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Physiological and nutritional responses of chickpea (*Cicer arietinum* L) to salinity

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Abstract: The aim of this study was to evaluate growth, nodulation, nitrogen fixation, and ionic repartition in 2 chickpea varieties, namely Chetoui and Amdoun, which are grown under salt stress. Inoculated seedlings were grown in a nutrient solution that lacked NaCl (control) or supplemented with 25 or 50 mM NaCl. Whole plant and nodule growth, nitrogen fixation and sodium, and chloride and potassium partitioning were subsequently evaluated. In comparison to Chetoui, Amdoun maintained a high level of whole plant and nodule growth and had a greater nitrogen fixation activity. Nitrogenase activity measurements demonstrated that nodule senescence was more precocious in Chetoui than in Amdoun. Genotypic differences were observed at the level of Na⁺, Cl⁻, and K⁺ distribution between organs: Amdoun showed the lowest leaf and nodule Na⁺ and Cl⁻ concentrations and the highest K⁺ concentrations in these organs, particularly under severe salinity (50 mM) condition. These findings suggest that the relative tolerance of Amdoun is linked to its ability to protect its photosynthetic and symbiotic apparatus against the toxic Na⁺ and Cl⁻ ions and to ensure an appropriate K⁺ supply of these organs.

Key words: *Cicer arietinum*, ionic repartition, N₂ fixation, salinity.

Introduction

Chickpea is one of the most important legume crops for human nutrition and has been traditionally cultivated in saline soils in arid and semi-arid regions (Rao et al. 2002). The agronomical importance of chickpea (*Cicer arietinum* L.) is linked to its high protein content (25%-29%) (Hulse 1991). Although chickpea is considered to be a salt-sensitive species, the selection and breeding of cultivars that can grow under saline conditions constitute a more effective option to minimize the repercussion of exposure to salinity (Ashraf and McNeilly 2004). In plants, salinity

drastically affects photosynthesis (Soussi et al. 1998), nitrogen (Mansour 2000; Santos et al. 2002) and carbon (Balibrea et al. 2003) metabolism, and disturbs plant nutrition causing accumulation of Na⁺ in plant tissues (Mengel and Kirkby 2001). A derailment of these physiological processes leads to a drastic decrease in plant growth and consequent reduction in crop yield. It is known that chickpea growth is significantly decreased even at low concentration of salt (20 mM) and recent studies have demonstrated a 71% reduction in growth in the presence of 25 mM NaCl (Sadiki and Rabih 2001).

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The establishment of the legume-*Rhizobium* symbiosis has been known to be salt sensitive (Rao et al. 2002). Garg and Singla (2004) used the nodulation capacity under stress as an index to select chickpea genotypes. Several studies point out that the nodule initiation appears to be more sensitive to salt stress than their further development (Zahran and Sprent 1986). However, Soussi et al. (1999) found that the tolerance of host plant to salt stress could be the determinant factor for this symbiosis. Once the legume-*Rhizobium* symbiosis is established, salinity can negatively affect nodule function by acting directly on the bacteroid through the inhibition of its nitrogenase activity (Cordovilla et al. 1994) and leghemoglobin accumulation (Sheokand and Dhandi 1995). In addition, salinity can exert additional deleterious effects by indirectly restricting the carbon supply from the host plant thereby inhibiting photosynthesis.

In saline soils, Ashraf and McNeilly (2004) suggested that the maintenance of a high tissue K^+/Na^+ ratio by plants may serve as a reliable criterion of salt-tolerance.

Since chickpea is indigenous to arid areas, some genotypes may have a certain degree of salt adaptation (Soussi et al. 1999; Rao et al. 2002). Differences in salt tolerance occur not only in different species, but also in different genotypes of the same species (Serraj 2002).

Several studies have focused on evaluating legume growth under salt stress. In spite of the large number of reports, studies aiming to determine the component of each toxic ion (Na^+ or/and Cl^-) that are involved in the disruption of the nodule integrity and the inhibition of the symbiotic nitrogen fixation are lacking. Most of the earlier reports on legumes assign the reduction of nodule growth and nitrogen fixation under salt stress to a variety of physiological parameters. Specifically, sodium and chloride ions may exert deleterious effects upon photosynthesis and may, therefore, restrict the supply of carbon to the nodules (Sadiki and Rabih 2001; Garg and Singla 2004). Exposure to salinity may also impact the diffusion and availability of oxygen to the bacteroids (Vadez et al. 1996; Garg and Singla 2004).

