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Growth, water relations, proline and ion content of in vitro cultured *Atriplex halimus* subsp. *schweinfurthii* as affected by CaCl_2

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ABSTRACT

Atriplex halimus subsp. *schweinfurthii*, a widely distributed perennial halophyte in the Algerian salt steppes, is of interest because of its tolerance to environmental stresses and its use as a fodder shrub for livestock in low-rainfall Mediterranean areas. This study reports the effects of salinity (0, 4, 8, 12, 16, and 20 g l⁻¹ CaCl_2) on the growth, succulence, proline and ion content of the species under in vitro conditions. Fresh and dry weight of plants increased with an increase in salinity. Optimal growth was recorded at 8 g l⁻¹ CaCl_2 ; growth declined with increased levels of salinity. Ca^{2+} , K^+ , Cl^- , and proline contents in plants increased, whereas Na^+ content decreased with an increase in salinity. Succulence of shoots and roots was significantly higher at CaCl_2 concentrations of 12 to 20 g l⁻¹ than at 8 g l⁻¹ CaCl_2 . Water potential of plants decreased with an increase in salinity. This plant can be used locally as a fodder for livestock and to stabilise sand dunes and rehabilitate salt soils.

Key Words: *halophyte; in vitro culture; ion content; proline; steppe; water potential.*

INTRODUCTION

Salinity affects more than 40% of the soils in the Mediterranean basin. Draining salinised soils or irrigating them with high quality water from remote sources is extremely expensive. Therefore, selecting plants tolerant to salinity is an alternative strategy for a sustainable agriculture in these marginal lands (Drevon et al., 2001). The increased cattle pressure on pastoral zones, deforestation, and extension of soil salinity are enormous problems in Algerian steppes (Le Houérou, 1995, 2000).

Many investigations on quantification of salt tolerance of plant species have been based on experiments in which NaCl was the predominant salt (Debez et al., 2004; Khan et al., 2005; Koyro, 2006). There have been few research reports on plant responses to CaCl₂. However, in soils and in groundwater in many areas of the world, including parts of Algerian steppes, CaCl₂ is present at higher concentrations than NaCl (Halitim, 1988). In Djelfa province (Algeria), CaCl₂ is the predominant salt in the steppe alkaline soils.

The use of halophytic plants in pasture and fodder production on saline soils is the only economically feasible solution available (Khan and Duke, 2001). *Atriplex* species (saltbushes) are dominant in many arid and semi-arid regions of the world, particularly in habitats that combine relatively high soil salinity with aridity (Ortiz-Dorda et al., 2005). Saltbushes have been used as a fodder for domestic livestock and for rehabilitation of degraded lands (sand dunes, saline/alkaline soils). *Atriplex* spp. are among a group of halophytes that complete their life cycle at high salinity levels and have the ability to accumulate high concentrations of micronutrients – much greater than the required minimum (Wilson et al., 2000; Ramos et al., 2004). *Atriplex* spp. has increased biomass production with salt increments in the growth medium ranging from 5 to 10 g l⁻¹ NaCl (Khan et al., 2000; Nedjimi et al., 2005). A similar promotion of growth has also been reported for other halophytic species (Debez et al., 2004; Ben Amor et al., 2005).

Salinity may decrease biomass production because it lowers plant water potential and causes specific ion toxicities or ionic imbalances in plants (Munns, 2002). Plants protect themselves from salt toxicity by minimizing Na⁺ uptake and its transport to the shoot (Blumwald, 2000; Tester and Davenport, 2003). Plants achieve osmotic adjustment under saline conditions via ion uptake or synthesis of osmotica or both (Parida and Das, 2005).

Halophytes have the ability to accumulate ions, such as Na⁺ and Cl⁻, in the vacuole so that the cytoplasm is maintained at substantially low ion concentrations, thereby avoiding inhibition of metabolic processes (Munns, 2002). One of the most common stress responses in plants is overproduction of different types of compatible organic solutes (Serraj and Sinclair, 2002). Compatible solutes are low molecular weight, highly soluble compounds that are usually non-toxic at relatively high concentrations. Generally, they protect plants from stress through different processes, including via contributing to cellular water economy, detoxification of reactive oxygen species, protection of membrane integrity, and stabilisation of enzymes/proteins (Ashraf and Foolad, 2006). Amino acid proline is known to occur widely in higher plants and normally accumulates in large quantities in response to environmental stresses (Kavi Kishore et al., 2005). In addition to its role as an osmolyte for water economy, proline helps stabilise sub-cellular structures (e.g., membranes and proteins), scavenge free radicals, and buffer cellular redox potential under stress conditions (Ashraf and Orooj, 2006).

