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Full Length Research Paper

Salt stress induced changes in germination, lipid peroxidation and antioxidant activities in lettuce (*Lactuca sativa* L.) seedlings

Mahmoudi Hela[1]*#, Nasri Nawel[1#], Tarchoune Imen[1], Zargouni Hanen[1], Ben salah Imen[2], Ben Massoud Raouia[1], Baatour Olfa[1], Kaddour Rym[1], Ben Nasri Ayachi Mouhiba[1], Hannoufa Abdelali[3], Mokhtar Lachaâl[1] and Zeineb Ouerghi[1]

[1]Unité de Physiologie et Biochimie de la Tolérance au Sel des Plantes, Département des Sciences Biologiques, FST, Campus Universitaire, 2092 Tunis, Tunisie.
[2]Laboratoire d'Adaptation des Plantes aux Stress Abiotiques, CBBC, BP901, 2050 Hammam-Lif,Tunisia.
[3]Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, 1391 Sandford Street, London, Ontario, Canada, N5V 4T3.

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Seeds of four lettuce (*Lactuca sativa* L.) genotypes viz., Great Lakes (GL), Paris Island cos, Kagraner Sommer (KS) and Isadora were assessed for their response to salt at the germination and seedling stages. The germination rate of the four varieties was comparatively studied under 0, 50, 100, 150 and 200 mM sodium chloride (NaCl) treatments. Differential response of genotypes to salt concentration was observed. Final germination percentage (FG%) decreased with increasing salinity in GL, Paris Island cos and Isadora varieties, and was annulated at the highest salt concentration in GL, the most sensitive variety. However, in the less sensitive, KS, FG% was decreased by 60% compared to the control at 200 mM. KS and GL varieties were selected as stress-tolerant and stress-sensitive varieties, respectively, and were used for further characterization. Dry weights (DWs) of radicles and hypocotyls were reduced by NaCl 100 and 150 mM in GL and only at 150 mM NaCl in KS. The activities of several antioxidant enzymes during germination were determined. Both varieties had the same malondialdehyde (MDA) content under 100 and 150 mM NaCl. The highest gaiacol peroxidise (GPX) activity was detected in GL seedlings at NaCl 50, 100 and 150 mM, respectively. Catalase (CAT) decreased at 50 and 100 mM NaCl, respecively. Total ascorbate (AsA + DHA) decreased in GL and increased significantly in KS. Our results suggest that tolerance of KS to salt may be due to a better protection mechanism against salinity induced oxidative damage relative to GL variety.

Key words: Germination, lettuce, NaCl, growth, antioxidant activities, ascorbate.

INTRODUCTION

Seed germination is a major factor limiting the establishment of plants under saline conditions (Ghavami and Ramin, 2007), and is the most critical phase in plant life (El- Keblawy and Al-Rawai, 2005) that is greatly influenced by salinity (Misra and Dwivedi, 2004).

Salinity may cause significant reductions in the rate and final germination percentage (FG%), which in turn may lead to uneven stand establishment and reduced crop yields (Foolad et al., 1999). Rapid, uniform, and complete germination is a prerequisite for successful transplant production and stand establishment in vegetable crops (Demir and Ermis, 2003). Several authors have shown that, pepper, is sensitive or moderately sensitive, to salinity during different growth stages (Pascale et al., 2003). It has also been shown that salinity caused a

*Corresponding author. E-mail: mahmoudihela@yahoo. Tel. +21624599739. Fax: +21671885480.

# These authors contributed equally to this work.

decrease in FG% of lettuce seeds (Nasri et al., 2010).

Salinity also induces numerous disorders in seeds and propagules during germination (Khan and Ungar, 1997). However, salt stress can stimulate formation of active oxygen species (AOS), such as superoxide, hydrogen peroxide (H_2O_2) and hydroxyl radicals. These activated oxygens injure the cellular components of proteins, membrane lipids and nucleic acids (Foyer et al., 1994). Saline environments are generally correlated with changes in plant lipid metabolism (Kuiper, 1985). Lipid peroxidation has been associated with damages provoked by a variety of environmental stresses (Hernandez et al., 2003). Polyunsaturated fatty acids (PUFA) are the main membrane lipid components susceptible to peroxidation and degradation. Malondialdehyde (MDA), which is the product of decomposition membrane PUFA, shows active accumulation under salt stress (Sudhakar et al., 2001).

