Growth and physiological parameters under salinity stress in *Lotus corniculatus*

Marjan Azarafshan and Nasser Abbaspour *

Department of Biology, Faculty of Science, Urmia University, Urmia, Iran

Abstract

Salinity is a main and common stress which decreases the amount of agriculture products and natural plants in many areas of the world. In this study, the effect of salt stress on three varieties of *Lotus corniculatus* 'Karaj', 'Jolfa', and 'Ardabil' was investigated. Plants were cultured in hydroponic condition with four NaCl treatments (0, 50, 100, 150 mM). The amounts of proline, glycine betaine, soluble sugars as well as Na^+ and Cl^- were analyzed. The results showed that sodium and chloride content in all varieties significantly increased in shoot ($p<0.05$). The amounts of proline and soluble sugars significantly increased in all varieties ($p<0.05$). However, increasing in NaCl concentration raised significantly glycine betaine content in leaves of the varieties ($p<0.05$). As far as the measured factors are concerned, it seems that 'Jolfa' and 'Karaj' have a higher capacity to tolerate salt stress particularly in 100-150 mM NaCl compared with 'Ardabil'.

Keywords: Lotus corniculatus; compatible solute; chloride; salt stress; sodium

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Introduction

Although all soils have a little amount of soluble salts which are essential for crops production, problems arise when the amount of salt rises to the levels where it is harmful for plants (Teimouri et al., 2005). Salt stress is reported mainly to change root environment condition, osmotic potential of soil solution, and normal balance of ions (Garg and Gupta, 1997). Normally, NaCl is the main salt that induces salt stress. Sodium ions are toxic for wild range of plants and some plants are affected by high concentration of chloride. When sodium enters into cytoplasm, plants activate their high affinity system for uptaking potassium so that they can absorb enough amount of this ion because the low amount of potassium caused by absorbing more sodium limits growth in plants (Jian-kang, 2001). Increase in chloride affects nutrition elements absorption which in turn causes imbalanced nutrition and decreases plants growth under salt stress. Salt tolerance is a positive characteristic for plants in saline soils. Salt tolerance includes two mechanisms: avoidance and resistance. Salt resistance includes producing compatible solutes, sodium compartmentation and ions exchange (Garg and Gupta, 1997).

Lotus corniculatus is a member of Papilonacea and is a pasture legume and has a low concentration of tannin so it is a suitable and delicious forage for grazers. High quality honey is made by pollens of this plant. However this plant

*Corresponding author
E-mail address: nabbaspour03@yahoo.com
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is used for revitalization and productiveness in low quality soils (Teimouri et al., 2005). Moreover, shoots of *Lotus corniculatus* have medicinal properties such as anti-spasm, anti-inflammatory and sedative (Mousavi, 2004). The aim of this study was investigation and evaluation of three *Lotus corniculatus* varieties responses under salt stress and analyzing their abilities for salt tolerance and/or resistance.

Materials and Methods

Plant material

The intact seeds with the same size of three varieties of *Lotus corniculatus* "Karaj, "Ardabil", and "Jolfa" with the ability of germination with a number of 80 to 90 percent (high viability) were provided from gene bank of Iran Research Institute of Forest and Rangelands (RIFR). The seeds (150 from each variety) were put on wet filter paper in petri dish under 27°C room temperature. Seed germination started three days after sowing. On the fourth day 72 seedlings same size from each varieties were selected and transferred to plastic pots with the aerated Hoagland solution (1/8 strength). The Hoagland solution of the plants was replaced in four days intervals. After fifteen days the plants were transferred to new Hoagland solution of (1/4 strength).

Salinity treatment

Two month old plants were treated with NaCl (0, 50,100 and 150 mM) in Hoagland solution for 2 weeks. Three replicates for each treatment and 6 plants for each replicate were taken into account. NaCl was added to the nutrient solution by incremental increases until the final concentrations were reached.

Growth parameters

After 2 weeks, plants were harvested and growth parameters including root and shoot dry weights and lengthes were measured.

