

Full Length Research Paper

Eco-physiological responses and symbiotic nitrogen fixation capacity of salt-exposed *Hedysarum carnosum* plants

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Nitrogen nutrition of *Hedysarum carnosum*, a pastoral legume common in Tunisian central and southern rangelands, largely depends on atmospheric nitrogen fixation. Yet, this process is greatly affected by environmental factors such as salinity. This study aimed to characterize the tolerance limits and the physiological responses of *H. carnosum* to salinity under symbiotic nitrogen fixation. Salt treatment was imposed by adding NaCl at different concentrations (0, 50, 100 and 200 mM) to the nutrient solution. Na⁺ content generally increased in the plant organs with increasing salinity in the culture medium. Especially, an excess accumulation of this cation was observed in leaves. Despite the fact that Na⁺ accumulation decreased plant growth, both nodulation and symbiotic nitrogen fixation capacity of *H. carnosum* appeared to be relatively salt-tolerant, owing to the plant capacity to maintain tissue hydration, control Na⁺ accumulation in shoots, and to conserve nodule efficiency to fix N₂. Taken together, our findings indicate that *H. carnosum* is a glycophyte that can tolerate moderate salinity (100 mM), suggesting its possible utilization (i) in the improvement of soil fertility and (ii) in saline pastures, where the survival of other fodder species is critical.

Key words: *Hedysarum carnosum*, nodulation, salinity, symbiotic nitrogen fixation.

INTRODUCTION

The economic importance of pastoral legumes is related to (i) their capacity to fix atmospheric nitrogen, thereby reducing agricultural cost through a reduction of fertilizer inputs and decreasing environmental contamination and (ii) their interest as an important source of protein for animal diet. Since salinity is an increasing problem on cultivated areas, it may be necessary to cultivate species and/or varieties that are naturally salt-tolerant. *Hedysarum carnosum* is an annual legume endemic to Tunisia and Algeria (Quezel and Santa, 1962), well adapted to arid and semi-arid climates, and with moderate aptitude to

cope with soil/water salinity (Hamza, 1977). Its nutritional value as a forage species is comparable to that of other pastoral plants such as *Medicago*, *Plantago* and *Rhanterium* spp. that grow in arid regions of North Africa (Floret and Pontanier, 1982). It is also characterized by its capacity to improve soil fertility by atmospheric nitrogen fixation. Indeed, this species could be used to compensate the lack of natural fodder resources and to exploit degraded lands with low fertility and/or high salinity. For instance, in Tunisian sabkhas, several *Hedysarum* species grow in association with halophytes.

Salinity decreases plant growth and consequently, yield via two ways: osmotic stress and ion toxicity (Munns, 2005). Osmotic stress caused by ions accumulation (mainly Na⁺ and Cl⁻) in the soil solution decreases the availability of water to roots; whereas, ion toxicity occurs when plant roots take up Na⁺ and/or Cl⁻ and when these ions accumulate to harmful levels in leaves, may lead to deficiencies of several nutrients (Tejera et al., 2006; Rejili, 2007). In legumes, salinity affects the Rhizobium-legume

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Abbreviations: SNF, Symbiotic nitrogen fixation; N, nitrogen; DAS, days after sowing; FW, fresh weight; DW, dry weight.

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symbiosis by reducing plant growth and available photosynthates (Salah, 2008). In addition, salinity impacts the nodule development (Gage, 2004) and decreases N_2 fixing capacity (Anthraer and Dubois, 2003). Different mechanisms of the symbiotic interaction as well as nodule development and metabolism are affected by salt stress, leading to the reduction in nodule number and limited nitrogen fixation (Saadallah et al., 2001). The limitation of symbiotic nitrogen fixation by salt stress has been attributed to a reduction in nodules respiration, through a decrease in oxygen diffusion to the infected zone (Serraj et al., 1995), the enhancement of reactive oxygen species accumulation in the nodules (Jebara et al., 2005), and also a limitation of bacteroid supply with the energetic substrates following reduced photosynthetic activity and nodule sucrose reduction (Gálvez et al., 2005; Lopez et al., 2008).

