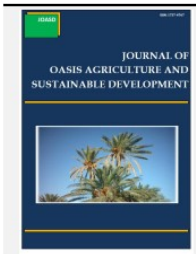

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The effects of increasing salinity levels on *Sulla carnosa* photosynthesis are mainly of stomatal nature

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Abstract

Sulla carnosa Desf. plants were subjected to 0, 100, 200, and 300 mM NaCl for 40 days. Leaf and stem growth as well as root and leaf water contents were not affected even at 300 mM NaCl, confirming the halophytic nature of this species. An accumulation of Na^+ in roots and at a higher magnitude in leaves together with a decline in K^+ concentrations suggest that Na^+ involvement in osmotic adjustment as a cheap osmoticum. This typical halophytic response together with the reduced transpiration rate by stomata closure may explain the ability of *S. carnosa* to maintain its water status. Interestingly, the stomatal limitations of photosynthetic activity did not affect F_v/F_m, F_0, and PSII energy distribution to photochemical process [Y(II)], regulated non-photochemical quenching [Y(NPQ)], and non- regulated non-photochemical quenching [Y(NO)], which suggests a high ability of this halophyte to cope with the energy expected to exceed the demand for its photosynthesis.

1. INTRODUCTION

Soil salinity is one of the most global threats affecting about 20% of the irrigated land (*c.a.* 6% of the total land) (Qadir et al., 2014; Gul et al., 2019). Although salt-affected soils greatly differ in their salinization level and the nature of accumulated salts (Arzani, 2008), it is assumed that sodium (Na^+) salts, mainly sodium chloride (NaCl), are the most widespread types (Arzani and Ashraf, 2016). Soil salinity is continuously decreasing crop yields due to its detrimental effects on plant growth and development through water deficit, cytotoxicity (excessive accumulation of salt ions within cells), nutritional disturbances, and oxidative stress (Isayenkov and Maathuis, 2019). Unfortunately, most crop plants are salt-sensitive and referred to as 'glycophytes' in comparison with 'halophytes' that are able to complete their life cycle in saline environments where about 99% of salt sensitive species cannot survive because of high salt concentrations (Panta et al., 2014).

Since plant biomass production is proportional to CO_2 assimilation (Pan et al., 2020), maintaining the performance of the photosynthetic apparatus is among the most important mechanisms evolved by plants to overcome salt stress. Hence, 'salt excluders' tend to reduce the rate of salt ion accumulation in photosynthetic organs, whereas 'salt includers' translocate salt ions to shoots where they are well-sequestrated and consequently used in osmotic adjustment as cheap osmotica (Mian et al., 2011). But, once the compartmentalization process is disturbed, salt ions substantially accumulate in the cytosol and chloroplast stroma, and consequently affect leaf photochemistry (Pan et al., 2020). Salt-induced limitations in the photosynthetic processes are of two different kinds: (i) stomatal limitations due to stomata closure that reduces CO_2 uptake and

increases photorespiration in C_3 plants, and (ii) non-stomatal limitations due to the inhibiting effects of both salt ions and the salt-induced overproduced reactive oxygen species (ROS) on metabolic processes (Percey et al., 2016). These detrimental effects of salinity on photosynthesis often observed in glycophytes are less pronounced or absent in halophytes under moderate salinity conditions, and photosynthetic activity is even improved in some obligate halophytes at optimal salt concentrations (Rabhi et al., 2012).

The halophytic legume *Sulla carnosa* Desf. is endemic to Tunisia and Algeria (Chennaoui-Kourda et al., 2007). Under moderate salinity/sodicity, it was shown to desalinize the soil that it is grown on by diluting Na^+ within its shoot tissues (Jlassi et al., 2013). The aim of the present investigation was to study the ability of *S. carnosa* to maintain the integrity of its photosynthetic apparatus under increasing salinity levels.

2. MATERIAL AND METHODS

2.1. Plant material and culture conditions

Seeds of *S. carnosa* were collected from Karkar sabkha (Centre-East of Tunisia). They were mechanically scarified then sown on inert sand and irrigated with tap water for 25 days. Thereafter, they were irrigated with a diluted Hewitt's (1966) nutrient solution. On the 42nd day after sowing, 4 treatments were applied: 0 (control), 100, 200, and 300 mM NaCl.

