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 the species *H. coronarium* and *H. criniferum.* Plastic pots with the sand were used for the experiment. 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. Both species were grown at four replication in green house condition. 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 CO2 concentration in both species were affected by salt stress. The lowest amount parameters measured 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 suggest that *H.coronarium*  is relatively better protected under salt stress conditions than  *H. criniferum*.

Keywords: Biochemical responses, Legumes, Physiology, Salt stress

1. **Introduction**

Environmental stresses are among the most limiting factors to crop plant productivity. Salinity is one of the most detrimental ones (Berrichi et al., 2010) and it is increasing worldwide (Chinnusamyet al., 2005) due to several causes, among them low rainfall, high surface evaporation, irrigation with saline water and poor irrigation practices. In Iran in the arid and semi-arid areas saline and alkaline soils are expanding covering 12.5% (204800 km2) of the total area (Akhani and Ghorbanli, 1993) and solutions to this issue are needed. The main salt present I these kind of soils is sodium chloride and it is well known that the majority of plants with economic importance are susceptible to to it at different levels. 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; Lauchli, 1986; Marchner, 1995; Sairam and Tyagi, 2004). Salinity stress has a major impact on plant growth and development (Cheong and Yun, 2007) due to the disruption of several processes where photosynthesis and cell division are seriously affected(Munns, 2002;Meloni et al., 2003). The efficiency of photosynthesis is reduced because of effects onchlorophyll content, photosynthetic enzymes, carotenoids (Stepien andKlobus, 2006). stomata closure leading to a reduction of intercellular CO2 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). *Hedysarumcoronarium* 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 (upto 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 (Ghahraman,1354) have shown good response in germination atmore than 200 mMNaCl(keshavarz et al., 2012) and both species may be options for saline areas; however, there is a lack of research on the performance of this species. Our research focuses on these two glycophytic legumes species with aim to determine the effects of salinity stress on physiological and biochemical traits.

**2. Materials and methods**

**2.1. Growth conditions and treatments**

A greenhouse experiment was conducted from December 2011 to May 2012 at the Faculty of Natural Resources and Marine Science of Tarbiat Modares University of Iran. Two species,  *H. coronarium* and *H. criniferum* were selected for study salinity responses (Table 1). Five salinity levels (0.81, 10.67, 20.33, 22.66 and 26.59 ds/m) were arranged in a 4-replicate completely randomized design as research treatments. 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 pods with river sterile sand. Plants were nourished with Hoagland’s nutrient solution for 4 months in controlled conditions. All nutrient solutions were renewed every week. The average day and night temperatures were 30±5 ºC and 15±3 ºC, respectively. The relative humidity ranged from 30 to 35%. 120 days after planting when the plants were in the vegetative stage, salinity stress was applied adding NaCl. Treatments started from 10.67 ds/m and increased stepwise by every other day 10.67 to reach 26.59 ds/m. Control plants were kept well-watered with no addition of NaCl.

Table 1

The primarycharacteristicsof seedsof 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 |

**2.2. 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 CO2 concentration (Ci) of upper mature leaves were measured with a portable LCpro+ Photosynthesis System (ADC BioScientific Limited UK) under greenhouse conditions (PAR average was 2100 µmol m-2 s-1 and leaf temperature was 30-35 ºC). Measurements were taken at 10-12 a m.

**2.3. Relative water content**

Relative water content (RWC) was determined on leaf tissues excised in the morning (around 9:00 am). Excised leaves were measured for fresh weight (FW), and then rehydrated in a water-ﬁlled Petri dish at room temperature. Turgor weight (TW) was measured by allowing full rehydration (16 h), removing all water on the leaf surface, weighing, and drying at 70 ºC for 48 h to determine DW. The relative water content was calculated from the following equation, RWC = 100[(FW - DW)/(TW - DW)].

