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ORIGINAL ARTICLE

Is salinity tolerance related to osmolytes accumulation in *Lygeum spartum* L. seedlings?

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Abstract *Lygeum spartum* L. (Poaceae) is a plant of commercial relevance used as raw material for manufacturing paper. This species is a newly found salt tolerant species, but its physiological responses to salinity are poorly understood. The effect of salt stress (50 and 100 mM NaCl) on growth, leaf water relations, soluble sugars and free amino acids in *L. spartum* has been investigated. Fresh and dry weights were reduced significantly above 50 mM NaCl. Transpiration, water potential (Ψ_ω) and osmotic potential (Ψ_π) decreased with elevated NaCl. No change was observed in the turgor potential (Ψ_τ). Subsequently, the composition of free amino acids estimated by high pressure liquid chromatography (HPLC) indicated a significant increase in free amino acid content. It appears that valine was the main amino acid accumulated significantly by the plants for both NaCl treatments. However, tyrosine levels decrease by salt treatment compared to control. Contents of Na^+ and Cl^- increased with an increase in salinity. The concentration of Na^+ of salinized plants (100 mM NaCl) was ~70-fold greater than that measured in control plants, and this was associated with significant reductions in leaf K^+ and Ca^{2+} concentrations. In addition, a significant accumulation of soluble sugars, probably associated with osmotic adjustment and protection of

Abbreviations: HPLC, high pressure liquid chromatography; Ψ_π, osmotic potential; Ψ_τ, turgor potential; Ψ_ω, water potential.

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membrane stability, occurred in roots of salinized plants. Based upon these results, a possible physiological role of soluble sugars and free amino acids was suggested in *L. spartum* to maintain turgor.

1. Introduction

Salinity is an important factor limiting agricultural productivity in arid and semiarid regions of Algeria (Nedjimi and Daoud, 2009a). Reclaiming these lands for forage crops is too costly for most countries to afford. A declining base of arable farmland and an increasing demand for forage and fibre warrant the need for utilization of the naturally salt tolerant species in irrigated and non-irrigated agriculture (Reddy et al., 2008).

Plants exposed to salt stress undergo changes in their environment. The ability of plants to tolerate salt is determined by the multiple biochemical pathways that facilitate retention and/or acquisition of water, protect chloroplast functions, and maintain ion homeostasis. Essential pathways include those that lead to synthesis of osmotically active metabolites, specific proteins, and certain free radical scavenging enzymes that control ion and water flux and support scavenging of oxygen radicals (Parida and Das, 2005).

Many plant species respond rapidly to stressors by increasing the concentration of compatible solutes involved in osmoregulation and in protection of proteins and membranes in conditions of low water potential (Munns and Tester, 2008).

The direct domestication of salt tolerant plants represents an alternative approach to expand cultivation onto unfavourable land, and it can be envisioned as a strategy complementary to the genetic engineering of salt tolerance in glycophytes. Therefore, for most species, their potential economic use as cultivated plants and their adaptability to agronomic conditions have not been fully assessed.

Lygeum spartum L. (Poaceae) is a native species in the Algerian salt steppes (Fig. 1). The plant is of interest because of its tolerance to environmental stresses and its use as a fodder grass for livestock in low-rainfall Mediterranean areas (Nedjimi, 2009). This species can tolerate extreme conditions of aridity, salinity and high temperatures (Le Houérou, 1995). Its extensive root system plays a significant role in preventing desertification by stabilizing the sand (Pugnaire and Haase, 1996). It may thus be a suitable candidate for the phytostabilization of acid mine tailings (Conesa et al., 2007). It has been used as a fodder for domestic livestock and for rehabilitation of degraded lands (gypsum and calcareous soils) (Garcia-Fuentes et al., 2001). In North Africa, it was used as raw material for manufacturing paper (FAO, 1992).

Figure 1 *Lygeum spartum* L. (Poaceae).

L. spartum adapts very well to arid and semi-arid soils and shows high growth capacity under these conditions (Nedjimi, 2009; Nedjimi et al., 2010). However, physiological response of *L. spartum* to salt stress is not well understood although salinity is often associated with the environment where it is grown. In previous works, Nedjimi (2009) demonstrated *L. spartum* as moderately tolerant to salinity. Until now, such a comprehensive investigation on *L. spartum* possible compatible solutes has not been done. The hypotheses tested were (1) that free amino acid accumulates in stressed *L. spartum* to high levels which can be important for osmotic adaptation, and (2) that salinity was able to induce soluble sugar accumulation in this species. The study aims to add arguments in favour of a significant contribution of osmolytes to the salinity tolerance of *L. spartum*.

