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Full Length Research Paper

Combined effect of hormonal priming and salt treatments on germination percentage and antioxidant activities in lettuce seedlings

Mahmoudi Hela[1]*[#], Zargouni Hanen[1#], Tarchoune Imen[1#], Baatour Olfa[1], Nasri Nawel[1], Ben Massoud Raouia[1], Zaghdoudi Maha[1], Abidi wissal[1], Huang Jun[2], Hannoufa Abdelali[3], Lachaâl Mokhtar[1] and Ouerghi Zeineb[1]

[1]Unité de Physiologie et Biochimie de la Tolérance au Sel des Plantes, Département des Sciences Biologiques, FST, Campus Universitaire, 2092 Tunis, Tunisia.
[2]Agriculture and Agri-Food Canada, 107 Science Place, Saskatoon, SK, Canada.
[3]Agriculture and Agri-Food Canada, 107 Science Place, Saskatoon, SK, Canada.

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Hormonal priming is a pre-sowing treatment that improves seed germination performance and stress tolerance. To understand the physiology of hormonal priming and its association with post priming stress tolerance, we investigated the effect of hormonal priming with increasing gibberellic acid (GA_3) concentrations (0, 3, 4.5 and 6 mM) on seedling growth and antioxidant system in lettuce. Germination percentage was higher in lettuce seedlings derived from primed seeds. Radicle and hypocotyl length and dry weight were reduced by salt treatment to a greater extent in non-primed than in primed seeds. Hormonal priming with 4.5 mM GA_3 induced the most dramatic decreases in electrolyte leakage (EL) and malondialdehyde (MDA) levels. NaCl increased catalase (CAT) activity in primed and non-primed seeds. The total ascorbate level remained constant in both primed and non-primed seeds under NaCl constraint. These results suggest that hormonal priming might have increased the salt tolerance of lettuce seeds through enhancing the activities of antioxidant enzymes and reducing the membrane damage as estimated using EL and MDA biomarkers.

Key words: Ascorbate, germination, hormonal priming, lettuce, salinity.

INTRODUCTION

Seed germination can be a major factor limiting the establishment of plants under saline conditions (Ghavami and Ramin, 2007). It is the most critical phase in plant life (El-Keblawy and Al-Rawai, 2005), and it is greatly influenced by salinity (Misra and Dwivedi, 2004). Salinity can affect germination and seedling growth either by creating an osmotic pressure that prevents water uptake or by toxic effects of sodium and chloride ions (Bajehbaj, 2010). Salt-induced osmotic stresses are responsible for both inhibition or delayed seed germination and seedling establishment (Almansouri et al., 2001). Decreased water uptake during imbibition and excessive uptake of ions was found to contribute to NaCl sensitivity during germination and seedling establishment in cowpea (Murillo-Amador et al., 2002). Seed priming is a technique in which seeds are partially hydrated until the germination process begins, prior to radicle emergence (Bradford, 1986). Priming allows the metabolic processes necessary for germination to occur without actual germination. The beneficial effects of priming were demonstrated in many

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

Abbreviations: NP, Non-primed; **P,** primed; **EL,** electrolyte leakage; **CAT,** catalase; **GPX,** gaiacol peroxidase; **AsA,** reduced ascorbate; **DHA,** dehydroascorbate; **MDA,** malondialdehyde.

[#]These authors contributed equally to this work.

crops (Mehmet et al., 2006). In addition, primed (P) seeds usually exhibit an increased germination rate, greater germination uniformity, greater total germination percentage (Basra et al., 2005).

