HIGHLIGHTING THE MECHANISMS BY WHICH PROLINE CAN CONFER TOLERANCE TO SALT STRESS IN *CAKILE MARITIMA*

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Abstract

Cakile maritima is an oleaginous halophyte growing in the sandy dunes along the Tunisian coast. In order to investigate the role of proline in inducing high salinity tolerance (200 and 400 mM NaCl) in this halophyte, we studied several aspects of the salt responses of *C. maritma* under exogenous proline supply (20 mM). Salinity levels above 100 mM, reduced growth, photosynthetic activity, and quantum yield of photosystem II (ΦPSII), while increasing the non photochemical quenching (NPQ). Significant inhibition of the linear electron transport rate (ETR) was also observed in plants grown at 400 mM NaCl. In addition, polyphenol content, total antioxidant and DPPH scavenging activities increased due to increasing salinity stress, and the concentration of malondialdehyde (MDA) also increased. The application of proline counteracted all these adverse effects of salt stress in plants grown at 200 mM NaCl, while it improved some of these physiological attributes at 400 mM NaCl. In addition, contribution of Na^+ for the osmotic adjustment decreased in the leaves of salt treated plants supplied with proline exogenously. Exogenous application of proline induced the accumulation of potassium, proline and soluble carbohydrates in salt stressed plants, particularly at 400 mM. This explained the reason of growth enhancement induced by proline application. All together, our results showed that the beneficial effect of exogenous proline on the response of *C. maritima* to salinity was due to its role in the protection of chloroplast structures, antioxidant defenses and osmotic adjustment.

Key words: *Cakile martima*; Exogenous proline; Osmoregulation; Osmoprotection; Induced tolerance; Salt stress.

List of abbreviations: Relative Growth Rate (RGR), Net photosynthetic rate *(A),* Stomatal conductance *(g_s)* Transpiration *(E),* Total soluble carbohydrats (TSC), Proline (Pro), malonyldialdehyde (MDA), DPPH 1,1-diphenyl-2-picryl-hydrazyl, Gallic acid equivalents (GAE) Relative water content (RWC), fresh weight (FW), turgid weight (TW), Dry weight (DW), Osmotic potential (Ψs), Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Photosystem II (PSII), PSII maximum quantum yield of photochemistry (Fv/Fm), Relative quantum yield of PSII (Φ_{PSII}), Non photochemical quenching (NPQ), Electron transport rate (ETR), Reactive oxygen species (ROS)

Introduction

Salinity is one of the major limiting factors for crop growth in large terrestrial areas of the world and substantially reduced their average yield by more than 50% (Athar & Ashraf, 2009; Flowers & Colmer, 2015). It disrupts water, ion homeostasis and photosynthetic activities such as the electron transport (Chaves *et al.*, 2009; Foyer & Shigeoka, 2011). These physiological changes results in damages to cellular structure and reduced growth. Salt tolerant plants are equipped with several resistance mechanisms to cope with salt stress like osmoregulation, ion homeostasis, antioxidant and hormonal regulation (Flowers & Colmer, 2015; Slama *et al.*, 2015; Song & Wang, 2015). For example, many halophytes have the ability to protect its photosynthetic tissue by sequestering sodium ion in vacuole. These salt ions were osmotically balanced in the cytoplasm by accumulation of low-molecular-weight called compatible solutes like betaine, proline and polyols (Szabados & Savouré, 2010). The osmoregulation defense mechanism results in a lowering of osmotic potential to take up water and maintain cell turgor to allow physiological process, such as stomatal opening, photosynthesis and cell expansion (Slama *et al.*, 2015). The osmotic adjustment is energetically a costly process and may thus affects on carbon partitioning for the growing phase (Roy *et al.*, 2014; Slama *et al.*, 2015). Besides, NaCl absorption and compatible solutes synthesis and their accumulation as osmoticum may require a high amount of ATP (Hare & Cress, 1997; Ashraf & Foolad, 2007). Due to salt-induced osmotic stress and ion imbalance, secondary stresses such as oxidative damage may also occur. This stress may induce loss of chloroplast activity, decrease photosynthetic rate and increase photorespiration rate which generate the formation of reactive oxygen species (ROS) (Mittler *et al.*, 2011; Foyer *et al.*, 2012; Foyer & Noctor, 2015), inducing cellular damage, metabolic disorders, and senescence processes (Miller *et al.*, 2010; Mittler *et al.*, 2011). Reactive oxygen species are scavenged by both enzymatic and non enzymatic antioxidant mechanisms. Moreover, enhanced biosynthesis of secondary metabolites protects the sub-cellular structures from oxidative effects (Abdul Jaleel *et al.*, 2007). Secondary metabolites such as polyphenols have a potential role in inhibiting membrane lipid peroxidation, modulating cell signal transduction pathways and program cell death (Rodrigo & Libuy, 2014). The antioxidant activity of phenolic compounds is due to their aromatic ring, bearing one or more hydroxyl substituent, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Bravo, 1998; Zrig *et al.*, 2011).