Gas exchange measurements. – After 39 days of treatment, net CO_2 assimilation rate (*A*), transpiration rate (*E*), and stomatal conductance (*gs*) measurements were performed at 10:00-12:00 am in fully-expanded leaves using a portable Licor gas analyzer (LC pro+). Water use efficiency (*WUE*) was calculated as follows: $WUE = A/E$.

2.2. Chlorophyll *a* fluorescence measurements

Chlorophyll *a* fluorescence parameters were also measured at the 39th day of treatment with a mini-PAM fluorometer (Walz). Fully-expanded leaves were dark-adapted for 30 min at ambient temperature and concentrations of oxygen (O_2) and carbon dioxide (CO_2), then minimal (F_0) and maximal (F_m) fluorescence values in the dark-adapted state as well as the maximum photochemical efficiency of PSII [$F_v/F_m = (F_m - F_0)/F_m$] were measured. Thereafter, leaves were light-adapted at 100-150 µmol photons m^{-2} s^{-1} for several minutes before measuring the steady-state fluorescence (F_s), the maximal fluorescence in the light-adapted state (F_m'), and the PSII operating efficiency [ϕPSII or Y(II) = $(F_m' - F_s)/F_m'$]. Two additional parameters, the regulated non-photochemical quenching [Y(NPQ) = $(F_s/F_m') - [(F_s/F_m)]$] and the non-regulated non-photochemical quenching [Y(NO) = F_s/F_m], were calculated from F_m, F_s, and F_m'. It is important to note that Y(II)+Y(NPQ)+Y(NO) = 1 (Klughammer and Schreiber, 2008).

2.3. Plant harvest and mineral analyses

On the 40th day of treatment, plants were harvested and rinsed with distilled water then cut into leaves, stems, and roots. All samples were dried with filter paper then weighed fresh and after oven-drying at 60°C until constant weight. For potassium (K^+) and sodium (Na^+) analyses, samples were ground into fine powder with porcelain mortar and pestle then digested with a 0.5% HNO_3 solution and filtered. K^+ and Na^+ concentrations were determined by a Corning 480 flame photometer (Deal, 1954)

2.4. Statistical analysis

Statistical analysis was carried out using the software package SPSS version 21.0 (SPSS Inc. Chicago, USA). Differences between treatments at a given salinity level were determined by a One-Way ANOVA according to Duncan's multiple range tests ($p \leq 0.05$ and by using a Student's t-test ($p \leq 0.05$).

3. RESULTS

3.1. Plant growth and tissue hydration

Each control plant showed in average a whole dry weight of 2.38 g distributed as follows: 1.09 g in leaves, 0.31 g in stems, and 0.98 g in roots (Table 1). Leaf growth was significantly reduced specifically at 300 mM NaCl with 38% of decrease relatively to control plants. At the same time, leaf tissue hydration was not significantly affected by salt

Table 1. Effects of increasing salinity levels (0, 100, 200, and 300 mM NaCl) on dry weights (DW) and water contents (WC) in *S. carnosa* plants.

NaCl (mM)	0	100	200	300
Leaf DW (g)	1.09 b	0.95 ab	0.79 ab	0.68 a
Leaf WC (ml H_2O. g^{-1} DW)	10.34	12.18	11.25	10.05
Stem DW (g)	0.31 ab	0.41 b	0.23 a	0.18 a
Stem WC (ml H_2O. g^{-1} DW)	13.20 b	9.31 a	8.33 a	8.10 a
Root DW (g)	0.98 c	0.62 b	0.26 a	0.36 a
Root WC (ml H_2O. g^{-1} DW)	9.39	8.50	9.49	7.16
Whole plant DW (g)	2.38 b	1.98 b	1.28 a	1.21 a
Root/shoot ratio	0.70 c	0.50 bc	0.26 a	0.46 ab

Values are means of 8 replicates. In each row, means followed by common letters are not significantly different according to Duncan's test at α=0.05

treatments. Under saline conditions, stem growth did not show a significant change but stem water content was reduced by 29-39%. As regards roots, their dry weight was decreased by 37% under 100 mM and up to 73% under higher salt concentrations, but no significant variation was recorded in their water content. Hence, the whole dry weight was affected only under 200 and 300 mM NaCl with a marked decline in root/shoot ratio.