Atriplex halimus subsp. *schweinfurthii* (*Chenopodiaceae*) is one of the most abundant perennial halophytes found in Algerian salt steppes in association with *Salsola vermiculata* and *Suaeda fruticosa* (Le Hou rou, 2004). It is reportedly highly tolerant to NaCl (Bajji et al., 1998), but there are no published reports on its tolerance to CaCl₂. This study was conducted to determine the effects of CaCl₂ on growth, water potential and ion accumulation of *A. halimus* subsp. *schweinfurthii* under in vitro conditions. We also investigated the induction of proline – a compatible solute – by salt, which could be responsible for protection against salt stress in this subspecies.

MATERIALS AND METHODS

The seeds of *A. halimus* subsp. *schweinfurthii* were collected from the area of El Mesrane in the province of Djelfa (Algeria) (chott Zahrez zone; 3°03'E longitude, 34°36'N latitude, and 830 m elevation). After removal of the fruiting bracts, seeds were surface sterilised for 30 s in 97% ethanol, followed by a treatments with 0.8% formaldehyde for 40 min and 5% calcium

hypochlorite for 20 min; they were finally rinsed three times with sterile deionised water (Bajji et al., 1998). The seeds were allowed to germinate in Petri dishes on two layers of sterilised filter paper. The filter paper was moistened every 24 h with 5 ml of sterile deionised water.

Five days after germination, seedlings (10 to 15 mm in height) were transferred to test tubes (one seedling per tube) containing 20 ml of Murashige and Skoog (1962) medium supplemented with vitamins of Morel and Wetmore (1951), 0.1mM Fe-EDTA, 20 g l⁻¹ sucrose (source of carbon), 8 g l⁻¹ of Agar (Bactoagar-Difco) and autoclaved for 20 minutes at 120°C and 150 kPa. Medium pH was adjusted to 5.8 before adding KOH (Margara, 1982). The fresh medium was amended with CaCl₂ (0, 4, 8, 12, 16, or 20 g l⁻¹). The seedlings were allowed to grow for 30 days in a controlled-temperature and -photoperiod culture chamber. The temperature was regulated at 25°C ± 1°C under an illumination of 170 μmol s⁻¹m⁻² provided by a series of fluorescent lamps. The photoperiod was 16 h of light and 8 h of darkness. The relative humidity was about 70%. Each treatment was applied to 10 plants in a completely randomized design.

To eliminate accumulation of salt on the surface, the plants were washed with distilled water. Dry mass (shoots and roots) was determined after drying for 48 h in a forced-draft oven at 60°C.

Proline and ion measurements were taken using 0.5 g of plant material that was boiled in 25 ml of water for 2 h at 100°C in a dry-heat bath. This hot-water extract was cooled and filtered using Whatman no. 42 filter paper, and proline concentration was measured according to Bates et al. (1973). One ml of the hot-water extract was diluted with distilled water for ion analysis. Chloride ion content was measured with Beckman specific ion electrode. Flame emission spectrophotometer JENWAY PFP7 model was used to determine the Na⁺ and K⁺ contents. Content of Ca²⁺ was determined via atomic absorption spectrophotometry. Water potential (Ψ_w) was measured using a plant moisture-stress instrument (PMS Instrument Co., Oregon, USA).

The measurements of shoot and root weights, ion contents, water potential, and proline were subjected to one-way analysis of variance (ANOVA) to test differences among means corresponding to CaCl₂ levels. When the general hypothesis was rejected (at $P < 0.05$), Newman-Keuls multiple-range test was applied to study differences among individual treatments via STATISTICA (STATISTICA, 2000). A linear regression of water potential against salinity concentration was conducted.

RESULTS

The one-way ANOVA indicated that both shoot fresh weight ($P < 0.0001$) and root fresh weight ($P < 0.001$) of *A. halimus* subsp. *schweinfurthii* plants were affected by salinity (Figures 1 and 2). Optimal shoot and root fresh weights were recorded at 12 and 16 g l⁻¹ CaCl₂, respectively. These parameters declined with a further increase in salinity (Figure 2).

