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Salinity Altered Profiles of Osmolytes in *Atriplex prostrata* (Chenopodiaceae) and *Plantago coronopus* (Plantaginaceae)

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Short Communication

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ABSTRACT

The aim of this study was to investigate the effects of salinity on the osmolytes (proline, glycinebetaine and sorbitol) in plants of *Atriplex prostrata* and *Plantago coronopus*. Collected seeds from Barranco Hondo (salt marshes, Jaen, Southern Spain) were grown in a growth chamber at concentration 0, 50, 100, 200 and 300 mM NaCl for 60 days in controlled conditions. Leaves were collected to carry out metabolic studies. *A. prostrata* showed a high germination and an optimum growth and only 200 and 300 mM by NaCl reduced the values, whereas *P. coronopus* showed a greater reduction in the growth, being more sensitive to salt stress. The leaves obtained of *A. prostrata* presented the proline as main osmolyte with significant changes where at 200 and 300 mM NaCl the increase was higher (4 and 10-fold compared to the control) followed by glycinebetaine. In the case of *P. coronopus* the proline was the predominant osmolyte, followed by sorbitol to a lesser extent. These results indicate that proline is used for both plants in the osmotic adjustment and protection of cellular structures being more efficient in the case of *A. prostrata.* The adaptive strategies of *A. prostrata* was increase the synthesis of proline and glycinebetaine and *P. coronopus* increased proline content and slightly sorbitol in order to survive better in saline conditions.

INTRODUTION

Salt stress is one of the major factors limiting growth and production of plants in various regions of the world [1]. In saline environments, the NaCl is usually the most injurious and predominant and saline stress is one of the most detrimental factors affecting germination of halophyte seeds and plant establishment [2]. An increase in salinity induces both a reduction in the percentage of germinating seeds and a delay in the initiation of the germination process and growth [3]. Salinity can alter seed germination and growth via an osmotic effect and/or ion toxicity [4]. The Atriplex genus (Chenopodiaceae) has several plant species that are capable to complete their life cycles under very harmful environmental conditions such as drought or high salinity. *Atriplex prostrata* is presented in semi-arid to sub humid areas of the Mediterranean [5]. These plants tolerant to salinity and they are able to uptake water by maintaining a high osmotic potential through the accumulation of inorganic and organic solutes. They are adapted to harsh environmental conditions and therefore are interesting for the identification of physiological mechanisms in abiotic stress resistance [6,7]. The genus Plantago (Plantaginaceae) can be a good model for comparative studies on the response to salt stress, since it includes species with considerable differences in their degree of tolerance. *Plantago coronopus* L. is a herbaceous, short-lived perennial rosette plant which mainly occurs in salt marshes and especially near the sea. This plant occurs in habitats of variable salinity, and shows an intermediate degree of salt tolerance [8].

In response to stress saline, many plants accumulate compatible metabolites in the cytoplasm of their cells in an attempt to combat the deficiency in water. Accumulation of compatible solutes is often regarded as a basic strategy for the protection and survival of plants under salt stress. These soluble compounds, including soluble carbohydrates, glycinebetaine, polyols and proline, which protect to plants against stress by cellular osmotic adjustment, detoxification of reactive oxygen species, protection of membrane integrity, and stabilization of enzymes/proteins [9]. Proline is known to occur widely in higher plants and

normally accumulates in large quantities in response to environmental stresses. In response to drought or salinity stress in plants, proline accumulation normally occurs in the cytosol where it contributes substantially to the cytoplasmic osmotic adjustment [9]. Glycinebetaine is an osmolyte localized in the cytoplasm of leaf cells in salt tolerant species above all Chenopodiaceae. This osmolyte protect higher plants against salt/osmotic stress and protecting the photosystem II complex, has protective effect for membranes against heat-induced destabilization and enzymes such as Rubisco [6,10]. Sorbitol is the sugar alcohol, this increase with external sodium chloride concentrations in the soil. Sorbitol is the dominant soluble carbohydrate in all Plantago species studied [8]. The objective of these experiments was to investigate the effects of NaCl on different osmolytes on plants grown with several saline concentrations. We investigate the induction by salt of several compatible solutes which are responsible of osmotic adjustment and protection against salt stress in these species, in order to provide a better understanding of the phenomenon of tolerance to salinity.