In the current investigation, we have studied the effect of salinity on the nodulation, growth; symbiotic nitrogen fixation, and mineral distribution in 2 Tunisian chickpea varieties, namely Amdoun and Chetoui. These varieties were previously demonstrated to have a contrasting behavior to salinity when they depend on mineral nitrogen nutrition: Amdoun is relatively tolerant and Chetoui is highly sensitive to salt (Sleimi et al. 1999).

Materials and methods

Plant growth and culture condition

This work was conducted on 2 local varieties of chickpea (*Cicer arietinum*): Amdoun and Chetoui. Healthy seeds of uniform size were disinfected with 2% calcium hypochlorite solution, and were germinated in sterile humidified perlite for 3 days at 25 °C in darkness. Seedlings were subsequently transferred to a glasshouse under natural light in 1 L pots filled with the following N-free nutrient solution: KH_2PO_4 (1.60 mM), $MgSO_4$ (1.50 mM), K_2SO_4 (1.50 mM), $CaSO_4$ (3.50 mM), H_3BO_3 (4 μ M), $MnSO_4$ (4 μ M), $ZnSO_4$ (1 μ M), $CuSO_4$ (1 μ M), $CoCl_2$ (0.12 μ M), $(Na)_6(Mo)_7O_{24}$ (0.12 μ M), and Fe-K-EDTA (45 μ M). As a means to ensure plant nodulation, 1 mL *Mesorhizbium ciceri* UPMC 835 inoculum containing approximately 10^{10} cells per mL was added. The *Mesorhizbium* strain was provided by the Laboratory of Legume-Microorganisms Interaction, Biotechnology Center, Borj Cedria Technopark. The pH of the solution was maintained to near neutrality with $CaCO_3$ (1 g l⁻¹).

During the first 2 weeks, i.e. before the development of functional nodules, the nutrient solution was supplemented with 2 mM urea. The addition of urea does not inhibit nodulation, and serves as a source of nitrogen after reserves in the cotyledons are exhausted (Drevon et al. 1994). Thereafter, the nutrient solution was renewed every week without the addition of urea and was aerated with a flow of 400 mL mn⁻¹ of filtered air via a compressor and “spaghetti tube” distribution system. After 2 weeks, plants of each variety were separated into 3 plots of 10 plants each. The first plot received nutrient solution without NaCl (control), while the other 2 batches received the same nutrient solution

containing 25 mM or 50 mM NaCl (EC: 2.65 ms cm⁻¹ and 5.3 ms cm⁻¹, respectively). After 4 weeks of treatment; the plants were separated into leaves, stems, roots, and nodules, dried at 60° C for 72 h, and then pulverized into a fine powder.

Nitrogen-fixation assays

Nitrogenase (EC 1.7.99.2) activity of the nodules (Acetylene Reduction Activity – ARA) was measured in situ as previously described (Drevon et al. 1988). To avoid nodule disturbance, the level of the solution was lowered to 40% of the bottle volume 1 day before the assay. The aliquots were analyzed for ethylene in a Hewlett Packard 4890 gas chromatograph equipped with a Poropak T Column and a flame ionization detector. The ARA values were computed as C₂H₄ concentration by flow rate and expressed as µmol C₂H₄ h⁻¹ plant⁻¹.

For N₂ determination, the samples were digested in hot concentrated H₂SO₄, as described by Kjeldahl. The symbiotic nitrogen fixation (SNF) was estimated as the difference between N₂ quantities (mmol plant⁻¹) at 17 and 45 days after germination.

Determination of ion content

After extraction in 0.5% HNO₃, K⁺, Na⁺, and Cl⁻ ions were measured as described by Pauwels et al. (1992). Chloride was assayed using a chloridometer (Buchler), while Na⁺ and K⁺ were quantified by flame emission photometry (Corning, UK).

Statistical analysis

Each treatment was analyzed with 10 replicates and a standard deviation (S.D.) was calculated at P < 0.05 and data are expressed as mean ± S.D. of the 10 replicates.

