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Effect of NaCl salinity on germination, physiological and biochemical parameters of *Plantago ovata* Forsk.

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Abstract

Since NaCl stress is a major limiting factor for plant production and growth, we investigated the effect of different salinity stress levels (control, 15, 25, 50, 100 and 200 mM NaCl) on some physiological and biochemical parameters of *Plantago ovata* for 30 days. The experiment was laid out as a completely randomized design in a factorial arrangement with three replications. Low concentrations of NaCl (0-25 mM) increased chlorophyll content as compared with control, however, this factor decreased gradually at high concentrations of NaCl (50-300 mM). The proline content increased significantly as NaCl concentration augmented. The accumulation of Na^+ increased in a concentration dependent manner with maximum of 205 mg kg^{-1} at 300 mM, which indicates the relative resistance ability of this plant to salinity. We conclude that *Plantago ovata* has physiological traits associated with accumulation of NaCl to relative high levels and it can be useful for restoring salinity and recovery of *the salinity-contaminated sites.*

Keywords: NaCl stress, Plantago ovata, physiological and biochemical parameters, Salinity.

Introduction

Medical plants are one of the nature treasures. They are considering as the most important natural resources, which have been used and given attention by human beings since many years ago. Increasing possibility of exposing human health under the toxicity of synthetic medicine and antibiotics, approach and interest to use plant medicine are increasing throughout the world (Satish *et al.,* 2003). Plants, under natural condition, are continually subjected to various tensions. Among these, salinity stress as one of the most significant bounding factors of agricultural products, act in most part of Iran and the world. High salinity effects may be observed in the whole plant as extremely decrease in products and even plant death.

Some plants employ some tolerate mechanism to resist salt entrance into the cells. After attacking salt and extending salinity stress in plant, all important processes such as photosynthesis, protein synthesis, energy, lipid metabolism, and finally plant growth, are affected (Munns & Tester, 2008). Studying on salinity effects on germination rate and percentage as well as initial root and shoot growth in agricultural plants shows that salinity stress is a reliable test to assess many resistant plant species in primary stages of growth (Ghoulam *et al.,* 2002). Generally, salinity stress induces delay in germination, decrease in germination percentage, rate of germination and seedling growth (Munns, 2002). Lack of plants germination in saline soils, due to high accumulation of salt in the area of sowing seed, is mediate by upward mowing soil solution and occurring salt accumulating in soil surface (Munns, 2002).

In order to maintain homeostasis during saline stress condition, plants exhibit physiological, biochemical and molecular responses at both the cellular and whole plant levels (Iyengar & Reddy, 1996). Ionic regulation of sodium and chloride, their partitioning, ion absorption and their allocation and increasing osmolites like proline as a compatible solute, are important mechanisms that plants applied to resist salinity (Satish *et al.,* 2003). In addition, the tolerance ability of some plants like rice (*Oryza sativa* L.) to salt stress depends on plant genotypes.

Plontago ovata is a medicinal-valuable plant, which its seeds and shell have a significant role in pharmacy as a laxative compound. In addition, recent researches have shown that *P. ovata* fiber plays an important role in declining blood cholesterol rate, lipid and sugar. It has valuable features including compatibility to dry and semi-dry climate condition, high production of effective materials and resistance to non-alive tensions especially dry tension (Agarwal & Pandey, 2004).

Applying salinity-resist plants considered as one of the most important effective methods in exploiting and increasing treatment in arid and semi-arid regions of the world (Upadhyay & Panda, 2005). Considering climate and environmental condition, produce salinity-resistant plants has a crucial role in producing effective medicinal materials and improving the quality of food industry. Therefore, we investigated the ability of *P. ovata* to grow in saline media and characterized the stress responses of the plant to NaCl salinity in order to increase our understanding of the distribution of this species in relation to salinity in nature. Specimens of *P. ovata* were grown at four concentrations of NaCl and the growth, morphology, various cell constituents as well as photosynthetic performance were monitored to assess the salinity stress responses. The study provides new insight into how *P. ovata* species response to increased concentrations of NaCl salinity, and supply information that can be used to evaluate the potential applicability of *P. ovata* for use in constructed land systems receiving saline polluted waters.

