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Physiological and biochemical adaptations of *Hedysarum coronarium* L. and *Hedysarum criniferum* Boiss to salinity stress

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Abstract
Salinity is one of the major environmental stresses that limit plant growth and productivity. In this study, the effects of salt stress on physiological and biochemical parameters were investigated in *Hedysarum coronarium* L. and *Hedysarum criniferum* species The NaCl treatments in Hoagland's nutrient solution were: Control (no salt: 0.81 ds/m NaCl), 10.67, 20.33, 22.66 and 26.59 ds/m. Plants were irrigated with Hoagland's nutrient solution during 4 months. Salt treatments were applied for 37 days. Gas exchange parameters, relative water content, proline, chlorophyll, carotenoids and stomata characteristics were measured. Data analysis showed that the measured parameters except intercellular CO_2 concentration in both species were affected by salt stress. The lowest amount parameters measured, relative water content, proline, chlorophyll, carotenoids and stomata characteristics were observed at 26.59 ds/m NaCl salinity. Proline and number stomata increased with increasing salinity in both species. In general, *H. criniferum* was more affected by salinity than *H. coronarium*. The results of this study suggested that *H.coronarium* is relatively better suited under salt stress conditions than *H. criniferum*.

Keywords: Growth, Hedysarum, Legumes, Physiology, Salt stress

Introduction

Environmental stresses are among the most limiting factors to crop plant productivity. Salinity is one of the most detrimental one (Berrichi *et al.,* 2010) and it is increasing worldwide (Chinnusamy *et al.,* 2005) due to low rainfall, high surface evaporation and irrigation with saline water. In the arid and semi-arid areas of Iran, saline and alkaline soils are expanding covering 12.5% (204800 km^2) of the total area (Akhani and Ghorbanli, 1993) and solutions to this issue are needed. The main salt present in this kind of soils is sodium chloride and it is well known that the majority of plants with economic importance are susceptible to it at different levels. The glycophytes, or nonhalophytes, to which most crop species belong, vary in response to salinity from very salt sensitive to moderately salt-resistant. Some glycophytes are able to adapt to salinization of the soil; however, salinization always lowers their productivity. The resistance of glycophytes to salts can be increased by saline hardening prior to sowing (Lauchli, 1986). Salinity stress affects glycophytic plants by lowering water potential of the root medium leading to a water deficit, toxic effects of ions, mainly Na^+ and Cl^- and imbalance in nutrient uptake or transport to shoot (Munns and Termaat, 1986; Dianati Tilaki *et al.,* 2011: Lauchli, 1986; Marchner, 1995; Sairam and Tyagi, 2004). Salinity stress has a major impact on plant growth and development (Cheong *et al*., 2007) due to the disruption of several processes where photosynthesis and cell division are seriously affected (Munns, 2002; Meloni *et al.,* 2003). A threshold for survival of *Artemisia herba-alba* to cumulative salt concentrations above 20 g salt per kg soil was recorded. Once salinity concentrations passed the threshold, survival decreased dramatically from 80% at 30 g salt per kg soil to 60% at 70 g salt per kg soil (Louhaichi *et al.,* 2015). The efficiency of photosynthesis is reduced because of effects on chlorophyll content, photosynthetic enzymes, carotenoids (Stepien and Klobus, 2006). Stomata closure leading to a reduction of intercellular CO_2 concentration and non-stomata factors. Different species of plants inherently possess different measures and capacities of coping with exposure to high salinity, and salt stress responses and tolerance vary among species (Munns and Tester, 2008). *Hedysarum coronarium* L. (sulla, French honey-suckle, Spanish sainfoin, Spanish esparcet) is a member of the Leguminosae family native to the Mediterranean basin, where it has been established as a forage crop (Benguedouar *et al.,* 1997) known to have tolerance to drought, salinity and alkaline pH (up to 9.6), well adapted

to marginal areas and basic clays (Gutierrez-Mas, 1983). *Hedysarum criniferum* Boiss (synonym: *Hedysarum ecbatanum* Beck.), an Iranian native perennial species have shown good response in germination at more than 200 mM NaCl (keshavarz *et al.,* 2012) and both species may be an option for saline area However, little research has been done in relation to the two species. Our research focuses on these two glycophytic legumes species with aim to determine the effects of salinity stress on physiological and biochemical traits.