Since *H. carnosum* is a native of semi-arid to arid zones (often affected by salinity), one may hypothesize that this species has evolved mechanisms enabling it to survive extreme abiotic constraints among which is salinity. The identification of *H. carnosum* cultivars tolerant to salinity could be helpful to enhance the productivity, and thereby, valorize salt-affected areas. Therefore, the main objective of this work is to investigate the effect of salinity on the annual fodder species *H. carnosum* under symbiotic nitrogen fixation (SNF) conditions, with a special emphasis on the plant nutrient relations and the nitrogen fixation capacity.

MATERIALS AND METHODS

Plant material and growth conditions

This study was carried on the dicotyledonous legume *H. carnosum* (Papilionaceae), frequent on salt lands. Seedlings with homogeneous development were harvested from Elkalbia sabkha in Kairouan (central Tunisia). Roots were then placed into a liquid inoculant containing the strain Hca 1 of specific *Rhizobium* (Benhizia et al., 2004). After 30 min, they were carefully transplanted into 1 l serum bottles by passing roots through the hole of a rubber stopper on the bottle neck, and cotton wool was fitted at the hypocotyl levels to maintain the root system suspended in the following nutrient solution (Vadez et al., 1996): 3.3 mM $CaCl_2$, 2.05 mM $MgSO_4$, 1.6 mM KH_2PO_4 , 1.25 mM K_2SO_4 , 4 μM H_3BO_3 , 6.6 μM $MnSO_4$, 1.55 μM $ZnSO_4$, 1.55 μM $CuSO_4$, 0.12 μM $CoCl_2$, 0.12 μM $NaMoO_4$, 40 μM Fe-EDTA. Nutrient solution was added with 1 ml inoculum of *Rhizobium* Hca 1 at transplanting and at the first replacement. It contained 1 g $CaCO_3$ per plant, 2 mM urea as starter nitrogen at transplanting and 1 mM urea at the first solution replacement. This nitrogen source prevents nitrogen deficiency that could occur between the exhaustion of cotyledon storage and the establishment of symbiosis. It does not inhibit the nodulation in common bean (Vadez et al., 1999). Thereafter, plants were grown in nitrogen (N)-free nutrient solution. Bottles were wrapped with aluminium foil to maintain darkness in the rooting environment and aerated with a flow of 400 ml min^{-1} of filtered air via a compressor and spaghetti tube distribution system. The nutrient solution was first replaced at 15 days after sowing (DAS) and subsequently each week. Thereafter, plants were grown in N-free nutrient solution. After the pre-treatment, an initial harvest was made and the remaining plants were maintained for 10 weeks on N-free nutrient solution added to 0, 50, 100, 200 mM NaCl and was renewed every week. The

experiments were performed in a glasshouse under controlled conditions: 30/25°C day/night temperatures, 16h photoperiod, and 75±5 / 90±5% day/night relative humidity.

Total nitrogen and ion contents

For chemical analyses, samples of fresh material were dried at 70°C for 72 h and ground to fine powder using a grinder with agate mortars. Total nitrogen was determined by the Kjeldahl method. Quantities of N fixed were calculated from the differences between whole-plant N content at the final and initial harvest. Ion extraction was achieved in 0.5% HNO_3 . Both Na^+ and K^+ were assayed by flame emission photometry (Corning, UK), whereas Ca^{2+} and Mg^{2+} contents were determined by atomic absorption spectrophotometry.

Chlorophyll content

Chlorophyll (a, b, and total) concentrations ($mg \cdot g^{-1}$ FW) were determined according to Torrecillas et al. (1984), using 100 mg (fresh weight (FW)) leaf tissue placed in a test tube containing 5 ml 80% acetone. After the sample was incubated at 4°C for 72 h and cooled in the dark. The absorbance of extracts was measured at 649 and 665 nm.

RESULTS

Plant growth and chlorophyll content

The plant growth was not affected by up to 100 mM NaCl (Figure 1A). Indeed, the plant expressed the same growth potentialities as compared to the control. At higher salinity levels (200 mM NaCl), plant biomass was about 44% lower as compared to the control treatment. This was associated with a decline in shoot and root biomass (Figures 1B and 1C). Chlorophyll (a, b, and total) content was not affected by up to 100 mM NaCl, but significantly decreased at 200 mM NaCl (Figure 2).