Materials and methods

Germination assay and seedling toxicity test

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Plantago ovata seeds were received from research institute of Pakan Bazr in Isfahan, Iran. The seeds in tiny nets were subjected to flowing water for 24 hours, in order to wash growth-suppressive materials especially mucilage then sterilized in 5% mercury chloride solution for 5 minutes. Two pieces of filter paper were placed on a Petri plate and moistened with 5.0 ml aqueous solution of NaCl. Controls were set up by moistening the filter paper with 5.0 ml deionized water. Fifty seeds of each genotype were placed in each plate, covered by lid, and incubated in a growth chamber with 14/10 h light/dark cycles; temperature was kept at 25 °C during the day and 20 °C during the night. Light intensity was around 280-µmol m^{-2} s^{-1}. The concentrations of NaCl were 0, 25, 50, 100, 200 and 300 mM and were prepared freshly. Counting germinated seeds performed from second day and every other day for two weeks. After 7 d of growth, shoot height was measured from the culm base to the tip of the longest leaf and root length was measured from the root-shoot junction to the tip of the longest root. Dry weights of seedling parts were measured after drying samples at 70°C in an oven until a constant weight is achieved.

Plant culture in hydroponics

Seeds of *Plantago ovata* were sterilized and germinated on acid washed sandy pots for 4 days. Germinated seeds were transferred to PVC perlite pots fertilized with 300 ml modified Hoagland solution containing (in mM): KNO_3, 0.5; Ca $(NO3)_2$. $4H_2O$, 0.5; KH_2PO_4, 0.1; $MgSO_4$. $7H_2O$, 0.2 and (in µM), Fa-EDDHA, 10; H_3BO_3, 10; $MnCl_2$. $4H_2O$, 2; $ZnSO_4$. $7H_2O$, 0.1; Na_2MoO_4. $2H_2O$, 0.1. Ten days later, the solutions were amended with seven NaCl concentrations (0, 5, 25, 50, 100, 200 and 300 mM) for another 14 days. The seedlings were grown in a growth chamber with 14/10 h light/dark cycles; temperature was kept at 26 ºC during the day and 20 ºC during the night. Light intensity was around 280 µmol m^{-2} s^{-1}. The nutrient solution was renewed twice a week and aerated continuously. Each treatment was replicated three times and Pots were randomly arranged during the growth period. On harvesting, the root and shoot biomass pot^{-1} (defined as the remaining aboveground portion of *P. ovata* plant) were measured after oven drying at 65 °C for 3 d.

Determination of photosynthetic pigments

Chlorophyll-a and -b contents in *P. ovata* leafs were determined after growing on fertilized hydroponic culture with NaCl. Chlorophyll-a and -b were determined spectrophotometrically. Leaf was cut into small pieces leaving away the midribs, mixed thoroughly and 0.25 g of the leaf was taken into a mortar to grind them finely by pastel with 25 ml of 80% cold acetone for 2 min. A small amount of Na_2CO_3 was added to the leaf before grinding to check degradation of pigments during grinding. The homogenate was filtered through filter paper (Whattman No.1) and was made a volume of 25 ml with 80% cold acetone. The optical density of each solution was measured at 663 and 645 nm against 80% acetone blank in 1.5 cm cell. The contents of chlorophyll a, b and total chlorophyll of each samples accounted by following formula.(V=chlorophyll content (ml), W=leaf weight (g), A=optical absorption of extract):

Chlorophyll a = 0.0127 A_{663} -0.00269 A_{645} (V/W)

Chlorophyll b = 0.0229 A_{645} -0.00468 A_{663} (V/W)

Total chlorophyll = 0.0202 A_{645}+0.00802 A_{663} (V/W)