Plant growth regulators are organic compounds, which are produced in very small amounts in plants and play an important role in determining growth, development and yield of crops, and are becoming essential tools in agricultural practices. One group of these growth regulators is gibberellins (GAs); plant hormones that regulate growth and development, including seed germination, stem elongation, leaf expansion and flowering time (Magome et al., 2004). The biosynthesis of GAs is regulated by both developmental and environ-mental stimuli (Hedden and Phillips, 2000). Of all GAs, gibberellic acid (GA_3) is the most active growth regulator, which promotes germination, internodal length, hypocotyl growth and cell division in cambial zone and increases the size of leaves, as well as, breaks seed dormancy. In cereal seeds, GA_3 stimulates hydrolytic enzymes that are needed for the degradation of the cells surrounding the radicle and thus speeds up germination by promoting the rate of seedling elongation (Rood et al., 1990). Although, priming improves the rate and uniformity of seedling emergence and growth particularly under stress conditions (Parera and Cantliffe, 1991), the effectiveness of different priming agents varies under different stresses and among various crop species (Iqbal and Ashraf, 2007). The response of plants to any given abiotic stress such as high salinity involves complex morphological, physiological and biochemical changes, including changes to germination, seedling growth, antioxidants, and endogenous hormones.

The purpose of this research was to evaluate the efficiency of employing hormonal priming with different GA_3 concentrations to mitigate salt stress effects on germination and seedling establishment of lettuce seedlings in relation to changes in antioxidant activities.

MATERIALS AND METHODS

Seeds of lettuce (*Lactuca sativa* L.) genotype Great Lakes were obtained from the Ministry of Agriculture of Tunisia. The Great Lakes genotype was identified as the most sensitive variety to NaCl in an initial screening of four lettuce varieties including Paris cos island, Kagranner Sommer, Isadora and Great Lakes (Mahmoudi et al., 2011). Homogenous seeds were surface sterilized with 95% ethanol for 5 min, and then rinsed 5 times with sterile distilled water. The seeds were subjected to seed priming with different GA_3 concentrations (0, 3, 4.5 and 6 mM) for 12 h. Primed seeds were air dried at room temperature. Aliquots of 25 seeds were then placed in 10 cm Petri dishes with double-layer filter paper in the presence or the absence of 150 mM NaCl. Germinated seeds were counted daily for up to six days.

Membrane permeability (electrolyte leakage)

Electrolyte leakage (EL) was determined as described by Quartacci et al. (2002). Fresh seedlings were placed in test tubes containing 25 ml double-distilled water at room temperature. After 1 h, the initial electrical conductivity of the medium (EC1) was measured using a digital conductimeter (Model: Metrohm). The samples were placed in liquid nitrogen and then returned in the same tube for one additional hour of shaking and the final electrical conductivity (EC2) was measured. EL was calculated using the formula: EL = (EC1/EC2)*100.

Malondialdehyde (MDA) assay

Malondialdehyde (MDA) content of seeds was measured according to the method of Du and Bramlage (1992), and used to determine the level of lipid peroxidation. Ground tissue (0.2 g) was homogenized with 2 ml of 0.1% trichloroacetic acid (TCA), and the crude extract preparation was centrifuged at 10,000 g for 20 min. A mixture of TCA, thiobarbituric acid (TBA) (1 ml) and an aliquot of the supernatant (1 ml) was heated at 95°C for 30 min and then quickly cooled on ice for 5 min. After cooling, the 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 lipids. The concentration of MDA was calculated from the absorbance values using an extinction coefficient of 155 mM cm^{-1}.

Determination of enzyme activity

Seeds of Great Lakes variety were moistened in Petri dishes with a 0 and 150 mM NaCl solution and were placed in an incubator in constant darkness at 25°C. After seven days, the seeds 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 tetra-acetic 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 the method of Bradford (1976) using bovine serum albumin as a standard.

Catalase (CAT) activity was determined by monitoring the disappearance of H_2O_2 according to the method of 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. Guaiacol peroxidase activity (GPX) was assayed using guaiacol as an electron donor, with a reaction mixture containing 20 mM phosphate buffer (pH 5.6), and 30% H_2O_2 according to Srinivas et al. (1999). 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.

