (DW) of roots and leaves were reduced by both sodic salts (Mahmoudi et al., 2010). This reduction was due to a decrease in root length, in leaf number, in rosette diameter and in total surface area. DW reduction was more significant with  $Na_2SO_4$  than with NaCl in both varieties (Table 1). However, total surface area decreased by 36 and 86% in comparison with the control plants in Verte, and by 48 and 84% in Romaine under NaCl and  $Na_2SO_4$ , respectively. Leaf number (Table 1) did not change in Verte treated with NaCl, but decreased significantly under  $Na_2SO_4$  in both varieties.

Growth of roots and leaves was similarly affected so that the roots/leaves ratio remained unchanged (Table 1)

### Sodium uptake and distribution

Independently of the treatments,  $Na^+$  was accumulated in both leaves and roots of Verte and Romaine (Figure 2) suggesting a possible saturation of the root tissues at approximately  $1 \text{ mmol.g}^{-1}$  root DW. The accompanying anion did not exert a significant effect on  $Na^+$  uptake in Romaine. However, sodium concentration reached high values [about  $3 \text{ mmol.g}^{-1}$  dry weight (DW)] in Romaine-treated plants. In Verte, the increase in  $Na^+$  concentration was more pronounced in response to  $Na_2SO_4$  ( $5 \text{ mmol.g}^{-1}$  DW) than to NaCl ( $3 \text{ mmol.g}^{-1}$  DW). Thus, Verte seemed inefficient or unable to limit the uptake or translocation of  $Na^+$  under  $Na_2SO_4$ .

### Effect of salts on $Na^+$ , $K^+$ and $Ca^{2+}$ accumulation

Under control conditions, roots of both varieties had similar contents of  $Na^+$  (Table 2). Under NaCl treatment, both lettuce varieties showed an increase in  $Na^+$  accumulation at equivalent concentrations of salt, but Verte roots showed a stronger capacity than Romaine roots to accumulate  $Na^+$  under  $Na_2SO_4$  treatment (Table 2).

Under control conditions, roots of both varieties had similar contents of  $Ca^{2+}$  and  $K^+$  (Table 2). Both salts decreased the  $K^+$  and  $Ca^{2+}$  levels in roots of both varieties especially under  $Na_2SO_4$  treatment. However,

**Table 1.** growth characteristics of Verte and Romaine plants grown in the presence of 0, 100 mM NaCl or 77 mM Na<sub>2</sub>SO<sub>4</sub> salts for 12 days.

Parameter	Genotype	Growth parameter		
		No treatment	100 mM NaCl	77 mM Na <sub>2</sub> SO <sub>4</sub>
Leaf number	Verte	8.50 <sup>a</sup> ± 0.57	7.5 <sup>a</sup> ± 0.57	6.17 <sup>c</sup> ± 0.43
	Romaine	9.00 <sup>a</sup> ± 0.00	8.00 <sup>b</sup> ± 0.00	6.17 <sup>c</sup> ± 0.43
Leaf area, cm <sup>2</sup>	Verte	31.75 <sup>a</sup> ± 2.73	20.40 <sup>b</sup> ± 2.45	4.50 <sup>c</sup> ± 0.54
	Romaine	30.86 <sup>a</sup> ± 2.43	16.00 <sup>b</sup> ± 1.01	5.07 <sup>c</sup> ± 0.52
Rosette diameter, cm	Verte	15.79 <sup>a</sup> ± 1.17	8.50 <sup>b</sup> ± 0.88	5.08 <sup>c</sup> ± 1.86
	Romaine	15.50 <sup>a</sup> ± 0.88	8.63 <sup>b</sup> ± 0.93	4.21 <sup>c</sup> ± 1.47
Root length, cm	Verte	22.75 <sup>a</sup> ± 2.43	18.83 <sup>a</sup> ± 4.33	14.17 <sup>b</sup> ± 1.84
	Romaine	20.33 <sup>a</sup> ± 1.39	18.08 <sup>a</sup> ± 2.58	15.00 <sup>b</sup> ± 1.62
R/L ratio	Verte	0.29 <sup>a</sup> ± 0.05	0.35 <sup>a</sup> ± 0.08	0.21 <sup>a</sup> ± 0.08
	Romaine	0.26 <sup>a</sup> ± 0.05	0.36 <sup>a</sup> ± 0.08	0.21 <sup>a</sup> ± 0.02

Means followed by different letters are significantly different at *P* inferior or equal to 0.05 as determined by two ways analysis (ANOVA). (mean ± S.E., n=6).

**Table 2.** Root ions concentrations and K/Na ratio of Verte and Romaine plants grown in the presence of 0, 100 mM NaCl or 77 mM Na<sub>2</sub>SO<sub>4</sub> salts for 12 days.