Nodulation and nitrogen fixation

Salt treatment led to a significant decrease in nodule dry weight (Figure 3A), which appeared to be more affected than nodule number (Figure 3B). Indeed, the latter was adversely affected only at 200 mM NaCl. Concerning N_2 fixing capacity, salt stress caused 77% decrease to the nitrogen quantity at 200 mM NaCl as compared to the control (Figure 3C). Nodule efficiency, evaluated by the N_2 fixed/dry weight (DW) nodule ratio (Figure 3D), represents the quantity of nitrogen fixed (g per nodule dry matter). This parameter was unaffected at 50 mM NaCl, whereas for 100 and 200 mM NaCl, the reduction was relatively important (about 17 and 52 % as compared to the control).

Nutrient relations

Sodium concentration of the different organs increased

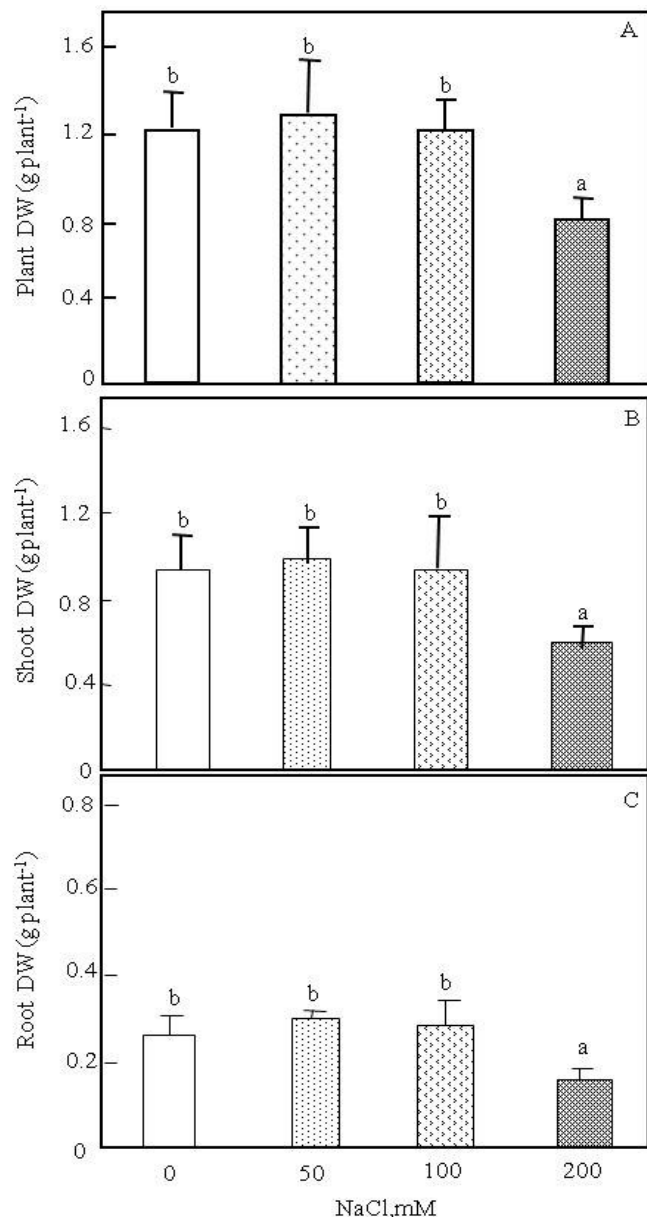


Figure 1. Effect of salt stress on plant (A), shoot (B), and root biomass (C) of *H. carnosum*. For each parameter, values (means of 8 replicates \pm SD) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

upon salt exposure, especially in leaves (4.5 mmol/g DW) (Figure 4). Sodium accumulation was generally lower in roots and nodules as compared to leaves. Salt treatment induced a significant reduction in potassium content, particularly in roots and nodules (Figure 4). In shoots, potassium content was only slightly decreased.