In addition, salinity significantly affected both shoot dry weight ($P < 0.0001$) and root dry weight ($P < 0.05$). Shoot dry weight peaked at 8 g l⁻¹ CaCl₂, showing a three-fold increase in biomass over the control (Figure 3). Root dry weight was significantly less at 0 g l⁻¹ and 20 g l⁻¹ CaCl₂ concentrations than at the other four concentrations (Figure 3).

Salinity significantly affected succulence in shoot ($P < 0.05$) and root ($P < 0.05$). Succulence, when expressed as a fresh weight/dry weight (FW/DW) ratio, increased in both shoots and roots with increases in salinity. Succulence did not change significantly between 12 and 20 g l⁻¹ CaCl₂ in shoots and between 8 and 20 g l⁻¹ CaCl₂ in roots (Figure 4).

Water potential ($R^2 = 0.93$, $P < 0.001$) of *A. halimus* subsp. *schweinfurthii* plants increased significantly with increased salinity, reaching -3.24 MPa at 20 g l⁻¹ CaCl₂ (Figure 5). Water potential was positively correlated with CaCl₂ concentration.



Figure 1. Effect of CaCl_2 on growth of in vitro plants of *Atriplex halimus* subsp. *schweinfurthii* on Murashige and Skoog (1962) medium; left: 0 g l^{-1} CaCl_2 (control), right: treatment with 8 g l^{-1} CaCl_2 .

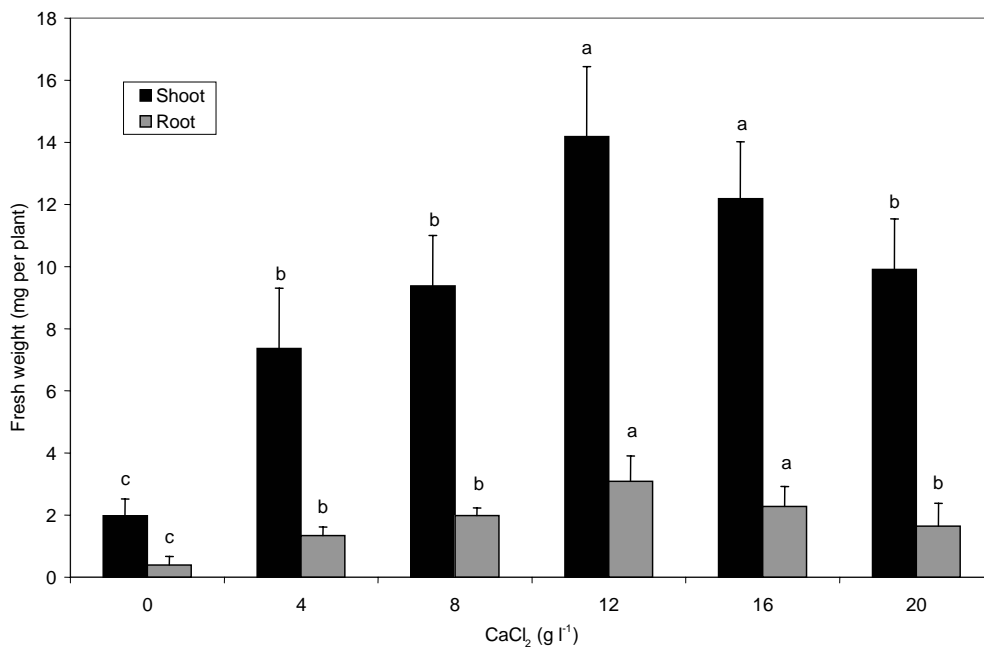


Figure 2. Effect of CaCl_2 ($0, 4, 8, 12, 16,$ and 20 g l^{-1}) on root and shoot fresh weights of *Atriplex halimus* subsp. *schweinfurthii* in vitro plants. Bars represent mean \pm standard error. Different letters above bars represent a significant difference ($P < 0.05$) between treatments.