Parameter	Genotype	Ions concentrations, mmol.g <sup>-1</sup> DW		
		No Treatment	100 mM NaCl	77 mM Na <sub>2</sub> SO <sub>4</sub>
Root Na <sup>+</sup>	Verte	0.38 <sup>c</sup> ± 0.21	1.52 <sup>b</sup> ± 0.12	2.36 <sup>a</sup> ± 0.03
	Romaine	0.38 <sup>c</sup> ± 0.02	1.16 <sup>b</sup> ± 0.24	1.67 <sup>a</sup> ± 0.08
Root K <sup>+</sup>	Verte	1.77 <sup>a</sup> ± 0.51	0.92 <sup>b</sup> ± 0.11	0.07 <sup>c</sup> ± 0.01
	Romaine	1.78 <sup>a</sup> ± 0.22	1.13 <sup>b</sup> ± 0.28	0.35 <sup>c</sup> ± 0.02
Root Ca <sup>2+</sup>	Verte	0.05 <sup>a</sup> ± 0.02	0.02 <sup>b</sup> ± 0.01	0.02 <sup>b</sup> ± 0.01
	Romaine	0.05 <sup>a</sup> ± 0.02	0.02 <sup>b</sup> ± 0.01	0.02 <sup>b</sup> ± 0.01
Root Na <sup>+</sup> +K <sup>+</sup> +Ca <sup>2+</sup>	Verte	2.19 <sup>a</sup> ± 0.45	2.47 <sup>a</sup> ± 0.13	2.45 <sup>a</sup> ± 0.03
	Romaine	2.21 <sup>a</sup> ± 0.22	2.31 <sup>a</sup> ± 0.47	2.04 <sup>a</sup> ± 0.10
Root K/Na ratio	Verte	0.82 <sup>c</sup> ± 0.10	50.69 <sup>a</sup> ± 5.23	5.77 <sup>b</sup> ± 0.55
	Romaine	0.82 <sup>c</sup> ± 0.02	66.33 <sup>a</sup> ± 5.43	36.08 <sup>b</sup> ± 1.20

(Mean ± SE., n=6). Means followed by different letters are significantly different at *P* inferior or equal to 0.05 as determined by two ways analysis (ANOVA).

accumulation of K<sup>+</sup> reduced by 48 and 96% in Verte roots, compared to 47 and 80% reduction in Romaine roots under NaCl and Na<sub>2</sub>SO<sub>4</sub> treatments, respectively. The decrease of Ca<sup>2+</sup> was similar under both treatments. A significant increase in root K/Na selectivity (Table 2) was found in Verte and Romaine under both salt treatments indicative of differential selectivity of

favourable nutrient ions over toxic Na<sup>+</sup> between Verte and Romaine.

### Water relationships

The maintenance of cell turgor by osmotic adjustment is

an important physiological adaptation to minimize the detrimental effects of salt stress (Munns et al, 1983).  $K^+$ ,  $Na^+$  and  $Cl^-$ , as main soluble inorganic intracellular ions participate in the osmotic adjustment in glycophytes. In varieties, the leaf (Figure 3A, B) and root (Figure 3C, D) water content decreased by  $Na^+$  salt treatments. Thus, large  $Na^+$  accumulation was probably associated with the absence of ion compartmentalization into the leaf and root cells, permitting its accumulation in the cytosol.

### Effect of NaCl and $Na_2SO_4$ on proline accumulation

Proline content was measured in both varieties under control and saline conditions. In control conditions, both varieties contained equivalent amounts of Proline in leaves but roots of Verte variety contained higher levels of Proline as compared to those of Romaine (Figure 4). In control conditions, both varieties contained equivalent amounts of Proline in leaves (Figure 4). Under salt stress, leaf Proline content increased significantly at 100 mM NaCl in Verte but remained unchanged in Romaine. Moreover, this increase was more pronounced in the presence of  $Na_2SO_4$  than of NaCl. However, leaf Proline content increased by 2.71- and 4.99-fold in Verte in the presence of 100 mM NaCl and 77 mM  $Na_2SO_4$  respectively. In Romaine, only an increase by 4.64-fold was observed in Proline content under  $Na_2SO_4$ .

In Verte roots (Figure 4B), Proline contents increased by 2.2-fold at 100 mM NaCl and remained unchanged under  $Na_2SO_4$  treatment, while in Romaine it increased by 2.8- and 2.2- fold in the presence of 100 mM and 77 mM  $Na_2SO_4$ , respectively. These results suggest that the relative sensitivity of proline accumulation to 100 mM NaCl in Verte compared to Romaine was related at least partially to transient leaf Proline accumulation.

### Salts effect on relative ion leakage ratio

Electrolyte leakage (EL) was used to assess membrane permeability. The EL index of leaves and roots of the both varieties progressively increased in response to salt treatments (Figure 5A, 5B). Relative to the control, salt treatments increased EL by 1.6- and 3.2-fold in Verte leaves and by 3.1- and 3.5-fold in Romaine leaves under 100 mM NaCl and 77 mM  $Na_2SO_4$ , respectively. In roots (Figure 5B), EL increased by 4.5 and 13.5 in Verte under 100 mM NaCl and 77 mM  $Na_2SO_4$  respectively. While in Romaine EL was increased by 6.7- and 11.4-fold compared to the control under both salt treatments.