Water content

Moderate salinity improved significantly water content of

the plant organs (Figure 5), the maximal values being reached at 50 mM NaCl for nodules and 100 mM NaCl for leaves, stems, and roots. Higher salinity (200 mM NaCl) had an adverse effect on tissue hydration, especially in roots, whereas no significant effect was observed on leaves, stems, and nodules.

DISCUSSION

Our results show that *H. carnosum* is a relatively salt-tolerant glycophyte, expressing maximal growth potentialities between 0 and 100 mM NaCl. Yet, 200 mM NaCl was detrimental for the plant growth. Both nodule and shoot growth (as dry weight) were more affected by salt than was root growth. The increased biomass allocation from shoots to roots under saline conditions was reflected in higher root/shoot DW ratio (data not shown), as previously found by Tejera et al. (2004) and Ben Salah et al. (2008). The increase in root/shoot DW ratio observed may be an adaptive trait to salinity, resulting in more efficient water and nutrient uptake under salt stress (Gorham, 1996).

The mean number of nodules per plant was less affected by salt treatment than nodule DW. This finding was also observed in *Phaseolus vulgaris* (Saadallah et al., 2001), chick pea (Tejera et al., 2005) and *Leucaena leucocephala* (Anthraper et al., 2003). According to Gordon et al. (1997), the decrease of nodule DW in the presence of salt could be related to a limitation of both root growth and the feeding of these organs with photosynthates, likely owing to (i) the decrease of photosynthetic activity under salt stress (Lopez et al., 2008) and (ii) the inhibition of enzymes associated with sucrose degradation (Gonzalez et al., 1998; Lopez et al., 2008). Concerning symbiotic nitrogen fixation, our data showed a significant decrease in nodule N₂ fixing capacity per plant under high levels of salt (200 mM NaCl), which is consistent with previous findings (Tejera et al., 2006). The negative relationship observed between N₂ fixed and nodule Na⁺ content (Figure 6A) suggests that Na⁺ accumulation in nodules may account for the reduction of N₂ fixation, as hypothesized by Faidi (2002) and Ashraf and Alweena (2003). The quantity of N₂ fixed was also dependent on leaf Na⁺ content (Figure 6B); it was significantly reduced by the increase of Na⁺ accumulation in leaves.

According to several authors, the inhibition of N₂ fixation under moderate salinity may be related to a decrease in bacteroid respiration (L'taief et al., 2006) and nodule leghemoglobin contents (Lopez et al., 2008). Indeed, salt stress increases cortical barrier to O₂ diffusion in the nodule, which enhances the activity of enzymes involved in anaerobic metabolism (Aydi et al., 2004). Therefore, such a barrier that was rigidified under salt conditions reduces N₂ fixation in stressed plants (Serraj et al., 1995). The inhibition of nitrogenase activity by salt stress may also be the consequence of the decrease in malate content

