

Comparative adaptations in halophytes (*Salicornia Europaea* L., *Puccinellia Distans* (JACQ.) parl., and *Atriplex olivieri* MOQ.) exposed to salt stress

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Abstract

Halophytic species show greatly different adaptation mechanisms in response to high salinity. The present study examines the effects of extreme salt (NaCl) application on three different halophytic plants (*Salicornia europaea* L., *Puccinellia distans* (Jacq.) Parl., and *Atriplex olivieri* Moq.). In order to evaluate the effects of salt stress, ten-day old halophyte seedlings were subjected to 0, 200, 400, 600 and 800 mM NaCl concentrations for two months. Growth, leaf water potentials, stoma situations and proline contents were evaluated. Chlorophyll and carotenoid content, soluble carbohydrates and hormone levels were also analyzed. *Puccinellia distans* and *Atriplex olivieri*'s growth was suppressed more than *Salicornia europaea*; up to 400 mM salt concentration. Physiological changes also included; significant proline accumulation, carbohydrates and phytohormone adjustments and changes in osmotic potential, protein profiles and stomata size. The results suggested that possesses strategies, commonly present in halophytic plants, to cope with high salt levels differ with halophytic plant species. In addition, *Salicornia europaea* can be used for halophytic adaptation studies as a model plant.

Keywords: halophytes, salt stress, adaptation, *Salicornia europaea*

Introduction

Salt tolerance is the ability of plants to cope with salt stress and survive their life cycle at soluble salt concentrations. Approximately 9.5 million hectares of the world's soil are primarily saline soil, without secondarily salinized soil in cultivated land. On the other hand, fresh water resources are limited and soil salinity increases with mistreat irrigation. Under these circumstances, researchers prompt the regarding of halophytes adaptation mechanism. ^[1] Many plants improve adaptation mechanisms either to prohibit salt from their cells or to tolerate its proximity with the cells. ^[2] Plants exhibit overabundance of physiological, biochemical and molecular mechanisms to cope with salt injuries. ^[3]

Abscisic acid is responsible for the modification of salt-stress-induced genes. Salt stress regulates polyamine biosynthesis and catabolism by acting as a cellular signal in hormonal pathways thereby regulating abscisic acid (ABA) in response to stress. ^[4] ABA-inducible genes are supposed to play a significant role in the mechanism of salt tolerance in rice ^[5]. As well-known function, ABA also triggers stomatal closure by rapidly altering ion fluxes in guard cells under environmental stress conditions.

Exposure of plants to salinity results in increased generation of reactive oxygen species, as by-products, which damage the cellular components. ^[6] Reactive oxygen species cause chlorophyll degradation and membrane lipid peroxidation, reducing membrane fluidity and selectivity ^[7]. In addition, proteins play an imminent role in plant stress response since they are directly involved in the acquisition of an enhanced stress tolerance ^[8].

Salicornia species are edible plants with plenty of minerals, and it can be used as a source of soda ash and soap making. Seeds are also used for to produce animal feedstuff and as a biofuel, feedstock on coastal land where the conventional

crops cannot be grown and able to accumulate and store salt within their leaves and stems which allows them to survive. Halophytes are plants growing on or surviving in saline conditions and their adaptation mechanisms are different from species to species. Therefore, in order to successfully understand salt tolerance in plants, the mechanism at each level must be studied individually. Thus, this study was undertaken to provide information on tolerance mechanism of three different halophytes grown under higher salinity levels.

Materials and Methods

Plant materials and salt treatments

Plant seeds used in study were collected from halophytic habitats, healthy and vigorous seeds were chosen. Seeds were surface sterilized and germinated in sterile petri dishes. Germinated seeds transferred to the pots (120 x 90 mm) with nonsterilized soil mixed with sand (soil; sand 3; 1 v/v). Pots were kept in phytotron at + 28 °C at 16 h daylight/8 h moonlight. Plants were exposed to salinity by adding NaCl (200, 400, 600, 800 mMol L⁻¹) to their irrigation water for two months. Control groups were irrigated with tap water at same volume (100 ml/d). Each treatment was performed in triplicate under same conditions. At the end of the growing period, plants were harvested for analysis and samples were kept in deep freezer at -80 °C until analyses.