### Root $H_2O_2$ accumulation under salt treatments

Under control condition, root  $H_2O_2$  content was 2-fold higher in Verte than in Romaine (Figure 6). Root  $H_2O_2$  content increased significantly in plants treated with NaCl

(3.1- fold and 9.0-fold in Verte and Romaine respectively). This increase was more pronounced in response to  $Na_2SO_4$ ; it reached the values of 3.6- and 10.1-fold in Verte and Romaine, respectively.

### Effect of isoosmotic salts on root antioxidative enzyme activities

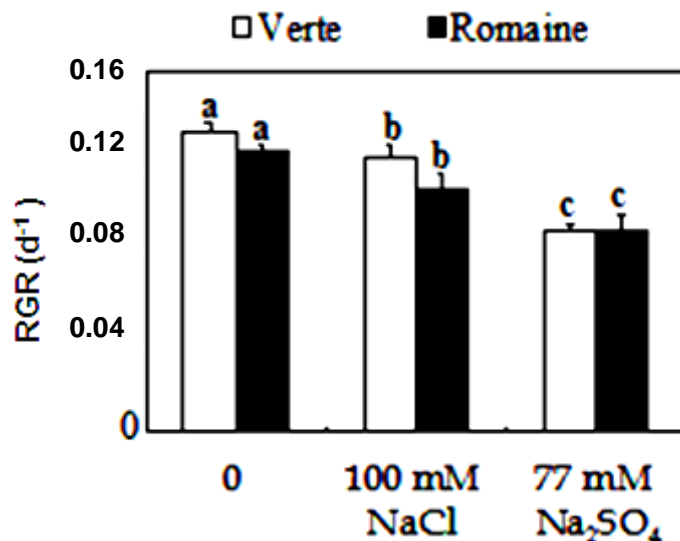
GPX activity (Figure 7A) increased by 193% in Verte roots treated with 100 mM NaCl, but decreased by 52% in roots treated with 77 mM  $Na_2SO_4$ . In Romaine variety, GPX activity did not change irrespective of salt treatment. CAT activity (Figure 7B) decreased by 75 and 66% in comparison with the control under 100 mM NaCl treatment, in Verte and Romaine, respectively. Treatment with 77 mM  $Na_2SO_4$ , did not cause any change in CAT activity in the roots of either variety.

APX activity (Figure 7C) remained unchanged in comparison with the control when plants were treated with 100 mM NaCl. However, increases of 14- and 13-fold in Verte and Romaine, respectively were observed when plants were treated with 77 mM  $Na_2SO_4$ .

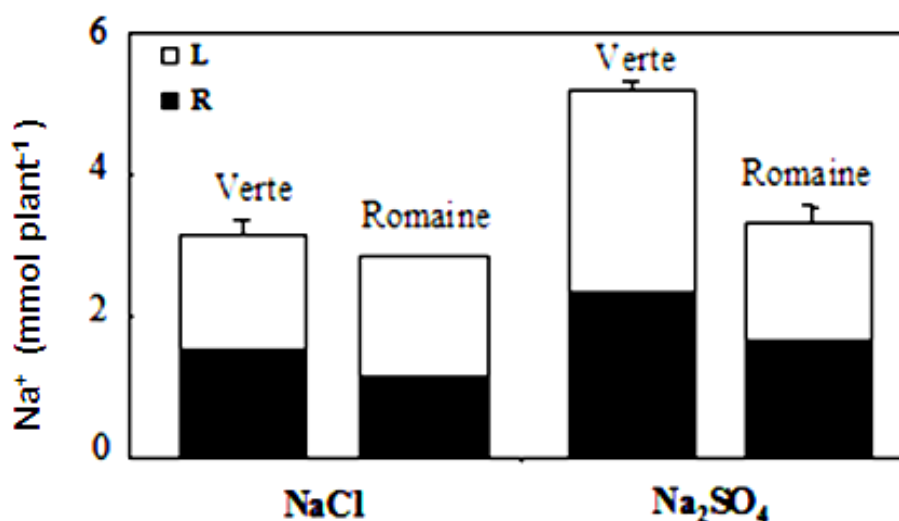
SOD activity represents the most activity as compared with the other three enzymes under control and treatment condition (Figure 7D). Under control condition, both varieties have the same SOD activity. Both NaCl and  $Na_2SO_4$  salts increased significantly SOD activity by 2.18- and 1.87-fold respectively in Verte. In Romaine, SOD activity increased by 2.71- and 3.45- fold under the same conditions.