Materials and Methods

Growth conditions and treatments: A greenhouse experiment was conducted from December 2011 until May 2012 and continued for 160 days, at the Natural Resources Faculty of Tarbiat Modares University of Iran. Two species of Hedysarum, *H. coronarium* and *H. criniferum* were selected for study salinity responses (Table 1). Treatments consists of five salinity levels (0, 10.67, 20.33, 22.66 and 26.59 ds/m) with 4-replicates in completely randomized design. Seeds were surface sterilized with 5% sodium hypochlorite for 5 min, subsequently washed several times with distilled water and air-dried before being used in the greenhouse experiments. Seeds were planted in plastic pots with 2 kg river sterile sand and plants in each pot were nourished with 20 mL Hoagland's nutrient solution every other day for 4 months in controlled conditions. The average day and night temperatures were 30±5 ºC and 15±3 ºC, respectively. The relative humidity ranged from 30 to 35%. When the plants were in the vegetative state (120 days after planting), salinity stress was applied adding NaCl. Treatments started from 10.67 ds/m and increased stepwise by every other day to reach 26.59 ds/m. Control plants were kept well-watered with no addition of NaCl.

Gas exchange measurements: Gas exchange measurements were carried out after 37d of salt treatment. The net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (E) and intercellular CO_2 concentration (Ci) of upper mature leaves were measured with a portable LCpro+ Photosynthesis System (ADC Bio-Scientific Limited UK) under greenhouse conditions (PAR average was 2100 µmol m^{-2} s^{-1} and leaf temperature was 30-35ºC). Measurements were taken between 10:00 and 12:00 AM.

Relative water content: Relative water content (RWC) was determined from 0.5 gram leaf tissues excised in the morning (around 9:00 AM) from 3 randomly selected plants per pot. Excised leaves were measured for fresh weight (FW), and then rehydrated for 16 h in a water-filled Petri dish at room temperature before measuring Turgor weight (TW). After drying at 70ºC for 48 h dry weight (DW) was measured. The relative water content was calculated from the following equation, RWC = 100[(FW - DW)/(TW - DW)].

Proline: Extraction and estimation of proline was conducted according to the procedure described by Bates *et al.* (1973). Plant material was frozen (-70ºC), and 300 mg per sample was homogenized in 10 ml of 3% (w/v) aqueous sulphosalicylic acid, then the homogenate was filtered through Whatman No.2 ûlter paper. Two milliliters of ûltrate was then mixed in a test tube with 2 ml acid ninhydrin and 2 ml glacial acetic acid, and incubated at 100ºC in a water bath for 1 h. The reaction was terminated by placing the mixture in an ice bath. It was then extracted with 4 ml toluene and the chromophore phase aspirated from the aqueous phase. The absorbance was read at 520 nm using a spectrophotometer.

Chlorophyll and carotenoids: Fresh tissue (0.2 g) of fully expanded leaf (4 leaves per plant) was sampled, and homogenized in 80% acetone and read using a UV/visible spectrophotometer at 470, 663, 652 and 645 nm. Total chlorophyll, chlorophyll a, b and carotenoid amounts were determined according to Litchtenthaler and Wellburn (1983) using the following equations.

Total chlorophyll (Total Chl): A652 x 27.8 x 20/mg leaf weight

Chlorophyll a (Chla): (11.75A663–2.35 A645) x 20/mg leaf weight

Chlorophyll b (Chl b): (18.61A645–3.96A663) x 20/mg leaf weight

Carotenoid (Car) = [(1000A470–2.27x Chla–81.4 Chlb)/227] x 20/mg leaf weight

Table 1. The primary characteristics of seeds of two species *H. coronarium* and *H. criniferum*

Species	Viability	Moisture	Origin	Thousand seed weight	Storage conditions
H. coronarium	95%	5.9%	Semirom (Iran)	4.7 g	Active refrigerating
H. criniferum	92%	7.3%	Chadgan (Iran)	14.7 g	Active refrigerating

Stomata characteristics: Stomata measurements were done in 3 randomly sampled leaves from each plant in lower epidermal cells using a light microscope. Samples of a very thin layer of the epidermis of the lower surface of leaves were prepared removing chlorophyll by bleach and distilled water treatments. The stomata parameters such as length, width and area of stomata were measured using the software Image Tools (Grant and Vatnick, 2004). Number of stomata per unit area (number of stomata/ mm^2) was counted by 40× objective lens and 10× eyepiece under light microscope.