**2.4. Proline**

Extraction and estimation of proline was conducted according to the procedures described by Bates et al. (1973). Plant material was frozen (-70 ºC), and 300 mg per sample was homogenized in 10 ml of 3% (w/v) aqueous sulphosalicylic acid, then the homogenate was altered through Whatman No.2 ﬁlter paper. Two milliliters of ﬁltrate was then mixed in a test tube with 2 ml acid ninhydrin and 2 ml glacial acetic acid, and incubated in a 100  ºC 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 (Lambda, Germany).

**2.5. Chlorophyll and carotenoids**

Fresh tissue (0.2 g) of fully expanded leaf 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: A652 × 27.8

Chlorophyll a= 11.75 × (A663) – 2.35 × (A645)

Chlorophyll b = 18.61 × (A645) – 3.96 × (A663)

Carotenoid = 1000× (A470) – 2.27 Chlorophyll a – 81.4 Chlorophyll b /227

**2.6. Stomata characteristics**

Stomata measurement was done in the sampled leaves, it was done in lower epidermal cells following the procedure described by the protocol for study on stomata parameters using an light microscope. Samples of a very thin layer of the epidermis of the lower surface of leaves were prepared removing chlorophyll leaf by bleach and distilled water treatments. They were placed on slides. Later, photos were taken and the stomata parameters were measured using the software Image Tools carefully 0.01µm (Grant and Vatnick, 2004). Number of stomata per unit area (number of stomata/mm2) were counted by 40×objective lens and 10m×eyepieces under light microscope.

**2.7. Experimental design and data analysis**

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

**3. Results**

**3.1. Gas exchange parameters**

In both plant species salinity affected negatively (p<0.0001) the values of gas exchange parameters (except for intercellular CO2 concentration). Both species had a decrease in net photosynthetic rate as salinity increased of 40% at the highest salt concentration (Table 2). Stomata conductance (Gs) decreased with the increase of salinity level in species both; but the decrease in *H*. *criniferum* was 77.8% and *H*. *coronarium* had a reduction of 23% compared to the control conditions (Table 2)*.*

Transpiration rate declined in response to salinity in both species showing *H*. *criniferum* higher E rate than *H*. *coronarium* at all treatments (Table 2).

Intercellular CO2 concentration (Ci) had not a consistent performance through salinity levels and no statistical differences were detected (Table 2).

**3.2. Relative water content**

Both plant species performed different in terms of RWC, having *H*. *coronarium*  higher values than *H*. *criniferum*. Salinity signiﬁcantly affected negatively the relative water content (p<0.0001) in both species, but *H*. *criniferum decreased*to a greater extent than *H*. *coronarium.*by aboutone–half.

**3.3. Chlorophyll and carotenoids**

### Differences between species (p<0.0001) were found for Chlorophylls where *H criniferum* showed higher values than *H*. *coronarium*. Both chlorophyl a and b were affected negatively by salinity, decreasing with increasing NaCl in the irrigation water. *H*. *criniferum* had decreases of 75.0 and 53.3% for Chl a and b respectively, while *H*. *coronarium* only had decreases of 22.8 and 31.0% for both chlorophylls, respectively. Total chlorophyll, consequently was affected, showing a decrease as salinity increased. In carotenoids both species were different in concentration, where *H*. *criniferum* had about twice than *H*. *coronarium*; nonetheless, *H*. *criniferum* could not hold this proportion at the highest salinity level where it was observed a fall close to cero, showing a higher sensitivity than *H*. *coronarium* (Table 3).

**3.4. Proline**

### Proline concentration increased in both species as salinity increased (p<0.0001) about four times compared to the control conditions (Table 3).

**3.5. Stomata characteristics**

Both, length and width of stomata were reduced by increasing salinity (p<0.0001). The length decreased 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<0.0001) 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).