Ion analysis

100 mg of the powdered dry matter of the aerial organ was weighed and poured in laboratory pipes and 10 ml distilled water was added to each pipe. The pipes were put in the boiling Ben Murray at the 100 centigrade degrees for an hour, and after cooling in the room temperature the pipes were centrifuged and the upper solution was transferred to a new pipe. Then, the volume of the new solution reached to 10ml with the distilled water. The new essence was used to measure the amount of sodium by flame photometer (Fater electronic 405,Iran).0.5ml aliquots were analyzed to measure tissue Cl^- concentration using chloride analyzer(Model 926,Sherwood scientific, UK).

Compatible solute contents

Soluble sugars assay

Soluble sugars were measure according to Dubois *et al*. (1956) method. 0.05 g of leaf dry matter was hemogenized in 5 ml 70% ethanol. The samples were kept in 5 degrees for a week. 1ml Phenol 5% and 5 ml pure sulfuric acid were added to 1ml of each supernatant. The absorbance of the solutions was read in 485 nm by spectrophotometer (Biochrom WPA S1200, UK). All chemicals and reagents were purchased from Merk Chemical Co. (Germany)

Proline assay

Proline measurement was done using the method of Bates et al. (1973). 0.04 g leaf dry matter was homogenized with 10 ml of 3% sulfo salicylic acid. Then, 2ml acetic acid glacial and 2 ml nin-hydryn (20 ml phosphoric acid 6M, 30 ml acetic acid glacial, 1.25 g nin-hydryn) were added to 2 ml of each sample and then were incubated in 95 °C for 1 hour. Ice cooled samples, were mixed with 4 ml toluene using vortex. The absorbance of the upper phase (colored solution) was read at 520 nm.L-proline was used as a standard. All chemicals and reagents were purchased from Merk Chemical Co. (Germany).

Glycine betaine assay

Glycine betaine content was determined according to Grieve and Grattan. (1983) method.

0.25 g leaf dry matter was shaken in 10 ml distilled for 24 hours. Then, the samples were filtered by filter paper(S&S 604) and then 0.25 ml of the extracts was diluted (1:1) with and 0.25 ml 2N sulfuric acid Aliquotes (0.5 ml) were cooled in ice for 1 hour and then 0.2 ml ($KI-I_2$) was added to each sample and stirred with vortex. The resulted solutions were kept in refrigerator for 16 hours at 4°C and then centrifuged with 6000g for 20 minutes. The upper solution is discarded and the periodide crystals were dissolved in 6 ml 1-2 dichloro ethan well with vortex. The absorbance for the resulted solutions was read at 365 nm. All chemicals and reagents were purchased from Merk Chemical Co. (Germany).

Statistical analysis

Data analysis was conducted using the SPSS (version 16.00). The difference between the means was calculated using the one-way ANOVA. The mean values of three replicates and the "Standard Error" of the means were calculated. GLM (General Linear Model) was used to determine the significance between different treatments and then Tukey's multiple range tests ($p<0.05$) was employed.

Results

The results obtained from the data analysis showed that stem length and shoot dry weight significantly ($p<0.05$) decreases with increasing sodium chloride concentration in all the varieties. So, indifferent levels of NaCl, "Karaj" and "Jolfa" showed higher stem length and shoot dry weight compared to "Ardabil" (Fig I).Salinity had obvious effects on Na^+ and Cl^- accumulation in shoots of varieties. Shoot Na^+ and Cl^- contents significantly ($p<0.05$) increased with increasing external NaCl concentrations. However, "Ardabil" variety showed a higher content of both Na^+ and Cl^- in compare to "Karaj" and "Jolfa".Also a significant difference($p<0.05$) was observed between the varieties particulary after 100mM external NaCl No obvious difference was observed in shoot Cl^- content between "Jolfa" and "Karaj" (Fig II).Salinity induced alterations in leaf osmoticum contents in all the varieties. In compare to "Ardabil", "Karaj" and "Jolfa" varieties showed higher contents of free reducing, sugars under salinity stress particularly in higher salinity levels. The results showed that leaf proline content significantly increased with increasing the salinity concentrations. The maximum proline content was observed in the highest salinity concentration (150mM).Among the varieties, the maximum leaf proline content was observed in "Karaj" and "Jolfa" .The difference between varieties was significantly ($p<0.05$) higher than 50 mM NaCl concentration (Fig III).