Salinity significantly affected Ca^{2+} ($P < 0.0001$), Cl^- ($P < 0.0001$), K^+ ($P < 0.0001$), and Na^+ ($P < 0.005$) content of plants. Contents of Ca^{2+} , K^+ and Cl^- increased in both shoots and roots with an increase in salinity. This increase was greater in shoots than in roots (Tables 1 and 2). The sodium content of plants decreased with an increase in salinity (Tables 1 and 2).

Salinity had a significant effect on proline content in shoots ($P < 0.0001$) and roots ($P < 0.0001$). In shoots, proline concentration substantially increased with an increase in

salinity (Figure 6). The results were similar for roots; however, the concentration of proline in roots was significantly lower than that in shoots (Figure 6).

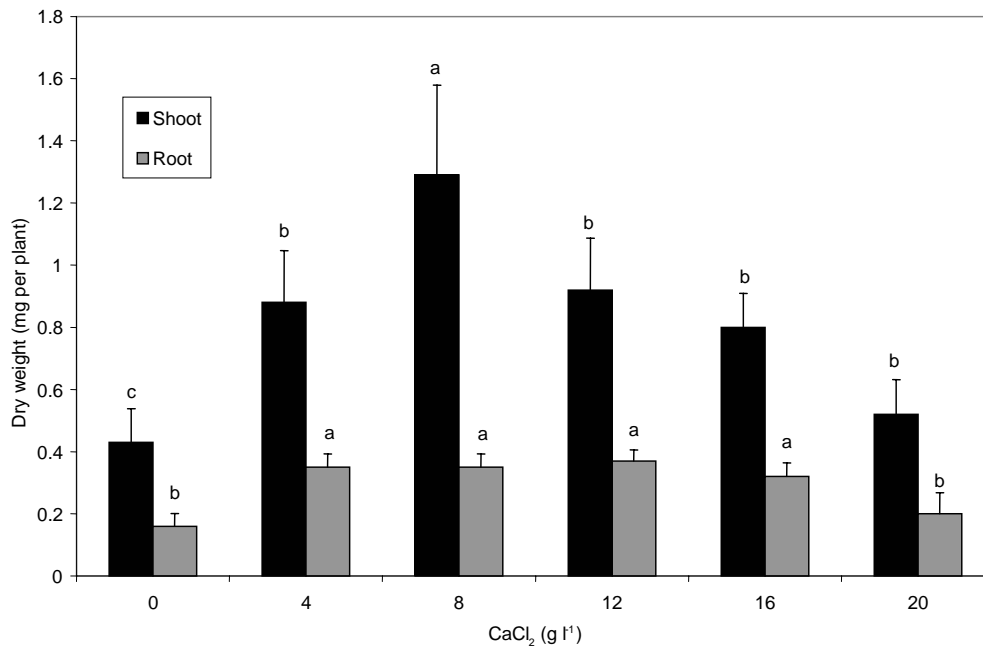


Figure 3. Effect of CaCl₂ (0, 4, 8, 12, 16, 20 g l⁻¹) on dry weight of *Atriplex halimus* subsp. *schweinfurthii* in vitro plants. Bars represent mean \pm standard error. Different letters above bars represent a significant difference ($P < 0.05$) between treatments.