Examination of stomata samples and leaf osmotic potential

When plants were harvested from the pots, leaves were preserved in formalin; acetic acid; ethanol (FAA: 1; 1;9) for microscopic examination ^[9]. Leaf osmotic potential was measured with Wescor 5520 osmometer. osmotic potentials were performed as Mega Pascal.

Determination of chlorophyll and carotenoid content

Fresh leaves were homogenized with a pestle in a mortar. Chlorophyll and carotenoids were extracted from leaves with 80 % acetone and absorbance values were measured at 450, 645 and 663 nm wavelengths for total chlorophyll content and carotenoids. The amount of total chlorophyll and carotenoids was calculated according to [10].

Quantification of proline contents

The method developed by [11] was used for the quantification of proline content. 0.5 g of plant material was homogenized using 10 ml of 40 % aqueous methanol. Homogenates were mixed with 2 mL of acid ninhydrin (1.25 g of ninhydrin + 30 mL of glacial acetic acid + 20 mL of 6 M phosphoric acid). The sample was heated for one hour at 100 °C in a water bath. Then 4 mL of cold toluene were added. This solution was mixed and read at 520 nm and the results were expressed as mol g⁻¹ fresh weight.

Determination of sugars

2 gr of sample was homogenized with 40 mL of methanol. The mixture was incubated on magnetic stirrer at 65 °C for 1 h. and centrifuged at 4 °C, 1300 rpm for 30 min. Methanol was removed by rotary evaporator. Extract was passed through Sep-Pack C18 cartridge. Filtrate by 0.45 µm membrane and injected into HPLC [12].

Extraction and determination of phytohormone levels

The analysis of phytohormones (Gibberellic acid and abscisic acid) was done according [13]. Battal *et al.* (2001). One gram of sample was mixed with 20 ml cold methanol and sonicated at Ultra Tissue Lysis. Methanol was removed at rotary evaporator. The extracts were dissolved in K₂HPO₄ buffer (pH=8.5) and centrifuged at 10,000 g for 1 h at 4°C. Samples were mixed well with PVPP and filtered through Whatman filter paper. The filtrates were load to Sep-Pack C 18 cartridges and extracts were injected into HPLC. [13].

Preparation of whole-cell proteins and SDS-PAGE analysis

One gram of fresh sample was mixed with 25µl of denaturing buffer (pH 6.8) containing 0.006 M Tris-HCl, 2.5 % glycerol, 0.5 % SDS and 1.25 % β-mercaptoethanol and boiled for 5min. After centrifugation for 3 min at 10,000 g. the supernatants were transferred into an eppendorf tube [14]. Electrophoresis was performed with discontinuous buffer system in UVP vertical electrophoresis Unit. The gel was scanned with densitometer and the molecular weight of each band was determined using one dimensional analysis of software (Lab Image ver.6.)

Data analysis

All data were evaluated by one-way ANOVA using SPSS 9.0 for Windows as well as the least significance difference and Post-hock significant difference tests. The experiments were repeated with three replicates for each treatment and comparisons with P<0.05 were considered significantly different

Results

The NaCl treatment did not show any effect on shoot length of *Salicornia europaea*. However exposure of 400 mM and further salt treatments decreased the shoot length of *Puccinellia distans* significantly. Shoot length of *Atriplex olivieri* was also decreased by increasing salt treatment concentration compare to control group. (Figure 1A). 200 mM of NaCl treatment did not cause a remarkable change in osmotic potential values whereas 400 mM and 600 mM salt treatments were decreased leaf osmotic potential significantly especially in *Atriplex olivieri* and *Puccinellia distans* plants (Figure 1. B). These data suggested that halophytes exhibits little or no growth stimulation at low salinity levels, while higher salinities reduced growth [15]. High salt stress set up osmotic homeostasis with water potential and ion distribution. This dispersion of homeostasis occurs not only at cellular level but also in whole plants levels [16]. 400 mM salt treatment had no significant effect on chlorophyll content of *Puccinellia distans*. Nevertheless, the chlorophyll content was decreased by 600 mM salt treatment. Chlorophyll content of *Atriplex olivieri* was stable up to 600 mM salt treatment but it was also reduced with 800 mM salt treatment. Finally chlorophyll content of *Salicornia europaea* showed uniform values in all salt treatment concentrations (Figure 1.C). Also 200-600 mM salt treatments were significantly decreased carotenoid content of *Puccinellia distans*. On the other hand carotenoid content of *Salicornia europaea* remained unchanged at all salt treatments compared to control group (Figure 1.D). Salt stress has direct and indirect effects on the chlorophyll content and photosynthetic efficiency of plants. The direct effects are achieved by regulating the activity and expression levels of enzymes involved in chlorophyll biosynthesis and photosynthesis, plants accommodate leaf morphology under saline conditions with reduced photosynthetic machinery, as well as reduction of chlorophyll content [16]. Salinity causes significant decreases in *Chl-a*, *Chl-b* and carotenoid in leaves of *Bruguiera parviflora* [3].

