

BRIEF COMMUNICATION

Effect of salinity on polyamines and ethylene in *Atriplex prostrata* and *Plantago coronopus*M. BUENO*, M^a. L. LENDÍNEZ, C. APARICIO, and M^a. P. CORDOVILLA*Department of Animal Biology, Plant Biology and Ecology, Faculty of Experimental Sciences, University of Jaén, Jaén E-23071, Spain***Abstract**

The aim of this study was to investigate the effects of salinity on germination, seedling growth, free polyamines (putrescine, spermidine, and spermine), and ethylene metabolism of two species (*Atriplex prostrata* Bouchér and *Plantago coronopus* L.) with different salt sensitivities. Seeds collected from Barranco Hondo (salt marshes, Jaén, southern Spain) were germinated at 0, 50, 100, and 200 mM NaCl in a growth chamber. The germination of *P. coronopus* seeds decreased considerably with an increasing NaCl concentration, however, seeds of *A. prostrata* showed high germination percentages (84, 87, and 80 %) at 0 (control), 50, and 100 mM NaCl, respectively, and only at 200 mM NaCl, the germination was reduced to 25 %. In the early phase of vegetative growth (8-d-old seedlings), the fresh mass increased in *A. prostrata* at 50 and 100 mM NaCl but the fresh mass of *P. coronopus* showed no significant differences. With respect to polyamines, there was a decrease of the putrescine and spermidine content at all the NaCl treatments, however, the spermine content increased and was much higher in *P. coronopus* than in *A. prostrata*. The ethylene, 1-aminocyclopropane-1-carboxylic acid content, and the 1-amino-cyclopropane-1-carboxylic acid synthase activity increased with the increasing NaCl concentration in *A. prostrata*, and only the ethylene content in *P. coronopus*. These results indicate that *P. coronopus* increased the free spermine content, whereas *A. prostrata* increased the ethylene biosynthetic pathway in order to survive in the saline conditions.

Additional key words: ACC, ACS, germination, growth regulators, halophyte, NaCl, putrescine, spermidine, spermine.

Salinity is one of the most important factors affecting seed germination. Genetics and the stage of development contribute to a greater or lesser tolerance of plants to salinity (Medgdiche *et al.* 2007). Salinity can alter seed germination and seedling growth *via* an osmotic effect and/or ion toxicity (Li *et al.* 2011). The detrimental effects of salinity on seed germination and seedling growth have been well characterized, but biochemical mechanisms controlling the establishment of seedlings are still poorly understood. The *Atriplex* genus (*Chenopodiaceae*) includes plants that can complete their life cycle under stresses, such as drought, high temperature, and high salinity (Khan and Gul 2006). *Atriplex prostrata* is an annual salt marsh species

growing in semi-arid to subhumid areas of the north Mediterranean and in arid zones of North Africa and the eastern Mediterranean (Le Houérou 1992). Also the genus *Plantago* (*Plantaginaceae*) presents a wide variety of species with different degrees of salinity tolerance. *Plantago coronopus* is a herbaceous and short-lived perennial rosette plant which mainly occurs in salt marshes. It is a weed common on all continents found on disturbed ground, waste places, and chalky banks especially near the sea (Yoshida and Tanaka 1997).

Polyamines (PAs) are small organic polycations found naturally in eukaryotic and prokaryotic cells where they have been associated with cell growth and development. The most abundant polyamines in plant

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Abbreviations: ACC - 1-aminocyclopropane-1-carboxylic acid; ACC oxidase - 1-aminocyclopropane-1-carboxylic acid oxidase; ACC synthase - 1-aminocyclopropane-1-carboxylic acid synthase; FM - fresh mass; HPLC - high performance liquid chromatography; PAs - polyamines; Put - putrescine; SAM - S-adenosyl-L-methionine; Spd - spermidine; Spm - spermine.