## DISCUSSION

Lettuce showed a moderate tolerance to NaCl constraint than to  $Na_2SO_4$ , as shown by the reduction of RGR and a number of morphological traits (leaf area, rosette diameter, leaf number, and root length) (Table 1, Figure 1)). Green leaves and dry matter production per plant were reported to be reduced with the increase in soil salinity (Bal and Dutt, 1984). Inhibition of the formation of leaf primordia under salinity stress could be the probable reason for low leaf number. Moreover, RGR provides a relative basis to compare plant growth rates of plants because it takes into account both the initial and the final plant weights over a specified time. Our results show that plant biomass (RGR), leaf number and leaf area (Table 1) were particularly affected by both salts. This finding suggests that salinity modified growth through its effects on leaf expansion and on the initiation of new leaves. Indeed, salt-induced growth reduction might be related to salt osmotic effects, which affect cell turgor and expansion (Rozema, 1991). Our growth parameters data indicate that the inhibitory effect of  $Na_2SO_4$  was stronger than that of NaCl. In contrast, in Sorghum bicolor (Khan et al., 1995), the inhibitory effect of NaCl on growth was more pronounced than that of  $Na_2SO_4$ . Sodium sulphate



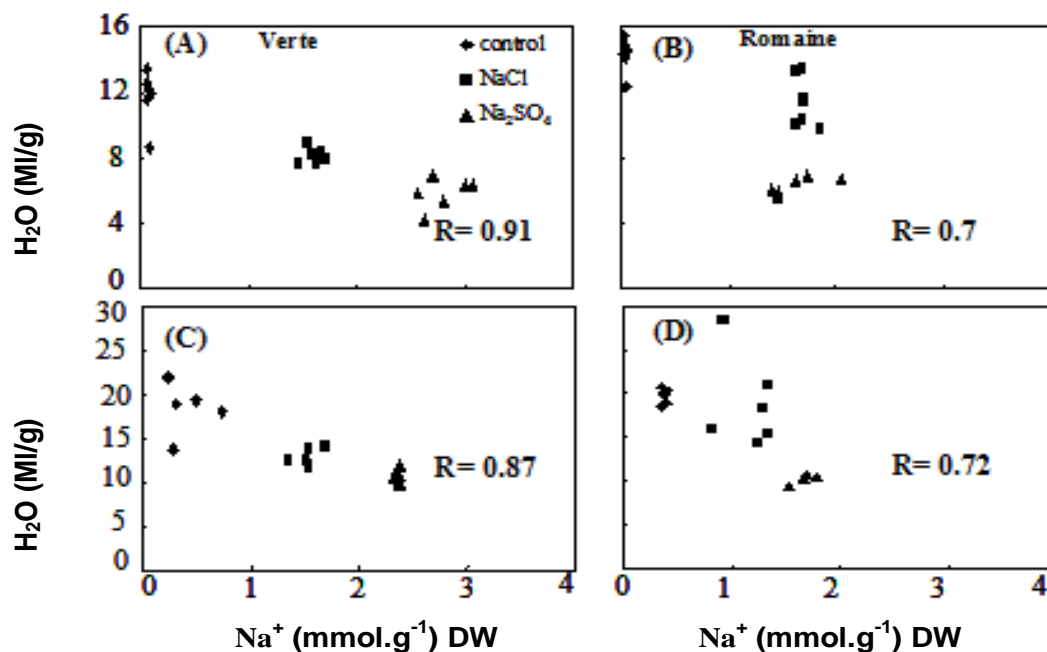
**Figure 1.** Relative growth rate (RGR, d<sup>-1</sup>) of Verte and Romaine. Plants were grown for 12 days in the presence of the indicated salts medium, 100 mM NaCl or 77 mM Na<sub>2</sub>SO<sub>4</sub>. Means of six replicates ± SE. R, roots; L leaves.



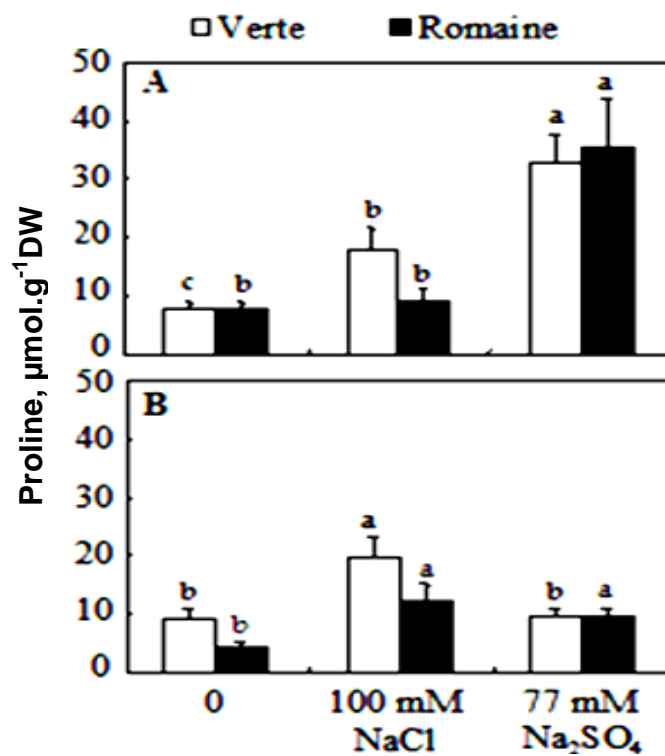
**Figure 2.** Na<sup>+</sup> content of the different plant organs of Verte and Romaine. Plants were grown for 12 days in the presence of the indicated salts medium, 100 mM NaCl or 77 mM Na<sub>2</sub>SO<sub>4</sub>. Means of six replicates ± SE. R, roots; L leaves.