Extraction and determination of proline

Free proline content was determined according to Bates *et al*. (1973). 500 mg of dry leafs tissue was homogenized in 10 ml of 3% (w/v) aqueous Sulfosalicylic acid acid and centrifuged at 10000 g for 10 min to remove debris. The supernatant (2 ml) was mixed with 2 ml of acid ninhydrin (625 mg ninhydrin in 15 ml glacial acetic acid and 10 ml of 6 M orthophosphoric acid) and 2 ml glacial acetic acid in a test tube and boiled at 100°C for 1 h and the reaction was terminated in an ice bath. The mixture was extracted with 4.0 ml toluene and mixed vigorously with a stirrer for 10-15 s. The chromophore containing toluene was aspirated from the aqueous phase and warmed to room temperature. The absorbance of the resulting organic layer was measured at 520 nm (Tomas 302, USA). The concentration of proline was estimated by referring to a standard curve prepared using L-proline.

Plant sodium analysis

At harvest, dried shoots of *P. ovate* were finely ground in a stainless steel miller. The powdered dry materials (0.1 g) were digested in 10 ml of 3% Sulfosalicylic acid. After 48 hours, the digests were filtered by Whattman filter-paper and then analysed using flame photometer. Reference standard for calibration of the flame photometer was made using 1000 mg l^{-1} (Beach leaves material FD8, Commission of the European Communities, Joint Research Centre ISPRA).

Statistical analysis

Statistical analysis was performed using SPSS statistical package version 16.0. One-way ANOVA was performed to test the significant differences for all measurable variables. Duncan's multiple range (DMRT) test was performed to compare among the groups for significant differences. Difference from control was considered significant as $P< 0.05$ or very significant as $P< 0.001$. All the values presented in this paper were expressed as the means of three replicates ± standard error (S.E).

Results and discussion

The effect of salinity on seed germination and the growth of *P. ovata* seedlings Seed germination indicate their reaction to environmental factors. Germination percentage of *P.ovata* seeds at different salinity treatments are shown in Fig 1. Germination percentage in different NaCl treatments initially increased and then decreased. As NaCl concentration was 100 mM in the medium, germination percentage increased 3.23% in respect of control, while this rate showed 6.45% decrease in respect of control when 300mM treatment was applied.

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Fig. 1. Effect of NaCl induced stress on germination percent of Plantago ovata Forsk. values followed by the same letter are not significantly different and vertical bars represent ± standard error.

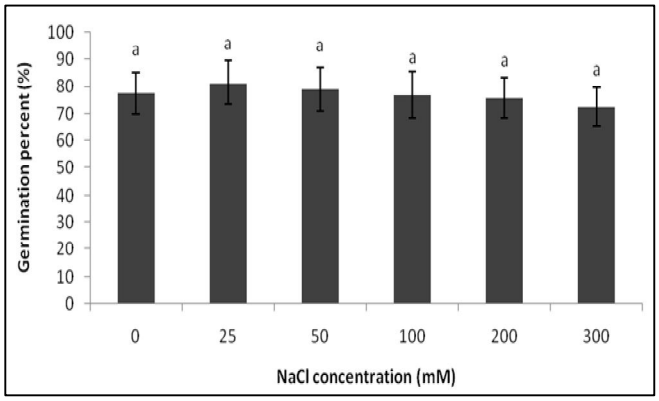


Fig. 2. Effect of NaCl induced stress on initial root and shoot length of Plantago ovata Forsk. Values followed by the same letter are not significantly different and vertical bars represent ± standard error.