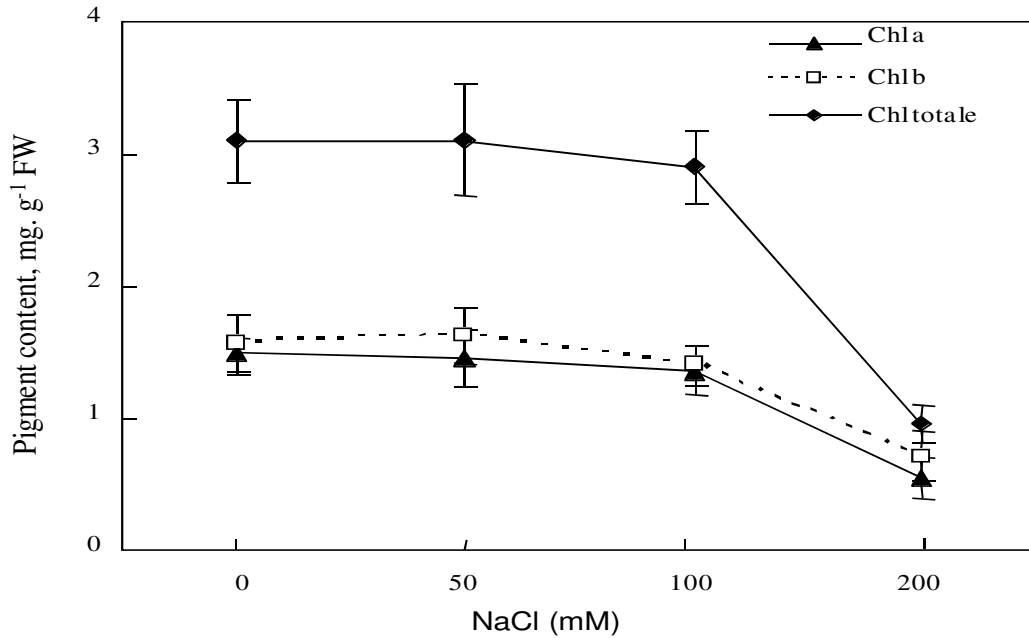


Figure 2. Effect of salt stress on chlorophyll content of *H. carnosum*.

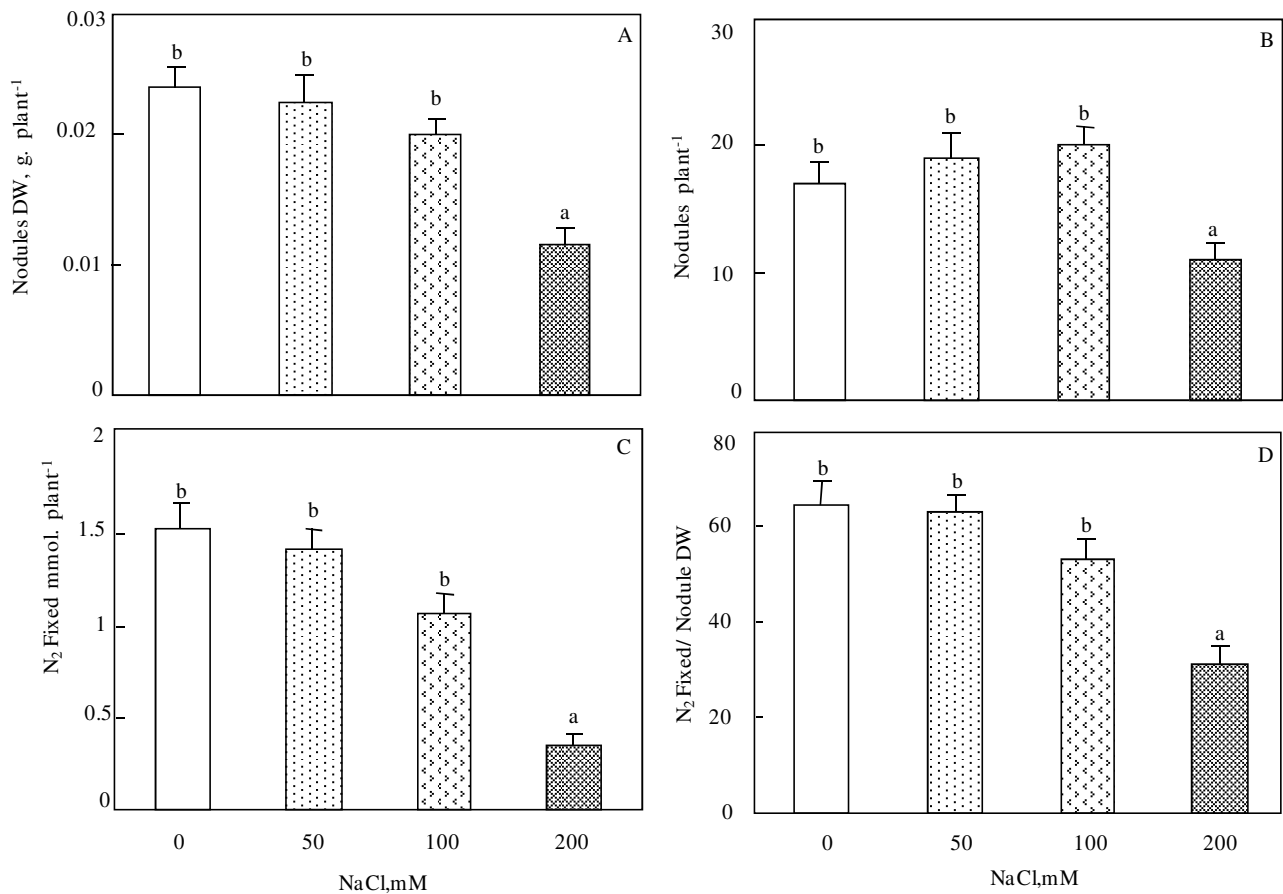


Figure 3. Effect of salt stress on Nodule biomass (A), nodule number (B), symbiotic nitrogen fixation (C), and Nodule efficiency (D) of *H. carnosum*. For each parameter, values (means of 8 replicates \pm SD) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