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tissues are putrescine (Put) and spermidine (Spd), whereas spermine (Spm) is found in lower or even trace concentrations (Kaur-Sawhney *et al.* 2003). Polyamines affect seed germination and may influence early responses of plant to salt stress (Zapata *et al.* 2004, 2008), and they also have a significant role in plant defense and adaptation to various abiotic or biotic stress conditions (Kaur-Sawhney *et al.* 2003). The PAs, Spd and Spm, and ethylene share a common precursor, S-adenosyl-L-methionine (SAM), and the biosynthetic relationship between these molecules is most often considered in terms of a competitive demand (Pandey *et al.* 2000). Ethylene is a gaseous hormone that regulates many aspects of the plant life cycle including seed germination (Matilla and Matilla-Vázquez 2008) and responses to biotic and abiotic stresses (Lin *et al.* 2009). Methionine (a common precursor) gives rise to ethylene via three key enzymatic reactions: 1) methionine is converted to SAM by S-adenosyl methionine synthetase (S-AdoMet synthetase); 2) 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) converts SAM to ACC; and 3) ACC oxidase (ACO) degrades ACC to release ethylene (Yang and Hoffmann 1984). The aim of this study was to investigate the effect of salinity on endogenous polyamines and ethylene metabolism in two plant species (located in saline marshes) with different sensitivities to salinity and in the early phase of vegetative development. This comparative study was carried out to observe modifications of these metabolites and their possible relation with tolerance to saline stress in these wild species in order to provide a better understanding of the phenomenon of tolerance to salinity.

Mature seeds were randomly collected from many plants in natural populations of *Atriplex prostrata* Bouché and *Plantago coronopus* L. in Barranco Hondo (salt-marshes, south Spain, 37°48'N, 3°43'W) in 2010. To avoid dormancy of seeds, these were stored dry and then stratified in cold (4 °C) for several months in order to break the dormancy (Baskin and Baskin 2001). Seeds of both species were incubated in 90-mm-diameter Petri dishes on two layers of *Whatman No. 1* filter paper moistened with 5 cm³ of a sterile water (control) or NaCl solutions (50, 100, and 200 mM). Each Petri dish contained 50 seeds. Germination was carried out in a growth chamber with a 16-h photoperiod, a photosynthetic photon flux density of 450 µmol m⁻² s⁻¹, day/night temperatures of 25/16 °C, and a relative humidity of 55 - 75 %. The seeds were considered germinated after the emergence of the radicle. The seed germination started after 24 h in both *A. prostrata* and *P. coronopus*, and a maximum germination percentage was found on day 8 in both the species. Therefore, fresh mass, content of Put, Spd, Spm, ethylene, and ACC, and ACC-synthase activity were measured in 8-d-old seedlings. It was rather difficult to obtain enough material for these analyses due to the low mass of the seedlings.

The technique of Smith and Davies (1985) was followed for the determination of free polyamines (Put, Spd and Spm). A fresh material (300 mg) was

homogenized with 0.2 M perchloric acid (1:4, m/v) containing 1,6-diamino-hexane (1:1, m/v) as an internal standard (100 µg g⁻¹ tissue). The homogenate was centrifuged at 27 000 g and 4 °C for 10 min and 0.1 cm³ aliquots of the supernatant was saturated with sodium carbonate and dansylated with dansyl chloride (10 mg cm⁻³ in acetone). The mixture was incubated at 60 °C for 1 h, then the solution of L-proline (100 mg cm⁻³) was added. After 30 min, the dansylated polyamines were extracted with toluene (HPLC grade). The toluene extract was dried under nitrogen and the residue was dissolved in acetonitrile (HPLC grade) and filtered through *Millipore* (Darmstadt, Germany) *HV-4* filters for an immediate analysis. An aliquot (0.02 cm³) of sample was injected into a reversed phase *Spheri-5 C₁₈ ODS* (8 µm, 4.6 × 220 mm) column. A *Shimadzu* (Kyoto, Japan) *LC-10A* HPLC equipped with a fluorescence spectro-photometer (the excitation and emission wavelengths were 252 nm and 500 nm, respectively) was used to quantify the dansyl derivatives. The same procedure was applied with standards.

For ethylene measurements, the method of Sánchez-Calle *et al.* (1989) was followed: a sample (500 mg) was aseptically transferred to a 5 cm³ flask containing 0.05 cm³ of distilled water. The flask was sealed with a silicone-rubber stopper and incubated in darkness at 30 °C. After 1 h, a 1-cm³ gas sample was injected into an *HP 5890 (series II, Hewlett Packard, Palo Alto, USA)* gas chromatograph fitted with a flame ionization detector and a 2 m × 4 mm stainless-steel column packed with 50 - 80 mesh *Poropak-R*. The N₂, H₂, and synthetic air flow rates were 50, 86, and 400 cm³ min⁻¹, respectively. Ethylene identification was based on the retention time compared with a C₂H₄ standard (purity 99.9 %).