In order to avoid these oxidative injuries, plants have developed enzymatic systems for scavenging these highly active forms of reactive oxygen species (ROS); superoxide is converted by superoxide dismutase enzyme into H_2O_2, which is further scavenged by catalase (CAT) and various peroxidases. Ascorbate peroxidase (APX) and gluthathione reductase (GR) also play a key role by reducing H_2O_2 to water through the Halliwell–Asada pathway (Noctor and Foyer, 1998). However, many reports suggest that the extent of oxidative cellular damage in plants exposed to abiotic stress is controlled by the capacity of their anti-oxidant systems (Scandalio et al., 2001).

Ascorbic acid (AsA) is an important component of the plant antioxidant system (Smirnoff and Wheeler, 2000) where it plays a protective role against ROS by scavenging free radicals and AOS that are generated during salt stress conditions (Hernandez et al., 2001), and those that are formed from photosynthetic and respiratory processes (Guo et al., 2005). AsA is linked to cell growth, being involved in the cell cycle and other mechanisms of plant cell growth and division, as well as acting as a co-factor for many enzymes (Lee and Kader, 2000). Various plant tissues are known to accumulate up to milimolar concentrations of L-AsA.

The aim of this study was to assess the response of lettuce seedlings to salinity by understanding the role of oxidative enzymes, viz, peroxidase, CAT and ascorbate.

MATERIALS AND METHODS

Lettuce seeds (500 g per variety) were obtained from the seed laboratory of the Ministry of Agriculture of Tunisia, which is the sole source of seeds in Tunisia. Seeds were surface-sterilized with 95% ethanol solution for 5 min, and thoroughly rinsed five times with distilled water. These cultures were kept under aseptic conditions for four days in the dark at 25°C. Seeds of four lettuce varieties were germinated in covered Petri dishes on two layers of filter paper moistened with 10 ml of treatment solution. Four NaCl concentrations (50 mM, 100 mM, 150 mM and 200 mM) were included in the germination tests. Each treatment was replicated four times each with 25 seeds for four consecutive days. The germinated seeds were counted every 24 h, and the germination test was ended after four days. The following traits were measured; germination percentage, MDA content, ascorbate level and antioxidant enzyme activities.

Plant material and final germination percentage

FG% was calculated as described by Nasri et al. (2010) based on the following equation;

FG% =100 × total germinated seeds/total number of seeds

Fresh weights (FW) of all samples were recorded. Four seedlings were cut into radicle, hypocotyl and cotyledons and then the sum of the different organs were put in 60°C for two days. The dry weight (DW) was then determined using a precision balance. Tissue water content was obtained from the (FW - DW/FW) ratio.

Malondialdehyde assay

Lipid peroxidation was defined by the content of MDA, according to the method of Du and Bramlage, 1992. 0.2 g sample of frozen leaves was ground to a fine powder with liquid nitrogen and extracted with 3 ml of cold ethanol. The crude extract preparation was centrifuged at 12,000 *g* for 20 min. A mixture of trichloroacetic acid (TCA), thiobarbituric acid, butylated hydroxytoluene, and an aliquot of supernatant was heated and the reaction was stopped by quickly placing the mixture in an ice-bath. The cooled mixture was centrifuged, and the absorbance of the supernatant was measured at 400, 500 and 600 nm. Thiobarbituric acid-reactive substances (TBARS) were measured as MDA, a degraded product of the lipid. The concentration of MDA was determined from the absorbance, by using an extinction coefficient of 155 mM cm^{-1}.

Determination of enzyme activity

Seeds of two lettuce varieties, Kagraner Sommer (KS) (tolerant variety) and Great Lakes (GL) (sensitive type) were moistened in Petri dishes with 0, 50, 100 and 150 mM NaCl solutions and were placed in an incubator in constant darkness at 25°C. After four days, the seedlings of each treatment and of the control were homogenised with 5 ml of extraction buffer containing 50 mM K phosphate buffer, pH 7.5, 100 mM ethylene diamine tetraacetic acid (EDTA), 5% polyvinyl pyrrolidone (PVP), 5% glycerol and 1 mM dithiothreitol (DTT). The homogenate was centrifuged at 15,000 *g* for 15 min, and the supernatant fraction was used to assay various enzymes. All steps in the preparation of enzyme extracts were performed at 4°C. Protein concentrations in the enzyme extract were determined by Bradford's method (1976) using bovine serum albumin as a standard.

CAT activity was determined by monitoring the disappearance of H_2O_2 according to the method outlined by Cakmak and Marschner (1992). The final reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0) and 2% H_2O_2. The activity was expressed as units (µmol H_2O_2 consumed per minute) per mg of protein.