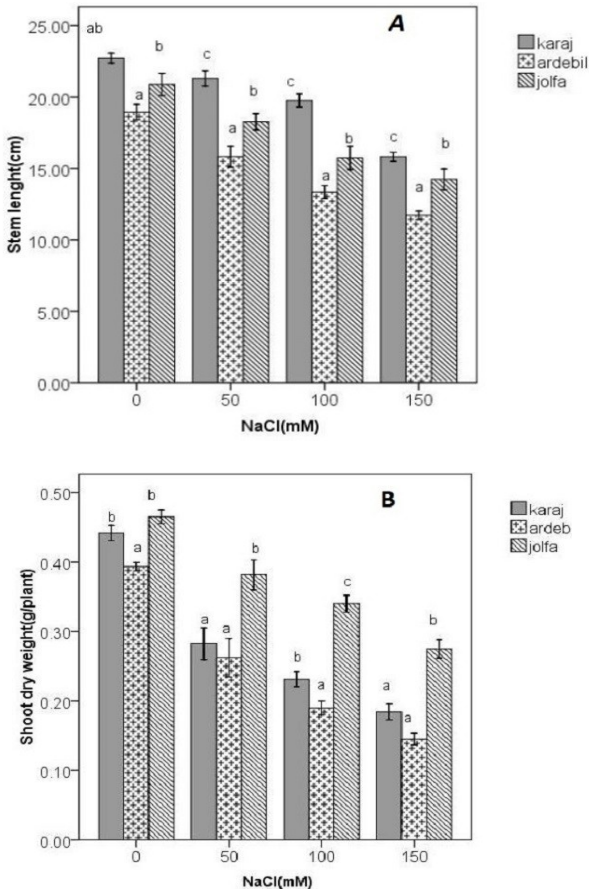

Fig. I. Stem length (A) and shoot dry weight (B) of the plants treated with different concentrations of NaCl for 2 weeks; Bars are ± SE of the means (n = 3) $p \leq 0.05$. Different letters indicate significant differences between varieties at each salt concentration.

Increasing the external salinity, leaf Glycine Betaine accumulation significantly ($p<0.05$) increased, in a way that the maximum amount of Glycine Betaine was observed at the highest salinity level (150 mM) and minimum

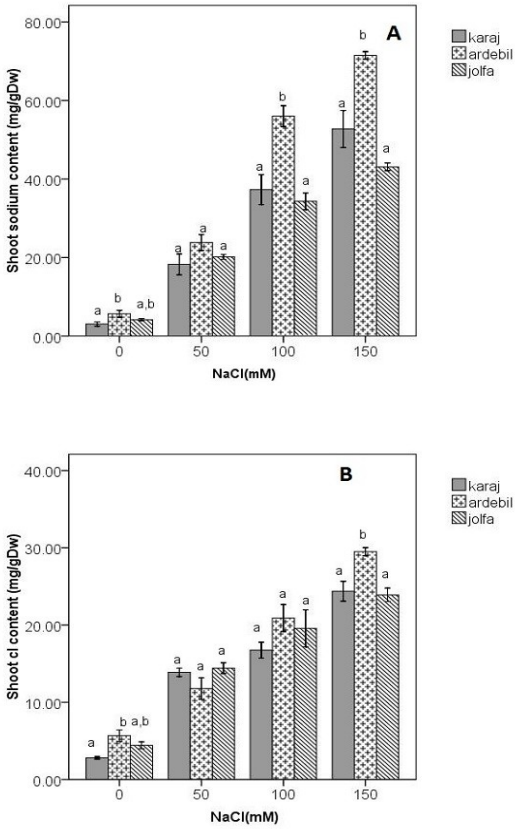

Fig. II Sodium content (A) and chloride content (B) treated with different concentrations of NaCl for 2 weeks. Bars are ± SE of the means (n = 3) tuky p ≤ 0.05. Different letters indicate significant differences between varieties at each salt concentration.

level was observed in control plants. Similar to sugar and proline contents, Glycine betataine accumulation in "Karaj" and "Jolfa" was significantly ($p<0.05$) higher than that of "Ardabil" when higher salinities (100 and 150 mM) were imposed in the plants growth medium (Fig. IV).