It is known that accumulation of free proline is a typical response of plants to salt stress. Proline is known as an osmoprotectant that stabilize protein structure, protects membranes from lipid peroxidation, and as an effective scavenger of hydroxyl (OH^-) and peroxide ion (Ashraf & Foolad, 2007). It provides protective effects to photosystem II (PSII) in thylakoid membranes (Szabados & Savouré, 2010). In addition, biosynthesis of proline requires NADPH and thus generates $NADP^+$ pool as electron acceptor of the photosynthetic electron transport chain (Hare & Cress, 1997). It also has a role in stress-induced phenolic synthesis, which exhibit antioxidant activities (Shetty, 1997). It has been suggested that proline synthesis stimulates biosynthesis of phenolics via shikimate and phenylpropanoid pathways (Shetty, 1997). Although trangenics of osmoprotectants showed a degree of salt tolerance, accumulation of osmoprotectants in these transgenics was far below than the desired protective level. Exogenous application of osmoprotectants is possible alternative way to improve salt tolerance and crop production (Ashraf & Foolad, 2007; Athar *et al.*, 2015; Khalid *et al.*, 2015). The ameliorative and osmoprotective role of proline has been extensively shown in over-producing proline transgenics (Molinari *et al.*, 2007) or in experiments utilizing exogenous application of proline (Ali *et al.*, 2007; Athar *et al.*, 2009). Apart from beneficial effects of proline, its high concentrations and its time of application were found to be highly toxic for some type of species and depend on plant developmental stage (Ashraf & Foolad, 2007).

Cakile maritima is an annual C_3 fleshy-leaf oilseed halophyte reveals potential for economical (biofuel) and therapeutic use and which strictly colonizes the sandy habitats, ranging from arid to humid climate (Debez *et al.*, 2006; Debez *et al.*, 2012). Generally *C. maritima* is salt-sensitive at the early developmental stages (Debez *et al.*, 2006) and become euhalophyte at the vegetative stage (it require 50-100 mM NaCl for its normal growth) (Debez *et al.*, 2006). Growth was dramatically limited by higher salinity levels (300-500 mM NaCl). It accumulates Na^+ for maintenance of ion homeostasis. At high internal salt concentration, thylakoidal reactions were greatly disturbed but it has the ability to maintain PSII functional integrity by increasing non photochemical quenching (NPQ) (Debez *et al.*, 2012). In addition, Megdiche *et al.* (2009) showed that the salt tolerance of *C. maritima* is correlated with high proline and polyphenol accumulation in the leaves. However, it is not known whether proline act as osmoprotectant or act as antioxidant molecule to confer salt tolerance in *Cakile maritima*. Thus, the aim of this present work was to determine through which physiological mechanism proline induce salt tolerance in *Cakile maritima*. The potential contribution of proline in the antioxidant defense was also investigated.