Results

Biomass production

Irrespective of the severity of salt stress, Amdoun produced more whole plant biomass than Chetoui. In the presence of 25 and 50 mM NaCl, respectively, the dry weight decreased by 14.5% and 16% in Amdoun and by 47% and 65% in Chetoui (Figure 1). These genotypic differences were also conserved at nodule growth level. Amdoun is usually more productive than Chetoui. In the presence of 25 and 50 mM NaCl,

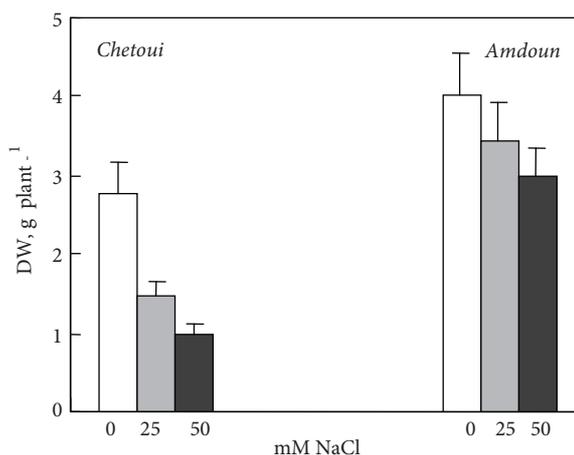


Figure 1. Effect of salt stress on growth of chickpea (*Cicer arietinum* L.) varieties. Data are means of 10 replicates (± standard error; P = 0.05).

respectively, biomass of the nodules decreased by 23% and 36% in Amdoun and by 55% and 67% in Chetoui (Figure 2a). Presence of salt also affected nodule initiation (Figure 2b). The number of nodules decreased by 40% and 44% in Chetoui and by 30% and 34% in Amdoun in the presence of 25 and 50 mM NaCl, respectively.

In order to quantitatively express genotypic variability, we calculated the sensitivity index (SI), estimated as the biomass decrease in stressed plants, as compared to control ones (SI = 100 × (DW_s - DW_c) / DW_c, Table 1).

The results indicated that for both salt concentrations, the SI values are more negative in Chetoui than those of Amdoun. These results lend further support to the observation that Amdoun is more tolerant to salt than Chetoui.

Symbiotic nitrogen fixation

In order to evaluate nitrogenase activity, we measured acetylene reduction activity (ARA) weekly in control and salt treated plants (Figure 3). Independently of the treatment, NaCl concentration in the medium and the plant age, Amdoun showed the highest ARA values. In Chetoui, the depressive effect of salt was observed after 4 weeks of treatment. In fact, ARA reaches the maximum after 3 weeks of treatment and then declines, indicating the senescence of symbiotic organs. In control plants, ARA continues to increase until the 4th week of

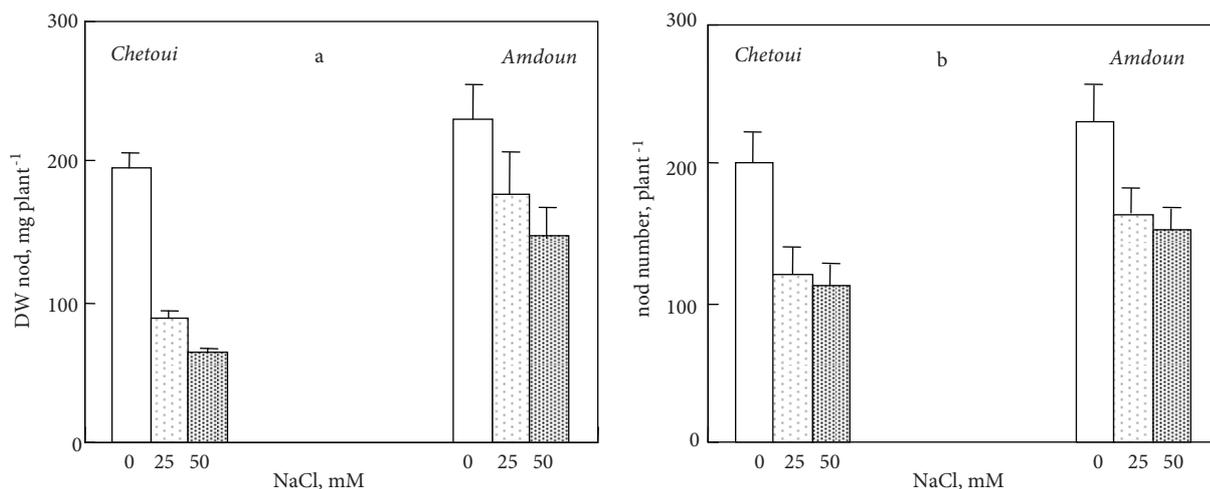


Figure 2. Effect of salt stress on growth (a) and number (b) of nodules in chickpea (*Cicer arietinum* L.) varieties. Data are means of 10 replicates (\pm standard error; $P = 0.05$).

