

Full Length Research Paper

Enhancement of compatible solute and secondary metabolites production in *Plantago ovata* Forsk. by salinity stress

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Accumulation of toxic ions in plant tissues modulates the levels of primary and secondary metabolites, which may be related to salinity tolerance. In this study, the effects of salt stress (0 to 300 mM) on proline and secondary metabolites (total flavonoids and total saponins) of *Plantago ovata* (Isabgol) were investigated. This experiment was conducted hydroponically in a NaCl spiked solution. The results indicated a significant increase in proline, total flavonoids and total saponins with the enlargement of NaCl in the medium. Increase in proline content suggests that compatible solutes may contribute to osmotic adjustment at the cellular level and enzyme protection stabilizing the structure of macromolecules and organelles. Increase in total saponins and total flavonoids suggest that the presence of these metabolites is related to increased salt tolerance of *P. ovata*. The increased synthesis of saponins and flavonoids seems to protect *P. ovata* from ion-induced oxidative stress, probably due to a common structural skeleton; the phenyl group of those metabolites. Collectively, our results indicate that *P. ovata* has physiological traits associated with resistance to salinity through accumulation of secondary metabolites to relative high levels and it can be useful for growing, in saline contaminated sites.

Key words: Flavonoid, *Plantago ovata*, salt stress, saponin.

INTRODUCTION

Soil salinity is a major environmental constraint to plant growth and productivity and is an especially serious problem in agricultural systems that rely heavily on irrigation (Munns, 2002). High salt concentration causes osmotic and ionic stress in plants (Le Rudulier, 2005). Salinity is expressed by a series of morphological, physiological, metabolic and molecular changes that cause delayed germination, poor stand establishment (Almansouri et al., 2001), high seedling mortality, stunted growth and lower yields (Allakhverdiev et al., 2000; Khan et al., 2010; Muhammad and Hussain, 2010). Plants have

evolved complex mechanisms for adaptation to osmotic and ionic stresses caused by high salt. These mechanisms include osmotic adjustment by accumulation of compatible solutes, such as proline, glycine, betaine, polyols, sugar alcohols and soluble sugars, and lowering the toxic concentration of ions in the cytoplasm by restriction of Na⁺ influx or its sequestration into the vacuole and/or its extrusion (Wuyts et al., 2006).

Plants produce a large variety of secondary products that their concentrations are strongly depending on the growing conditions and it is obvious that, especially, stress situations have a strong impact on the metabolic pathways responsible for the accumulation of the related natural products. Due to their capacity to scavenge reactive oxygen species, secondary products like flavonoids and saponins represent important radical scavengers. These aspects of the significance of

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secondary metabolites could contribute to the understanding of the high plasticity and variability of secondary metabolism. In a series of experimental observations, it could be shown that plants which are exposed to salt stress produce a greater amount of secondary plant products, such as phenols, terpenes as well as N and S containing substances such as alkaloids (Horborne and Williams, 2000; Stewart et al., 2001; Winkel, 2002; Mosaleeyanon et al., 2005; Couceiro et al., 2006).

Flavonoids are one of the largest classes of plant phenolics that perform very different functions in plant system, including pigmentation and defense (Horborne and Williams, 2000). They are reported as antioxidant agents by scavenging reactive oxygen species (ROSs), that function by virtue of the number and arrangement of their hydroxyl groups attached to ring structures. They have multiple biological activities *in vitro* and *in vivo*, such as antiadherence, antioxidant and anti-inflammatory. Their ability to act as antioxidants depends on the reduction potentials of their radicals and accessibility of the radicals (Rice-Evans, 2001; Heim et al., 2002).

Saponins belong to the secondary metabolites of mixed biosynthesis. They consist of a tri-terpene/steroid nucleus (the aglycone) with mono-or oligosaccharides attached to this core. They have been considered undesirable due to toxicity and their haemolytic activity. Literature suggests that leguminous saponins may possess anticancer activity (Shi et al., 2004; Zou et al., 2006; Gurfinkel and Rao, 2003), and is beneficial for hyperlipidemia (Shi et al., 2004). The adjuvant properties of certain saponins have been utilized in vaccines for many years (Rajput et al., 2007). The best studied are the soya saponins both in terms of epidemiology and *in vitro* and *in vivo* systems. There is evidence for saponin regulation of the apoptosis pathways enzymes (Akt, Bcl and ERK1/2), leading to programmed cell death of cancer cells (Ellington et al., 2006; Zhu et al., 2005).