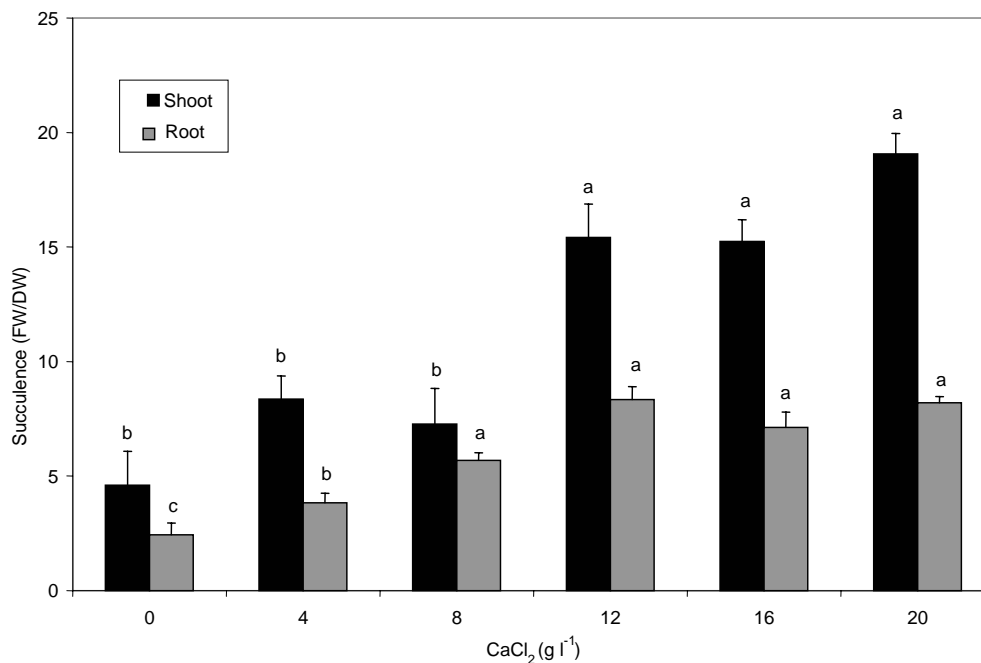


Figure 4. Effect of CaCl₂ (0, 4, 8, 12, 16, and 20 g l⁻¹) on the succulence (FW/DW) of *Atriplex halimus* subsp. *schweinfurthii* in vitro plants. Bars represent mean \pm standard error. Different letters above bars represent a significant difference ($P < 0.05$) between treatments.

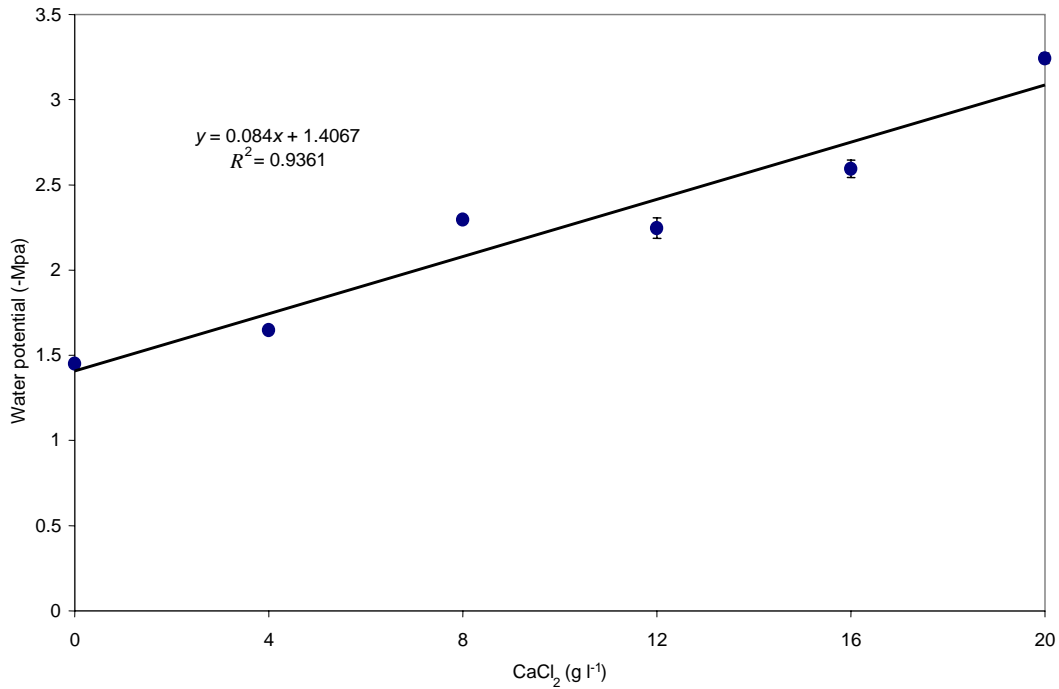


Figure 5. Effect of CaCl₂ (0, 4, 8, 12, 16, and 20 g l⁻¹) on water potential in *Atriplex halimus* subsp. *schweinfurthii* in vitro plants. Linear regression, means and their standard errors are shown.