Table 1. Sensitivity index (SI) and Nodule efficiency (NE, $\text{mmol N g}^{-1} \text{DW nod}$) in chickpea (*Cicer arietinum* L.) varieties subjected to salt stress.

NaCl, mM	Chetoui			Amdoun		
	0	25	50	0	25	50
SI		-47.5 ± 4.17	-64.4 ± 3.81	-13.7 ± 0.6	-25.6 ± 1.13	
NE	13.9 ± 0.75	12.4 ± 0.57	5.4 ± 0.39	27.3 ± 0.55	22.5 ± 0.51	11.8 ± 0.39

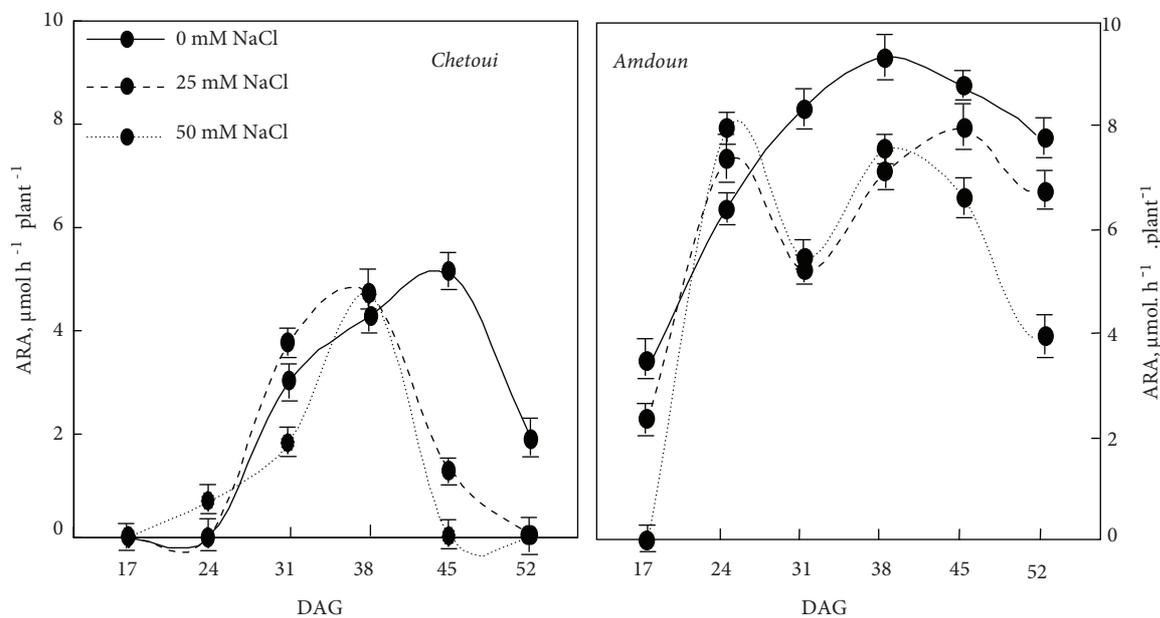


Figure 3. Effect of salinity on acetylene reduction activity (ARA) in chickpea (*Cicer arietinum* L.) varieties in relation to plant age. Data are means of 9 replicates (\pm standard error; $P = 0.05$).

treatment. In Amdoun, the maximum ARA is attained after 3 weeks of treatment in control as well as treated plants. Differences between the treatments appear after 2 weeks of treatment with a high level of ARA in control plants. After 4 weeks, ARA declines in all plants but remains at high level.

In both chickpea varieties, ARA decreased in all plants after 5 weeks of treatment indicating the onset of nodule senescence. However, even at this late stage, the nodules in Amdoun remained functional, whereas those of Chetoui were found to be senescent.

Symbiotic Nitrogen Fixation (SNF) was also estimated as the quantity of N fixed during the treatment. Independently of salt stress intensity, Amdoun was found to be more efficient than Chetoui in fixing nitrogen (Figure 4). The SNF was 1.6 times (in control plants and in the presence of 25 mM NaCl) and 1.2 times (in the presence of 50 mM NaCl) higher in Amdoun than in Chetoui.

Sodium and Chloride distribution

Independently of NaCl concentration in the medium, in Amdoun, we observed a decreasing gradient of Na^+ from roots to shoots. At a concentration of 25 mM NaCl, Na^+ accumulation was 10 folds higher in roots than in leaves (Figure 5).