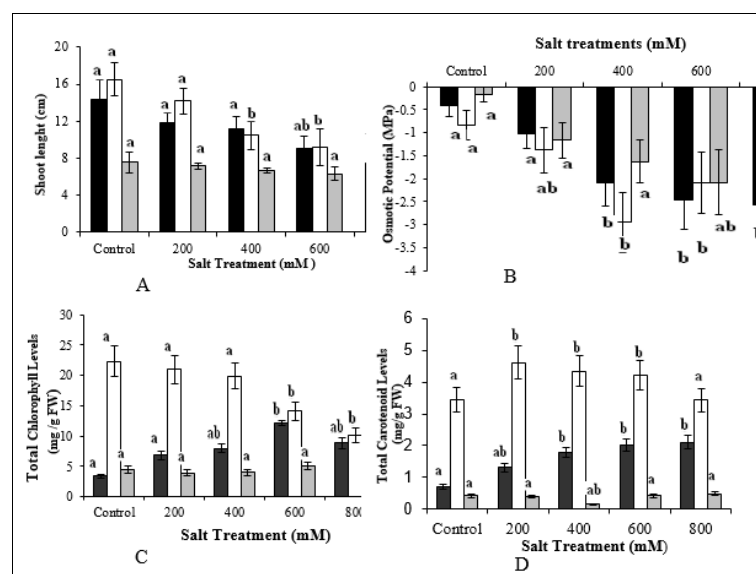


Fig 1: The effects of salt treatments on shoot lengths (A), osmotic potential (B), total chlorophyll (C) and caretonoid (D) of halophyte plants. Means ± S.E., n =5. Bars with different letters are significantly different at P<0.05. (■ *Atriplex olivieri*, □ *Puccinellia distans*, ▒ *Salicornia europaea*)

The comparison of stoma size of control plants with 800 mM salt treatment groups of halophyte plants were shown in Fig. 3.A,B,C. Stoma size did not show prominent differences in *Puccinellia distans* but the stoma size of *Atriplex olivieri* and *Salicornia europaea* significantly decreased in 800 mM salt treatment compared to control group (Figure 2). These adverse stress factors result in an

enhanced stomata closure, a decreased photosynthesis rate and a reduced plant growth. The plant ability to cope with stress underlies plant salinity tolerance. The plant has to behave physiologically embarrassment for growth on saline substrate with regulation of CO₂ and H₂O exchange by stomata [17].