Plantago ovata (Isabgol) is an annual herb cultivated as a medicinal plant, generally grown in India, Pakistan and Iran (Khaliq et al., 2011). It has been used in medicines since ancient times, but it has only been cultivated as a medicinal plant in recent decades (Wolver et al., 1994; Handa and Kaul, 1999). Its seed contains mucilage, fatty oil, large quantities of albuminous matter, the pharmacologically inactive glucoside, namely Aucubin (C₁₃ H₁₉ O₈ H₂O) and a plantiose sugar (Chevallier, 1996). Since salt stress is one of the most serious factors limiting the productivity of different crops and especially quantity and quality of their metabolic (secondary plant products) products to a greater extent, it would be possible to enhance a wide variety of useful metabolites in plant through applying different environmental stresses (Cevallos-Casals and Cisneros-Zevallos, 2003). It is assumed that accumulation of secondary metabolites enhances the *P. ovata* capacity for salt tolerance. Thus, the objective of this research was to study the effect of

salt stress on the proline as total free amino acids and flavonoids and saponin as total phenolic content of *P. ovata*.

MATERIALS AND METHODS

Plant, growth conditions and treatments

Mature seeds of *P. ovata* were sterilized in 70% ethanol for 1 min, 0.1% mercuric chloride for 5 min, followed by three washes in sterile distilled water. After sterilization, seeds were germinated into pots filled with perlite. The uniform seedlings were fed with modified 10% Hoagland nutrient solution containing: 0.2 mM KH₂PO₄, 0.8 mM Ca(NO₃)₂·4H₂O, 1 mM KNO₃, 0.4 mM MgSO₄·7H₂O, 15 μM FeEDHA, 10 μM H₃BO₃, 3 μM MnCl₂·4H₂O, 0.2 μM ZnSO₄·7H₂O, 0.2 μM CuSO₄·5H₂O and 0.1 μM Na₂MoO₄·2H₂O. Plants were grown in growth room with 16/8 h light/dark cycles, day/night temperature of 26/20°C and light intensity approximately 280 μmol m⁻² s⁻¹. The nutrient solution was renewed every week. Two weeks later, the solutions were amended with 8 NaCl concentrations (0, 25, 50, 100, 200 and 300 mM) for another 4 weeks. Every third day, the perlite was flushed with deionized water to prevent a potential toxic build up of nutrient salts in the substrate and plants received 200 ml of the appropriate solution. At harvest, plants were divided into root and shoot fractions. Root tissue samples were rinsed twice in deionized water to remove surface contaminants. Air dried samples were cut with stainless steel scissors, weighed and ground in mortar to obtain homogeneous samples.

Proline quantification

Proline was quantified according to the method described by Bates et al. (1973). Shoot samples (0.1 g) from each group were homogenized in 3% (w/v) sulfosalicylic acid and then the homogenate was centrifuged. The mixture was heated at 100°C for 1 h in a water bath after the addition of ninhydrin and glacial acetic acid. The reaction was terminated on ice bath and was extracted with 4 ml of toluene. The extract was vortexed for 20 s and the chromophore containing toluene was aspirated from the aqueous phase and absorbance was determined photometrically at 520 nm (Tomas 302, USA) using toluene for a blank. Finally, proline content of the plant sample were measured based on mg/g of dry weight (DW).

Flavonoids determination

Flavonoids content was determined with modified aluminum chloride colorimetric method as described by Woisky and Salatino (1998). 250 mg of shoots were third extracted with ethanol (20 ml) under reflux in water bath at 80°C for 2 h and then was filtered. 0.5 ml of ethanol extracts were separately mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). The final results were calculated as μg/g shoot DW. Quercetin was used to make the calibration curve. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank.

Saponin's estimation

For determination of total saponins content, the dry shoot powder

was extracted using 80% ethanol, and supernatant was collected after twice centrifugation. To 0.5 ml of ethanol solution of sample, 0.5 ml of vanillin and then 5 ml of sulfuric acid were added and were mixed well in an ice water bath. The mixture was warmed in a bath at 60°C for 10 min, and then was cooled in ice-cooled water. The absorbance of the mixture was recorded against the blank at 450 to 700 nm with a Shimadzu UV-160A spectrophotometer (Kyoto, Japan) and results were measured based on mg/g shoot DW.