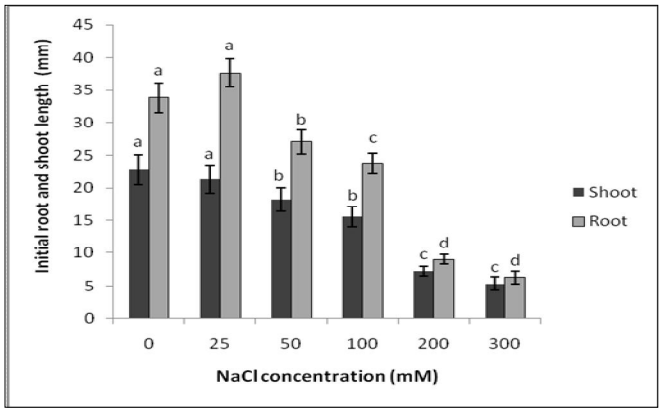


Fig. 3. Effects of NaCl induced stress on initial root and shoot dry weight of Plantago ovata Forsk. Values followed by the same letter are not significantly different and vertical bars represent ± standard error.

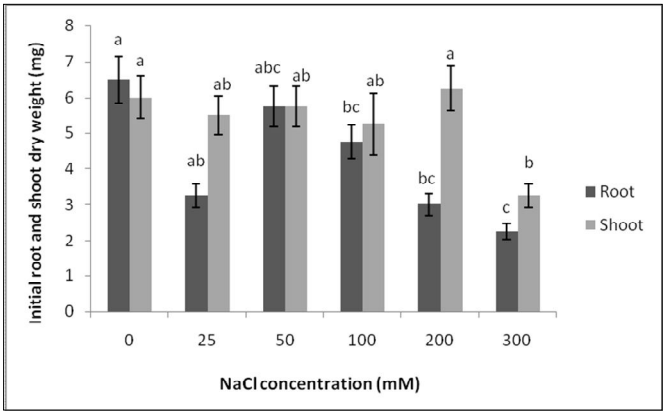


Fig. 4. Effect of NaCl induced stress on root and shoot dry weight of Plantago ovata Forsk. Values followed by the same letter are not significantly different and vertical bars represent ± standard error.

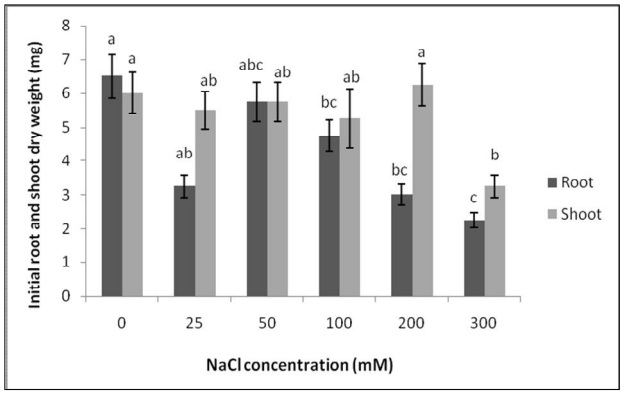


Fig. 5. Effect of NaCl induced stress on chlorophyll content of Plantago ovata Forsk. Values followed by the same letter are not significantly different and vertical bars represent ± standard error.

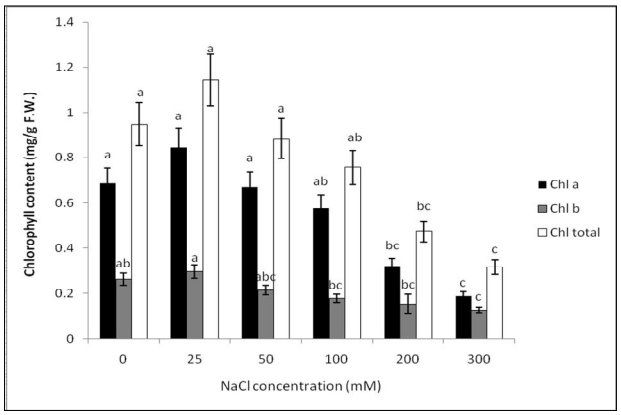
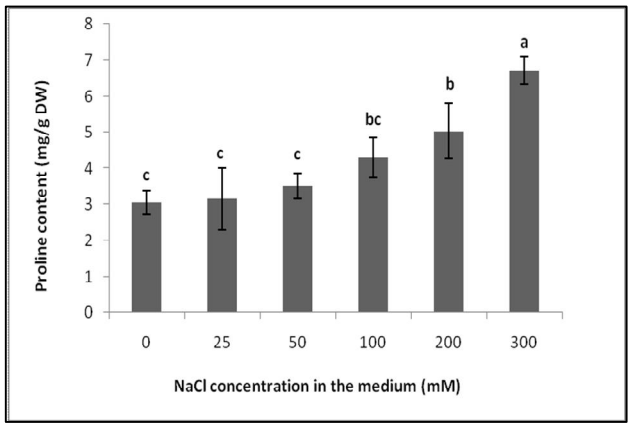