Gaiacol peroxidase (GPX) activity was assayed according to Srinivas et al. (1999) using guaiacol as an electron donor. The reaction mixture contained 20 mM phosphate buffer (pH 7.0), and 30 mM H2O2. The increase of absorbance, due to tetraguaiacol formation, was recorded at 470 nm. One unit of peroxidase activity catalyzes the oxidation of 1 µmol of guaiacol.

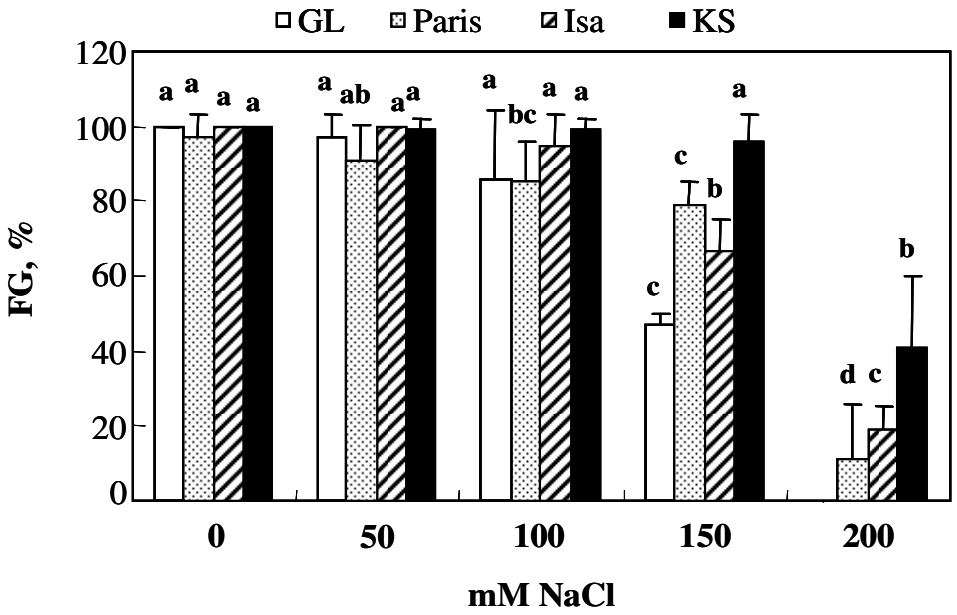

Figure 1. Effect of different NaCl concentrations (0, 50, 100, 150 and 200 mM) on the FG% of four lettuce varieties (GL, Paris island cos, Isadora and KS) after four days of treatment.

Ascorbate determination

AsA content was assayed as described by Kampfenkel et al., (1995). Aliquots of the fresh material (0.25 g) were homogenized in ice-cold 6% (w/v) TCA, using a cold mortar and pestle. The total and reduced ascorbate were determined in the supernatant after centrifugation at 15,000 *g* for 10 min at 4°C. A red colour resulted from the complex of bipyridine and iron(II) ion (Fe^{2+}) caused by reduction of iron(III) ion (Fe^{3+}) to Fe^{2+} by AsA and was measured at 525 nm. AsA + DHA was determined through a reduction of dihydroascorbate (DHA) to ascorbate by 10 mM DTT. Excess DTT was removed with 4% (w/v) N-ethylmaleimide (NEM). A standard curve covering the range of 10 to 50 µmol ascorbate was used.

Statistical analysis

Results were expressed as the means of indicated replicates across each parameter. Significant differences between treatments were analyzed using analysis of variance (ANOVA) and mean comparison with Duncan test (Statistica®). Values were calculated at the $p \leq 0.05$ probability level.

RESULTS

Effects of NaCl on final germination and daily germination percentages

The germination percentage decreased with increasing salt concentration (Figure 1). Among the salinity levels, the highest germination was observed at 100 mM and the lowest at 200 mM. Germination kinetics and germination percentages of seeds of the four lettuce varieties (Figures 1 and 2) were over 85% in 0, 50 and 100 mM NaCl. At 150 mM NaCl, final seed germination percentages were significantly decreased by 53, 33, and 21%, respectively in GL, Isadora, Paris island cos varieties, but not considerably affected in KS (4%). The highest reduction of germination was found at 200 mM, at which the FG% decreased to 100, 81, 89 and 59%, respectively in GL, Isadora, Paris island cos and KS. The two lettuce varieties KS and GL showed contrasting behaviour in response to 200 mM NaCl constraint; as KS was more tolerant and GL was more sensitive, compared to the other lettuce varieties used in our experiment. These two varieties were thus used in this investigation.