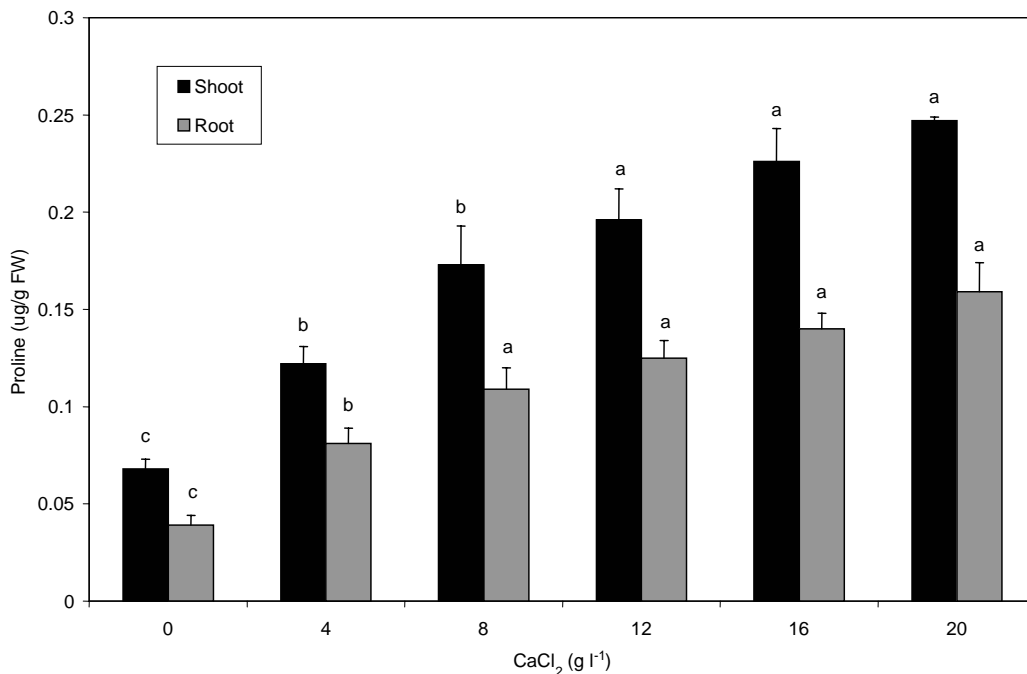


Figure 6. Effect of CaCl₂ (0, 4, 8, 12, 16, and 20 g l⁻¹) on the proline content in *Atriplex halimus* subsp. *schweinfurthii* in vitro plants. Bars represent mean \pm standard error. Different letters above bars represent a significant difference ($P < 0.05$) between treatments.

Table 1. The effect of CaCl₂ on the concentration of cations and anions in shoots of *Atriplex halimus* subsp. *schweinfurthii*. Values represent means ± standard error.

CaCl ₂ (g l ⁻¹)	Na ⁺ [μmol (g FW) ⁻¹]	K ⁺ [μmol (g FW) ⁻¹]	Ca ²⁺ [μmol (g FW) ⁻¹]	Cl ⁻ [μmol (g FW) ⁻¹]
0	102±0.17c*	27±1.13a	54±1.03a	33±0.35a
4	63±0.20b	30±0.09a	75±1.13b	45±0.54b
8	35±0.17a	32±1.00a	80±0.64b	53±0.26b
12	34±0.39a	35±0.88a	93±0.65b	66±0.26c
16	31±0.33a	160±1.00b	130±0.95c	98±0.19d
20	27±0.11a	411±1.03c	185±0.88c	188±0.55d

* Means across rows, followed by different letters, are significantly different at 0.05 probability level according to the Newman-Keuls multiple-range test.

Table 2. The effect of CaCl₂ on the concentration of cations and anions in roots of *Atriplex halimus* subsp. *schweinfurthii*. Values represent means ± standard error.

CaCl ₂ (g l ⁻¹)	Na ⁺ [μmol (g FW) ⁻¹]	K ⁺ [μmol (g FW) ⁻¹]	Ca ²⁺ [μmol (g FW) ⁻¹]	Cl ⁻ [μmol (g FW) ⁻¹]
0	36±1.82c*	27±0.03a	33±0.64a	21±0.54a
4	33±0.10b	27±0.02a	47±0.24b	37±0.64b
8	29±0.12b	32±0.06a	58±0.03b	48±0.13b
12	26±0.17b	35±0.04b	66±0.41b	58±0.61b
16	24±0.15b	40±0.02b	93±0.51c	79±0.38c
20	18±0.68a	222±0.01c	126±0.11d	112±0.41d

* Means across rows, followed by different letters, are significantly different at 0.05 probability level according to the Newman-Keuls multiple-range test.