Experimental design and data analysis: The experimental design was two factorial (species and salinity levels), arranged in a completely randomized design with 4 replications and 50 seedlings in each replicate. The data were statistically analyzed by the SPSS, version 16, computer program. The difference between the means was compared using Duncan's multiple range test at $P<0.05$.

Results and Discussion

Gas exchange parameters: The gas exchange parameters (except intercellular CO_2 conc.) were affected negatively ($P<0.01$) with increase in salinity in both the plant species. A decrease in net photosynthetic rate with increased salinity was observed (Table 2). Stomata conductance (Gs) also decreased with the increase of salinity level in both the species. But the decrease in *H. criniferum* was 77.8% and 23% in *H. coronarium* compared to the control (Table 2). Transpiration rate declined in response to salinity in both species with *H. criniferum* showing higher rate than *H. coronarium* for all treatments (Table 2). Intercellular CO_2 concentration (Ci) had not a consistent performance through salinity levels and no statistical difference was detected (Table 2).

Photosynthesis as the main path for energy absorption is the basis of all vital functions and is severely affected by salinity. Under normal conditions, 98% of plants that absorb water from the roots, lose it by stomata through the transpiration phenomenon (Heidarisharifabad, 2001). But in the face of salinity stress, according to Leung *et al*., (1994) and Cramer and Quarrie (2002) absicic acid (ABA) is produced which causes stomata closure preventing further loss through transpiration (Chaves *et al.,* 2009). Limitation of stomata conductance and transpiration is a defense mechanism to cope with too much salt with their negative consequences for plants (Clark *et al.,* 1990). The regulation of transpiration has an important role in controlling ion accumulation in stems, because salt transport occurs via the transpiration stream (Benzarti *et al.,* 2012). Reduction of gas exchange can be one strategy for reducing salt concentration in leaves and helps to extend the life of the plant by keeping salts below toxic levels (Everard *et al*., 1994). As the gas exchange is affected, then photosynthesis is reduced probably due to a reduction in plant available water at high salinity (Chartzoulaki *et al*., 2002). Accumulation of Na^+ and Cl^- at cell membranes also is a further cause of limiting photosynthesis (Munns, 1993; Neumann, 1999). All these aspects explain the trends found in the present research.

Relative water content: Both plant species performed differently in terms of relative water content (RWC), with *H. coronarium* having higher values than *H. criniferum*. Salinity showed signiûcant negative effect on the relative water content ($P<0.01$) in both the species, with more decrease of RWC in *H. criniferum* (Table 2). Relative water content better reflects the stomata status and leaf transpiration. Leaf water status is intimate related to several leaf physiological variables, such as leaf turgor,

Table 2. Gas exchange parameters and RWC% of *H. coronarium* and *H. criniferum* as affected by NaCl in the irrigation water (Mean of four replicates ± SE)

Species	NaCl(ds/m)	Pn(µmol $m^{-2} S^{-1}$)	Gs(mol $m^{-2} S^{-1}$)	E(mmol $m^{-2} S^{-1}$)	Ci(µmol $m^{-2} S^{-1}$)	RWC(%)
H. coronarium	Control (0)	3.87±0.051 b	0.26±0.004 f	5.26±0.090 c	336.71±0.886 a	87.63±0.662 a
	10.67	2.52±0.051. e	0.23±0.006 g	4.58±0.050 d	335.61±1.184 a	88.51±0.875 a
	20.33	2.41±0.061 e	0.21±0.004 h	3.82±0.056 e	336.42±0.762 a	83.63±0.488 b
	22.66	2.41±0.031 e	0.20±.006 h	2.85±0.074 g	336.73±1.785 a	82.05±0610 b
	26.59	2.34±0.025 e	0.20±0.004 h	2.46±0.071 h	334.73±1.398 a	82.95±0.169 b
H. criniferum	Control (0)	4.06±0.031 a	1.40±0.009 a	11.43±0.090 a	335.74±1.478 a	66.55±0.829 c
	10.67	3.56±0.070 c	0.45±0.008 b	5.76±0.039 b	335.11±1.624 a	65.03±0.676 c
	20.33	2.84±0.018 d	0.38±0.008 c	4.72±0.095 d	334.49±0.346 a	62.35±0.699 d
	22.66	2.69±0.087 d	0.34±0.007 d	3.75±0.053ef	334.69±1.682 a	61.42±0.715 d
	26.59	2.42±0.092 e	0.31±0.006 e	3.56±0.044 f	335.30±0.937 a	37.62±0.271 e