For determination of ACC content and ACC-synthase activity, the method of Chi *et al.* (1991) was followed: a frozen tissue (1 g) was homogenized at 4 °C with 1 cm³ of an extraction medium containing a 100 mM potassium phosphate buffer (pH 8.5), 1 mM dithiothreitol (DTT), 25 µM pyridoxal 5'-phosphate, and 5 % (m/v) polyvinyl-pyrrolidone (PVPP). The homogenate was centrifuged at 19 000 g and 4 °C for 20 min. The supernatant was retained and centrifuged again before it was assayed for ACC according to the method of Lizada and Yang (1979). The protein content of the supernatant was determined according to the method of Bradford (1976) using bovine serum albumin as standard. For ACC-synthase assay, 0.02 cm³ of the supernatant was incubated at 30 °C for 15 min in a 0.6 cm³ reaction mixture consisting of 400 µM SAM and 10 µM pyridoxal 5'-phosphate in a 100 mM potassium phosphate buffer (pH 8.5). The amount of ACC formed was determined according to the method of Lizada and Yang (1979). One unit of enzyme activity was defined as that which converts 1 nmol of SAM to ACC per hour at 30 °C. A standard curve of ACC from *Sigma* (St. Louis, USA) were also constructed.

An experimental layout was a randomized block design. Results were subjected to the one-way analysis of

variance. The seed germination tests, fresh masses and ethylene production values were means of six replicates per treatment. Five replicates were performed for Put, Spd, Spm, ACC content, and for ACC-synthase activity. Differences between means were assessed using the Tukey's multiple-range test (*Statgraphic Centurion XVI*).

Although the seed germination started after 24 h in both the species (data not shown), *A. prostrata* showed on day 8 a maximum germination of 84, 87, and 80 % at 0, 50, and 100 mM NaCl, respectively, without significant differences among the concentrations used (Table 1). Only at 200 mM NaCl, the germination reduced to about 25 %. No further germination was observed up to 20 d (data not shown). At the same time, the germination percentage of *P. coronopus* decreased from 70 % (control) to 26 and 5 % under 50 and 100 mM NaCl, respectively, and 200 mM NaCl completely inhibited germination (Table 1). In *A. prostrata*, significant increases in fresh mass of the whole seedlings were observed at 50 and 100 mM NaCl, whereas in *P. coronopus*, only a slight increase at 50 mM NaCl (Table 1). The results suggest that *A. prostrata*, in contrast to *P. coronopus*, possessed early adaptive mechanisms that could allow it to grow in 50 and 100 mM NaCl and tolerate the exposure to 200 mM NaCl. In *P. coronopus*, a slower growth is a general adaptive feature for survival under stress, and the germination percentage is drastically inhibited (Vicente *et al.* 2004). Similar results have been found in *Cakile maritima* and *Arabidopsis thaliana* (Ellouzi *et al.* 2011). Halophytes increase the water content at medium salt concentrations and, therefore, they increase fresh mass. One of the basic mechanisms for survival under salt stress conditions relies on the compartmentalization of toxic

ions (Na^+ and Cl^-) in vacuoles which allows an osmotic adjustment avoiding the inhibition of metabolic processes in the cytoplasm (Türkan and Demiral 2009). Hassine *et al.* (2008) have shown that proline, glycine-betaine, and sugars may be involved in an osmotic adjustment in *A. halimus*; Vicente *et al.* (2004) described the increase of sorbitol in *P. crassifolia*.