Material and Methods

The seeds of *C. maritima* were collected from natural population at sandy beaches, 20 km to the north of Tunis. Plants were grown in a greenhouse under controlled conditions with following specification 16-h light/8-h dark regime; 440 $\mu mol.m^{-2}s^{-1}$ photosynthetically active radiation

(PAR); 25± 5°C temperature, and 60±10% relative humidity. Seeds were sterilized for 5 minutes with sodium hypochlorite solution (1%), and then rinsed thrice with distilled water. Sterilized seeds were allowed to germinate in pots filled with sand. On seed germination, seedlings were daily watered for 4 weeks with Long Ashton nutrient solution (Messedi *et al.*, 2004). Then, plants were divided into two groups. In the first one, plants subjected to salt treatments (100, 200, 400 mM NaCl) and to the second group of plants 20 mM proline was applied as a foliar spray. Salinity concentrations were gradually increased by 50 mM daily to reach the maximum salinity levels. As *Cakile maritima* is an obligatory halophytes (Debez *et al.*, 2006), in our experiment plants treated with 100 mM NaCl considered as controls. Absence of salinity in these kinds of plants can be described as deficient (Flowers & Colmer, 2015). Six plants were used for each treatment. Two harvests were made, at the beginning of the treatment and four weeks later. The measured parameters were fresh and dry matter production, gas exchange, chlorophyll fluorescence, malondialdehyde (MDA) content, relative water content, leaf osmotic potential (ψs) and Na^+, K^+, Cl^-, free proline, and total soluble carbohydrates (TSC) concentration in tissues.

Growth activity: Growth was estimated by two parameters: dry matter production (following desiccation at 60°C for 72 hours) during treatment estimated as difference between dry weight at final and initial harvest and Relative Growth Rate (RGR) corresponding to the amount of biomass accumulated by 1g of biomass per unit of time. This parameter was calculated following formulae as $\Delta Ln (\Delta W)/ \Delta t$, where ΔW is the change in dry weight, Ln is natural logarithm, and Δ represents the difference between final and initial value.

Gas exchange and chlorophyll fluorescence measurements: Gas exchange attributes such as net CO_2 assimilation rate (*A*), stomatal conductance (g_s), intracellular CO_2 concentration (*Ci*), and transpiration rate (*E*) were determined on the upper leaves from the youngest and well developed leaf by using an open type and portable infra red gas analyzer (LCA-4, ADC, Hoddesdon, England). Chlorophyll fluorescence was measured using a pulse amplitude modulated chlorophyll fluorescence meter (OS1-FL). Leaves previously used for the measurement of photosynthetic rates were dark adapted for 30 minutes and then used for chlorophyll fluorescence measurements (Genty *et al.*, 1989). The minimal (Fo) and maximal (Fm) Chla fluorescence were assessed in leaves after 20 min of dark adaptation. The maximum quantum efficiency of PSII photochemistry was calculated as Fv/Fm = (Fm - Fo)/Fm. The relative quantum yield of PSII (Φ_{PSII} or actual PSII efficiency) at steady-state was calculated as APSII = (F0m - Fs)/F0m, where Fs and F0m are fluorescence at steady-state and maximum fluorescence in the light, respectively. Non-photochemical quenching of fluorescence (NPQ) was calculated as NPQ = (Fm – Fm')/Fm' according to Maxwell & Johnson (2000). ΦPSII was used for calculation of the linear electron transport rate as ETR = $\Delta F/Fm' \times PPFD \times 0.5 \times 0.84$, where PPFD is incident photosynthetic photon flux density; 0.5 is distribution of energy between the two

photosystems; and 0.84 is fraction of light energy absorbed by leaf. All chlorophyll fluorescence measurements were taken from 10:00 am to 12:00 pm.