MATERIAL AND METHODS

Plant Material and Growth Conditions

A. prostrata (Boucher) and *P. coronopus* (L.) plants were grown from seeds collected in a salt marsh Barranco Hondo (salt-marshes, south Spain, 37°48'N, 3°43'W) in September of 2010. Seeds were germinated in sand in an incubator with a 12 h photoperiod with an irradiance of 20.0 µmol (photons) m^{-2} s^{-1}, 400-700 nm and a dark/light temperature of 15/25°C. Plants were transferred to pots containing a mixture of peat, coconut fibre and sand (approximately 3:2.1), and kept in a greenhouse with controlled minimal and maxime temperatures of 15°C and 25°C, respectively. Salt treatments with solutions of 0, 50, 100, 200 and 300 mM NaCl (NaCl dissolved in half strength Hoagland and Arnon's solutions) [10] were applied on plants, which were maintained in independent trays for each treatment. Watering was carried out by periodically adding the corresponding salt solution (or water, for the control plants) to the trays, ensuring that the pots were maintained wet throughout the experiment. After 60 days all plants were harvested and leaves utilized for metabolic studies [6,8,10].

Identification of Osmolytes in Leaves

Leaves of plants of two months of growth were utilized for proline determination [11]. Proline was extracted from samples of 200 mg fresh weight with 4 ml of 3% (w/v) sulfosalicylic acid and centrifuged at 1.000 × *g* for 10 min. One volume of the supernatant was mixed with one volume of freshly prepared ninhydrin acid (2.5% w/v) and one volume of glacial acetic acid, and incubated at 100°C for 1 h in darkness. The reaction was stopped by cooling the sample on ice and the sample was extracted with two volumes of toluene. The absorbance of the organic phase was determined at 520 nm (VARIAN spectrophotometric Cary 4000 UV-VIS), using toluene as a blank. The proline concentration was determined using a standard curve. Leaves of plants of two months of growth were utilized for glycinebetaine measurements [6,12]. Leaves (200 mg) were mixed with 5 ml distilled water and the crude extracts applied to a small column (1.6 ml) containing an AG1X8 resin (200-400 mesh; OH-form Bio-Rad). The column was dried down by centrifugation (3 min, 4°C, 300 *g*) and then washed with 875 µl of distilled water. Extracted glycinebetaine was quantified method [12], after HPLC (High Performance Liquid Chromatography) separation on a Spherisorb 5 ODS2 column (250 mm × 4.6 mm) preceded by a precolumn (10 mm × 1 mm) packed with the same phase. The mobile phase contained 13 mM sodium heptane sulphonic acid and 5 mM Na_2SO_4 in deionized water (pH adjusted to 3.7 with 1 N H_2SO_4) at a flow rate of 0.8 ml min^{-1}. Detection was by a UV detector (Bio-Rad 1801 UV monitor) and quantification was performed with HPLC system (Bio-Rad Chromatography Software). Leaf material of two months old was utilized for determination of sorbitol [8]. Leaf material (200 mg fresh weight) was resuspended in 1 ml water, after grinding in liquid nitrogen. The samples were incubated at 95°C for 10 min, cooled on ice and centrifuged at 4°C for 5 min to remove debris, and the supernatants were filtered through Sep-Pak Plus C-18 solid phase cartridges (Waters). The soluble sugar fraction (mono and oligosaccharides) was separated by chromatography in an HPLC anion exchange column (Hamilton RCX-10, 250 mm × 4.1 mm), coupled to a pulsed electrochemical detector (Waters 464). Elution was carried out in an isocratic flux of 100 mM NaOH, and quantification was done by peak integration and comparison with sugar standards (1 mM aqueous solutions of sorbitol).

Statistical analysis

The experimental layout was a randomized block design. Forty plants were grown for each plant species and four replicates were performed for proline, glycinebetaine and sorbitol. Results were subjected to one-way analysis of variance. Differences between means ($P<0.05$) were assessed by Tukey's multiple-range test (Statgraphics Centurion XVI provided by the University of Jaen).