Different letters for each species show significant differences among salinity levels and species based on Duncan's test at *P<0.01*.

growth, stomata conductance, transpiration, photosynthesis and respiration (Kramer and Boyer, 1995). Osmo regulation is a symptom in response to osmotic stress and under conditions of water scarcity caused by any stress, the osmotic potential is reduced resulting in a lower relative water content in leaves (Basra and Basra, 1990) which could be the situation in the present study.

Chlorophyll and carotenoids: For all treatments, differences between species (P<0.01) were found for Chlorophyll where *H. criniferum* showed higher values than *H. coronarium*. Both total chlorophyll and chlorophyll a and b were affected negatively by salinity, decreasing with increasing NaCl in the irrigation water. The chlorophyll a and b were decreased by 75.0 and 53.3% in *H. criniferum* and 22.8 and 31.0% in *H. coronarium*, respectively The carotenoid concentration varied in both the species with *H. criniferum* had about twice concentration than *H. coronarium*; nonetheless, *H. criniferum* could not hold this proportion at the highest salinity level where it was observed a fall close to zero, showing a higher sensitivity than *H. coronarium* (Table 3). Several pigments such as chlorophyll and carotenoids present in chloroplasts are some of the internal factors that hold a major role in photosynthesis (Doganlar *et al.,* 2010). Carotenoids are responsible for quenching off singlet oxygen (Knox and Dodge,1985). Salt affects photosynthetic components such as enzymes, chlorophyll and carotenoid contents (Sultana *et al*., 1999). Decreasing concentration of chlorophyll a and chlorophyll b might be due to the formation of proteolytic enzymes (*i.e*. chlorophyllase) that are responsible for the degradation of chlorophyll and/or damaging the photosynthetic apparatus (Tuna *et al.,* 2008). Salt stress causes leaf necrosis which results in reduction of optical absorption and optical degradation of chlorophyll pigments (Sai-Kachout *et al.,* 2009). Chlorophyll content is one of the parameters of salt tolerance in crop plants (Srivastava *et al.,* 1988). Our results agreed with several reports of decreased content of chlorophyll and carotenoids by salinity as reported in a number of other glycophytes (Gadallah, 1999; Agastian *et al.,* 2000).

Proline: Proline concentration increased in both species as salinity increased (P<0.01) by four times compared to the control conditions (Table 3). Proline accumulation is one of the adaptations of plants to salinity. It has also been widely advocated that proline accumulation serve as a parameter of selection for salt stress tolerance (Bates, 1973; Ramanjulu and Sudhakar, 2001). A positive correlation between the magnitude of free proline accumulation and salt tolerance was detected in several plant species (Irigoyen *et al.,* 1992; Misra and Gupta, 2005).

Stomata characteristics: Both, length and width of stomata were reduced (P<0.01) with increase in salinity. The length decreased by 20.3 and 19.2% for *H. coronarium* and *H. criniferum,* respectively; while width decreased by 32 and 12.9%, respectively. This reduction in size was compensated by an increase (P<0.01) in the number of stomata per unit area where there was an increase of 59.8 and 35.4% for *H. coronarium* and *H. criniferum*, respectively (Table 4). Plant leaves usually optimize their gas exchange by altering stomata pore openness, stomata aperture size, stomata frequency (stomata density and stomata index), and stomata distribution pattern, which are regulated by environmental factors (Lake *et al.,* 2002; Hetherington and Woodward,