Effect of NaCl on seedling growth of lettuce varieties

In order to define salt stress tolerance or sensitivity of seedlings, the effects of NaCl treatment on DWs of the cotyledons, hypocotyls and radicles were tested (Table 1). In GL variety, the DW of radicles and hypocotyls showed no significant changes at 50 mM NaCl, but diminished at 100 and 150 mM. At higher salinity (150 mM), radicle growth was more affected than was hypocotyl growth. Radicle biomass was diminished by 65% as compared with the control (no salt), whereas hypocotyls biomass was diminished only by 40%. In KS variety, the DW of radicles and hypocotyls was affected only at 150 mM. The DW of cotyledons increased progressively with increasing NaCl concentrations. Radicle water content was poorly diminished in GL, but increased in KS under 150 mM NaCl whereas, hypocotyl and cotyledon dehydration increased with increasing NaCl concentrations especially in GL.

Table 1. Effect of different NaCl concentrations (0, 50,100 and 150 mM) in DW (mg) and the water content (ml.g^{-1}DW) in radicle, hypocotyl and cotyledons of two lettuce varieties GL and KS.

Concentration	Radicle		Hypocotyle		Cotyledon	
	DW	H_2O	DW	H_2O	DW	H_2O
Great Lakes						
Control	0.58^{a}±0.08	20.47^{a}±3.74	1.23^{a}±0.26	27.02^{a}±3.58	1.12^{a}±0.22	6.51^{a}±2.06
50 mM NaCl	0.53^{a}±0.11	18.75^{a}±3.21	1.35^{a}±0.17	24.18^{a}±2.77	1.37^{ab}±0.13	5.06^{b}±0.27
100 mM NaCl	0.42^{b}±0.11	22.28^{a}±3.11	0.93^{b}±0.11	19.17^{b}±2.11	1.53^{b}±0.23	3.60^{c}±0.68
150 mM NaCl	0.20^{c}±0.00	18.50^{a}±4.72	0.73^{b}±0.24	6.78^{c}±2.93	1.92^{c} ±0.28	1.62^{d}±0.42
Kagranner Sommer						
Control	0.55^{a}±0.06	16.14^{a}±2.45	1.28^{ab}±0.08	25.96^{a}±3.30	1.38^{a}±0.22	6.15^{a}±0.66
50 mM NaCl	0.63^{a}±0.11	13.76^{a}±3.01	1.48^{a}±0.04	23.82^{a}±1.82	1.67^{ab}±0.17	5.04^{b}±0.48
100 mM NaCl	0.55^{a}±0.17	14.13^{a}±4.64	1.23^{b}±0.33	18.35^{b}±4.45	1.73^{b}±0.29	4.27^{b}±0.59
150 mM NaCl	0.38^{b}±0.04	21.51^{b}±3.96	0.83^{c}±0.08	18.51^{b}±8.36	2.42^{c}±0.54	2.32^{c}±0.37

Values are means of six replicates ± SE. Means followed by different letters are significantly different ($P \leq 0.05$) as assessed by Duncan's multiple range tests.

Effect of NaCl on lipid peroxidation

Membrane lipid peroxidation was estimated in seedlings after four days of NaCl treatment (Figure 3), with MDA content used as its index. MDA levels were elevated in the KS variety more than GL under control conditions. On the 4th day of treatment, the seed MDA contents increased by 2-, 3,4- and 2.8-fold compared to the control seed of GL in the presence of 50, 100 and 150 mM NaCl, respectively; whereas, in KS, NaCl increased the MDA content by 1.7- and 1.3-fold under 100 and 200 Mm, respectively. At 50 mM NaCl, the MDA content decreased by 65% compared to the control seedling.

Salt effect on antioxidant enzyme activities

Effects of NaCl on two antioxidant enzyme activities (CAT and GPX) in the two lettuce varieties seedlings are presented in Figure 4. The important GPX activity in GL seedlings was observed at NaCl 100 mM. However, at this concentration, the increase represented 2.21-fold compared to the control. GPX activity decreased significantly with increasing NaCl concentrations in KS. This decrease was 66, 72 and 75% under 50, 100 and 150 mM NaCl, respectively. The increased GPX activity in GL (sensitive variety) might enable plants to protect themselves against salt stress.