3.2. K^+ and Na^+ concentrations and K^+/Na^+ ratio

Fig. 1 shows the variations of K^+ and Na^+ concentrations as well as K^+/Na^+ ratio in leaves, stems, and roots of *S. carnosa* under increasing salinity levels, all expressed as Log2(treatment/control). In leaves and roots, it is evident that all salt treatments led to a significant decrease in K+ concentrations, accompanied by a notable increase in Na+ accumulation, with a more pronounced effect observed in leaves compared to roots. Consequently, K^+/Na^+ ratio was substantially decreased in both organs, the effect being more pronounced in leaves. In stems, these parameters did not show significant changes under the overall salt treatments.

Under 100 mM NaCl, both K^+ and Na^+ concentrations significantly increased in stems, resulting in a significant variation in K^+/Na^+ ratio especially at 300 mM NaCl. Beyond this salt concentration, K^+ concentration was stabilized under control treatment, while Na^+ concentration significantly increased but with a lower magnitude than those observed in leaves and roots.

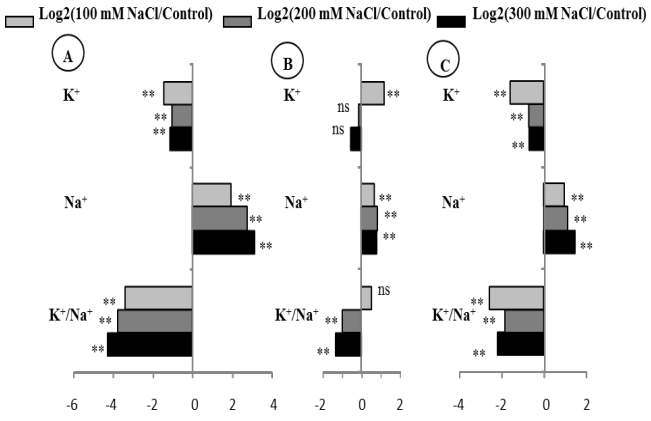

Fig. 1. Effects of increasing salinity levels (0, 100, 200, and 300 mM NaCl) on K^+ and Na^+ concentrations and K^+/Na^+ ratio in leaves (A), stems (B), and roots (C) of *S. carnosa* plants. Data are presented as Log2 (treatment/control).

*Bars are means of 5 replicates. ns – no significant difference with the control; ** –P≤0.01 according to Student's t test.*

3.3. Gas exchange

Gas exchange was substantially affected by salinity (Fig. 2). Net CO_2 assimilation rate (*A*) was diminished by 48-60% under saline conditions with no significant difference between 100, 200, and 300 mM NaCl treatments. A more pronounced decline of 62-70% was recorded in stomatal conductance (*gs*) under saline conditions. Transpiration rate (*E*) decreased significantly by 61% with the increasing salinity under 300 mM NaCl.However, although the substantial effects of salinity on *A*, no improvement was observed in water use efficiency (*WUE*).

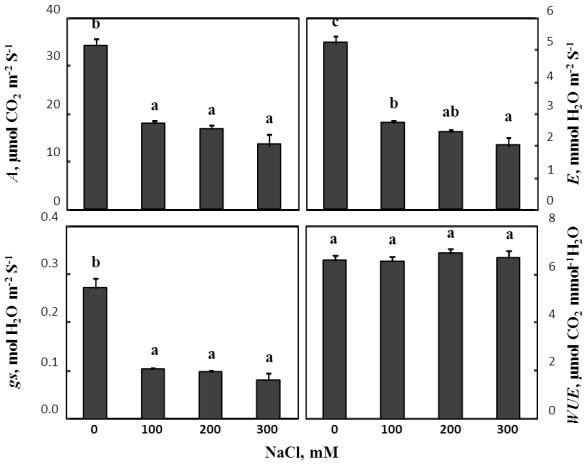

Fig. 2. Effects of increasing salinity levels (0, 100, 200, and 300 mM NaCl) on gas exchange parameters in *S. carnosa* plants.