Statistical analyses

This experiment was laid out in a completely randomized design (CRD) with three replications. Data were analyzed statistically by the Statistical Package for Social Sciences (SPSS) 16. One-way analysis of variance (ANOVA) was performed to test the significant differences for all measurable variables. Duncan's multiple range test (DMRT) was performed to compare among the groups for significant differences.

RESULTS

Free proline concentration

The results pertaining to the effect of NaCl on proline content is as shown in Figure 1. The 25 and 50 mM of NaCl did not have significant effect on the proline content of Isabgol. There was an almost linear increase in proline accumulation with increasing concentrations of NaCl from 25 to 300 mM, with the greatest increase in proline content in a medium that contains 300 mM NaCl (Figure 1). At the highest NaCl concentration, proline accumulation increased by 2.2 fold in comparison with the control.

Total flavonoids content

Total flavonoid content varied with different solution salinity treatments. Statistical analysis indicates that NaCl concentration had significant effect on flavonoids content of *P. ovata* ($P < 0.05$). Significant induction of flavonoids was observed with increase of NaCl concentration. The maximum flavonoid content increased with increasing salinity supply levels from 50 mM and dramatically increased at NaCl levels higher than 200 mM (Figure 2). The highest increase was noticed under higher experimented doses and it reached to about 1.63, 1.99, 2.05, 2.12 and 2.57% of the control in the presence of the 25, 50, 100, 200 and 300 mM NaCl concentration, respectively (Figure 2).

Saponin accumulation

Saponins are triterpenoid and a type of secondary metabolites that are produced by plant species like *P. ovata*. The saponin accumulation of *P. ovata* was

increased in a concentration dependent manner with a maximum at 300 mM. The mean saponin content in the leaves of the *P. ovata* was 147.5, 151.5, 153.2, 168.2, 192.2 and 244.6 mg/g in control, 25, 50, 100, 200 and 300 mM of NaCl treatments, respectively. The mean saponin content in the shoot of the *P. ovata* did not affect significantly up to 100 mM NaCl in the nutrient solution. At higher level of NaCl treatment (300 mM), total saponin content was increased at 1.65 time of control (Figure 3).

DISCUSSION

Proline accumulation under salinity

Synthesis and accumulation of compatible solutes and secondary metabolites in plants is regulated in time and space, which is mediated by abiotic environmental factors like drought, light intensity, mineral nutrition and salinity (Wink and Schimmer, 1999). Since *P. ovata* is semi salt resistant, it is assumed that accumulation of secondary metabolites enhances its capacity for salt tolerance. Therefore, the harvestable parts of *P. ovata* which is named as shoot (stem and leaf) were selected for quantification of proline and secondary metabolites. Accumulation of amino acids and proline is a stress response from the perspective of altered photosynthetic metabolism. These solutes appeared to behave as osmoprotectant. Proline acts as an osmoticum, a protective agent of enzyme and cellular structure and a storage compound of reducing nitrogen for rapid regrowth after stress are relieved. It was also approved that proline could react with hydroxyl radicals thereby protecting lipids, DNA, proteins and macromolecular structure from degradative reactions leading to cell destructions during salinity stress (Orthen et al., 1994).

The results of the present study are in agreement with earlier reports on free proline accumulation under salinity stress (Misra et al., 2002). The results of this study revealed that under salinity stresses, *P. ovata* accumulated proline at higher extent than the stress-sensitive plants. These findings suggest that proline is a stress resistance marker in this plant species. Identical statements were reported in several other species (Alvarez et al., 2003; Ehsanpour and Fatahian, 2003; Khaliq et al., 2011). Thus, proline can serve as an organic nitrogen reserve ready to be used after stress relief to sustain both amino acid and protein synthesis (Trotel et al., 1996; Sairam and Tygai, 2004).

Flavonoids accumulation

Flavonoids are one of the largest classes of plant phenolics that perform very different functions in plant system including pigmentation and defense (Kondo et al., 1992; Hahlbrock and Scheel, 1989). The application of