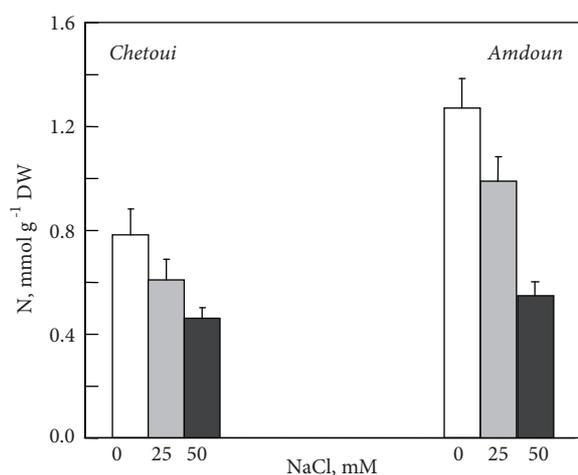


Figure 4. Effect of salinity on symbiotic nitrogen fixation in chickpea (*Cicer arietinum* L.). Data are means of 10 replicates (\pm standard error; $P = 0.05$).

Further, in spite of their close association with the roots, the nodules appeared to be perfectly protected against the accumulation of Na^+ (17% of root charge). Even at 50 mM NaCl, the nodules did not accumulate more than 51% of the root charge. These results indicate that Amdoun is able to protect its leaves and nodules against Na^+ . In contrast, Chetoui did not have a similar mechanism of protection. In fact, in this variety, in the presence of 25 mM and 50 mM NaCl, Na^+ accumulation was nearly the same in roots and leaves, whereas nodules accumulated about 75% of root charge of Na^+ .

In contrast to Amdoun, Chetoui systematically accumulates more Cl^- in all the parts of the seedlings (Figure 6). In both varieties, leaves were found to accumulate more chloride than the roots. The nodules in Amdoun also appeared to protect their symbiotic organs much more against Cl^- than Chetoui. In addition, the results showed that in leaf tissue, the level of chloride accumulation exceeded those of sodium. It is known that this repartitioning of ions is an exclusive feature in some glycophytes, specifically chickpea.

Potassium nutrition

Figure 7 illustrates the potassium repartition in plant organs. In leaves, K^+ accumulation increased slightly when plants were subjected to 25 mM NaCl. With 50 mM NaCl, plants of Amdoun continued to accumulate potassium at a high level. The leaf K^+ concentration at 50 mM NaCl was 1.5 times higher than those at 25 mM NaCl. On the other hand, Chetoui plants exhibited an opposite behavior. With an increase in salt concentration, K^+ accumulation decreased significantly. In nodules, K^+ accumulation decreased at 25 mM NaCl, while at 50 mM NaCl, potassium concentration increased 4-fold in Amdoun and 2-fold in Chetoui.

In Amdoun roots, we noted a gradual decrease in accumulation of potassium with increasing NaCl concentrations. In comparison to control, the nodules accumulated 60% K^+ at 25 mM NaCl and only 40% K^+ at 50 mM NaCl. In Chetoui, a completely opposite trend was observed with respect to K^+ accumulation.