Table 2

Gas exchange parameters and RWC% of *H. coronarium* and *H. criniferum* as affected by NaCl in the irrigation water. Mean of fourreplicates ± 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 (0.81) | 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 (0.81) | 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 | .031±0.006 e | 3.56±0.044 f | 335.30±0.937 a | 37.62±0.271 e |

Table 3

Chlorophyll, carotenoidsand proline concentration of *H. coronarium* and*H. criniferum* as affected by NaCl 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 (0.81) | 10.24±0.0.123 d | 4.19±0.153 d | 14.91±0.189 d | 1.53±0.050c | 28.39±0642i |
|  | 10.67 | 8.89±0.185e | 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 | 8.35±0.136ef | 3.24±.069e | 11.59±0.196 f | 1.03±0.024d | 89.29±0.256 d |
|  | 26.59 | 7.90±0.125f | 2.89±0.200 e | 10.52±0.579 g | 1.04±0.115d | 125.10±0.855 b |
| *H. criniferum* | Control (0.81) | 17.27±0.360a | 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 |

Table4

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/mm2)** |
| *H. coronarium* | Control (0.81) | 28.23±0.364 b | 25.91±0.613 a | 138.85±0.607i |
|  | 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±0.366ef | 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 (0.81) | 29.42±0.103 a | 21.38±0.439bc | 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±1.042ef | 230.52±0.439 a |

**4. Discussion**

**4.1. Gas exchange parameters**

Photosynthesis as the main path for energy absorption is the basis of all vital functions and is severely affected by salinity (Leclerc, 2003). Under normal conditions, 98% of plants that absorb water from the roots, losing it by stomata through the transpiration phenomenon (Heidari, 2001). But in the face of salinity stress, according to Leung (1994) and Cramer and Quarrie (2002) absicicacid (ABA) is produced which causes stomata closure preventing further loss through transpiration (Levitte, 1980; Iyengar and Reddy, 1996; Lee et al., 2004; Chaves et al., 2009; Heidari, 2001). Limitation of stomata conductance and transpiration is a defense mechanism to cope with too much salt with their negative consequences for plants (Flanagan and Jefferies, 1989; Clark et al., 1999). 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 (Yeo et al., 1985; Munns, 1993; Neumann, 1997). All these aspects may explain the trends found in the present research.

**4.2. Relative water content**

Relative water content better reflects the stomata status and leaf transpiration (Heidari, 2001). Leaf water status is intimately related to several leaf physiological variables, such as leaf turgor, 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, 1997) which could be the situation in this research.

**4.3. Chlorophyll and carotenoids**

Several pigments such as chlorophyll and carotenoids present in chloroplasts are someof 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). Carotenoids are accessory pigments in photosynthetic systems and their levels are altered during physiological and pathological conditions. Besides their function a slight harvesting pigments that contribute to photosynthesis, the carotenoids have another function in thylakoids lamellae which protects chlorophylls against oxidative destruction by O2 when irradiance level is high (Aono et al., 1993). Salt affects photosynthetic components such as enzymes, chlorophyll and carotenoid contents (Sultana, 1999). Decreasing concentration of chlorophyll a and chlorophyll b may 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). The decrease of chlorophyll synthesis may be due to a decrease of δ-aminolevulinic acid dehydratase (ALAD) activity under environmental stress (Vajpayee et al., 2000). All this conduct to a decrease in chlorophyll content due to an increase of chlorophyll degradation or/and a decrease of chlorophyll biosynthesis (Santos, 2004). Chlorophyll (Chl) content is one of the parameters of salt tolerance in crop plants (Srivastava et al., 1988). Our results agree with several reports of decrease content of chlorophyll and carotenoids by salinity as reported in a number of glycophytes (Gadallah, 1999; Agastian et al., 2000).

4.4. Proline

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; Hare and Cress, 1997; Nanjo et al., 1999; Ramanjulu and Sudhakar, 2001). A positive correlation between the magnitude of free proline accumulation and salt tolerance has been detected in several plant species (Irigoyen et al., 1992; Misra and Gupta, 2005). Proline is able to stabilize proteins, DNA as well as membranes (Matysik et al., 2002).

4.5. **Stomata characteristics**

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, 2003). According to these results stomata length and stomata width were reduced, while stomata intensity was increased by 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). During a salt stress, the plant has to close their stomata due to water loss (Chatrath et al., 2000; Robinson et al., 1983).

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