RESULTS AND DISCUSSION

Germination started at 24 h in both species, but the salt drastically inhibited growth at 200 and 300 mM NaCl in *A. prostrata* although growth reduction is greater in *P. coronopus* (data not shown). The behavior of *A. prostrata* was typically of halophyte, proving to be resistant to the salinity treatment while *P. coronopus* showed to be more sensitive to salt stress.

Leaves of *A. prostrata* (60 days of growth of plants) and exposed to saline treatments of 50, 100, 200 and 300 mM NaCl respect to a control (water) were utilized for determination of 2 osmolytes (proline and glycinebetaine). In general, proline levels

were very high in *A. prostrata* with ranging between 0.25 µmol g^{-1} FW (50 mM NaCl) to 3 µmol g^{-1} FW (300 mM NaCl) **(Figure 1),** the control showed a similar value to 50 mM; it was observed an increase of proline content of 4-fold (200 mM NaCl) and 10-fold (300 mM NaCl) compared to untreated plants (P<0.05). In the case of glycinebetaine **(Figure 2)**, it was shown that the level of this osmolyte was increased by salinity as increased the NaCl concentration although the values to 200 and 300 mM NaCl were lower than those found in proline (2.15 µmol g^{-1} FW in 300 mM NaCl) (P<0.05). One of the basic mechanisms for survival under salt stress is the compartmentalization of toxic ions (Na^+ and Cl^-) in the vacuoles, which allows osmotic adjustment avoiding the inhibition of metabolism in the cytoplasm [9]. Proline is an osmolyte contributes to the osmotic adjustment and to occur widely in higher plants. On the other hand, the high concentration of Na^+ salts increased the glycinebetaine accumulation in *A. prostrata*; this osmolyte play an important role beside to proline in maintaining osmotic balance and/or act as an osmoprotectant in Atriplex plants treated with Na^+ salts. It has also been shown that proline, glycinebetaine and sugar may be involved in osmotic adjustment and protection of cellular structures in *A. halimus* [6]; It was reported that both osmolytes (proline and glycinebetaine) may alleviate the deleterious impact of salt stress on antioxidant enzymes (catalase and ascorbate peroxidase) but proline is more effective than glycinebetaine [6]. The best growth of Chenopodiaceae family, under saline stress conditions is due, in part, to the production of these osmolytes (proline and glycinebetaine), which are involved in osmotic adjustment and protection of cellular structures. Glycinebetaine also appears to be directly involved in the protection of chloroplast structures or repair of salt-induced damage [13]. Proline and glycinebetaine provided a protection against saline stress decreasing the level of reactive oxygen species (ROS), lipid peroxidation and increasing antioxidant enzymes activities and cell redox balance [14,15].

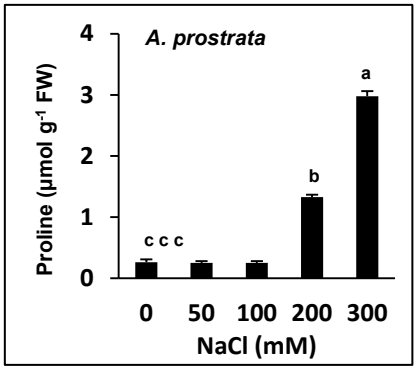

Figure 1. Proline content in 60 days plants of *A. prostrata* treated with 0, 50, 100, 200 and 300 mM NaCl. Data presented are mean ± S.E. of four replicates. Bars with the different letter are significantly different in multiple range comparison (Tukey's test, P<0.05).