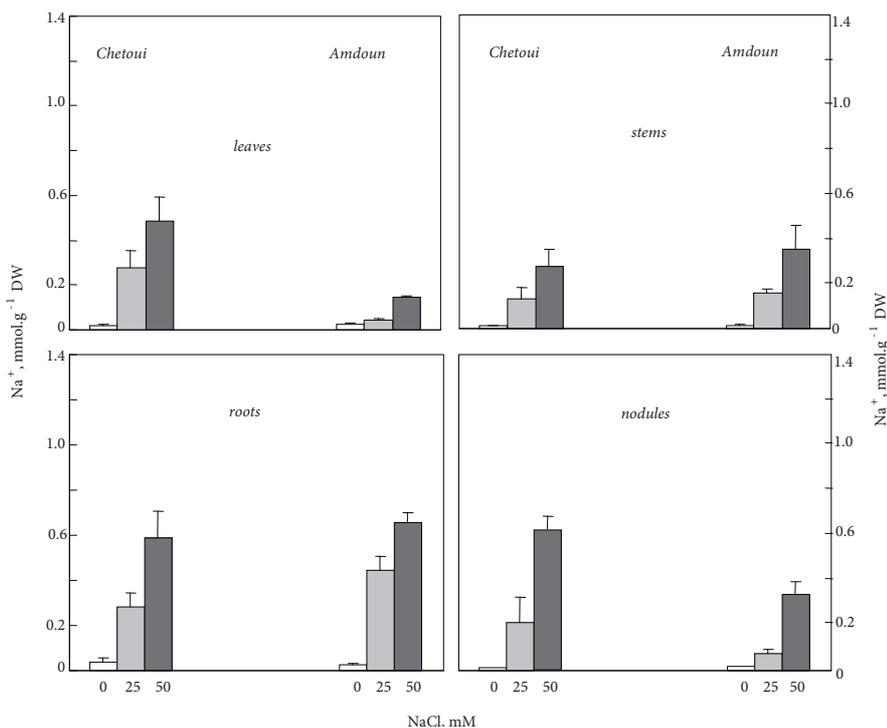


Figure 5. Sodium accumulation in response to salinity in different plant organs of chickpea (*Cicer arietinum* L.) varieties. Data are means of 10 replicates (\pm standard error; $P = 0.05$).

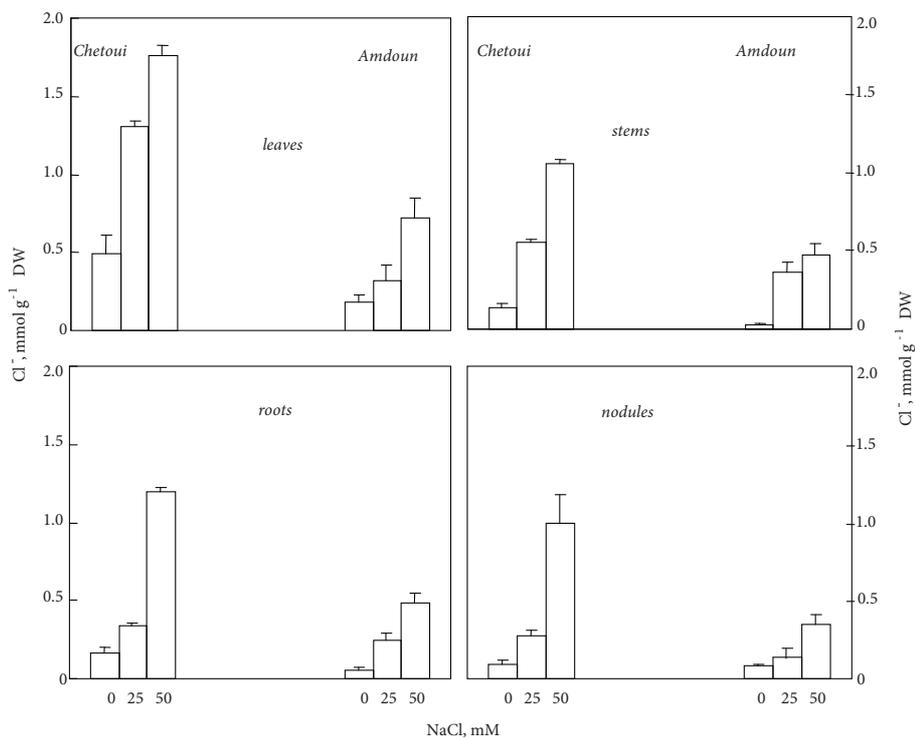


Figure 6. Effect of salinity on chloride accumulation in leaves, roots, stems, and nodules of chickpea (*Cicer arietinum* L.) varieties. Data are means of 10 replicates (\pm standard error; $P = 0.05$).