Ascorbate determination

Ascorbic acid content was assayed as described by Kampfenkel et al. (1995). The aliquots of the fresh material (0.25 g) were homogenized in ice-cold 6% (w/v) TCA, using a cold mortar and pestle. Total and reduced ascorbate (AsA) 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 Fe^{2+} caused by reduction of Fe^{3+} to Fe^{2+} by ascorbic acid and was measured at 525 nm. Total ascorbate was determined through a reduction of dehydroascorbate (DHA) to AsA by 10 mM DTT. Excess DTT was removed with N-ethylmaleimide (NEM) 4% (w/v). A standard curve covering the range of 10 to 50 µmol AA was used.

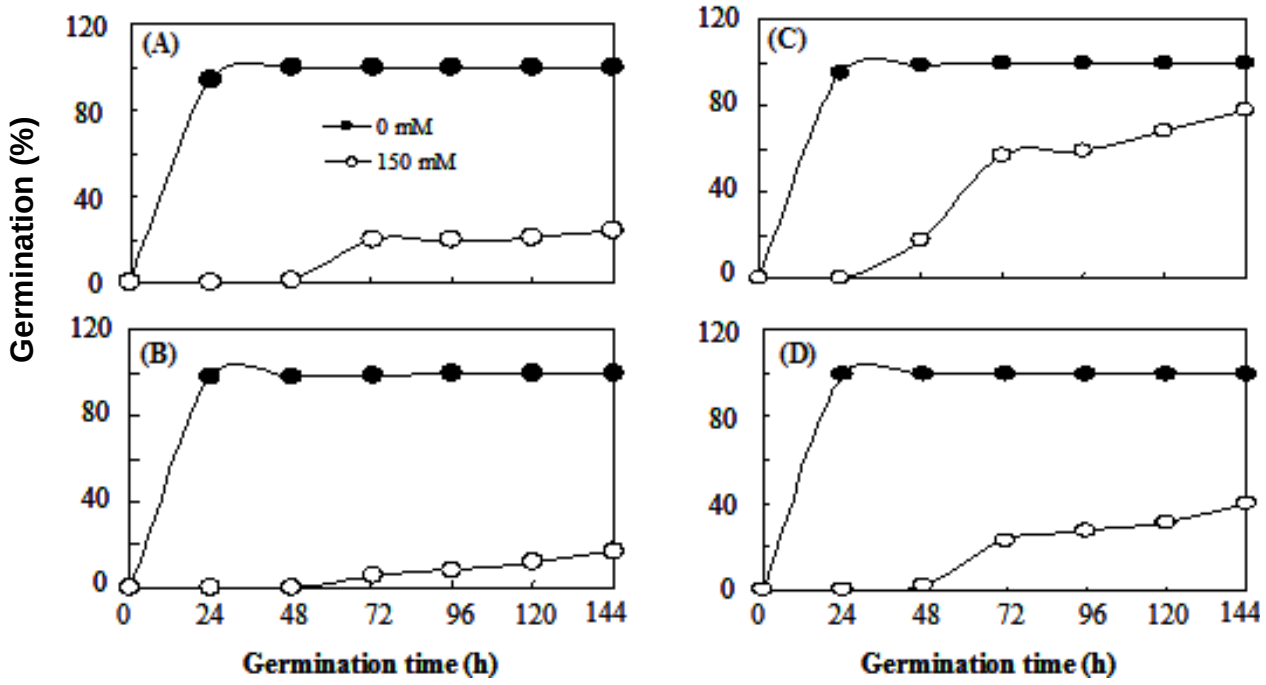

Figure 1. Germination curves of NP (A) and P of GL variety seeds (B, C and D), treated with 3, 4.5 and 6 mM GA_3, under 0 and 150 mM NaCl, respectively, for six days. Data are the mean of four samples of 25 seedlings each.