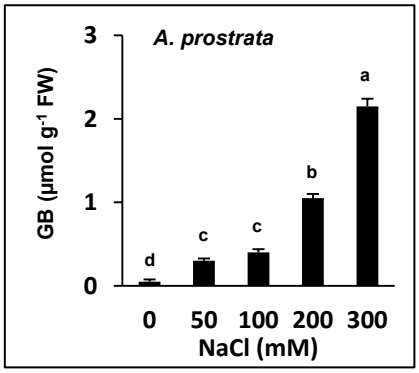

Figure 2. Glycinebetaine content (GB) in 60 days plants of *A. prostrata* treated with 0, 50, 100, 200 and 300 mM NaCl. Data presented are mean ± S.E. of four replicates. Bars with the different letter are significantly different in multiple range comparison (Tukey's test, P<0.05)

P. coronopus treated with 50 and 100 mM NaCl showed proline level too low **(Figure 3)**, although with 300 mM the level of proline was 300 nmol g^{-1} FW (100% respect to the control) (P<0.05), although these values always were lower that *A. prostrata*. Under salinity conditions, it has also been shown that the increase in the proline accumulation in two species with different sensibility to salt was higher in the tolerant one (*A. prostrata*), which implies the involvement of proline accumulation in the osmotic adjustment during salinity. In *P. coronopus* is possible that some other compound(s) could be used for osmotic balance with these levels of salinity. Several authors describe the importance of sorbitol as osmolyte compatible in the Plantaginaceae family [8,16]. For this, it was evaluated the levels of sorbitol in leaves **(Figure 4).** The level of this osmolyte was diminished respect to control and only was recovered at 300 mM NaCl (50 nmol g^{-1} FW). However, it has shown that *P. coronopus* need sorbitol for osmotic adjusted in the root and this could explain because is diminished in the leaves; in this way, *P. coronopus* can store important amounts of salt in the leaves, while maintaining high concentrations of nutrients and soluble carbohydrates in the roots [16]. This sugar is accumulated in cytosol maintaining osmotic balance with ions sequestered in the vacuole, acted as a compatible solute. Along with the proline play an important role by scavenging ROS generated by salt stress. Sorbitol has been discovered like dominant soluble carbohydrate in all Plantago species studied [8,16].

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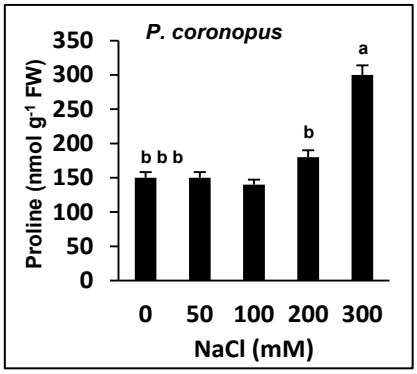

Figure 3. Proline content in 60 days plants of *P. coronopus* treated with 0, 50, 100, 200 and 300 mM NaCl. Data presented are mean ± S.E. of four replicates. Bars with the different letter are significantly different in multiple range comparison (Tukey's test, *P*<0.05).

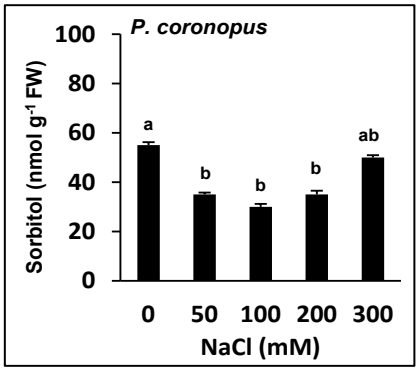

Figure 4. Sorbitol content in 60 days plants of *P. coronopus* treated with 0, 50, 100, 200 and 300 mM NaCl. Data presented are mean ± S.E. of four replicates. Bars with the different letter are significantly different in multiple range comparison (Tukey's test, *P*<0.05)

CONCLUSION

The present work demonstrates that the response to salinity of *A. prostrata* and *P. coronopus* was different. It has been shown that the salt stress remarkably enhanced proline accumulation in the two species although the highest proline content was found in *A. prostrata* (more resistant to salt), which implies that proline accumulation contribute osmotic adjustment and protection cellular membranes during salinity. Salinity also caused an increase in glycinebetaine levels so it can be considered another important osmolyte of *A. prostrata.* Sorbitol was found in *P. coronopus*, although in leaves was not presented with important values, however is considered interesting in root and other organs of Plantaginaceae families.

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