2. Materials and methods

2.1. Plant material and growth conditions

The seeds of *L. spartum* were collected from the area of *Aïn Maâbed* in the province of *Djelfa* (Algeria) (2°39′E longitude, 34°50′N latitude and 934 m elevation). Seeds were pre-hydrated with aerated, de-ionised water for 12 h and germinated in vermiculite, at 28 °C in an incubator, for 2 d. They were then transferred to a controlled-environment chamber with a 16 h light–8 h dark cycle and air temperatures of 25 and 20 °C, respectively. The relative humidity (RH) was 60% (day) and 80% (night) and photosynthetically active radiation (PAR) was 400 μmol m^{-2} s^{-1}, provided by a combination of fluorescent tubes (Philips TLD 36 W/83, Germany and Sylvania F36 W/GRO, USA) and metal halide lamps (Osram HQI.T 400 W, Germany).

After 7 d, the seedlings were placed in 15-L containers (six plant per container) with continuously aerated, modified Hoagland nutrient solution (Hoagland and Arnon, 1938): $Ca(NO_3)_2$ (2 mM), K_2HPO_4 (0.5 mM), $MgSO_4$ (0.5 mM), H_3BO_3 (25 μM), $MnSO_4$ (2 μM), $ZnSO_4$ (2 μM), $CuSO_4$ (0.5 μM), $(NH_4)_6Mo_7O_{24}$ (0.5 μM), Fe-EDDHA [Fe-ethylendiamino-di(o-hydroxyphenylacetic) acid] (20 μM).

The solution was replaced completely every week. After 13 d (when plants were 20 d-old), plants were treated with 0, 50 and 100 mM NaCl. The experiment was set up as a "Completely Randomized Design". Each treatment was replicated five times and each replicate included six plants (i.e., 30 plants per treatment). Dry and fresh weights, water potential (Ψ_ω), osmotic potential (Ψ_π), turgor potential (Ψ_τ) and amino acids were measured after 30 d of the treatments, when plants were 50 d.

2.2. Measurement of fresh and dry weights

Plants were harvested and total fresh weight (FW) was determined. The total dry weight (DW) was measured after the samples had been dried at 65 °C for 72 h.

2.3. Plant transpiration

For plant transpiration measurement, each pot containing one plant was covered with a plastic bag, secured around the stem base. The water transpired was estimated under controlled light from the weight loss over a 6-h period (10:00–16:00 h) corresponding to high natural sunlight period. The mean transpiration rate (per g FW) was calculated based on the amount of transpired water and total fresh weight at sampling time.

2.4. Leaf water relations

The leaf water potential (Ψ_ω) of the most recent fully-expanded leaves was measured using the pressure chamber technique (Turner, 1988). The same leaves were put into plastic bags and rapidly frozen with liquid nitrogen. They were subsequently thawed and pressed to extract the cell sap. The osmotic potential (Ψ_π) of the leaf sap was calculated after measuring sap osmolarity using an automatic, freezing-point depression osmometer (Digital Osmometer, Roebling, Berlin), by van't Hoff equation (Nobel, 1991):

$$\Psi\pi = nRT.$$

where n = mosmol, R = 0.083 and $T = t^{a}$ ambient temperature (K).

Turgor potential (Ψ_τ) was calculated as the difference between leaf water potential and osmotic potential.

2.5. Chemical analysis

Leaves samples were put in Eppendorf tubes with holes at the bottom and rapidly frozen with liquid nitrogen. These tubes were then centrifuged twice into assay tubes, at 4000 *g* for 4 min (4 °C), in such a way that all the sap was extracted from the samples. For the ion analysis, 25 μL of cell sap was filtered, diluted and injected into a Dionex-D-100 ion chromatograph with an Ionpac AS124-4 mm (10–32) column and an AG 14 (4 × 50 mm) guard column. Chloride (Cl^-) was measured with Chromeleon/Peaknet 6.40 chromatography software, by comparing peak areas with those of known standards. The sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) concentration in the cell sap, determined by atomic absorption spectrometry (905AA, GBC, Australia), was measured for extract aliquots diluted with a $LaCl_3$ + CsCl solution.