Table 1. The effect of different GA_3 concentrations (0 to 6 mM) and NaCl treatments on daily germination of GL seedlings after six days.

NaCl (mM)	Final germination percentage			
	0	3 mM GA_3	4.5 mM GA_3	6 mM GA_3
0	100	100	100	100
150	24	17	78	40

Means of six replicates ± SE. Means followed by different letters are significantly different ($P \leq 0.05$) as determined by analysis of variance (ANOVA).

RESULTS

Effect of priming and salinity treatments on germination and seedling growth

Under control conditions, final germination (FG) curves reached a plateau after 24 h of germination (Figure 1). However, there were considerable differences in the value of maximum FG percentages (FG% max) in the presence of 150 mM NaCl (Table 1 and Figure 1). The percentage of seed germination decreased in response to NaCl in both primed (P) and non-primed (NP) seeds. Meanwhile, the germination percentage in P seeds at all GA_3 concentrations, except 3 mM was higher (Figures 1B, C and D) than in that of NP seeds (Figure 1A). However, the most improved germination was observed in seeds treated with 150 mM NaCl and 4.5 mM GA_3. For this reason, two GA_3 concentrations (0 and 4.5 mM) were chosen to analyze the effect of salt on growth parameters and enzymatic activities at 150 mM NaCl six days after sowing. Under control conditions, radicle biomass was higher in P seeds compared to NP ones (Table 2). This result suggests that hormonal priming enhanced seedling growth. In the presence of 150 mM NaCl, radicle growth was restricted in NP seeds by 33% compared with the no salt control, and by 29% in P. NaCl had no significant effect on hypocotyle or cotyledon growth. Radicle elongation was still more sensitive to salt inhibition as salt diminished radicle length by 3.1-fold in NP seeds and by 2.9-fold in P seeds. Hypocotyl elongation was more important in both control and NaCl 150 mM in P seeds as compared to that of NP. However, NaCl decreased hypocotyl elongation by 8-fold and 1.6-fold compared to their controls in NP and P seeds, respectively. Water content was also reduced by NaCl, 150 mM in hypocotyl and cotyledon for both NP and P seeds, suggesting tissue dehydration occurred following exposure to relatively high NaCl.

Table 2. Effect of hormonal priming with GA_3 (0 or 4.5 mM) in dry weight (mg), the water content (ml.g^{-1}DW) in radicle, hypocotyl and cotyledons and radicle and hypocotyle length (cm) of Great Lakes seedlings grown under NaCl treatment (0 and 150 mM).

NaCl (mM)	GA_3 (mM)	Organ							
		Radicle			Hypocotyl			Cotyledon	
		DW	H_2O	Length	DW	H_2O	Length	DW	H_2O
0	0	0.45^a ± 0.06	24,49^a ± 5.31	1.46^a ± 0.34	1.27^a ± 0.12	42.48^a ± 1.61	3.29^a ± 0.14	1.02^a ± 0.16	6.13^a ± 0.42
	4.5	0.75^a ± 0.06	20.69^a ±7.42	1.55^a ± 0.40	1.33^a ± 0.08	38.15^a ± 2.32	3.89^a ± 0.25	0.80^a ± 0.10	10.09^a ± 0.92
150	0	0.30^b ±0.07	26.11^a ± 7.47	0.47^b ± 0.10	1.18^a ±0.07	28.78^b ±1.06	0.41^b ± 0.11	0.87^a ±0.10	3.58^b ± 2.00
	4.5	0.53^b ± 0.05	25.18^a ± 7.25	0.54^b ± 0.14	1.66^a ± 0.20	17.49^b ± 1.82	1.50^b ± 0.21	1.05^a ± 0.15	4.69^b ±0.83

Means of six replicates ± SE. Means followed by different letters are significantly different ($P \leq 0.05$) as determined by analysis of variance (ANOVA).