and sodium chloride have been found to have very different effects on plant growth, pigment concentration, protein metabolism, cellular structure (Strogonov, 1973), nutrient interactions (Franklin et al., 2002b), and water relations (Renault et al., 2001; Redfield and Zwiazek 2002). Plants may avoid accumulation of potentially toxic ions in leaf tissues by restricting ion uptake or translocation, and our data suggest that lettuce has a limited ability to regulate both processes for both Na<sup>+</sup> and Cl<sup>-</sup>. In our conditions, the capacity of the roots to sequester ions appears to be exceeded after 2 weeks of

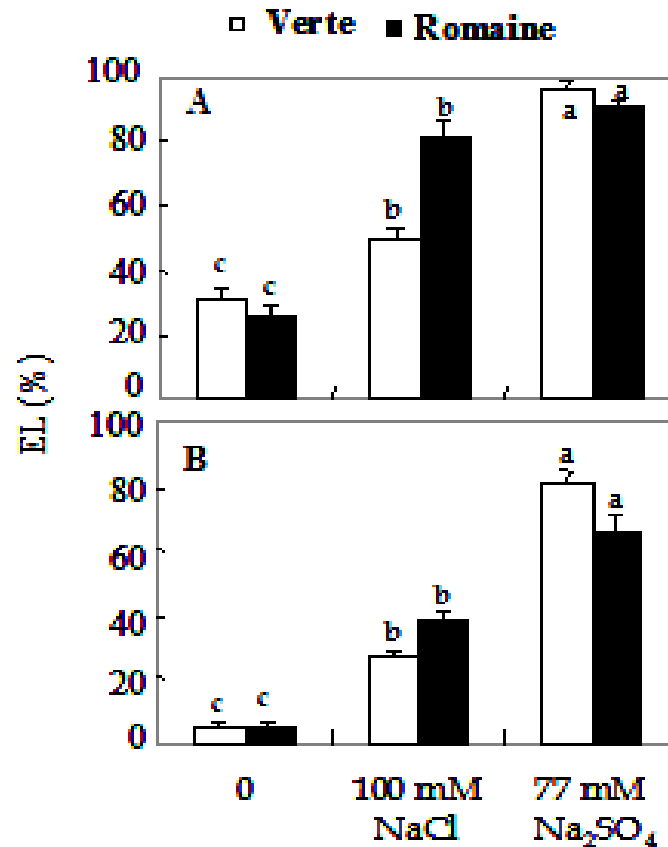
treatment (Figure 2), resulting in increases of Na<sup>+</sup> accumulation in roots and led to the dehydration of this organ (Figure 3, illustrated by high correlation R) especially in Verte under Na<sub>2</sub>SO<sub>4</sub> despite the high proline accumulation (Figure 4). The important increase in the proline contents of leaves and roots of both varieties seemed to be related to a neo-synthesis and an inhibition of the catabolism of this compound (Abdul Jaleel et al., 2007). Our results showed that RGR decreased significantly with salt treatment. This result could indicate that proline accumulation would occur at the expense of



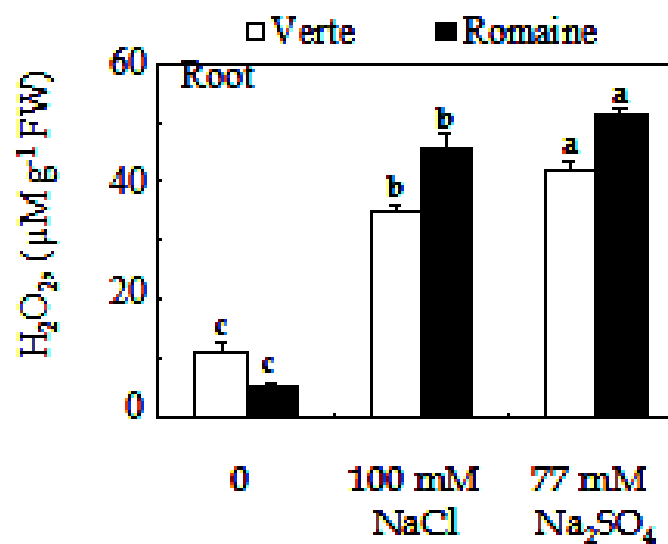
**Figure 3.** Relationship of leaf (Figure 3A, B) and root (Figure 3C,D) hydration (ml H<sub>2</sub>O. g<sup>-1</sup> DM) to Na<sup>+</sup> concentration (mmol. g<sup>-1</sup> DM) in the two lettuce varieties cultivated on the absence (control) or on presence of 100 mM NaCl or 77 mM Na<sub>2</sub>SO<sub>4</sub>. Individual value of six replicates.