After analyzing the content of polyamines, it was observed that the most abundant polyamine was Spm followed by Spd and Put in *P. coronopus*. However, in *A. prostrata*, the most abundant was Spd followed by Put and Spm. In the control seedlings, the Put content was the same in *A. prostrata* and *P. coronopus* (Table 1). The salt treatments reduced the content of Put by 66 % (50 mM NaCl), 79 % (100 mM NaCl), and 88 % (200 mM NaCl) in *A. prostrata*, whereas in *P. coronopus*, the degree of reduction was less than 52 % (50 and 100 mM NaCl). The highest content of Spd was found in the *P. coronopus* control seedlings (Table 1), and its content was reduced by 27 % and 30 % at 50 and 100 mM NaCl, respectively. In *A. prostrata*, the Spd content decreased only at 100 and 200 mM NaCl (Table 1). The salinity caused a significant increase in the Spm content in *P. coronopus* (Table 1), however, an increase in the Spm content in *A. prostrata* was significant only at 200 mM NaCl and all values were much lower than in *P. coronopus* (Table 1). A better protective effect of Spm in comparison to Spd could be accounted for its longer chain and a greater number of positive charges which allow a greater neutralization and stabilization of membranes. The PAs may also act indirectly by elevating the content of antioxidants or activating the gene expression of antioxidant enzymes (Tang and Newton 2005, Mallik *et al.* 2011). Our results seem to indicate

Table 1. The effect of NaCl (0, 50, 100, and 200 mM) on seed germination, fresh mass (FM), content of putrescine (Put), spermidine (Spd), spermine (Spm), ethylene (C_2H_4), and ACC, and ACC-synthase activity (ACS) in *A. prostrata* and *P. coronopus*. All parameters were measured in 8-d-old seedlings. Means $\pm$ SE, $n = 5 - 6$. Different letters within the same row indicate statistically different means according to the Tukey's test ($P < 0.01$). n.g. - no seed germination.

		Control	50 mM NaCl	100 mM NaCl	200 mM NaCl
<i>A. prostrata</i>	germination [%]	84.0 $\pm$ 1.2a	87 $\pm$ 2.14a	80 $\pm$ 2.49a	25 $\pm$ 1.91b
	FM [mg (seedling) ⁻¹]	3.40 $\pm$ 0.07b	4.88 $\pm$ 0.08a	4.98 $\pm$ 0.12a	3.52 $\pm$ 0.20b
	Put [nmol g ⁻¹ (FM)]	68 $\pm$ 3.85a	23 $\pm$ 1.94b	14 $\pm$ 1.30bc	8 $\pm$ 0.92c
	Spd [nmol g ⁻¹ (FM)]	110 $\pm$ 3.85a	112 $\pm$ 5.19a	78 $\pm$ 4.04b	37 $\pm$ 1.89c
	Spm [nmol g ⁻¹ (FM)]	13 $\pm$ 0.19b	14 $\pm$ 0.66b	18 $\pm$ 2.83b	41 $\pm$ 2.50a
	C ₂ H ₄ [pmol g ⁻¹ (FM) min ⁻¹]	37 $\pm$ 8.0c	100 $\pm$ 17b	114 $\pm$ 22.0b	151 $\pm$ 23a
	ACC [pmol g ⁻¹ (FM)]	90 $\pm$ 1.0c	160 $\pm$ 1.0b	160 $\pm$ 1.00b	250 $\pm$ 3.00a
	ACS [pmol g ⁻¹ (FM) min ⁻¹]	3.7 $\pm$ 0.01c	4.5 $\pm$ 0.07b	4.5 $\pm$ 0.07b	5.0 $\pm$ 1.00a
<i>P. coronopus</i>	germination [%]	70 $\pm$ 5.17a	26 $\pm$ 5.87b	5 $\pm$ 1.26c	n.g.
	FM [mg (seedling) ⁻¹]	3.78 $\pm$ 0.10a	4.36 $\pm$ 0.20a	3.92 $\pm$ 0.22a	-
	Put [nmol g ⁻¹ (FM)]	68 $\pm$ 5.63a	36 $\pm$ 4.42b	30 $\pm$ 2.92b	-
	Spd [nmol g ⁻¹ (FM)]	179 $\pm$ 2.64a	131 $\pm$ 4.11b	125 $\pm$ 1.40b	-
	Spm [nmol g ⁻¹ (FM)]	195 $\pm$ 3.78c	241 $\pm$ 3.99b	264 $\pm$ 4.04a	-
	C ₂ H ₄ [pmol g ⁻¹ (FM) min ⁻¹]	23 $\pm$ 4.00c	57 $\pm$ 6.00b	90 $\pm$ 6.00a	-
	ACC [pmol g ⁻¹ (FM)]	90 $\pm$ 5.00a	90 $\pm$ 5.00a	100 $\pm$ 4.00a	-
	ACS [pmol g ⁻¹ (FM) min ⁻¹]	3.7 $\pm$ 0.07a	3.7 $\pm$ 0.07a	3.7 $\pm$ 0.07a	-