Under control conditions, GL had much higher CAT activity compared to KS. In response to NaCl stress, CAT activity in GL seedlings decreased significantly by 37 and 41% compared to the control in the presence of 50 and 100 mM NaCl, respectively while a significant increase by 38% was observed in response to 150 mM NaCl. In KS seedlings, the CAT activity showed an increase of 41% in the presence of 150 mM NaCl. However, at 50 and 100 mM NaCl, the CAT activity was not affected. Our result suggests that H_2O_2 elimination in GL under salt stress may be due to higher GPX activity at 100 mM and to CAT activity at 150 mM NaCl.

Salt effect on ascorbate content

AsA + DHA content (Figure 5) was higher in GL than in the KS variety. Salt treatment changed the AsA content of both varieties. However, AsA + DHA showed a significant decrease by 51, 55 and 51% in GL seedlings, respectively in the presence of 50, 100 and 150 mM NaCl. Contrary, in KS seedlings, salt increased significantly. The AsA + DHA rose by 210, 360 and 262% under 50, 100 and 150 mM NaCl, respectively, compared to the control. Moreover, increases of 61 and 22% of AsA content were observed in GL under 100 and 150 mM NaCl, respectively, while increases of 1.5-, 6.7- and 5.5-fold were observed in KS. Important to note are the increases of 3.3 and 5.3-fold in the AsA/DHA ratio in the presence of 100 and 150 mM NaCl, respectively in KS seedlings, and of 2.5- and 2.12-fold in the presence of 50 mM and 150 mM NaCl in GL.

DISCUSSION

Soil salinity is a prevalent abiotic stress for plants, with retarded growth being a common response to salinity. Plant growth is one of the most important agricultural indices of salt stress tolerance as indicated by many studies (Parida and Das, 2005). The effect of salinity on seed germination is due to an osmotic effects and/or ion toxicity. However, variation of adaptive mechanisms exists in different species (Rehman et al., 1996). Salt

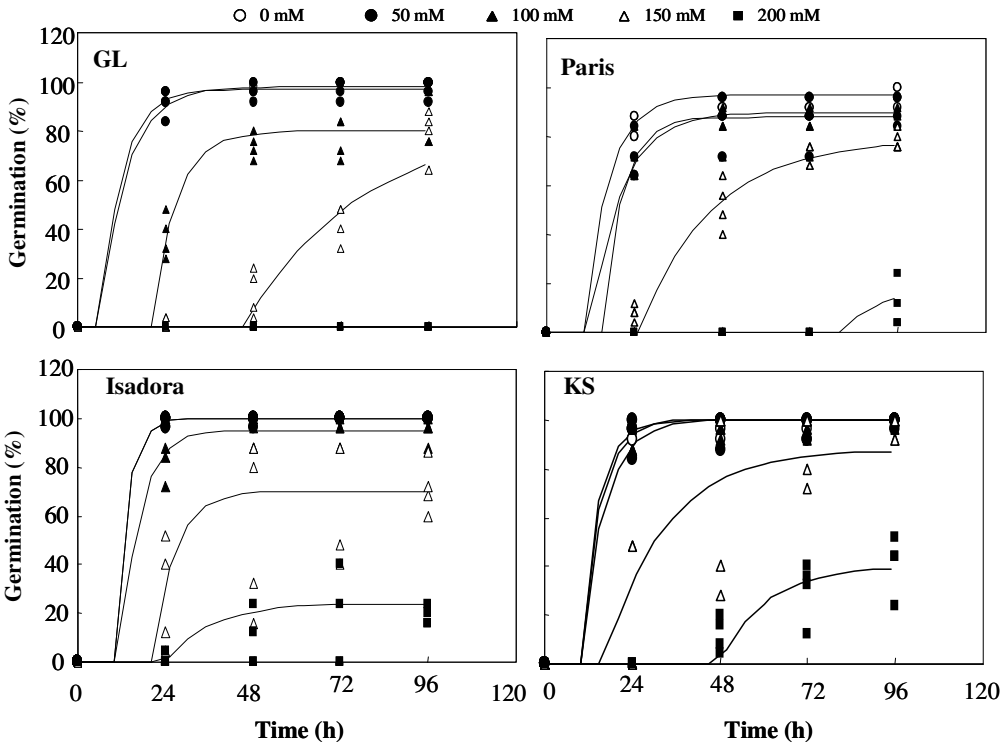

Figure 2. Germination curves of four lettuce varieties (GL, Paris island cos, Isadora and KS) under 0, 50, 100, 150 and 200 mM NaCl for foyr days (**n**, 4).