Total phenolic content: Air-dried leaves were ground into powder and total phenols were extracted by agitating 2.5 g dried powder of leaves with 25 ml of methanol-water mixture (8:2, v/v) for 30 minutes. Phenolic extracts from all samples were kept at 4°C for 24 h and then filtered through a Whatman filter paper No. 4. All leaf extracts were evaporated under vacuum to dryness. They were stored at 4°C until analysis began. Total phenolics in the leaves were measured following Singleton's method with slight modification (Dewanto *et al.*, 2002). In an aliquot (0.125 ml) of sample extract, 0.5 ml of distilled water and 0.125 ml of the Folin-Ciocalteu reagent was added. After 3 min, 1.25 ml of saturated sodium carbonate solution (7 g/100 ml) was added and made its final volume 3 ml. The reaction mixture was incubated at 23°C in the dark for 90 min and its absorbance was measured at 760 nm. Total phenolics was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE g^{-1} DW).

Determination of total antioxidant capacity: The total antioxidant activity was measured following Prieto *et al.* (1999). It was measured by adding 0.3 ml of leaf extract to 3 ml of reagent solution containing 0.6 M H_2SO_4, 28 mM sodium phosphate and 4 mM ammonium molybdate. The reaction mixture was incubated at 95°C for 90 min. after which reaction mixture cooled to room temperature. The absorbance of reaction mixture was measured at 695 nm against a blank. The antioxidant activity was expressed as the number of equivalents of ascorbic acid and BHT.

DPPH radical–scavenging activity: The DPPH radical-scavenging activity from leaf extract was measured as hydrogen donating potential (Hatano *et al.*, 1988). In extracted sample (1 ml), 0.25 ml of methanolic solution of 0.2 mM DPPH was added and incubated for 30 min in dark. The absorbance of the resulting solution was then read at 517 nm. The radical scavenging activity was expressed as IC_{50} (μg ml^{-1}) using the following equation:

$$DPPH^{\cdot} \text{ scavenging effect (\%)} = [(A_0-A_1)/A_0]*100$$

where A_0 is the absorbance of blank, and A_1 is of the sample.

Measurement of lipid peroxidation: Lipid peroxidation was measured as amount of thiobarbituric acid reactive metabolites (Sreenivasulu *et al.*, 2000). Fresh leaf samples weighing 0.1 g were ground in 10 ml of 0.1% (w/v) trichloroacetic acid solution. The grounded leaf extract was centrifuged at 10,000 x g for 20 min. An aliquot of the supernatant was added to 4 ml of 20% trichloroacetic acid solution containing 0.5% thiobarbituric acid. The resultant solution was heated at 95°C for 30 min, and then quickly cooled on ice. Afterwards, the samples were centrifuged at 10,000 x g for 10 min. The absorbance was measured at 532 and 600 nm and subtracted non-specific values at 600 nm from values at 532 nm. The concentration of thiobarbituric acid reactive metabolites or MDA content was calculated using an extinction coefficient of MDA (155 $mM^{-1}cm^{-1}$).

Leaf water relation: Relative water content (RWC) was calculated in the same leaves using the following equation:

$$RWC\ (\%) = 100\ x\ (FW - DW\ /\ (TW - DW),$$

where fresh weight (FW) was determined within 2 h after harvest, turgid weight (TW) was obtained by immersing leaves in distilled water for 24 h, and leaves were carefully blotted on tissue paper and turgid weight was recorded. Dry weight was measured after oven drying samples at 60°C for 48 h. Osmotic potential (Ψs) was determined from frozen leaves. Leaf samples (100 mg) were frozen in liquid nitrogen immediately after harvest, crushed with glass rod and centrifuged at 15000 x g for 10 min and supernatant or cell sap was collected. From collected cell sap leaf osmotic potential was measured using vapor pressure osmometer (Osmomat 030, Genotec), and converted from mOsmol kg^{-1} to MPa using the formula:

$$\Psi s\ (MPa) = -\ c\ (mOsmol\ kg^{-1})\ x\ 2.58\ 10^{-3} \text{ according to}$$
the Van't Hoff equation.