Bars are means of 5 replicates ± SE. Those sharing common letters are not significantly different according to Duncan's test at α=0.05. A– net CO_2 assimilation rate; E– transpiration rate; gs– stomatal conductance; WUE– water use efficiency.

3.4. Chlorophyll a fluorescence

F_v/F_m ratio was maintained at 0.79-0.80 under 0 and 100 mM NaCl and increased slightly but significantly at higher concentrations (Table 2). This was mainly due to a decrease in F_0 values under 200 and 300 mM NaCl as F_m showed no significant differences between treatments.

Table 2. Effects of increasing salinity levels (0, 100, 200, and 300 mM NaCl) on chlorophyll *a* fluorescence parameters in *S. carnosa* plants.

NaCl (mM)	0	100	200	300
F_0	573 b	521 ab	476 b	500 b
F_m	2744 a	2586 a	2548 a	2728 a
F_v/F_m	0.79 a	0.80 a	0.81 b	0.82 b

Values are means of 8 replicates. In each row, means followed by common letters are not significantly different according to Duncan's test at α=0.05.

PSII energy distribution indicated that photochemical process [Y(II)] was not affected by salinity and used 62-65% of the energy (Fig. 3). Interestingly, the energy lost *via* the non-regulated non-photochemical quenching [Y(NO)] was reduced from 10% in the control to 6-7% in salt-treated plants. The safer regulated non-photochemical quenching [Y(NPQ)] exhibited a slight increase under 200 mM NaCl.

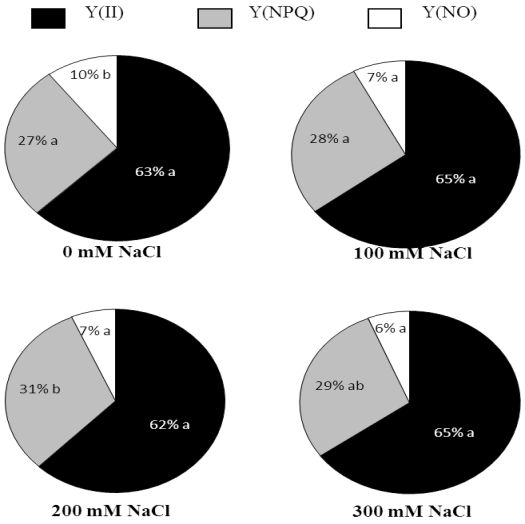

Fig. 3. Effects of increasing salinity levels (0, 100, 200, and 300 mM NaCl) on PSII energy distribution to photochemical process [Y(II)], regulated non-photochemical quenching [Y(NPQ)], and non-regulated non-photochemical quenching [Y(NO)] in *S. carnosa* plants. *Values are means of 8 replicates.*

4. DISCUSSION

A salinity of 100 mM NaCl was shown to affect growth in glycophytes although their differential responses to salt stress (Munns and Tester, 2008). In the present investigation, the whole plant dry weight of *S. carnosa* plants subjected to 100 mM NaCl was as high as that of the control. This legume is an indifferent halophyte (that can grow on both salt-affected and non-salt-affected soils) (Jlassi et al., 2013), and consequently does not require salt for its optimal growth. Beyond 100 mM NaCl, whole plant dry weight was affected, which was due to a decrease in root biomass production. Consequently, root/shoot ratio was significantly reduced in plants subjected to 200 and 300 mM NaCl. Such a change in biomass distribution under these conditions may be explained by: (i) a preferential photoassimilate allocation to shoots as an adaptive mechanism of *S. carnosa* to the stress, or (ii) a salt-induced inhibition of photoassimilate transport from leaves to roots. According to Maggio et al. (2007), although several works considered the increase in root/shoot ratio as a general response to salinity, it was demonstrated theoretically and experimentally that its decrease may enhance salt tolerance *via* the restriction of toxic ion flux to shoots and consequently by the delay of the tolerance threshold onset.