Effect of priming and NaCl treatments on electrolyte leakage and membrane integrity

The extent of membrane damage in lettuce seedling was estimated by MDA and EL content. MDA levels of seeds were determined to evaluate lipid peroxidation (Figure 2A). In the absence of salt, MDA levels were similar in NP and P seeds (5 µmol.g^{-1}FW). In NP seeds, MDA content increased by 1.3-fold in the presence of 150 mM NaCl. However, MDA level decreased by 1.8-fold in P seeds as compared to control (0 mM NaCl). The same pattern was observed with EL index (Figure 2B). In NP seeds, the EL index remained unchanged after six days of germination, but it decreased significantly in P seeds. These results suggest that hormonal priming might have alleviated the membrane damage induced by salt.

Effect of priming and NaCl treatments on antioxidant activities

To determine the response of lettuce to salt-induced oxidative stress, GPX and CAT activities, AsA contents were measured in seedlings grown with or without 150 mM NaCl. There were no significant differences in GPX activity between both NP and P seeds grown under non-NaCl conditions. Salt-stress caused an increase in GPX activity in both P and NP seeds. This increase was highest in P seeds. GPX activities in NP seeds and P seeds treated with 150 mM NaCl, were three- and six-fold higher, respectively, than those measured in control seedlings (Figure 3A). Under non-salt control conditions, P seeds had the highest activity of CAT than the NP seeds. Salt treatment increased CAT activity in both NP and P seeds, compared with their control groups. CAT activities increased similarly by 1.4-fold in NP and P seeds treated with salt compared to those measured in control seedlings (Figure 3B).

Effect of priming and NaCl treatments on ascorbate content

The P seeds with 4.5 mM GA_3 exhibited a greater accumulation of AsA than NP ones under both control and NaCl conditions (Figure 4A). The NaCl treatment had no effect on the total ascorbate content (Figure 4B), or the AsA/DHA ratio (Figure 4C) in NP and P seeds.

DISCUSSION

The effect of salinity on seed germination is due to an osmotic effect and/or ion toxicity. Salt stress can induce both a reduction in seed germination and a delay in initiation of the germination process in glycophytes and to a lesser extent in halophytes (El-Keblawy and Al-Rawai, 2005). It is evident from our results that NaCl salinity caused growth inhibition in lettuce seedlings leading to decreases in the germination percentage and dry weight. Exogenous application of GA_3 was previously reported to increase the seed germination rate in different plant species (El-Bakkosh, 2001; Zeinalabedini et al., 2009). At 150 mM NaCl, the maximum germination rate was observed in seeds primed with 4.5 mM GA_3. This result suggests that GA_3 priming can induce salt tolerance in lettuce seeds. Furthermore, seed priming with GA_3 might caused acceleration of metabolic reactions before germination and made germination of cultivated seeds possible under salinity stress (Varma et al., 1984). However, in the present study, it was