2.6. Free amino acids analysis

The analyses were carried out with an HPLC/MS system consisting of an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA), μ-well plate auto-sampler and a capillary pump, connected to an Agilent Ion Trap XCT Plus Mass Spectrometer (Agilent Technologies) using an electrospray (ESI) interface.

Standards, with known concentrations of each amino acid (0.1, 0.5, 1, 10, 25 and 50 μM), and samples were prepared in the mobile phase A, consisting of water/acetonitrile/formic acid (89.9:10:0.1), and passed through 0.22-μm filters. Then, 5 μl of each standard or sample was injected onto a Zorbax SB-C18 HPLC column (5 μm, 150 × 0.5 mm, Agilent Technologies), thermostatted at 40 °C, and eluted at a flow rate of 5 μl min^{-1}. The mobile phase B, consisting of water/acetonitrile/formic acid (10:89.9:0.1), was used for the chromatographic separation. The elution consisted of 5 min of 0% B, a linear gradient from 0% to 10% B in 10 min and 10% B for 5 min. The column was equilibrated with the starting composition of the mobile phase for 20 min before each analytical run. The UV chromatogram was recorded at 210 nm with the DAD module (Agilent Technologies).

The mass spectrometer was operated in the positive mode, with a capillary spray voltage of 3500 V and a scan speed of 26,000 $(mz^{-1})\,s^{-1}$ from 50 to 250 mz^{-1}. The nebuliser gas (He) pressure was set to 15 psi and the drying gas was set to a flow of 5 l min^{-1}, at 350 °C.

The chromatogram of each amino acidic ion from both standards and samples was extracted and the peak area was quantified using the Data Analysis programme for LC/MSD Trap Version 3.2 (Bruker Daltonik, GmbH, Germany). The peak area data of the standards were used for calculation of the calibration curve, from which the concentrations of each amino acid in the samples were obtained.

2.7. Soluble sugars analysis

Total soluble sugars content in leaf sap was measured according to the phenol–sulfuric acid of Dubios et al. (1956) method.

2.8. Data analysis

Data were analysed statistically, using the SPSS 7.5 software package, by ANOVA and by Tukey's multiple range test, to determine differences between means.

3. Results

Fresh weight ($P < 0.001$) and dry weight ($P < 0.01$) of *L. spartum* plants were affected by salinity. Growth parameters mentioned above decreased significantly at high NaCl concentration. These parameters did not change significantly at 50 mM NaCl (Table 1). In addition, transpiration in *L. spartum* L. seedlings declined significantly ($P < 0.01$) with the increase of salinity (Table 1).

Analysis of leave sap revealed that the water potential (Ψ_ω) ($P < 0.001$) and osmotic potential (Ψ_π) ($P < 0.01$) were decreased by the high-NaCl treatment (100 mM NaCl) (Fig. 2). However, there were no significant differences ($P > 0.05$) between the turgor potential (Ψ_τ) values of the control and treated plants (Fig. 2).

Salinity significantly affected Na^+ ($P < 0.0001$), K^+ ($P < 0.0001$), Ca^{2+} ($P < 0.0001$), and Cl^- ($P < 0.005$) contents of plants. Contents of Na^+ and Cl^- increased with an increase in salinity. The concentration of Na^+ in leaves sap of plants treated with 100 mM NaCl was ~70-fold greater than that measured in control plants. Seedlings also accumulated significantly higher Cl^- than their corresponding controls (Fig. 3). The calcium (Ca^{2+}) and potassium (K^+) contents of plants decreased with an increase in salinity (Fig. 3).

Table 1 Effects of NaCl on FW and DW, transpiration and of *Lygeum spartum* L. grown in hydroponic conditions. Values represent means ± standard error ($n = 5$).

NaCl (mM)	FW (g plant^{-1})	DW (g plant^{-1})	Transpiration (g H_2O g plant^{-1})
0	1.61 ± 0.19 a	0.35 ± 0.05 a	0.88 ± 0.15 a
50	0.81 ± 0.22 ab	0.29 ± 0.03 ab	0.65 ± 0.13 b
100	0.67 ± 0.27 b	0.22 ± 0.03 b	0.49 ± 0.05 c

Different letters in the same column indicate significant difference at the 5% level according to the Tukey's multiple range test.