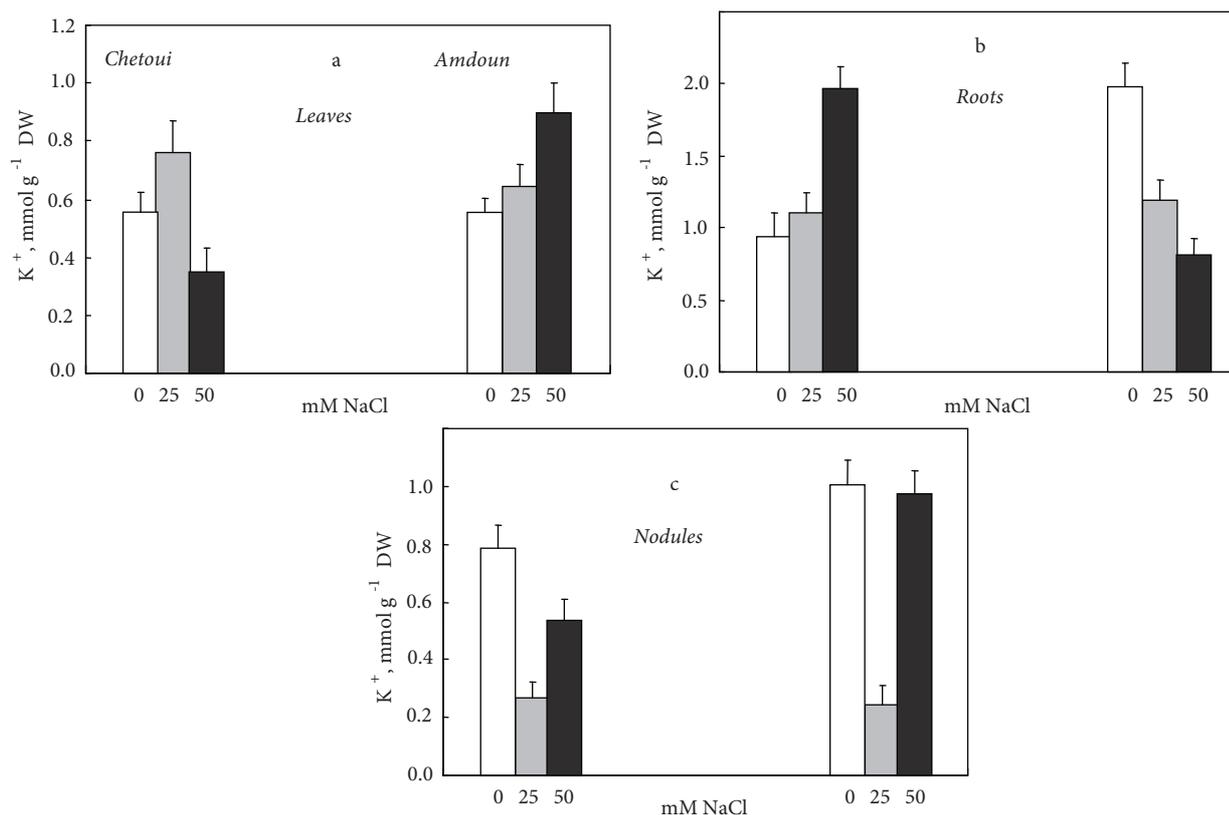


Figure 7. Potassium accumulation in leaves (a), nodules (b), and roots (c) of chickpea (*Cicer arietinum* L.) varieties. Data are means of 10 replicates (\pm standard error; $P = 0.05$).

Discussion

Our results demonstrate that although salinity affects whole plant growth in chickpea, distinct genotypic differences were observed between the 2 varieties. Specifically, Amdoun expressed a relatively high potential for growth even under salt stress, whereas the growth of Chetoui was severely hindered. In addition to negatively affecting the host plant, salt stress also reduced nodule growth and number in the both varieties. However, the deleterious effect was more pronounced in Chetoui than in Amdoun. Earlier, Velagaleti and Marsh (1989) demonstrated that the reduction of nodule weight resulted from the reduction of carbohydrate translocation towards nodules that follows the shoot growth inhibition and the decrease of intrinsic photosynthetic capacity. In fact, it has been documented that salt stress limits plant productivity in legumes through diminished photosynthetic efficiency, nitrogen fixation and carbon metabolism (Ferri et al. 2000; Garg and Singla

2004). However, results of other investigators suggest that the reduction of nodule growth is more linked to an inhibition of activities of the enzymes that are involved in the hydrolysis of sucrose (Anthon and Elmerich 1990; Robertson and Taylor 1995).

In chickpea, we have also observed that salinity reduces symbiotic nitrogen fixation. This effect was more pronounced in Chetoui than in Amdoun concomitant with a restriction of nodule growth and performance. Indeed, the nodule efficiency, which is expressed as the amount of fixed nitrogen per unit of nodule biomass (Table 1), was inhibited at 50 mM NaCl (-50%) in comparison with control plants. However, it remained 2-fold higher in Amdoun than in Chetoui. The decline of nitrogen fixation under environmental stresses, including salinity, has been reported in several studies (Hunt and Layzell 1993; Sadiki and Rabih 2001; Garg and Singla 2004; Abdelly et al. 2005; Tejera et al. 2006) and may be due to: (i) a preferential degradation of the leghemoglobin