**Figure 4.** Effect of salt on leaf Pro (Figure 4A) and root pro (Figure 4B) contents. Seedlings of Verte (empty bar) and Romaine (filled bar) lettuce were grown in the presence of 0, 100 mM NaCl or 77 mM Na<sub>2</sub>SO<sub>4</sub> for 12 days. Error bars represent standard error of the means of healthy leaves randomly selected from six plants for each genotype and treatment.

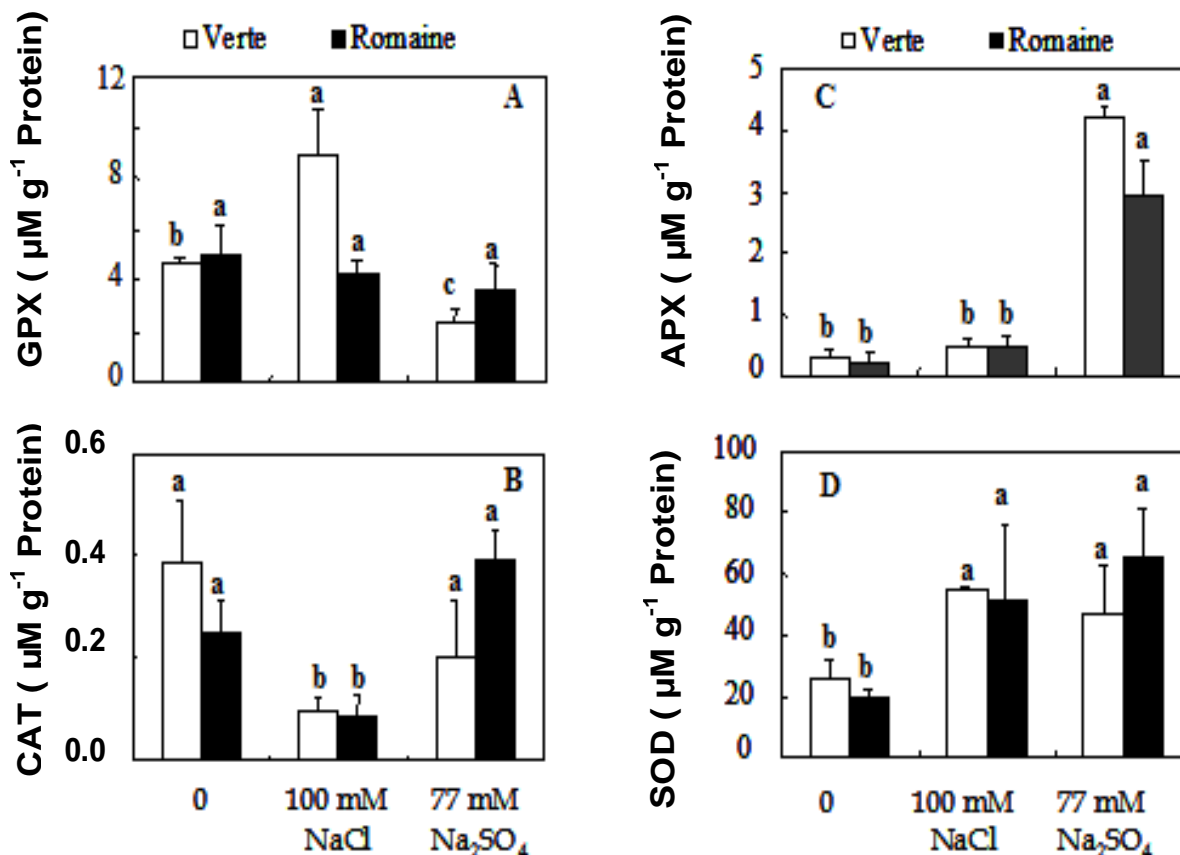


**Figure 5.** Effect of salt on electrolyte leakage and in leaves (A) and roots (B). Seedlings of Verde (empty bar) and Romaine (filled bar) lettuce were grown under 0, 100 mM NaCl or 77 mM Na<sub>2</sub>SO<sub>4</sub> for 12 days. Error bars represent standard error of the means of 5 replicates for EL.