Table 3. Chlorophyll, carotenoids and proline concentration of *H. coronarium* and *H. criniferum* as affected by Na Cl in the irrigation water (Mean of four replicates ± SE)

Species	NaCl (ds/m)	Chlorophyll a (mg g^{-1} FW)	Chlorophyll b (mg g^{-1} FW)	Total chlorophyll (mg g^{-1} FW)	Carotenoid (mg g^{-1} FW)	Proline (mg g^{-1} FW)
H. coronarium	Control (0)	10.24±0.0123d	4.19±0.153 d	14.91±0.189 d	1.53±0.050c	28.39±0642 i
	10.67	8.89±0.185 e	3.36±0.133 e	12.72±0.309e	0.99±0.079d	70.70±0.510 f
	20.33	8.96±0.115e	3.04±0.045e	12.35±0.184ef	1.10±0.016d	78.91±0.462 e
	22.66	8.85±0.136 ef	3.24±.069e	11.59±0.196 f	1.03±0.024d	89.29±0.256 d
	26.59	7.90±0.125 f	2.89±0.200 e	10.52±0.579 g	1.04±0.115d	125.10±0.855 b
H. criniferum	Control (0)	17.27±0.36a	7.01±0.215b	31.72±0.291a	3.14±0.056a	37.20±0748 h
	10.67	15.11±0.157 b	7.61±0.115 a	31.14±0.253a	2.94±0.046b	49.71±0621 g
	20.33	15.06±0.341b	7.69±0.158a	28.29±0.032b	2.86±0.046b	88.66±0.661 d
	22.66	11.78±0.154 c	6.55±0.136c	17.65±0.517c	2.78±0.092 b	105.89±0.985 c
	26.59	4.31±0.299 g	3.27±0.179e	12.65±0.296e	0.22±0.064 e	146.27±0.445 a

Different letters for each species show significant differences among salinity levels and species based on Duncan's test at P*<0.01*.

Table 4. Stomata characteristics of *H.coronarium* and *H. criniferum*as affected by NaCl in the irrigation water (Mean of four replicates ± SE)

Species	NaCl (ds/m)	Stomata length(µm)	Stomata width (µm)	Stomata intensity (number/mm^2)
H. coronarium	Control (0)	28.23±0.36 b	25.91±0.613 a	138.85±0.607 i
	10.67	25.84±0.147 d	19.45±0.535 de	145.70±0.633 h
	20.33	23.17±0.381fg	18.59±0.366 ef	205.84±1.177 d
	22.66	22.33±0.098 g	17.13±0.227 f	215.20±1.413 c
	26.59	22.48±0.509 g	17.62±.616 f	221.12±1.361 b
H. criniferum	Control (0)	29.42±0.103 a	21.38±0.439 bc	170.18±1.133 g
	10.67	27.15±0.398 c	22.38±0.311 b	187.87±0.969 f
	20.33	24.24±0.366 e	19.88±0.349cde	197.83±1.092 e
	22.66	23.83±0.387ef	20.35±0515 cd	216.16±1.396 c
	26.59	23.76±0.085ef	18.62±1.042 ef	230.52±0.439 a

Different letters for each species show significant differences among salinity levels and species based on Duncan's test at *P<0.01*.

2003). According to these results stomata length and stomata width were reduced, while stomata intensity was increased under salinity stress. The results suggest that the number of stomata was increased in order to adapt to saline conditions by plants. In addition, plants had smaller stomata than the control plants due to reduced plant growth. The effect of salinity on photosynthesis and growth is complex. Photosynthesis is limited by both stomata and non-stomata factors of salt-stressed plants. Stomata conductance is more sensitive to salinity than the non-stomata components of photosynthesis. Stomata conductance is a sensitive indicator of the osmotic stress because stomata closure is often a rapid initial response to salt stress and it is reduced immediately with the onset of salinity, indicating that it responds to the osmotic stress generated by the salt outside the roots (James, 2008).

Conclusion

Salinity stress affected the physiological and biochemical parameters in both *Hedysarum coronarium* and *Hedysarum criniferum* with pronounced effect in *H. criniferum*. The results of this study suggested that *H.coronarium* is relatively better suited under salt stress conditions than *H. criniferum*.

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