Fig. 6. Changes in proline concentration of Plantago ovata Forsk. as affected by NaCl induced stress after 4 weeks of exposure to stress. Each value is the mean of three replicates and vertical bars represent ± standard error.



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High level of soil salinity can significantly suppress seed germination and growth in halophyte and glycophyte plants. This suppression is because of potential osmotic effects and especial ionic toxicity. Some plants severely damaged in salinity and others can stay alive in saline condition and /or even make profit of it (Safarnejad *et al.,* 1996). The mechanism of salinity resistance in plants is complex, and consists of corresponding impact between molecular synthesis, enzyme activity, and membrane interchange (Munns, 2002).

The result of this study showed that *P. ovat*a has a high tolerance in high salt concentrations, and its germination rate does not decrease as salt increase (Singh & Pal, 2000). Germination percentage in seeds treated with 25, 50, and 100 and 200 mM NaCl increased in respect of control treatment. This probably regulated by hormone signals, which originated from roots. However, in plants treated with 300mM NaCl, decrease in germination rate is due to inner damages, which take place by the impact of accumulating high amounts of salt in seedling .The high salt tolerance in *P. ovat*a can be having a genetic source.

Effect of salinity on initial root and shoot length

The effect of salinity on *P. ovat*a initial root and shoot length is represented in Fig. 2. According to the statistic analysis, NaCl treatments have a significant effect ($p<0.01$) on initial root and shoot length. The initial root first increased then significantly reduced with increasing concentrations of NaCl (Fig 2). Initial root reduced by 20, 29, 73, and 82% at 50, 100, 200 and 300 mM treatments, respectively.

The initial shoot length of *P. ovata* was also decreased significantly with increasing salinity in the medium. However, salinity caused greater root length reduction in *P. ovata* especially at the mid concentration range (Fig. 2).

The effect of salinity on plant growth and development are including toxicity, lack of equilibrium in nutrient elements, and destruction osmotic regulation. Since root is responsible to absorb water and nutrients and it is the first organ encountered whit salinity stress, it was affected more than shoot against salinity. Results of this study confirming these results that an increase in salinity in water and soil environment causes an extreme decline in the growth of upper organs and stem of plants and this induces so much damage in plant functions (Rengasamy, 2006). The same researches were done on growth of barley and wheat in the salinity stress media, which resulted in a decrease in stem length, and development of upper organs under salinity stress (Pessarakli *et al*., 1991). High NaCl concentrations in the growth medium of plants generate primary and secondary effects that negatively affect plant growth and development. Primary effects are ionic toxicity and osmotic stress. Ionic toxicity occurs because high concentrations of Na^+ and Cl^- in the cytoplasm of cells disturb several biochemical and physiological processes, and osmotic stress is induced by the lowering of the water potential causing turgor reduction and cellular water loss. Secondary effects of NaCl stress include inhibition of K^+ uptake, membrane dysfunction and generation of reactive oxygen species in the cells (Rout & Shaw, 2001; Ghoulam *et al*., 2002; Agarwal & Pandey, 2004; Upadhyay & Panda, 2005).

Plants which are involved in salinity may have especial compatibility against membrane damage through two-layer lipid components of fat acid of plasma membrane. Generally, inner cells and tissues of plants exposed to lower level of salt (NaCl) compared to external solution, and in salinity tolerance boundary of each plant the nourishing and osmotic impacts are lower, compared to direct damages of salinity on plant growth (Bernstein *et al.,* 1993).