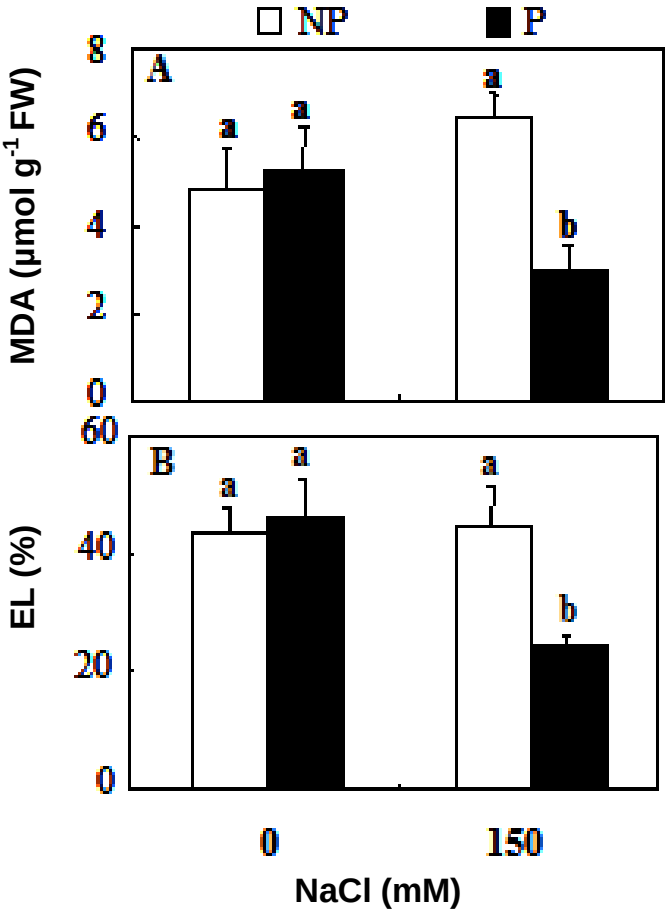

Figure 2. Effect of NaCl on MDA content (µmol g^{-1}FW, n=12) and EL in NP (filled bar) and P seedlings (empty bar) lettuce germinating under 0 and 150 mM NaCl for six days. Means of six replicates ± SE. Means followed by different letters are significantly different ($P \leq 0.05$) as determined by analysis of variance (ANOVA).

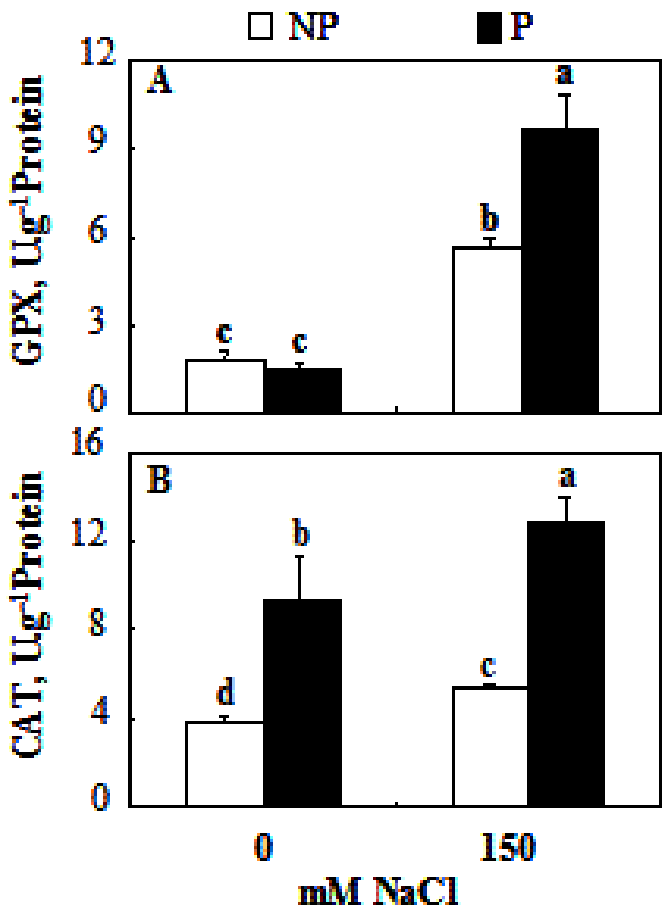

Figure 3. Effect of NaCl on antioxidant activities (U.mg^{-1}P, n=4) in NP (filled bar) and P seedlings (empty bar) lettuce germinating under 0 and 150 mM NaCl for six days. Means of four replicates ± SE. Means followed by different letters are significantly different ($P \leq 0.05$) as determined by analysis of variance (ANOVA).