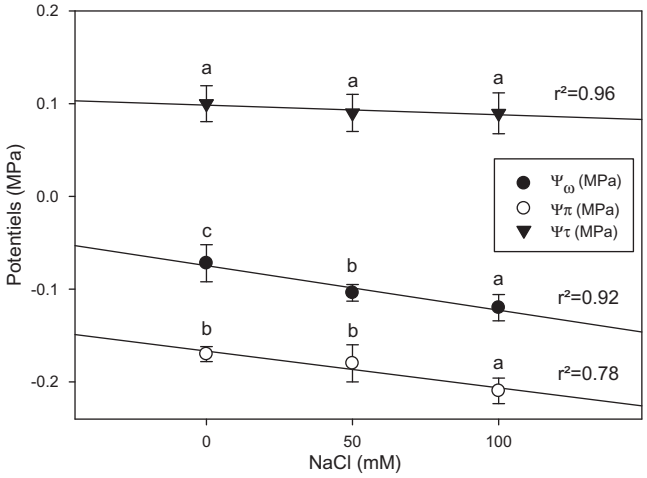

Figure 2 Effects of NaCl on water potential (Ψ_ω), osmotic potential (Ψ_π) and turgor potential (Ψ_τ) of *Lygeum spartum* L. grown in hydroponic conditions. Data represent means ± SE ($n = 5$). Values with different letters are significantly different ($P < 0.01$, Tukey's test).

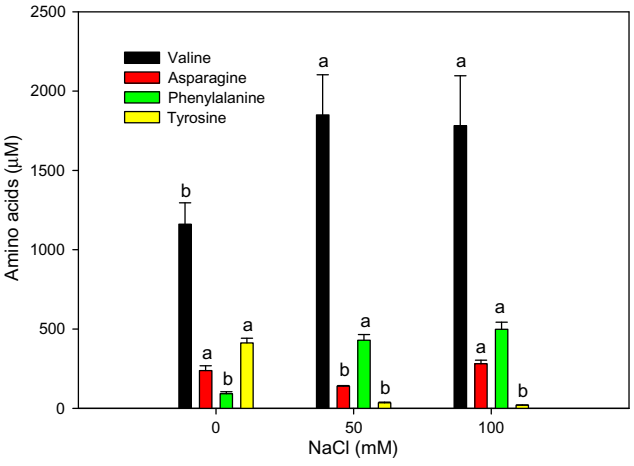

Figure 4 Effects of NaCl on amino acids content of *Lygeum spartum* L. grown in hydroponic conditions. Data represent means ± SE ($n = 5$). Values with different letters are significantly different ($P < 0.01$, Tukey's test).

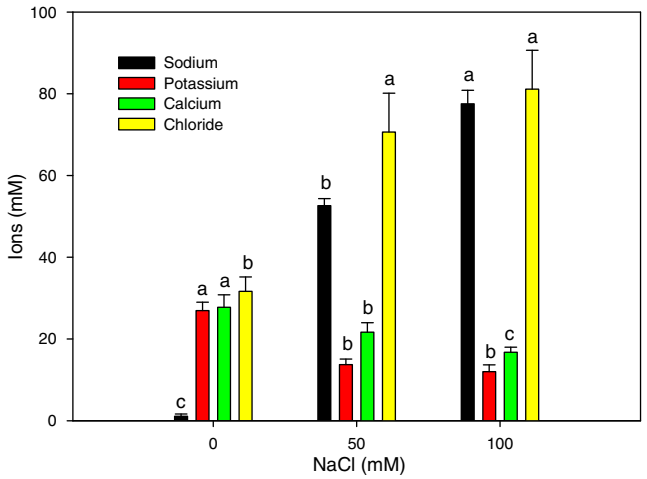

Figure 3 Effects of NaCl on Na^+, K^+, Ca^{+2} and Cl^- contents of *Lygeum spartum* L. grown in hydroponic conditions. Data represent means ± SE ($n = 5$). Values with different letters are significantly different ($P < 0.01$, Tukey's test).

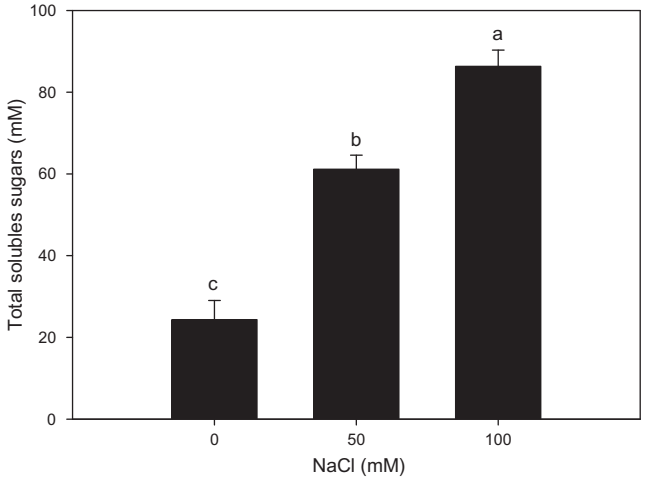

Figure 5 Effects of NaCl on total soluble sugars content of *Lygeum spartum* L. grown in hydroponic conditions.