The effect of salinity stress on initial root and shoot dry weight

The results of initial root dry weight showed that means of initial root dry weight decreased in plants treated with 25mM NaCl, but increased in 50 Mm treatments (Fig 3). This increase had a descending trend in treatments of 100, 200 and 300 mM, respectively, so that the mean of initial root weight involved a significant decline (65.38%) in 300 mM treatment, compared to the control. Dry weight of initial shoot in *P. ovata*, also, had a similar trend, but its variability rate was less in similar treatment, compared to initial root. Initial shoot dry weight increased by 8.33%, 4.77%, 12.5% and 4.17% at 25, 50, 100 and 200 mM treatments, respectively, while it was decreased by 45.83% at 300 mM treatment.

Effect of salinity stress on root and shoot dry weight

Studying dry weight of *P.ovata* roots affected by different salinity treatments showed that root dry weight was increased associated with increasing saline from 25 to 300 mM in the medium (Fig 4). Therefore, in plants growing on 200 mM NaCl treatment, morphological brandishing roots of plant were completely perceptible and showed the highest rate of dry weight in roots. One-side variance analysis and Tukey test between means of subjects shows that the variance among treatments is not significant ($p>5\%$).

Similar response pattern to salinity supply levels were noted for shoot dry weight samples treated with 25 - 100 mM NaCl, while in 200-300 mM saline treatments shoot dry weight were decreased compared to the control plants. Generally, the results showed that *P. ovata* is resistant in high contents of salt and has high tolerance to salinity. The cells of sublime plants usually are in contact to hydroponic solution only through the contact point between plant and soil, directly in the site of plasma membrane of epiderm and shell cells of root (Munns & Tester, 2008).

Effect of salinity stress on chlorophyll content in P. ovata leaf

The contents of Chl-a, Chl-b and total Chl all first displayed increasing trend and then decreasing trend with

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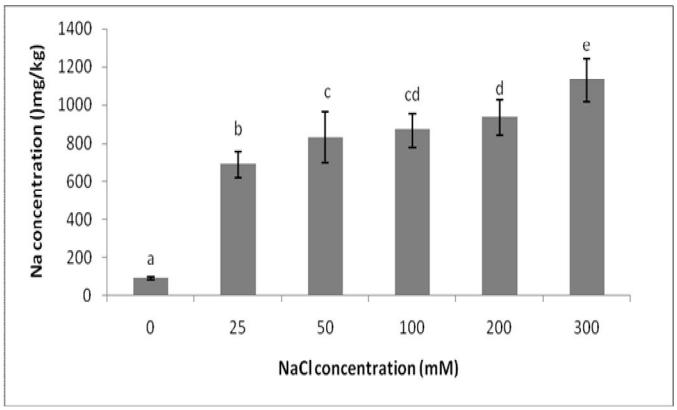
Fig. 7. Effect of NaCl induced stress on Na concentration of Plantago ovata Forsk. Values followed by the same letter are not significantly different and vertical bars represent ± standard error.



the increase of NaCl concentration (0-300 mM) (Fig 5). They increased by 14.3%, 6.3% and 9.6%, respectively, when the concentration was 25 mM. However, they were reduced by 73.21%, 52.31% and 66.12%, respectively, at the highest concentration (30 mM) (Fig. 5). The mean chlorophyll content in the leaves of the *P. ovata* did not affect significantly up to 25 mM NaCl in nutrient solution.

Primary effects of salinity stress consist of ionic toxicity and osmotic stress. Sodium accumulation in adult and aged leaves cells reduces photosynthesis rate, so transmission of Xylose from adult to young leaves and root reduced, and because of two movements, sodium accumulated (Hopkins, 1999). Decrease photosynthesis in low salinities is because of closing stomas, and in strong salinities is because of biochemical destructions. Salinity induces destruction of chloroplast structure, loss of stability in pigments with protein, and carotenoids have been affected and optical suppression reinforced. Moreover, the high activity of *chlorophylls* enzyme which activated by salinity lead to chlorophyll reduction (Parida & Das, 2005). The content of chlorophyll-a which typically considered as pre-construct of chlorophyll b-affected by salinity more than chlorophyll- b.