reasoned that hormonal priming might have diminished the inhibiting effects of salinity on seed germination and seedling growth of lettuce. Similar results were reported by Kim et al. (1993), Coale (1991) and Hays (1992), who indicated that seed soaking in GA_3 solution increased the emergence rate in rice. Although, these results are not in line with the findings of Hardegree and Vactor (2000) who reported that osmotic priming of wheat seeds improved the germination rate, GA_3 had no effect on the emergence rate. Our results show that GA_3 priming increased hypocotyl length, as well as, radicle and hypocotyle biomass. Increased root length by GA_3 indicates that GA_3 stimulated hydrolytic enzymes that are needed for the degradation of the cells surrounding the radicle and thus, speeds up germination by promoting seedling elongation (Rood et al., 1990). These results are in agreement with findings reported by Jett et al. (1996), who showed that root growth rates of matric primed seeds were significantly higher than either osmotic or non primed seedlings at most temperatures. Rapid growth of hybrids is associated with increased content of endogenous GA_3, which promotes seedling vigour, increases shoot height and weight and enhances grain yield as reported by Rood et al. (1990).

A consequence of salt-stressed plants is generation of excessive reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals (Masood et al., 2006). These radicals can damage vital cellular macromolecules (for example, via denaturation of proteins, mutation of DNA, and/or peroxidation of lipids). However, at optimal concentrations, ROS can also promote seed germination and early seedling growth (Barba-Espín et al., 2010, 2011a; Kranner et al., 2010; Müller et al., 2009). Plants have evolved both enzymatic and non-enzymatic mechanisms to scavenge ROS (Asada, 1999). CAT is an important antioxidant enzyme that converts H_2O_2 to water in peroxysomes (Fridovich, 1989). In this organelle, H_2O_2 is produced from β-oxidation of fatty acids and photorespiration (Morita et al.,