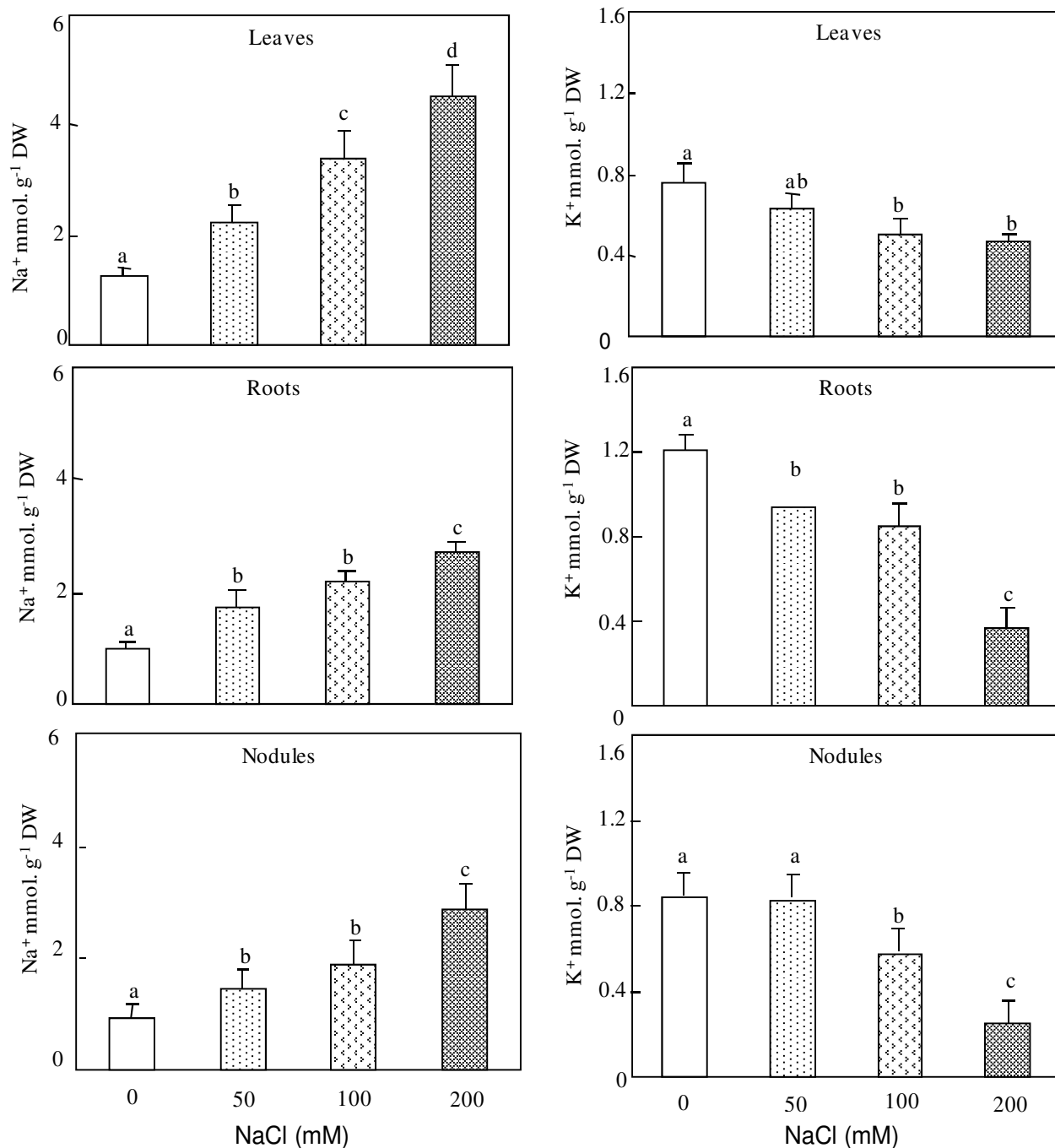


Figure 4. Effect of salt stress on Na⁺ and K⁺ accumulation in *H. carnosum*. For each parameter, values (means of 8 replicates \pm SD) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

in nodules (Delgado et al., 2006), which is the preferred respiratory substrate for bacteroids.