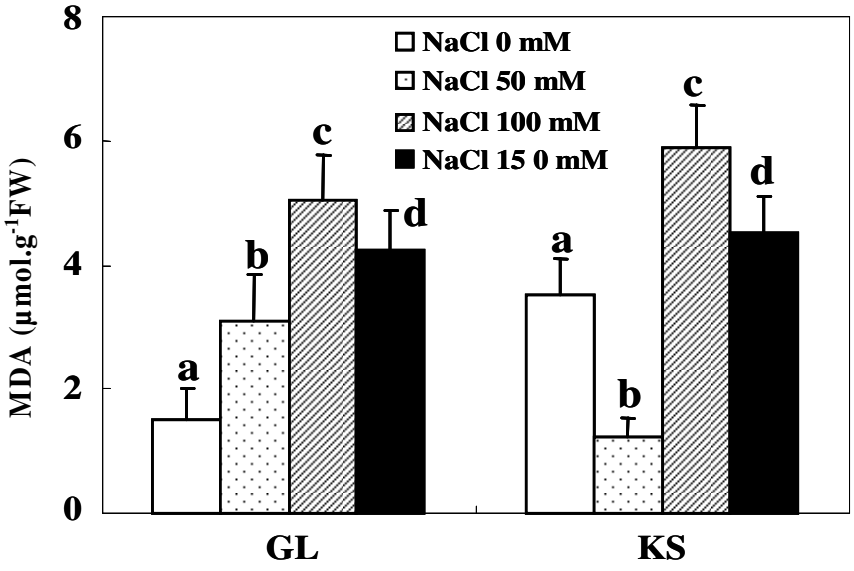

Figure 3. Effect of NaCl on MDA content (mmol g-1FW, n=12) in Great lakes seedlings and Kagranner sommer seedlings lettuce germinating under 0, 50, 100, 150 and 200 mM NaCl, for 4 days.

stress can induce both a reduction in seed germination and a delay in the initial germination process in glycol-phytes and to a lesser extent in halophytes (El-Keblawy, 2004). The increase in salinity does not only decrease germination, but also delays the initial germination rate (Hajar et al., 1996). In this study, the latency for radicle emergence augmented with salt concentration, notably under 150 mM NaCl in GL seeds (Figure 2). It was hypothesized that the presence of NaCl even at low concentrations, could contribute to a decrease in the

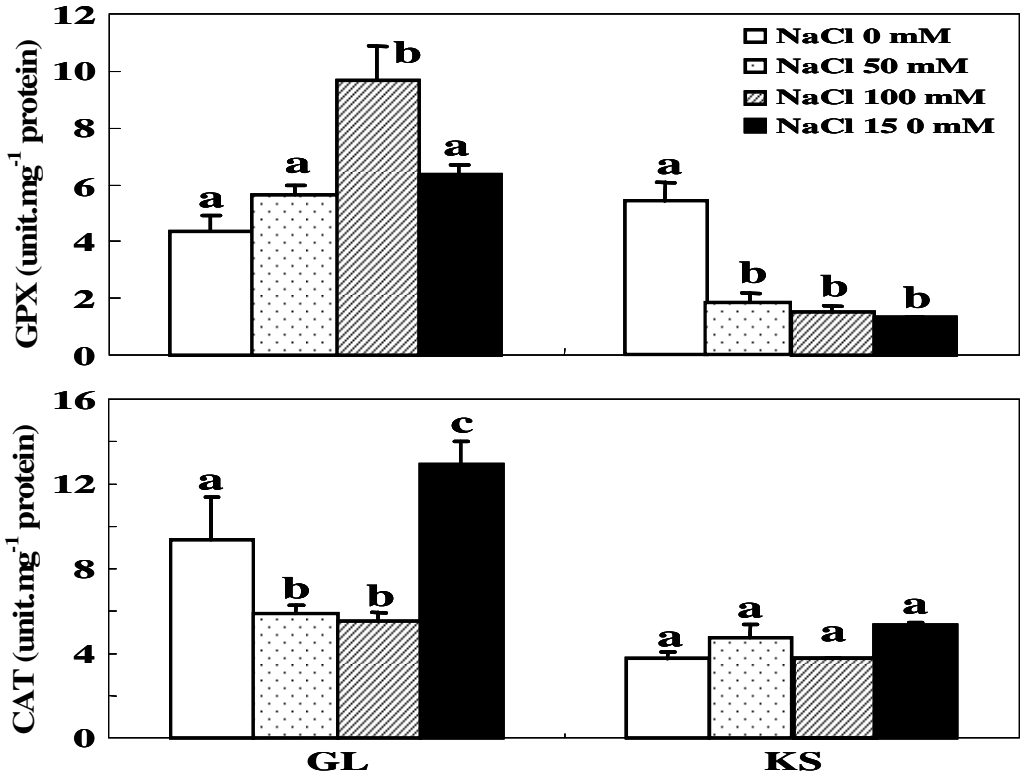

Figure 4. Effect of NaCl on antioxidant activities (U.mg-1P, n=4) in Great lakes seedlings and Kagranner sommer seedlings lettuce germinating under 0, 50, 100, 150 and 200 mM NaCl, for 4 days.