Except in stems that showed a decrease in water content under saline conditions, plants maintained their tissue hydration unaffected even at the highest salinity level. This can be attributed to the substantial decline in stomatal conductance that lowered transpiration rate. Indeed, stomata closure is considered as a quick adaptive mechanism to salt stress (Vysotskaya et al., 2010; Reef and Lovelock, 2015) together with the reduction of stomatal density (Shabala et al., 2013; Zhu et al., 2015). However, the salt-induced decrease in stomatal conductance drastically reduced net CO_2 assimilation rate. Our results are in agreement with those of Medini et al. (2019) who found a diminution of *A* due to a decline of *gs* in the glycophyte barley (*Hordeum vulgare* L.) as well as in its halophytic relative sea barley (*H. marinum* Huds.) under 150 and 300 mM NaCl. Despite the marked decrease in photosynthetic activity, this indifferent halophyte did not improve *WUE*. Studying the relationship between gas-exchange parameters and stomatal structural changes in five desert grasses, *Aeluropus lagopoides*, *Cymbopogon jwarancusa*, *Lasiurus scindicus*, *Ochthochloa compressa*, and *Sporobolus ioclados*, each taken from two populations with different salinities. Naz et al. (2010) concluded that several structural modifications of stomata such as their density, their area, and their degree of encryption under saline conditions were responsible for their photosynthetic adaptation to salt stress.

S. carnosa showed differential variations in potassium status and sodium accumulation under saline conditions amongst its organs. Stems were the only organs to maintain their K^+/Na^+ ratio due to less Na^+ accumulation and no significant decrease in K^+ concentration. By contrast, a significant increase in stem K^+ concentration was recorded, which can be explained by a higher inhibiting effect of Na^+/K^+ antagonism on K^+ translocation from stems to leaves than on its translocation from roots to stems. Unlike several glycophytes in which K^+/Na^+ ratio is considered as a key salt tolerance strategy (Anschütz et al., 2014; Himabindu et al., 2016), it was found to decrease in several halophytes (Daoud et al., 2001; Yıldıztugay et al., 2011). Indeed, halophytes are able to use Na^+ in osmotic adjustment and keep K^+ for specific metabolic processes such as the regulation of leaf nyctinastic movement and stomata movements, the activation of a multitude of enzymes, the loading and unloading of sugars in phloem, the translocation of nitrate (as a

counter-ion), the regulation of cytosolic pH, the stabilization of membrane potential, and the trafficking of proteins to specific vacuoles (Assaha et al., 2017). In this way, potassium use efficiency is increased (Rabhi et al., 2010).

The use of Na^+ for osmotic adjustment allows halophytes maintain a positive turgor pressure but it needs successful Na^+ (and Cl^-) sequestration into vacuoles, while organic solutes such as sucrose, sugar alcohols, proline, and glycinebetaine are probably accumulated only in cytoplasm (Flowers et al., 2015). In the present study, the maintaining of a constant tissue hydration in leaves and roots suggests that Na^+ was successfully sequestered into vacuoles. In leaf cells, this probably avoided ROS overproduction as indicated by the absence of any substantial decrease in F_v/F_m often considered as an indicator of 'stress' in leaves (Murchie and Lawson, 2013). In addition, F_0 was not decreased under saline conditions, suggesting the absence of any damage by ROS excessive accumulation to D1 protein (Murchie and Lawson, 2013), a key protein in the reaction center of PSII (Bose et al., 2013). Moreover, PSII energy distribution revealed no decrease in the photochemical process [Y(II)] or the safe quenching mechanism [Y(NPQ)]. This indicates that *S. carnosa* leaves were able to manage the energy collected by PSII antennae theoretically exceeding the demand for its photosynthesis considering the marked decrease in net CO_2 assimilation due to the decrease in stomatal conductance.

5. CONCLUSION

Taken together, the present findings showed that salt treatments decreased *S. carnosa* root growth, while its shoot growth was not affected. The effect was not osmotic since, except a decrease of 29-39% in stem water content under saline conditions, the remaining plant organs maintained their water content unchanged even at the highest salinity level. This may be explained by the marked decrease in transpiration rate *via* stomata closure as well as the use of sodium in osmotic adjustment. The stomatal limitations of photosynthetic activity did not disturb the functional integrity of PSII, which suggests a high ability of this halophyte to cope with the energy expected to exceed the demand for its photosynthesis.

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