NaCl impaired the nutritional status of *H. carnosum*. Indeed, Na⁺ accumulation in shoots was accompanied by a significant decrease in K⁺ concentrations. It has been suggested that Na⁺ and K⁺ compete for uptake at the plasma membrane level (Carpaneto et al., 2004). Hence, limited absorption of potassium impairs plant growth and

development as this cation functions as a cofactor of various reactions. Salt stress also caused a significant decrease in the chlorophyll a and b contents at salinities exceeding 100 mM NaCl, hence confirming previous reports (Parida and Das, 2005; Hameed and Ashraf, 2008). The inhibitory effect of salt on chlorophyll could be due to the inhibition of specific enzymes involved in the synthesis of the photosynthetic pigments (Strogonove

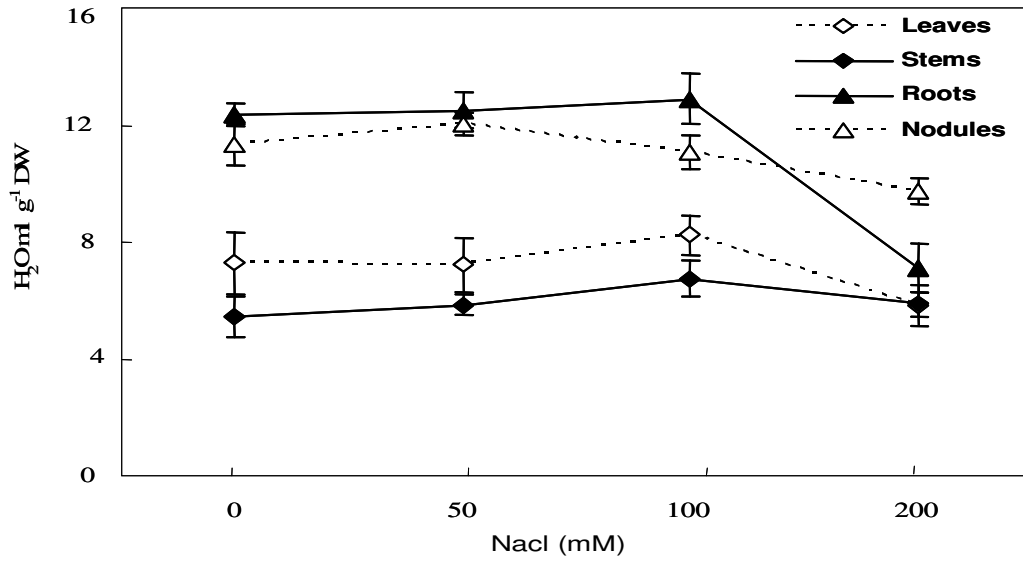


Figure 5. Effect of salt stress on water content of *H. carnosum*.