Ion analysis: At the harvests, leaf dry weights were measured, after desiccation for 48 h at 60°C. Sodium, potassium were assayed by flame emission photometry (Corning, UK) after nitric acid extraction (HNO_3, 0.5%) of the finely grounded dry matter. Chloride was determined on the same extract by coulometry (Haake Büchler chloridometer).

Determination of organic solutes: For organic solute analysis, leaves were washed with double distilled H_2O from which 100 mg sample was taken. Free proline was extracted from leaves and estimated spectrophotometrically following method of Bates *et al.* (1973). For total soluble carbohydrats (TSC) determination, fresh leaves were boiled in 80% ethanol and then dried in a Turbovap LV evaporator (Zymark Corp, Hopkinton, MA, USA). Dried leaf extract was re-dissolved in 1 ml of distilled water and centrifuged at 20000 $\times$ g for 10 minutes. Total soluble carbohydrates were determined by reacting ethanol extracts with anthrone and thiourea reagents. The leaf extract (100 μl) was added to 3 ml of assay medium which contains 1.08 M H_2SO_4, 1.09 mM thiourea and 2.1 mM anthrone. The reaction mixture was warmed for 10-15 minutes at 100 °C and absorbance was read at 620 nm. A calibration curve was made using D-glucose as a standard. TSC was expressed as $\mu mol\ g^{-1}$ DW after correction for leaf water content.

Contribution of solutes to osmotic adjustment: The relative contribution of each solute to osmotic potential was estimated using the equation:

$$\% \text{ of osmolality} = \frac{\text{Solute content (mmol } kg^{-1} \text{ water tissue)/}}{\text{Osmolality (mosmol } kg^{-1} \text{ solvent),}}$$

where the osmolality was determined directly in the tissue sap.

Statistical analysis: The obtained data were subjected to correlations and analysis of variance (ANOVA) using SPSS 11.0. Means from treatments were compared using Fisher's least significant difference (LSD) test (p<0.05). Correlation coefficients were partitioned into path coefficients following Dewey & Lu (1959), which helps in assessing direct and indirect impact as well as relative importance of causal factors.

Results

Growth: Relative Growth Rate (RGR) corresponding to the rate of increase in total dry weight per unit of plant dry weight was significantly reduced when plants were subjected to 200 and 400 mM NaCl (Fig. 1A). Exogenous application of 20 mM proline markedly enhanced growth in salinized plants grown at 200 mM NaCl, but exogenous application of proline was not effective in alleviating the adverse effects of high salinity stress (400 mM NaCl). The response of biomass production during treatment by *Cakile maritima* was similar to those of the RGR (Fig. 1B).

Photosynthetic parameters: photosynthetic rate (A), stomatal conductance (g_s), transpiration (E): Upon exposure to salinity, a marked reduction in g_s was registered which was accompanied by a similar decline in both A and E. The externally supply of proline increased net photosynthetic rate, stomatal conductance and the transpiration rate (Fig. 2).

Chlorophyl fluorescence: PSII maximum quantum yield of photochemistry (Fv/Fm), PSII yield (Φ_{PSII}), non photochemical quenching (NPQ), electron transport rate (ETR): In control plants (with 100 mM NaCl), PSII maximum quantum yield of photochemistry (estimated as Fv/Fm), was close to 0.8. This parameter slightly declined in plant irrigated with 200-400 mM NaCl to reach 0.73. No change was recorded in this parameter in plants treated by 20 mM proline (Fig. 3A). PSII yield (Φ_{PSII}) decreased with

NaCl concentration (0.25 at 400 mM NaCl), whereas it increased with proline supply to attain approximately 0.67 at 200 mM NaCl and 0.42 at 400 mM NaCl (Fig. 3B). NPQ was significantly enhanced in plants treated with salt. The exogenous application of proline decreased values of this parameter which remained stable compared with that in the control (Fig. 3C). The linear ETR was significantly inhibited in plants cultivated at 400 mM NaCl. This effect was strongly eliminated with proline supply and this parameter reached a high value at 200 mM NaCl (Fig. 3D).