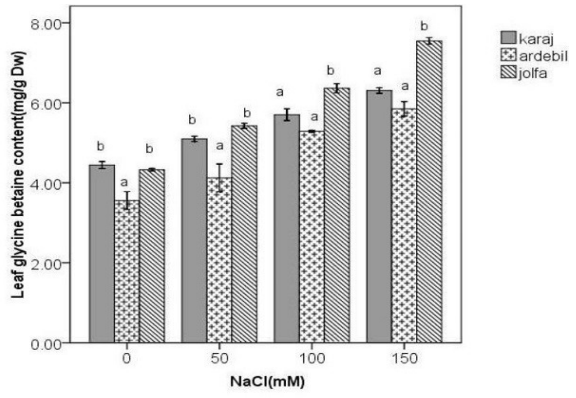

Fig.IV. Glycine Betaine in plants treated with different concentrations of NaCl for 2 weeks; Bars are ± SE of the means (n = 3) tuky p ≤ 0.05. Different letters indicate significant differences between varieties at each salt concentration.

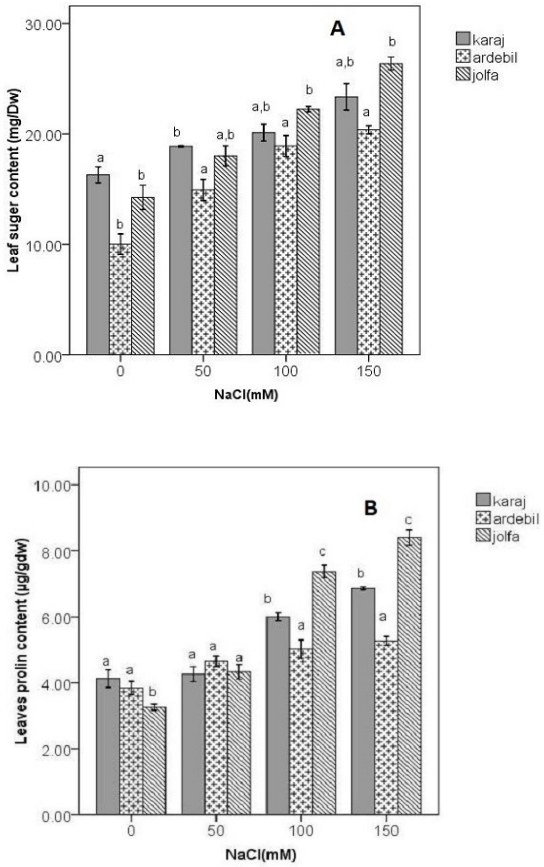

Fig. III. Sugar content (A) and proline content (B) of the plants treated with different concentrations of NaCl for 2 weeks; Bars are ± SE of the means (n = 3) tuky p ≤ 0.05. Different letters indicate significant differences between varieties at each salt concentration.

Discussion

Growth is the most basic sign of metabolic activity of a plant. One of the first phenotypic symptoms that are caused because by the stress is the qualitative and quantitative changes in growth. The plant growth reduction is the result of salinity stress because of the decrease in the photosynthesis amount in unit leaf area. Growth prevention happens because of the high accumulation of salt in shoot and root, and subsequent osmosis and ionic effects of salinity on plants (Nedjimi, 2011). In addition, decreasing the plant length can be due to metabolic activities such as meiosis, enzyme activities (mostly the protein synthesis is affected), and plant hormones (Amira and Qados, 2011). The result obtained from this research showed that salinity had a significant effect