(Delgado et al. 1993), (ii) a decrease in the activity of enzymes involved in tissue protection against reactive oxygen species (Sheokand et al. (1995), and (iii) an inhibition of ammonium assimilation pathways, particularly as a consequence of a decrease in glutamine synthetase activity (Cordovilla et al. 1994). Results of other investigators attribute the decline of nitrogenase activity to the limitation of oxygen diffusion in the nodules. For examples, Serraj and Drevon (1995) and Serraj et al. (1995) showed that NaCl inhibits nitrogenase activity and respiration of the nodules by decreasing the availability of oxygen. Drevon et al. (1994) demonstrated that the limited conductance of oxygen in the nodules is responsible for the inhibition of nitrogenase activity. This conclusion is supported by studies in which an increase of the oxygen pressure in the medium of nodulated roots suppressed the inhibitory effect of the saline stress on the activity of nitrogenase (Serraj et al. 1995; Vadez et al. 1996). Although these studies provide new information, they do not propose a mode of salt action on nodule function. In particular, the relationship between nodule ionic status and nodule functioning is not well established. Our results showed that the tolerant variety (Amdoun) accumulated Na⁺ mainly in roots, which contribute to osmotic adjustment. It is also reasonable to postulate that this mechanism may ensure a limited Na⁺ toxicity in leaves and nodules. In fact, leaf Na⁺ concentration did not exceed 10% and 17% of Na⁺ concentration in roots at 25 and 50 mM NaCl, respectively, whereas nodule Na⁺ concentration did not exceed 21% and 51% of roots Na⁺ concentration at 25 and 50 mM NaCl. In contrast, in Chetoui variety, both the leaves and the nodules, which are involved in symbiotic nitrogen fixation, were found to be overloaded with Na⁺. Indeed, the level of Na⁺ in leaves and nodules

reached 97% and 82% of the Na⁺ concentration of the roots at 25 mM NaCl, and 75% and 72% of root Na⁺ concentration at 50 mM NaCl. These results are in agreement with those of Rogers et al. (1993) and Tejera et al. (2006). As far as Cl⁻ is concerned, it appears that, in contrast to Na⁺, this element is apparently accumulated in preference over Na⁺ in all the organs of both varieties subjected to salt stress (Figure 5). However, the level of Cl⁻ in Chetoui generally exceeded those found in Amdoun, which is in conformity with the relatively high salt tolerance of this variety.

Although the values of K⁺/Na⁺ ratio (Table 2) decreased with salinity in both varieties, K⁺/Na⁺ ratios obtained from leaves and nodules were usually higher than those obtained from roots of Amdoun. An inverse behavior was observed in Chetoui. The results indicated that the latter variety (Chetoui) accumulates less Na⁺ in roots than in leaves and nodules, and as a result, photosynthetic and symbiotic apparatus are relatively more prone to the toxic effect of salt. In agreement with our studies, Tejera et al. (2006) observed that the sensitive cultivar of chickpea accumulate less Na⁺ in roots than in shoots.

Our results suggest that the Na⁺ accumulation in roots can contribute to the osmotic adjustment in these organs, which have an efficient mechanism of salt vacuolar compartmentation. However, the accumulation of this ion in leaves and nodules appears to be correlated with salt sensitivity suggesting a toxic effect of this ion. In these organs, osmotic adjustment is likely to be ensured mainly by K⁺. Indeed, our results showed that the relatively more salt tolerant variety Amdoun exhibited the highest K⁺ concentration in nodules and leaves, particularly under high salinity condition. In addition, since an

Table 2. Effect of salt on potassium/sodium ratio (K/Na) in leaves, roots, and nodules of chickpea (*Cicer arietinum* L.) varieties.

NaCl, mM	Chetoui			Amdoun		
	0	25	50	0	25	50
Leaves	25.4 ± 3.17	3.03 ± 0.48	0.7 ± 0.17	23.0 ± 1.74	14.4 ± 1.0	1.4 ± 0.16
Roots	22.0 ± 2.67	3.9 ± 0.64	3.1 ± 0.19	76.3 ± 6.8	2.7 ± 0.19	1.2 ± 0.07
Nodules	65.0 ± 4.05	1.3 ± 0.28	0.9 ± 0.12	56.6 ± 5.53	3.5 ± 0.47	2.8 ± 0.44

increased Na⁺ and Cl⁻ accumulation in nodules was found to be related to the fall of their functional integrity, as shown by a decline in the nodule efficiency (estimated by the amount of fixed nitrogen per g nodule DW), we suggest that an apoplastic Na⁺ and Cl⁻ accumulation may have a role in cell dehydration and water accumulation in intercellular spaces. Such a situation may lead to the limitation of O₂ diffusion towards bacteroids and a reduction of symbiotic nitrogen fixation. This assumption is in conformity with the limitation of the symbiotic nitrogen fixation by low O₂ availability observed at several legumes subjected to salt stress.

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