Amino acids were also analysed in the extract cell sap. When individual amino acids were studied, only the amounts of some of them changed as a consequence of the treatments (Fig. 4). Valine was a major amino acid increased significantly for both NaCl treatments. An increase in phenylalanine was also observed for both treatments, compared with control plants. However, a general and similar decrease in tyrosine levels occurred in the saline treatments compared to control (Fig. 4).

After 30 d of treatments, salinity had a significant effect on total soluble sugars content in *L. spartum* plants ($P < 0.05$).

Total soluble sugars content substantially increased with an increase in salinity (Fig. 5).

4. Discussion

The present study showed that FW and DW of *L. spartum* L. were decreased significantly with the increase in NaCl treatments (Table 1). Similar results have been reported for other grasses (Bai et al., 2008; Gulzar and Khan, 2006; Muscolo et al., 2003). Thus, the growth reduction observed in plants subjected to salt stress often results from direct effects (toxicity of ions accumulated in tissues) and/or from indirect effects (limitation of mineral and water acquisition) (Nedjimi and Daoud, 2009b; Nedjimi, 2009).

Transpiration rates of our plants decreased significantly with the increase of salinity (Table 1). This may be due to the possibility that lowered water potentials in the roots can trigger a signal from root to shoot. This signal might be hydrostatic or involve organic molecules such as ABA, as previous authors have claimed (Zhang and Davies, 1991).

Osmotic adjustment involves the net accumulation of solutes in cells in response to a fall in the water potential of their environment. As a consequence of this net accumulation, the cell osmotic potential is lowered and turgor pressure tends to be maintained (Marcum and Murdoch, 1992). The reductions observed in osmotic potential (Ψ_π), when treatments were applied, were well related to reductions in water potential (Ψ_ω), but had no effect on turgor (Fig. 2). The fact that there were no changes in turgor in the plants, in response to the treatments of our experiment, indicates a certain level of osmotic adjustment (Munns, 2002; Nedjimi et al., 2010).

The presence of high concentrations of Na^+ and Cl^- in the nutrient solution produced a high uptake of these ions and contributed to their increased flux into the xylem (Fig. 3), suggesting that these were the inorganic solutes involved in osmotic adjustment (Munns and Tester, 2008). This effect has been reported in other Poaceae as well, e.g., in *Sporobolus virginicus* (Marcum and Murdoch, 1992), *Oryza sativa* (Alam et al., 2002), *Hordeum vulgare* (Shabala et al., 2005) and *Iris lactea* (Bai et al., 2008).The reduction of growth induced by salinity (Table 1) was probably associated with the toxic effect of the accumulation of Na^+ and Cl^- in plant tissues and reduction of absorption of K^+ and Ca^{2+}. High Na^+ concentration interferes with intracellular K^+ and Ca^{2+} accumulation presumably by competing for the same sites of influx (Tester and Davenport, 2003. Therefore, the key for tolerance might be synchronisation between the high rate of ion transport to the shoot and ion compartmentation by the leaf cells (Munns et al., 2006).

In our experiments, *L. spartum* accumulated a large amount of Na^+ and Cl^- ions and lower amount of Ca^{2+} and K^+. First the similar physicochemical structures of Na^+ and K^+ mean that Na^+ competition at transport sites for K^+ entry into the symplast may result in K^+ deficiency. Secondly, cytoplasmic Na^+ competes for K^+ binding sites and hence inhibits metabolic processes that crucially depend on K^+ (Maathius and Amtmann, 1999). Under saline conditions, high levels of external Na^+ not only interfere with Ca^{2+} acquisition by the roots, but also may disrupt the integrity of root membranes and alter their selectivity (Grattan and Grieve, 1999). Since maintaining an adequate supply of Ca^{2+} in saline soil solutions is an important factor in controlling the severity of specific ion toxicities, particularly in crops which are susceptible to Na^+ and Cl^- injury (Nedjimi and Daoud, 2009b).