DISCUSSION

This study showed that *Atriplex halimus* subsp. *schweinfurthii* was a highly salt-tolerant species. Fresh weights of shoots and roots were stimulated at 12 and 16 g l⁻¹ CaCl₂ (Figure 2). The 8 g l⁻¹ CaCl₂ concentration was most conducive to plant growth (dry weight). An important finding was that plants could survive at 20 g l⁻¹ salinity (Figure 3) and this might be a suitable concentration to use to select highly salt-tolerant plants. Studies have shown that *Atriplex* spp., such as *A. nummularia*, *A. griffithii* and *A. hortensis*, could survive under highly saline conditions; with optimal growth occurring at 5 to 10 g l⁻¹ NaCl (Khan et al., 2000; Wilson et al., 2000; Ramos et al., 2004).

Succulence is anatomical adaptation, which, by increasing the vacuolar volume, permits the accumulation of large amounts of water and dissolved ions in both shoots and roots (Munns, 2002). Exposure to salinity concentrations has been shown to increase the tissue water content of halophytes (Khan et al., 2005; Lee et al., 2005). Our results showed that the optimal growth of *A. halimus* subsp. *schweinfurthii* occurred at 8 g l⁻¹ CaCl₂ and that the highest succulence value was achieved at 20 g l⁻¹ CaCl₂ (Figure 4). Water potential of *A. halimus* subsp. *schweinfurthii* plants decreased with an increase in salinity levels (Figure 5). This suggested that the enhancement of the dry matter production from 0 to 8 g l⁻¹ CaCl₂ was due primarily to ion uptake. Halophytes are characterised by their ability to adjust tissue water potential to a level that is lower than that of the soil water potential of their habitat (Zhang et al., 1999; Zhu, 2001).

In halophytes, water content and the ability to make osmotic adjustments have been seen as important determinants of growth response (Maggio et al., 2000; Kurkova et al., 2002; Ben Amor et al., 2005; Benlloch-Gonzalez et al., 2005). It would appear that the growth response at moderate salinities might be largely the consequence of an increased throughput of solutes required to derive cell expansion (Khan et al., 2001). At high salinities, growth reduction might be caused by a reduced ability to make osmotic adjustments as a result of saturation of solute uptake system (Munns, 2002). Other factors, such as nutrient deficiencies (Munns, 2005), may also play an important role. Concentrations of Ca^{2+} and Cl^- in shoots and roots of *A. halimus* subsp. *schweinfurthii* were regulated by ion accumulation, because an increase in external CaCl_2 from 0 to 20 g l^{-1} resulted in a significant increase in internal Ca^{2+} and Cl^- content at higher salinities (Tables 1 and 2). Similar results were reported for *Lycopersicon esculentum* (Caines and Shennan, 1999) and *Triticum durum* (Davenport et al., 1997). Our results indicated that calcium, potassium, and chloride concentration in shoots and roots increased, whereas Na^+ content decreased with an increase in salinity. *Atriplex halimus* subsp. *schweinfurthii* showed higher affinity for K^+ than for Na^+ (Figure 7). This selectivity K^+/Na^+ ratio is considered to be a selection criterion for identifying salt-tolerant plants (Maathius and Amtmann, 1999; Grieve et al., 2004).