Our results of decrease in chlorophyll content corroborated with the findings of Rahman *et al.* (2007) who also found a decrease in chlorophyll content with NaCl stress in five widely cultivated rice (*Oryza sativa* L.). The loss in chlorophyll content can consequently lead to disruption of photosynthetic machinery. Chlorosis symptoms appeared at the highest NaCl supply, which could be a direct result of the action of increased salinity concentrations on membranes (Smith *et al*., 2010). The decline in chlorophyll content in plants exposed to NaCl stress is believed to be due to: (a) breakage and swelling of thylakoid membrane (Marin *et al.,* 1993); (b) inhibition of important enzymes, such as δ-aminolevulinic acid dehydratase (ALA dehydratase) and protochlorophyllide reductase (Van & Clijsters, 1990) associated with chlorophyll biosynthesis; (c) strong oxidation of photochemical apparatus (Somashekaraiah *et al*., 1992); (d) reduction in chloroplast density and size, phosphorus deficiency, or reduced Mg, Fe and Mn transport (Benavides *et al*., 2005).

Effect of NaCl on proline accumulation

To determine whether proline accumulates in response to salinity the content of free proline was measured. The result showed an increase of 0.74, 1.032 and 1.23 fold in proline content was observed when treated with 100, 200 and 300 mM NaCl stress, respectively (Fig 6).

Compartmentalization of Na^+ and Cl^- into the vacuole, and the accumulation of organic solutes, such as sugars, and amino acids, that do not inhibit metabolic processes, in the cytoplasm, is a common mechanism of maintaining intercellular homeostasis. Proline is one of the so-called 'compatible compounds' that are commonly found in high concentrations when plants are exposed to salt stress (Dluzniewska *et al*., 2007; Wang *et al.,* 2007; Pagter *et al*., 2009). In the present study, the content of proline indicating that *P. ovata* has an appropriate capacity to sequester Na^+ and Cl^- in the vacuoles and to synthesize proline as a compatible compound.

Sodium accumulation

The salinity treatments significantly affected ion uptake and concentrations of cations in the tissues (Fig 7). The concentration of Na^+ in the plant tissue was especially affected, as concentrations were more than 11 times higher in the highest salinity treatment compared with the control treatment (Fig. 7). Therefore, it concentrations increased as expected with the increase of Na^+ concentrations in the growth medium.

Ionic toxicity of Na^+ and Cl^- generally occurs at concentrations in the cytoplasm exceeding 100 mM where most enzymes start to become inhibited (Munns, 2002). Ionic toxicity, due to Na^+ and Cl^- accumulation in the cytoplasm, did not seem to be of major importance for *S. natans*, as the difference in Na+ concentration in the tissues between plants grown at 50 mM NaCl, where RGR was not affected, and 100 and 150 mM NaCl, where RGR was strongly reduced, was only minor. Rather, disturbance of the K+ acquisition resulting in very high Na^+/K^+-ratios in the tissues were presumably a main factor responsible for the salt injury, as has also commonly been found for both terrestrial and aquatic plants (Ashraf & Sultana, 2000; Rout & Shaw, 2001; Parida *et al*., 2005; Pagter *et al*., 2009).

In conclusion, *P. ovata* is a salt-tolerant species having efficient measures to cope with exposure to high salinity. The growth rate of the plant is not affected at NaCl salinities up to 200 mM but in the 300 mM treatment older leaves became first yellow green and then brown indicating marked injury of old leaves and the roots shorter and fewer. The salts that unavoidably are taken up even at low salinities accumulate in the old leaves, eventually to toxic levels where the leaves die. However, because new leaves are produced at a higher rate than

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for plants not exposed to salt, the plants maintain a high relative growth rate even when exposed to low salinities.

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