Polyphenol, total antioxidant activity and DPPH scavenging activity: The results given in Table 1 showed that the polyphenol content, the total antioxidant and DPPH scavenging activities increased significantly in the leaves of plants treated with 200 mM NaCl. Exogenous application of proline augmented the accumulation of polyphenols in control plants treated with 100 mM NaCl. The total antioxidant activity doubled under proline supply reaching approximately 16 mg GAE g^{-1} DW in plants treated with 200 mM NaCl. The better antiradical activity with the lowest IC50 was noted in plants supplemented with 20 mM of Proline.

Lipid peroxidation: Lipid peroxidation levels in leaves of *C. maritima* measured as the content of MDA, are given in Fig. 4. A significant increase in MDA was observed with increases in NaCl concentration. Addition of 20 mM proline to plants treated with 200 and 400 mM NaCl reduced the MDA content by 20% (Fig. 4).

Water relations: relative water content (RWC) and osmotic potential (Ψ_s): High salt treatment (400 mM) caused a significant decrease in relative water content (RWC) (Fig. 5A). In the presence of proline the effect of salt was partially alleviated essentially in plants submitted to 200 and 400 mM NaCl. Also, the osmotic potential decreased in plants enriched by exogenous proline especially under 400 mM NaCl (Fig. 5B).
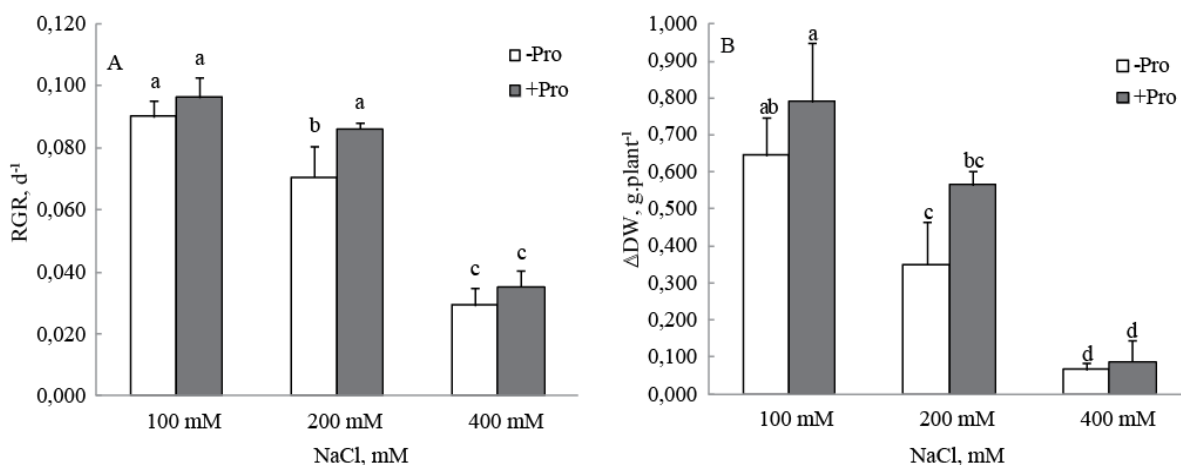

Fig. 1. Effect of proline supply (20 mM) on the leaves growth of *Cakile maritima* grown under different concentrations of salinity (100, 200, 400 mM NaCl). (A) Represents the mean relative growth rate (RGR). This parameter corresponds to the biomass production per unity of dry matter and per unity of time. (B) Represents biomass production during four weeks of salt treatment. The values showed means 4 replicates ± SE (standard error) per treatment. For each parameter, means followed by at least one same letter were not significantly different at p<0.05.

Table 1. Effect of proline supply on total polyphenol, antioxidant activity in *C. maritima* grown under different concentrations of salinity during four weeks.