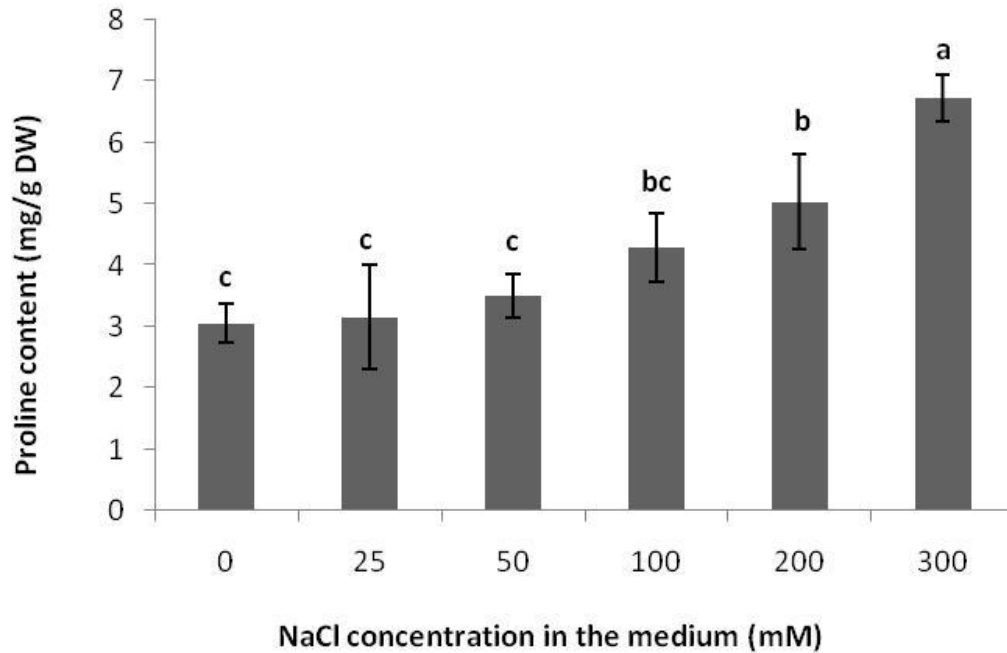


Figure 1. Changes in proline concentration of *P. ovata* Forsk. as affected by NaCl induced stress after 4 weeks of exposure to stress. Each value is the mean of three replicates and vertical bars represent \pm standard error.

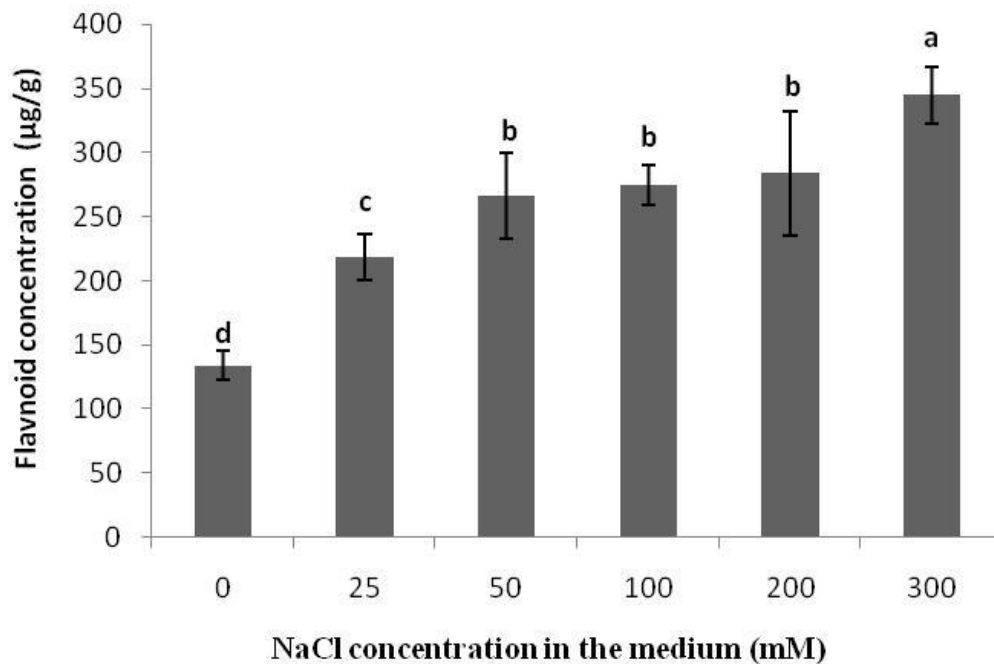


Figure 2. Effect of NaCl induced stress on flavonoids concentration of *P. ovata* Forsk. after 4 weeks of exposure to stress. Each value is the mean of three replicates and vertical bars represent \pm standard error.