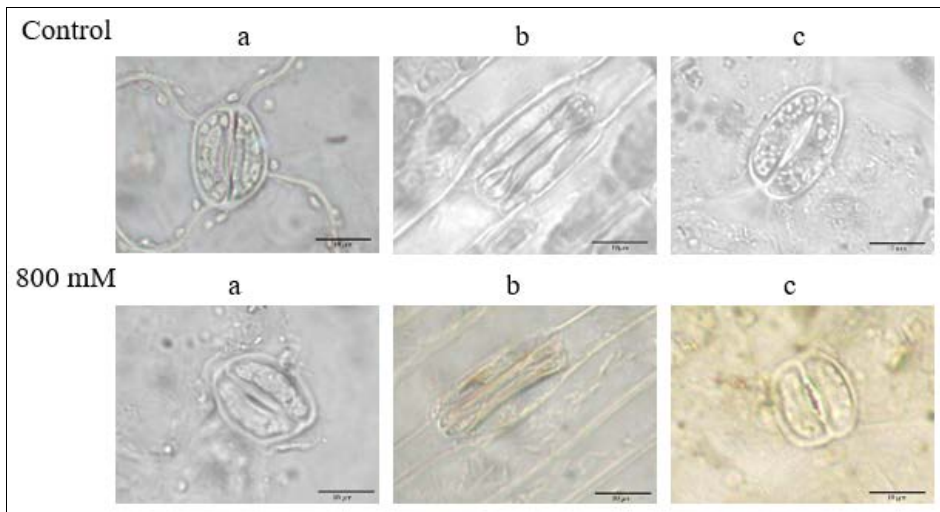


Fig 2: The effects of 800 mM salt treatments on stoma size a: *Atriplex olivieri* b: *Puccinellia distans* c: *Salicornia europaea*

Free proline content in leaves of *Atriplex olivieri* plants exposed to 600 mM salt treatment reached to the highest level and showed six times higher accumulation than leaves that of the leaves of control group. In *Puccinellia distans*, proline accumulation was started at 200 mM salt treatment and the highest proline accumulation in *Puccinellia distans* was determined in 800 mM NaCl treated group. Free proline content in leaves of *Salicornia europaea* did not change in all

salt treatments (Figure 3A). Proline accumulation increase salt resistance of economic crops [18]. Proline can preserves protein structure and activity with reducing enzyme denaturation by inactivating hydroxyl radicals and other reactive chemical species. Accumulation of free amino acids like as proline reduces osmotic potential in cytoplasm and provide to maintaining homeostasis under environmental stress [19].

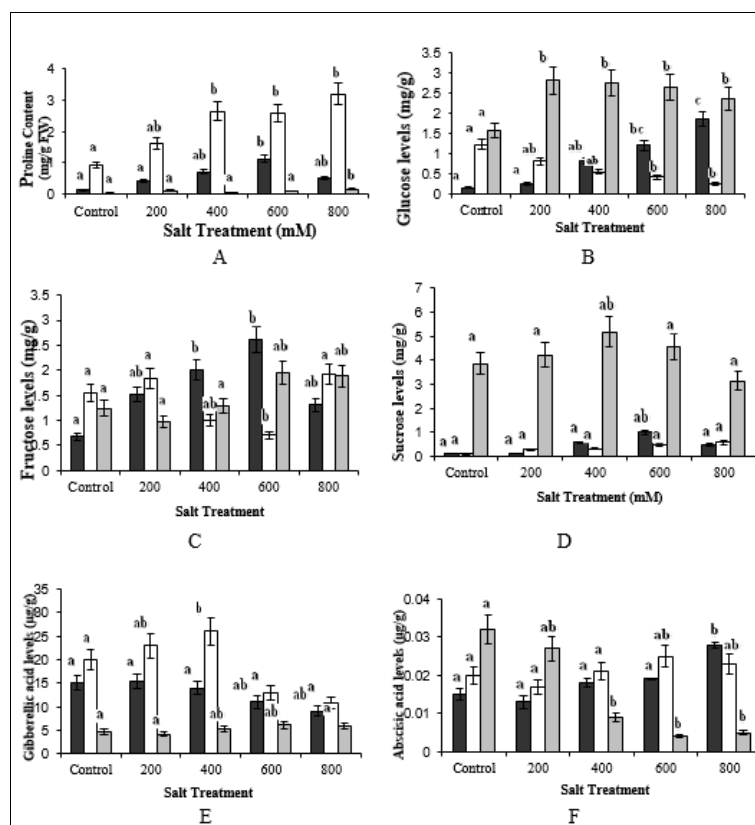


Fig 3: The effects of salt treatments on Proline (A), glucose (B), fructose (C), sucrose (D) Gibberellic acid (E), Abscisic acid (F) of halophyte plants. Means ± S.E., n = 5. Bars with different letters are significantly different at P < 0.05. (■ *Atriplex olivieri*, □ *Puccinellia distans*, □ *Salicornia europaea*)

Exposure of 800 mM NaCl, *Atriplex olivieri* crucially increased glucose level. Although increment of salt treatment significantly increase glucose levels of *Salicornia europea*, also significant decreases were observed with increasing of salt concentration in *Puccinellia distans* compared to non-salinized control groups (Fig.6.A). NaCl treatment enhanced total sugar and proline accumulation, prevents toxic and nutrient deficiency effects of salinity. Nevertheless in some plant species salinity may increase the but decrease in others [20] [21].