internal osmotic potential of germinating structures (Huang and Lui, 2002). At 150 mM NaCl treatment, a significant decrease in the germination percentage of GL was observed. At 200 mM NaCl concentration, the FG% of KS seeds was much higher than that of GL seeds, and the early germination process of KS seed was found to be significantly delayed (Figure 2). These results indicate that at the germination stage, GL was more sensitive, while Paris Island cos and Isadora could adapt to a stronger salt stress. KS seeds possessed an important mechanism of delaying initiation of the germination process in order to adapt to the saline environment. Inhibition or delay in germination under saline conditions is due to an osmotic effect (Gosset et al., 1994), which limits the uptake of water during seed germination (Flowers et al, 1986) by obstructing membrane, or cytosolic enzymes and hormones (Huang and Lui, 2002). The covering test showed that transferring seeds from 200 mM NaCl to distilled water can increase the germination percentage of the four lettuce varieties after four days to 100%. This result suggests that reduction in seed germination percentage in lettuce was due to the osmotic effect of salt.

Salinity (NaCl) adversely affected seedling growth parameters of the four lettuce varieties. Radicle and hypocotyle presented the most reduction of DW and of water content than cotyledon at 100 mM and 150 mM NaCl concentrations, in GL, and only at 150 mM in KS (Table 1). This might be due to toxic effects of the sodium ion (Na^+) and/or chloride (Cl^-) in seed tissues as well as unbalanced nutrient uptake induced by salt.

There is increasing evidence that membrane injury under salt stress is related to a higher production of highly toxic ROS (Shalata and Tal, 1998). Determining the MDA concentration and hence, the extent of membrane lipid peroxidation, has often been used as a tool to assess the severity of oxidative stress (Ben et al., 2005). Our data showed that, after four days of treatment of the two different varieties of lettuce seeds with different NaCl concentrations, membrane lipid peroxidation was induced and MDA contents in the seeds were much higher than in those of the control. The increase in the content of H_2O_2 due to salt stress was the principal cause of peroxidation of lipid membrane, thus disrupting its permeability. Mansour (1998) reported that the reduced membrane damage of onion leaf tissues under salinity stress could be explained by a reduction of concentrations of H_2O_2 and of MDA, in leaf tissues. Our results are in agreement with those of Sairam and Srivastava (2002), who reported increases in the amount of MDA with the increase in salt stress in the salt-sensitive cultivar as compared to the tolerant cultivar of rice and in *Lemna minor* roots.

Several studies have demonstrated that salt-tolerant species increase their antioxidant enzyme activities and antioxidant contents in response to salt stress, while salt-