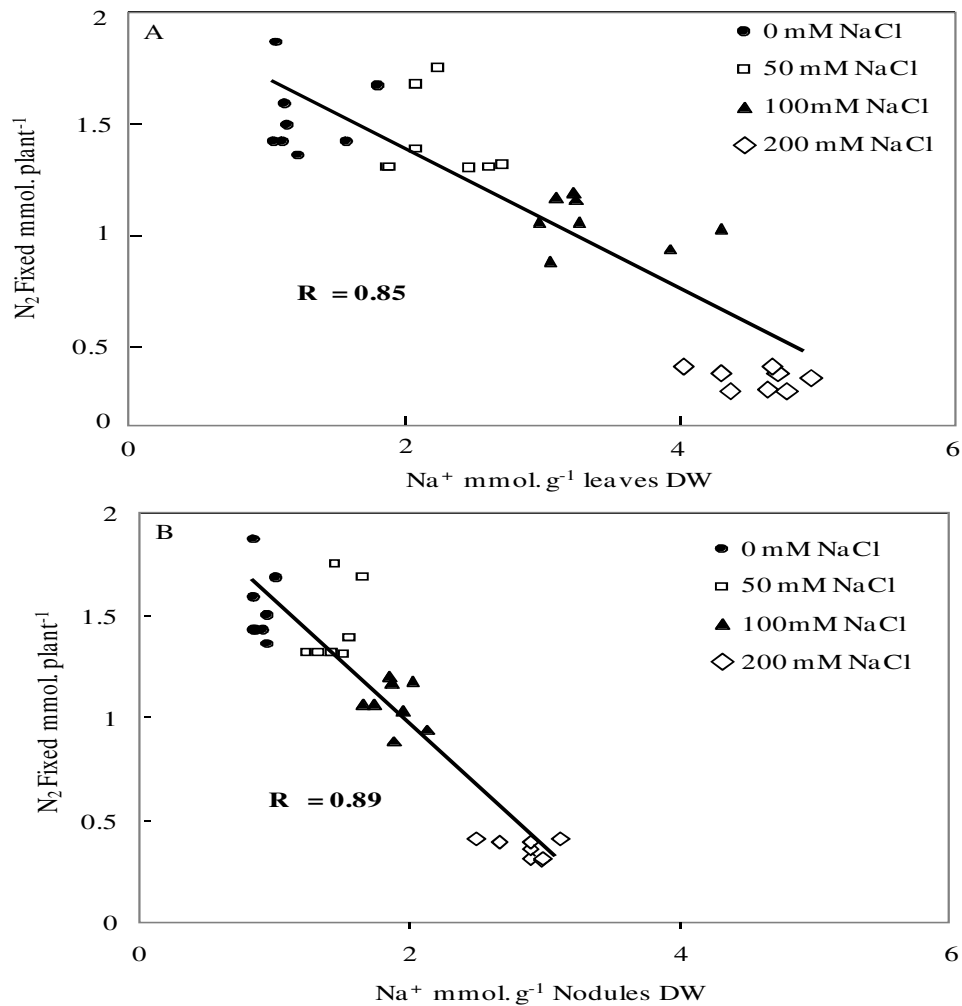


Figure 6. Relationship between symbiotic nitrogen fixation and Na⁺ accumulation in leaves (A) and nodules (B) of *H. carnosum*. Data are individual values of plants (8 replicates).

et al., 1970).

Several studies showed that plants adapt to the saline constraint by using one or several mechanisms to maintain a low cellular/tissue Na^+ concentration. These include sodium exclusion, sodium compartmentalization, and sodium secretion (Zhang et al., 2001). Sodium transport out of the cell can take place by the operation of plasma membrane-bound Na^+/H^+ antiports. Transport mechanisms can also actively move Na^+ across the tonoplast into the vacuole, to remove the toxic effect of these ions on cytosol metabolic activities. These ions, in turn, act as an osmoticum within the vacuole (Shi et al., 2000; Mansour et al., 2003). The present study shows that in order to avoid the toxic effects of Na^+ , which was highly accumulated following salt application, *H. carnosum* probably achieved sodium compartmentalization, as shown by the improved water status content up to 100 mM NaCl. Such a mechanism reflects probably an inclusive behavior of this species and its aptitude to use the dominant ion (Na^+) for the osmotic adjustment and for specific functions, such as enzyme activation, photosynthesis and stomata movements (Maeser et al., 2001). Our results also suggest that the growth reduction observed at higher salt concentration (200 mM NaCl) was not due to an osmotic effect, but rather the consequence of a massive accumulation of toxic ions and/or of limited nutrient absorptions (ionic effect).

As a whole, it seems that the relative tolerance of *H. carnosum* to moderate salinity is associated to its capacity to maintain tissue hydration, to control Na^+ flux in shoots, and to maintain high efficiency of nodule to fixed N_2 . These preliminary findings are in favor of the introduction of this plant in salt-affected pastures.

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