GA3 levels of *Puccinellia distans* reached to maximum value in 400 mM salt treatment but decreased with increasing NaCl concentration. However, no significant difference was determined between the control and salt treatments in leaves of *Salicornia europea* (Fig.7.A). GA3 altered the pattern of distribution and accumulation of ions in shoots and roots under salt stress [22].

Salt treatments altered leaf ABA concentrations in all halophytic species. Salt treatment in *Atriplex olivieri*, especially 800 mM NaCl treatment led to a significant increase on ABA concentration. Salt stress increases biosynthesis and accumulation of abscisic acid in higher plant [23]. The increased ABA content was accompanied by an improvement in K^+/Na^+ ratio leading to increased salt tolerance.

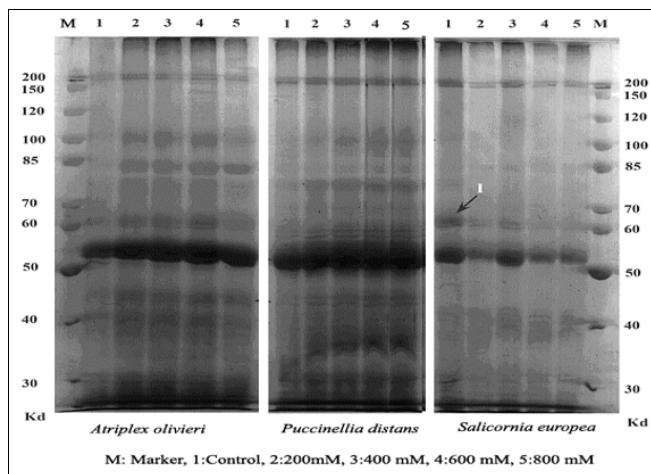


Fig 4: Protein profiles in leaves of halophyte plants in response to different NaCl concentration.

The protein band at molecular weight of 52 kDa (Kilo Dalton) was considered as a structural protein and its concentration was altered with different salt treatments. These protein band's concentrations were increase with increment of salt treatment in *Atriplex olivieri* and *Puccinellia distans* plants. However, in *Salicornia europea*, the band was found to be different in the concentration of 200 mM, 400 mM and 800 mM salt applications. The double protein bands in molecular weights of 58/59 kDa and 43/44 kDa were observed in all salt treatments obviously in *Puccinellia distans*, however they were not visible in control group. The protein band in molecular weight of 84 kDa was determined only in the salt treatments of *Atriplex olivieri*. Also molecular weight of 60 kDa protein band was seen in control group of *Salicornia europea* plant. Nevertheless the concentration of band was decreased with increasing salt treatments (Figure 4). In barley, salinity induces six new proteins in roots and also SDS-PAGE analysis in peanut

(*Arachis hypogaea* L.) display that plants exposed to NaCl show induction (127 and 52 kDa) or repression (260 and 38 kDa) in the synthesis of a few polypeptides.

In the present study demonstrated that *Salicornia europea* is a highly salt-tolerant species that is able to survive and grow at salinities up to 800 mM salt treatment. Adaptation mechanism of plants response to salt stress is either to exclude salt from cells or to tolerate with physiological progresses such as photosynthesis and protein synthesis [24]. Scientific articles about various salt-tolerance mechanisms can be found in and many reports emphasize the important role of halophytes in improving saline soil conditions and the cultivation of salt-tolerant crops [25] [26].

Conclusion

Regarding to the fluctuated data provided from three different halophytic plants, bring to mind the question of whether halophytes require saline conditions for their existences. It was also suggested that the physiological mechanism leading to salt tolerance in halophytes are regulated in such a way that allow them competitive advantages to salt stress over other plants not only with single adaptation mechanism but also with their genetics. However adaptation mechanisms vary from plant to plant species. This study has also demonstrated that *Salicornia europea* show the best tolerance mechanism, comparison with the *Puccinellia distans* and *Atriplex olivieri*. Combining with all studies about halophyte adaptation mechanism, in this study we aimed to force the halophyte plants to understand their tolerance limit and this will generate crop plants able to tolerate and grow in high salt concentrations.

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