($p<0.05$) on decreasing the shoot length in all varieties. Reviewing salinity tolerance mechanisms in the plants, Tester and Davenport. (2008), reported that the osmotic stress in the first stage of the salinity treatment causes decreasing in cellular water content and shoot lengthening. Even after recreating the osmosis balancing and osmosis pressure of the cells, their development and lengthening is progressing slowly.In saline conditions, with increasing the salt concentrations in nutrient solution, water potential decreases and consequently cells water draining prevents plant growth. Also, with shortening and falling the leaves the plant production decreases and as a result small amount of material will be absorbed by the cells which causes the decrease of cells number and dimension, and finally the dry weight of the plant will be diminished (Sibole et al., 1998). In the present study, salinity had a significant effect on the shoot dry weight of the varieties. Rejili et al. (2007), reported that the dry weight of both studied population from *Lotus certicus* decreased with increasing the salinity. Increasing sodium accumulation in root, osmotic potential decreases and this factor causes the plasmolysis and decreases nutrient absorption on the root surface. Sodium absorption increases with increasing the salinity stress. Extreme increasing of sodium in cytoplasm causes the extreme depolarization of cell membrane and prevents the metabolic processes (Bor et al., 2003). In the present study, with increasing salinity concentration, sodium content in the shoot increased in the varieties. In a study on the relationship between growth and ionic status in response to salinity in two forage plants of *Lotus cornicolatus* and *Lotus tenuis,* Teakel et al. (2006), reported that salinity causes the accumulation of sodium in leaves and root and this increase in *Lotus cornicolatus* was higher than *Lotus tenuis*. Chloride concentration may cause nitrogen and phosphorus deficiencies in plants. Indeed, chloride accumulation decreases nitrate reductase enzyme activity and thereby low nitrate ion absorption causes the growth decreasing in the plant. Meanwhile, chloride and sodium ions accumulation is always simultaneous and their toxic effects on the plant are complementary (Teakel and Colmer, 2006). In the present study, shoot Cl^- content increased in all varieties with increasing external salinity. Also, from the view point of salinity tolerance, significant difference between the varieties was observed in NaCl concentrations higher than 100 mM. In a comparative investigating on ions and osmolites amounts in *Lotus* species, Sanches et al. (2011), reported that plants Cl^- content the chloride increased with increasing external salinity concentrations and in compare to other species, this increase was less in *lotus certicus* which is more tolerant than *L. corniculatus* and *L.tenuis*. Plants are able to tolerate salinity stress through the osmotic mechanisms to maintain their water status by accumulating metabolites such as proline and soluble sugars and some of the ions. Under the salinity stress conditions, soluble sugars and proline can act as osmostic protectants (Bray, 2003) .Increasing the soluble sugars in response to salinity may cause their less displacement from leaves. Slow consumption in leaves is resulted from growth decreasing and other changes like hydrolysis of starch. In the present study, the soluble carbohydrates amounts increased under the salinity conditions and this increase was observed in all the varieties and was more in "Karaj" and "Jolfa" varieties than "Ardabil". In a study on the mechanisms of tolerance salinity in *Lotus japonicus* and *Medicago truncatula* plants, Lopez et al. (2008), reported that with increasing the salinity the soluble sugars in both plants increased in the shoot and root and the amount of this increasing in aerial organs was specifically higher in *Lotus japonicus*. In addition to direct role in fixing the macromolecules and their hydration layers, proline shows an indirect protective act through its anti-oxidation characteristics. The anti-oxidation role of proline is in its ability to inactivate the hydroxyl radicals and other types of active oxygen which have been produced under the salinity stress and create disorders in transferring the electrons in chloroplasts and mitochondria, and in this way, proline protects the proteins and membranes against the harm. Another important factor which controls the proline amount in the plant is the decomposition or metabolism of proline. After stopping the salinity treatment, the amount of the accumulated proline decreases quickly and the

accumulated proline turns into glutamate during two biochemical stages (Jamil et al., 2005). In this study, the results showed that with increasing NaCl concentration proline content increased in leaves. Menzi et al. (2010), reported that with increasing the external concentration of NaCl , accumulation of of proline increased in all parts of three *Medicago sativa* cultivars.

Glycine betaine is one of the quaternary ammonium compounds (QACs) which its function as an osmolyte donate adaptation capacity for the cultivated plants under drought (Raul et al., 2003). It is reported that betaine is created through re-synthesis (*denovo*) during the water stress (Grieve and Grattan, 1983).This osmolyte is mainly accumulated in chloroplasts and it has an important role in protecting the thylakoids membrane of chloroplasts, thus it may effect on photosynthesis (Rogers et al., 2009). In the present study the amount of the leaves glycine betaine content increased in the varieties with increasing salinity. Bashir and Ashraf. (2003), reported that with increasing the salinity, the glycine amount in two varieties of *Phaseolos vulgaris* (sensitive to salinity) and *Sesbania aculeate* (resistant to salinity) has significantly increased in plant leaves (Bashir and Ashraf, 2003).

As a conclusion, regarding the results obtained in this study, the amount of Cl^- and Na^+ in "Jolfa" and "Karaj" varieties was less than Ardabil and the growth rate and physiological indices such as compatible solutes of "Jolfa" and "Karaj" were higher than "Ardabil", therefore, it seems that "Ardabil" showed a lower capacity to tolerate salt stress condition when compared to the other varieties studied in this experiment.

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