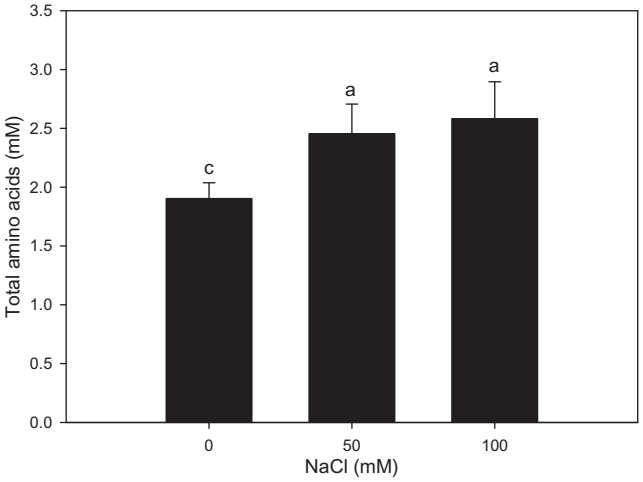

Figure 6 Effects of NaCl on total amino acids content of *Lygeum spartum* L. grown in hydroponic conditions. Data represent means ± SE ($n = 5$). Values with different letters are significantly different ($P < 0.01$, Tukey's test).

Osmotic adjustment, which is necessary for growth in a saline environment, may be accomplished by accumulation of inorganic and organic solutes. Inorganic ions are believed to be sequestered in the vacuoles, while organic solutes are assumed to be compartmentalised in the cytoplasm to balance the low osmotic potential in the vacuole (Munns and Tester, 2008). Massive accumulation of free amino acids under salt stress has been reported in many graminaceous species (Lutts et al., 1999; Morant-Manceau et al., 2004; Wang et al., 2007). In the present experiment with *L. spartum*, salinity increased the total amino acids, compared with the control plants (Fig. 6). However, when individual amino acids were studied, only an increase in leaf sap valine concentrations, to values nearly double those observed in control plants were reported (Fig. 5). The increase of valine was positively related to Cl^- ($r = 0.93$, data not shown) and Na^+ ($r = 0.85$, data not shown) accumulations.

Amino acids have been reported to accumulate in higher plants under salinity stress (Ashraf, 1994). The important amino acids include alanine, arginine, asparagines, glycine, serine, and leucine, together with the amino acid, proline, and the non-protein amino acids, citrulline and ornithine (Ashraf and Harris, 2004). Valine was also been reported to accumulate in plants in response to salt stress such as strawberry (Keutgen and Pawelzik, 2008), *Beta vulgaris* (Gzik, 1996) and *Phragmites australis* (Hartzendorf and Rolletschek, 2001).

Free amino acid accumulation in plants under salt stress has often been attributed to alterations in biosynthesis and degradation processes of amino acids and proteins (Hare et al., 1998). Considering that salinity increased the free amino acid content in sap leaves (Fig. 6), our results could be related to an increase in amino acid degradation or inhibition in synthesis jointly with reductions in degradation or increases in protein synthesis.

In this work, the contribution of total soluble sugars accumulation to osmotic adjustment was significant, since the total

soluble sugars content increased with an increase in salinity (Fig. 5). Similar results were obtained by Morant-Manceau et al. (2004), who reported that the concentrations of sugars change in response to salt stress in *Triticum dicoccum*. Carbohydrates such as soluble sugars (glucose, fructose, sucrose, fructans) accumulate under salt stress to accommodate the ionic balance in the vacuoles (Parida and Das, 2005; Ashraf and Harris, 2004). Their major functions are osmoprotection, osmotic adjustment, carbon storage, radical scavenging and stabilization of the structure of proteins such as Rubisco (Rejsiková et al., 2007). The activity of sucrose phosphate synthase increases under salt stress, whereas starch phosphorylase activity decreases (Dubey and Singh, 1999). Soluble sugar accumulation may be due to further transformation of starch to sugars or less consumption of carbohydrates by the tissues in saline conditions (Hare et al., 1998).

To conclude, the strategies of salt tolerance in *L. spartum* involve a delicate balance among ion accumulation, osmotic adjustment, soluble sugars and amino acids production and maintenance of pressure potential. Further studies on enzymatic and growth hormones are required to elucidate the biochemical mechanisms implied.

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