that SAM was diverted mostly towards the production of ethylene in *A. prostrata*, whereas *P. coronopus* produced less ethylene and, therefore, it might have more capacity to synthesize Spm. The accumulation of Spm has been described in salt-treated rice (Maiale *et al.* 2004), several vegetables (Zapata *et al.* 2003, 2004, and 2008), *Lotus glaber* (Sanazzaro *et al.* 2007), and maize (Jiménez-Bremont *et al.* 2007). The application of exogenous spermine partially protected injury under salt stress during sweet sorghum seed germination (Chai *et al.* 2010). The protective role of Spm against salt stress has been well established in *Arabidopsis* (Yamaguchi *et al.* 2006, 2007) using the mutant *Arabidopsis acl5/spms* which cannot produce Spm and is sensitive to drought and salinity but recovers when treated with Spm.

The salinity significantly increased ethylene production in both *A. prostrata* and *P. coronopus* (Table 1). In all the NaCl concentrations, the *A. prostrata* seedlings exhibited a higher ethylene production than the *P. coronopus* seedlings. *A. prostrata* showed an increase more than 300 % at 200 mM NaCl. The ACC content response to the salinity varied with the species (Table 1). The *A. prostrata* seedlings treated with 50, 100, and 200 mM NaCl showed an increase in the ACC content, whereas the *P. coronopus* ACC content showed no significant differences among the treatments. The response of ACC-synthase to the salinity was similar to that described for ACC (Table 1). In *A. prostrata*, the ACC-synthase activity increased significantly with the increasing NaCl concentration. On the other hand, in *P. coronopus*, no significant differences were observed. A number of studies have dealt with the interaction between ethylene and PAs (Pandey *et al.* 2000, Metha *et al.* 2002). In our case, it seems that there was competition between the metabolic pathways of ethylene and PAs due to their common precursor (SAM). The ethylene metabolism increased and the polyamine metabolism decreased in *A. prostrata*, although the latter way was not so clear for *P. coronopus*. It has been reported that NaCl increases ethylene production in various species (Zapata *et al.*

2003, 2007) and more recently in rice (Quinet *et al.* 2010). These researchers have shown that an increase in ethylene production is higher in salt-tolerant than in salt-sensitive cultivars demonstrating that the capacity to increase the ethylene production under salinity can provide a higher tolerance to salinity. More recently, Yang *et al.* (2013) have confirmed the key role of ethylene for improving *Arabidopsis thaliana* salt tolerance mainly via regulating K⁺ accumulation in the leaf and root. In addition, ethylene stimulates plasma membrane H⁺-ATPase activity to modulate ion homeostasis and salt tolerance (Wang *et al.* 2009). The ACC content and the ACC-synthase activity in *A. prostrata* were higher in the seedlings treated with NaCl, and this was accompanied by an increased ethylene production. However, the increase in the ethylene production in *P. coronopus* was not accompanied by an increase in the ACC-synthase activity and the ACC content. The fact that *P. coronopus* produced lesser amounts of ethylene than *A. prostrata* might be due to lack of stimulation of the ACC-synthase activity and the fact that SAM is diverted towards the synthesis of PAs (Spd and especially Spm).

In conclusion, the salinity caused a considerable inhibition of seed germination in *P. coronopus*, whereas a great inhibition of seed germination in *A. prostrata* was only at 200 mM NaCl demonstrating that *P. coronopus* was more sensitive to the salinity than *A. prostrata*. The salinity caused an increase in the Spm content in both the species although the values were much higher in *P. coronopus*, which could indicate a protective role of Spm in this species. On the contrary, NaCl stimulated ethylene production, ACC content, and ACC-synthase activity in *A. prostrata*. These results demonstrate a different protection against the salt stress in *P. coronopus* (more salt-sensitive) and *A. prostrata* (more salt-resistant). Therefore, the knowledge of the physiology of plants adapted to saline environments and their relation with growth regulators can help us better understand the mechanisms of salinity tolerance.

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