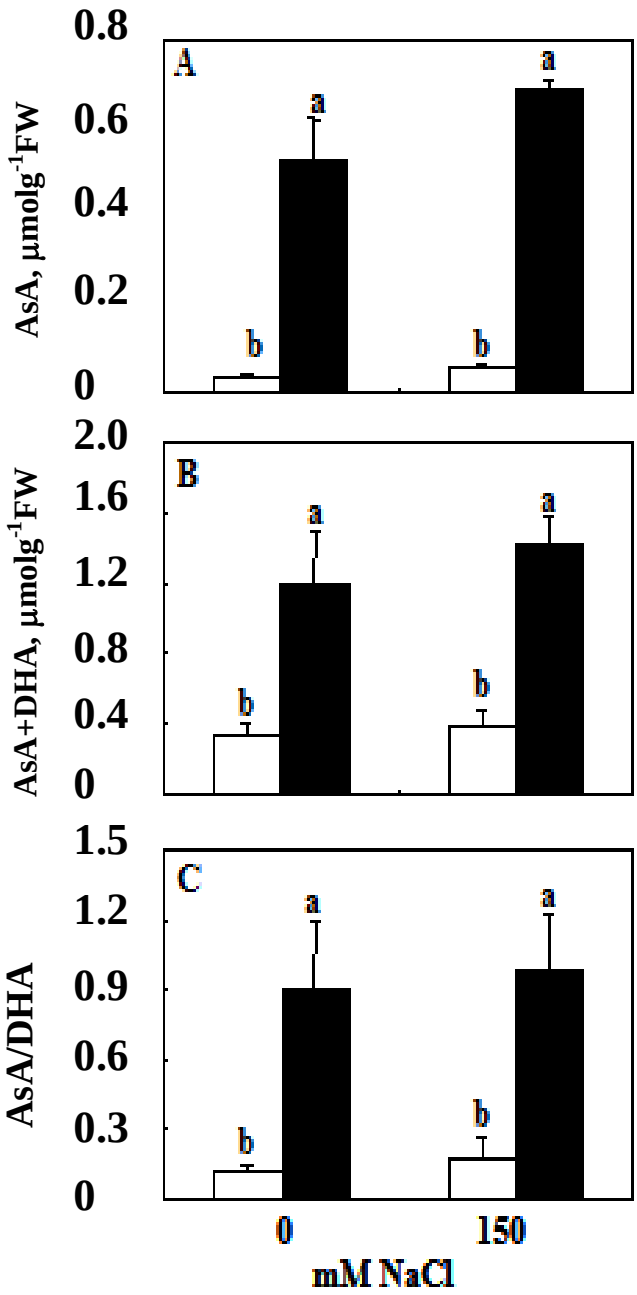

Figure 4. Effect of NaCl on total ascorbate (AsA+DHA, µmol.g^{-1}FW), ascorbate (AsA, µmol.g^{-1}FW) and AsA/DHA ratio in NP (filled bar) and P seedlings (empty bar) lettuce germinating under 0 and 150 mM NaCl for six days. Means of six replicates ± SE. Means followed by different letters are significantly different (*P*≤0.05) as determined by analysis of variance (ANOVA).

1994). Higher activity of CAT and ascorbate peroxidase decrease H_2O_2 level in the cell providing protection to membranes, mainly in the chloroplast, because several enzymes of the Calvin cycle are extremely sensitive to H_2O_2. A high level of H_2O_2 directly inhibits CO_2 fixation (Yamazaki et al., 2003).

In our experiments, we observed that CAT activity was not adequate in NP seeds, although, its activity increased with NaCl treatment, it was not sufficient for the complete scavenging of H_2O_2. On the other hand, CAT activity increased in P seeds compared to that of the control suggesting a negative relationship between CAT activity and MDA (Figures 2 and 3). This negative relationship was also confirmed by Esfandiari et al. (2007) and Shao et al. (2005). GA increased AsA contents that can be important during the germination process as an antioxidant molecule. In this sense, antioxidant mechanisms have been regarded as being of particular importance for the success of germination (Tommasi et al., 2001). Under our experiment, the total ascorbate remained constant in both P and NP seeds under NaCl constraint, suggesting that seedlings maintained equilibrium between AsA utilization and its synthesis.

Notably, MDA accumulation, which represents the level of lipid peroxidation and thus, the accumulation of ROS was also reduced during hormonal priming associated with an increase in activities of CAT and GPX. Mansour (1998) reported that reduced membrane damage of onion leaf tissues under salinity stress could be explained by a reduction in concentrations of H_2O_2 and MDA. Furthermore, the induction of GPX and CAT in GA_3-primed seeds correlated with a decrease in EL and MDA contents, suggesting a membrane protection under salt stress conditions. On the other hand, CAT is restricted to the peroxisome, and H_2O_2 can diffuse through the peroxisomal membrane into the cytosol (Del Río et al., 1988), thus, increasing the risk of oxidative damage in this compartment. Therefore, increased CAT activity could control H_2O_2 levels in the peroxisome during the stress period. In this sense, it was reported that salt stress increases H_2O_2 levels in both leaves (Barba-Espin et al., 2011b) and peroxisomes (Corpas et al., 1993). The aforementioned notion is in agreement with the well-document observation that GA priming enhances alternative antioxidant mechanisms via other hormones such as salicylic acid, which might be responsible for reduced lipid peroxidation in Arabidopsis exposed to salt or other abiotic stresses (Alonso-Ramı´rez et al., 2009; Lee and Park, 2010).

In conclusion, there is evidence that in most field and horticultural crops, priming led to improvement of germination and seedling establishment (McDonald, 2000). Improvement in seedling growth, development and establishment correlates with efficient water uptake of prime derived plants. In the present study, GA_3 priming increased seed germination percentage, decreased lipid peroxidation and enhanced antioxidant defenses. Therefore, GA_3 may be an efficient method to overcome seed germination problems and to improve seedling growth in the field, especially under salinity. Other studies that are in progress in our laboratory are aimed at investigating whether the beneficial effects of GA_3 priming persist beyond the later growth and development stages

of lettuce plants.

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