**Figure 6.** Root H<sub>2</sub>O<sub>2</sub> content of Verde (empty bar) and Romaine (filled bar) lettuce grown under 0, 100 mM NaCl or 77 mM Na<sub>2</sub>SO<sub>4</sub> for 12 d. Error bars represent standard error of the means of 4 independent replicates.





**Figure 7.** Root antioxidant activities of Verté (empty bar) and Romaine (filled bar) lettuce grown under 0, 100 mM NaCl or 77 mM Na<sub>2</sub>SO<sub>4</sub> for 12 days. Error bars represent standard error of the means of 4 independent replicates.

growth activity and thus it could not be sufficient by itself to confer salt tolerance (Poustini et al., 2007).

Our previous data (Mahmoudi et al., 2010) showed a marked increase of leaf and root MDA content of both varieties subjected to 100 mM NaCl or 77 mM Na<sub>2</sub>SO<sub>4</sub>, which is indicative of severe oxidative injury to membrane lipids in these plants due to the inadequate response of the antioxidative systems. The apparent increase in membrane EL (Figure 5) seems to parallel the increase in salt content of the leaves in both NaCl and Na<sub>2</sub>SO<sub>4</sub> treatments, and the movement of Na<sup>+</sup> is closely associated with the movement of the anion. The observed reduction in control over ion movement could result from high external levels of Na<sup>+</sup>, since high Na<sup>+</sup> levels can result in membrane depolarization and K<sup>+</sup> efflux (Jacoby, 1994), but this does not explain the differences between NaCl and Na<sub>2</sub>SO<sub>4</sub> treatments.

Salinity can affect growth and yield of plants and can also generate secondary oxidative stress that occurs when there is a serious imbalance between the production of reactive oxygen species (ROS) and antioxidant defence, leading to oxidative damage of macromolecules and, eventually, of cellular structure. In agreement with growth parameters, the production of H<sub>2</sub>O<sub>2</sub> increased

following the treatment (Figure 6), and was more significant in Na<sub>2</sub>SO<sub>4</sub> than in NaCl treatments in both lettuce varieties, indicative of cellular damage. The increased production of H<sub>2</sub>O<sub>2</sub> with both isoosmotic salts could be due to a significant decrease of GPX in Verté under Na<sub>2</sub>SO<sub>4</sub> and CAT activities in Roots of Verté and Romaine under NaCl (Figure 7A, C). Although, APX activity was significantly increased in roots of both varieties in response to Na<sub>2</sub>SO<sub>4</sub> associated with a significant increase in SOD activity under NaCl and Na<sub>2</sub>SO<sub>4</sub> treatments associated to an increase of H<sub>2</sub>O<sub>2</sub> level. These findings suggest a general depression of the antioxidant enzymatic system in lettuce roots.

In conclusion, our data indicate that both salts induced oxidative damage in *Lactuca sativa* L. The damage inflicted by salinity on plant physiology and development was shown to be caused by osmotic stress due to high concentrations of Na<sup>+</sup> ion in organs; leaves and roots associated with a nutritional disturbance (reduction of K<sup>+</sup> and Ca<sup>2+</sup> accumulations) and the toxic effect promoted by Na<sup>+</sup> accumulation in plant tissues. However, despite concomitant increases in the activities of some antioxidant enzymes such as APX and SOD, an increase of H<sub>2</sub>O<sub>2</sub> level was observed, indicative of cellular damage and a

general depression of the antioxidant enzymatic system in lettuce roots.

## ACKNOWLEDGEMENTS

This work was supported through a Tunisian-Canadian Cooperation Initiative. Ministry of Agriculture of Tunisia provided the lettuce seeds.

**Abbreviations:** **RGR**, Relative growth rate; **GPX**, gaiacol peroxidase; **CAT**, catalase; **SOD**, superoxide dismutase; **EL**, electrolyte leakage.