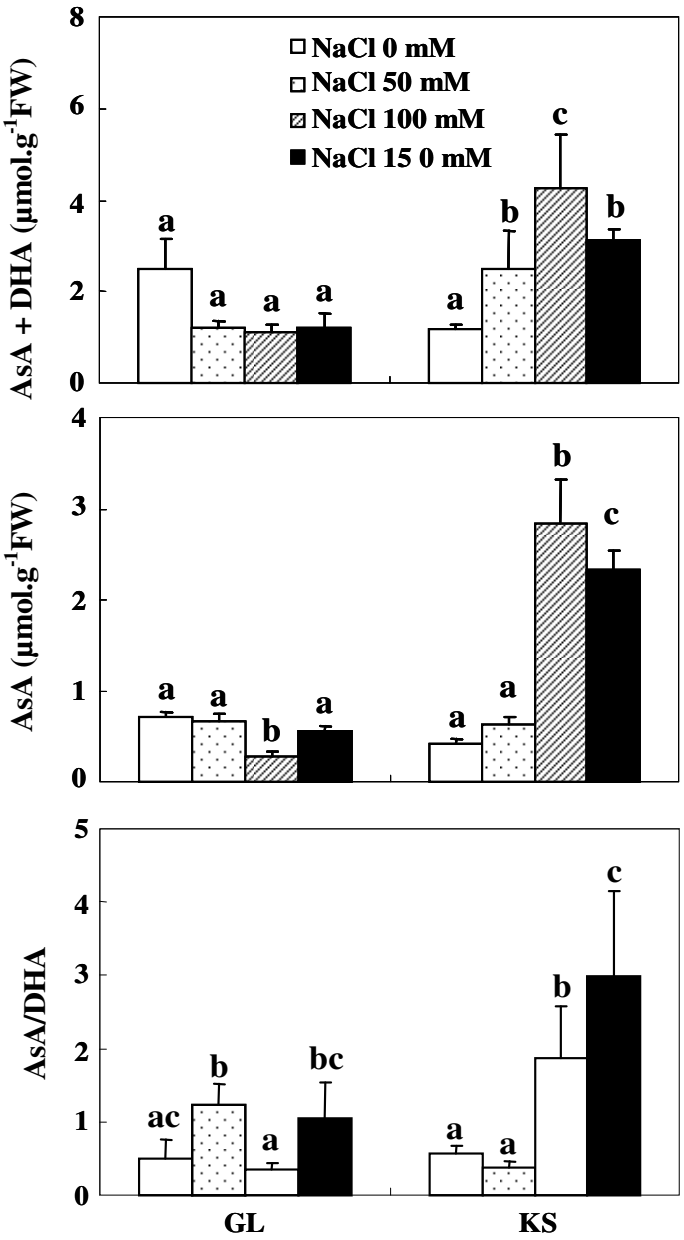

Figure 5 Effect of NaCl on total ascorbate (AsA+DHA, µmol.g-1FW), ascorbate (AsA, µmol.g-1FW) and AsA/DHA ratio in Great lakes seedlings and Kagranner sommer seedlings lettuce germinating under 0, 50, 100, 150 and 200 mM NaCl, for 4 days.

sensitive species fail to do the same (Shalata and Tal, 1998). The increase in the activity of antioxidant enzymes may be due to the enhanced levels of oxygen (O_2) produced under stress. This shows that an efficient defence mechanism might be involved in the increase of the antioxidant levels. GPX is widely distributed in higher plants where it is involved in various processes, including lignification, auxin metabolism, salt tolerance and heavy metal stress (Passardi et al., 2005; Gao et al., 2008). Therefore, GPX has often served as a parameter of metabolism activity during growth alterations and environmental stress conditions (Gao et al., 2008). Contrary to our finding, salinity led to an increase of GPX activity in GL seedlings, and to a decrease in KS seed-lings, after been treated with salt for four days (Figure 1). This suggests that salinity induced more production of O_2^- in GL which was counterbalanced by GPX increasing activity.

Generally, NaCl treatment increased AsA content in KS seedlings (salt tolerant variety), but decreased it in GL (salt sensitive variety). Higher AsA contents under salt stress were also reported in salt-tolerant pepper species

(Aktas, 2002) and citrus species (Arbona et al., 2003). The AsA + DHA decrease under NaCl constraint in GL seedlings, suggest that rate of utilization of AsA exceeded its synthesis.

Conclusion

We concluded that anti-oxidative enzyme activities play a protective role against salt-stress, and the anti-oxidative defence mechanisms were effective in providing resistance to salt-stress in lettuce at the stages of seed germination and seedling growth. The increase in the activity of antioxidant enzymes may be due to the higher levels of O_2 produced under stress. This shows that an efficient defence mechanism might be involved in the increase of the antioxidant levels.

Abbreviations

CAT, Catalase; **GPX,** gaiacol peroxidise; **MDA,** malondialdehyde; **FG%,** final germination percentage; **DW,** dry weight; **GL,** Great Lakes; **KS**, Kagraner Sommer; **NaCl,** sodium chloride; **AOS,** active oxygen species; **PUFA,** polyunsaturated fatty acids; **ROS,** reactive oxygen species; **H_2O_2,** hydrogen peroxide; **APX,** ascorbate peroxidase; **GR,** gluthathione reductase; **FW,** fresh weights; **TBARS,** thiobarbituric acid-reactive substances; **EDTA,** ethylene diamine tetraacetic acid; **DTT,** dithiothreitol; **TCA,** trichloroacetic acid; **NEM,** N-ethylmaleimide; **ANOVA,** analysis of variance; **O_2,** oxygen; **AsA+DHA,** total ascorbate; **DHA,** dihydroascorbate.

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