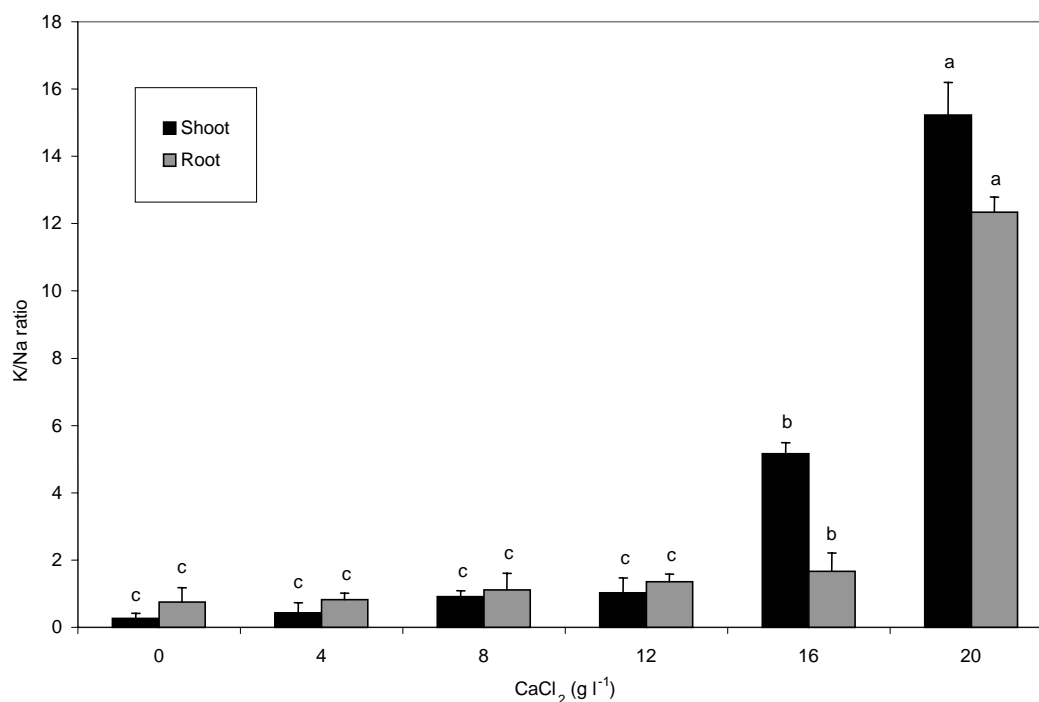


Figure 7. Effect of CaCl_2 (0, 4, 8, 12, 16, and 20 g l^{-1}) on K^+/Na^+ ratio in *Atriplex halimus* subsp. *schweinfurthii* in vitro plants. Bars represent mean \pm standard error. Different letters above bars represent a significant difference ($P < 0.05$) between treatments.

Incorporation of Ca^{2+} into the root environment increases salt tolerance of plants (Epstien, 1998), reduces the absorption of Na^+ through antagonism (Davenport et al., 1997), and increases absorption of K^+ (Epstien, 1998). In addition, Ca^{2+} improves growth of plants subjected to salt stress by facilitating osmotic adjustment (Girija et al., 2002) and modifying intracellular K^+/Na^+ ratio (Grieve et al., 2004).

Halophytes are able to compartmentalise toxic ions in the vacuole under salt stress conditions. This response requires the accumulation in the cytoplasm of alternative, non-toxic "compatible" osmotically active solutes to maintain water economy (Hare et al., 1998). Generally, salt stress induces proline accumulation in many halophytes (Brown and Pezeshki, 2006; Koyro, 2006; Song et al., 2006). Accordingly, a significant accumulation of

proline in shoots and roots of *A. halimus* subsp. *schweinfurthii* was observed (Figure 6). Compatible solutes appear to have additional functions during the stress response, acting as "osmoprotectants" either by direct stabilisation of protein and membrane structures under dehydration conditions or by protecting the cell against oxidative stress as scavengers of reactive oxygen species (Zhu, 2001; Maggio et al., 2002). Proline, which occurs widely in higher plants, accumulates in larger amounts than other amino acids do in salt-stressed plants (Claussen, 2005; Parida and Das, 2005; Ashraf and Foolad, 2006). However, the role of proline in osmoregulation and salt tolerance has been questioned. Lutts et al. (1996) found that proline did not take part in osmotic adjustment in salt-stressed rice (*Oryza sativa* L.) and its accumulation seemed to be related to injury rather than an indication of salt tolerance.

Salt tolerance in *A. halimus* subsp. *schweinfurthii* could involve a delicate balance among ion accumulation, osmotic adjustment, proline production, and maintenance of pressure potential and growth. At relatively high salinities, a significant reduction in growth occurs because of plant's inability to make osmotic adjustments, and specific ion toxicities can cause a significant reduction in growth.

In summary, *Atriplex halimus* subsp. *schweinfurthii* was a highly salt-tolerant perennial halophyte in salt zones of Algerian steppes, where a major issue is the irregularity of fodder resources. This plant has the ability to complete its life cycle under very high saline media. It can accumulate a large amount of Ca^{2+} and Cl^- to achieve osmotic balance across the soil-water-plant gradient.

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