salt stress enhances the concentration of flavonoid content in harvestable parts of *P. ovata* which is two

times of the control in the presence of the 200 mM NaCl concentration. It may be due to the inductions in

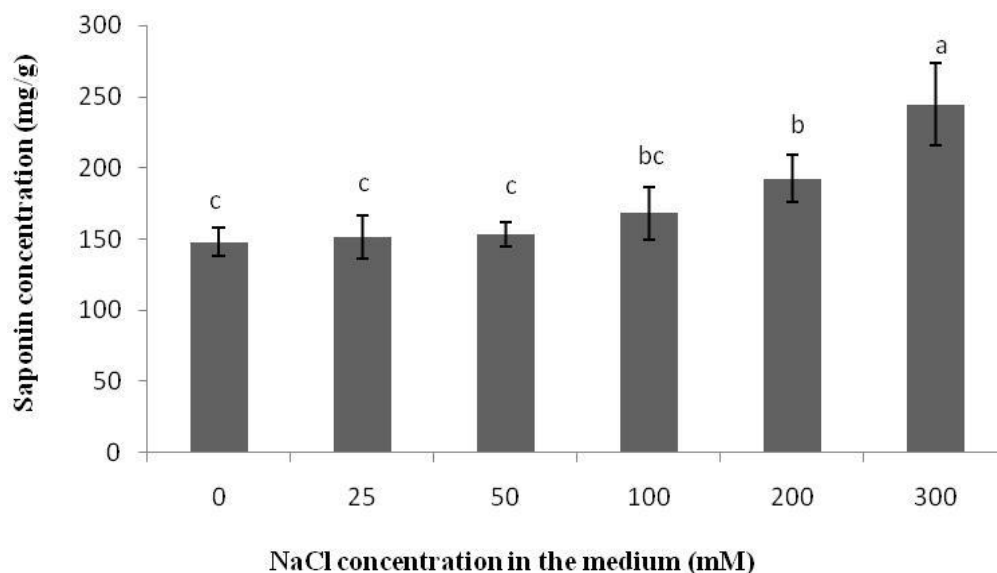


Figure 3. Effect of NaCl induced stress on saponin concentration of *P. ovata* Forsk. after 4 weeks of exposure to stress. Each value is the mean of three replicates and vertical bars represent \pm standard error.

enzymatic activity occurring under salinity condition, thereby favoring the production of different flavonoid compounds. Nonstructural carbohydrates like flavonoids then tend to accumulate and thus trigger the synthesis of carbon based defensive substances. These results indicate that flavonoid production is one of the key secondary metabolite that *P. ovata* produce to tolerate salinity stress.

Saponin content

The natural role of saponins in plants is likely to be in defense against pathogens. Also, saponins are used as drugs, foaming agents and test modifiers. Based on our results, the saponin accumulation in *P. ovata* is induced by salinity stress. This means that there may be some correlation between saponins and salinity environments, but this correlation may not be as direct as that between biology and environment, and needs more exploration to elucidate.

Regarding secondary metabolites accumulation in plants affected by stress, it should be taken as consideration that environmental stress like salinity reduces the growth of most plants. Plants that suffer salinity stress generate a high oversupply of reduction equivalents. The corresponding strong reduction power (massive amounts of $\text{NADPH} + \text{H}^+$) seems to enhance the synthesis of highly reduced compounds, like isoprenoids, phenols or alkaloids. Accumulated natural products also prevent massive generation of oxygen radicals and the corresponding damage by photo inhibition (Xin et al., 2011). These results indicate that *P.*

ovata is tolerant to mild and high salinity stress by accumulating certain proteins and secondary metabolite, though, the overall photosynthesis is reduced.

Generally, plant secondary metabolism and its metabolites result from the response and adaptation to different environmental stresses during long process of evolution, and hence, the production of secondary metabolites closely relate to environmental factors (including biotic and abiotic factors). The present study highlights the importance of the effects of salt stress on proline and secondary metabolites of *P. ovata* which mediated its resistance to salinity. Our results suggest that the accumulation of osmolytes (proline) and secondary metabolites (flavonoids and saponins) may have an important role in osmotic adjustment, prevent the generation of oxygen radicals and protect the cells from damaging effects which lead to its resistance to salinity. Moreover, improving the content of some active compounds like flavonoids in *P. ovata* is the most important pharmaceutical value. More extensive studies will be required to know the mechanisms of *P. ovata* resistance to salinity and the crucial roles of plant secondary metabolites in this story.

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