## REFERENCES

- Abdul Jaleel C, Gopi R, Sankar B, Manivannan P, Kishorekumar A, Sridharan R, Panneerselvam R (2007). Studies on germination, seedling vigour, lipid peroxidation and proline metabolism in *Catharanthus roseus* seedlings under salt stress. *South Afr. J. Bot.* 73: 190-195.
- Aebi H (1984). Catalase *in vitro*. *Method of Enzymol.* 105: 121-126.
- Ashraf M, McNeilly T (1990). Responses of four Brassica species to Sodium chloride. *Environ. Exp. Bot.* 30(4): 475-487.
- Bates LS, Waldren RP, Teare ID (1973). Rapid determination of free proline for water-stress studies. *Plant Soil*, 39: 205-207
- Beak KH, Skinner DZ (2003). Alteration of antioxidant enzyme gene expression during cold acclimation of near-isogenic wheat lines. *Plant Sci.* 165: 1221-1227
- Cakmak I, Marschner H (1992). Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. *Plant Physiol.* 98: 1222-1227.
- Cramer GR, Alberico GL, Schmidt C (1994). Salt tolerance is not associated with the sodium accumulation of two maize hybrids. *Australian J. Plant Physiol.* 21: 675-692.
- Gara LD, Pinto MC, Tommasi F (2003). The antioxidant systems vis-à-vis reactive oxygen species during plant-pathogen interaction. *Plant Physiol. Biochem.* 41: 863-870.
- Ghorbanli M, Ebrahimzadeh H, Sharifi M (2004). Effects of NaCl and mycorrhizal fungi on antioxidative enzymes in soybean. *Biol. Plant.* 48: 575-581.
- Hoagland DR, Arnon DI (1950). The water-culture method for growing plants without soil. *Calif. Agric. Exp. Station Circ. Berkeley* 347: 1-32.
- Jacoby B (1994). Mechanisms involved in salt tolerance by plants. In: Pessaraki M (ed) *Handbook of Plant and Crop Stress*. Marcel Dekker, New York, pp. 97-124
- Khan AH, Ashraf MY, Naqvi SSM, Khanzada B, Ali M (1995). Growth, ion and solute contents of sorghum grown under NaCl and Na<sub>2</sub>SO<sub>4</sub> salinity stress. *Acta Physiol. Plant.* 17: 261-268
- Kim SY, Lim J, Park MR, Kim YJ, Park TI, Seo YW, Choi KG, Yun SJ (2005). Enhanced Antioxidant Enzymes Are Associated with Reduced Hydrogen Peroxide in Barley Roots under Saline Stress. *Biochem. Mol. Biol.* 38: 218-224
- Kotchoni SO, Kuhns C, Ditzer A, Kirch HH, Bartels D (2006). Overexpression of different aldehyde dehydrogenase genes in *Arabidopsis thaliana* confers tolerance to abiotic stress and protects plants against lipid peroxidation and oxidative stress. *Plant Cell Environ.* 29: 1033-1048.
- Leopold AC, Willing RP (1984). Evidence for toxicity effects of salt on membranes. In: Staples RC, Toenniessen GH, editors. *Salinity Tolerance in Plants*. New York: John Wiley Sons. pp. 67-76
- Lutts S, Kinet JM, Bouharmont J (1996). NaCl-induced senescence in leaves of rice *Oryza sativa* L.) cultivars differing in salinity resistance. *Ann. Bot.* 78: 389-398.
- Munns R, Greenway H, Kirst GO (1983). Halotolerant eukaryotes. In: OL Lang, PS Nobel, CB Osmond, H Ziegler (eds): *Encyclopaedia of plant physiology*, vol. 12C. New Series. Springer, New York, 59-135
- Munns R (2005). Genes and salt tolerance: bringing them together. *New Phytologist.* 176: 645-63.
- Muscolo A, Sidari M, Panuccio MR (2003). Tolerance of kikuyu grass to long term salt stress is associated with induction of antioxidant defenses. *Plant Growth Regul.* 41: 57-62.
- Neto ADA, Prisco JT, Eneas-Filho J, Abreu CEB, Gomes-Filho E (2006). Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. *Environ. Exp. Bot.* 56: 87-94.
- Poustini K, Siosemardeh A, Ranjbar M (2007). Proline accumulation as a response to salt stress in 30 wheat (*Triticum aestivum* L.) cultivars differing in salt tolerance. *Genet. Res. Crop Evol.* 54: 925-934.
- Redfield E, Zwiazek JJ (2002). Drought tolerance characteristics of black spruce (*Picea mariana*) seedlings in relation to sodium sulfate and sodium chloride injury. *Can. J. Bot.* 80: 773-778
- Renault S, Crosser C, Franklin JA, Zwiazek J.J (2001). Effects of NaCl and Na<sub>2</sub>SO<sub>4</sub> on red-osier dogwood (*Cornus stolonifera* Michx) seedlings. *Plant Soil.* 233: 261-268
- Rozema J (1991). Growth, water and ion relationships of halophytic monocotyledonae and dicotyledonae: a unified concept. *Aquat. Bot.* 39: 3-16.
- Sairam RK, Srivastava GC (2002). Changes in antioxidant activity in sub-cellular fraction of tolerant and susceptible wheat genotypes in response to long term salt stress. *Plant Sci.* 162:897-904
- Silvana BD, Gallego SM, Benavides MP, Tomaro ML (2003). Behaviour of antioxidant defense system in the adaptive response to salt stress in *Helianthus annuus* L. cells. *Plant Growth Regul.* 40: 81-88.
- Scandalios JG (1993). Oxygen stress and superoxide dismutase. *Plant Physiol.* 101: 7-12
- Strogonov BS (1973). Structure and function of plant cells in saline habitats: New trends in the study of salt tolerance. Translated from Russian by A. Mercado. Translation edited by B. Gollek. John Wiley & Sons, New York
- Zhu JK (2001). Plant salt tolerance. *Trends Plant Sci.* 6: 66-71.