	Polyphenol contents (mg GAE g^{-1}DW)		Antioxidant activities (mg GAE g^{-1}DW)		DPPH scavenging activity, IC$_{50}$ (µg.ml^{-1})	
	-Pro	+Pro	-Pro	+Pro	-Pro	+Pro
100 mM	1.625d	3.575c	5.94d	6.21c	340a	240b
200 mM	4.750b	4.917a	7.93b	12.3a	240b	180c

The values showed means 4 replicates ±SE (standard error) per treatment. For each parameter, means followed by at least one same letter were not significantly different at p<0.05
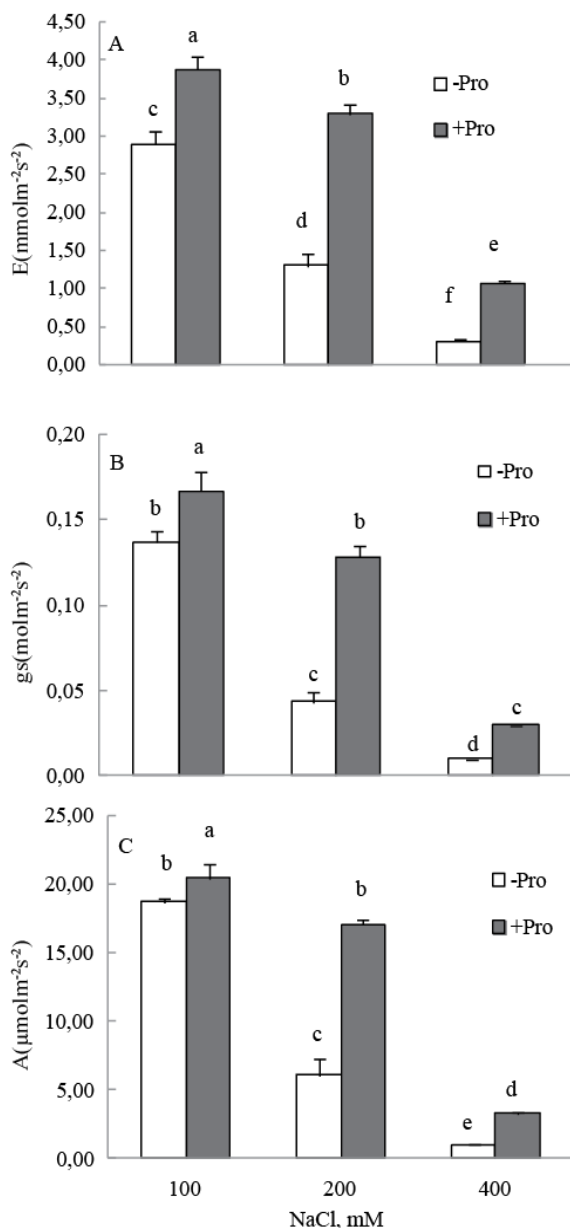
Fig. 2. Effect of proline supply (20 mM) on transpiration rate, E (A), stomatal conductance, gs (B) and net photosynthesis rate, A (C) in *Cakile maritima* grown under different concentrations of salinity (100, 200, 400 mM NaCl) during four weeks. The values showed means 4 replicates ± SE (standard error) per treatment. For each parameter, means followed by at least one same letter were not significantly different at p<0.05.

Ion content: Large concentrations of sodium and chloride were found in leaves of plants treated with 200 mM and 400 mM NaCl reaching 3.7 mmol g^{-1}DW and 2.5 mmol g^{-1}DW, respectively (Fig. 6A, B). Salt treated plants supplied with 20 mM of proline showed increased accumulation of both Na$^+$ and Cl$^-$ in the leaves to attain respectively 4.3 mmol g^{-1}DW and 3.9 mmol g^{-1}DW under 400 mM NaCl (Fig. 6A, B).

The supply of proline did not change K concentrations in plants treated with 100 and 200 mM NaCl However, plants treated with 400 mM showed higher potassium concentration under proline supply compared to control plants (without proline) (Fig. 6C).

Proline content: Leaf proline content was higher in the plants treated with NaCl. The values of proline increased with an increase in the concentration of the salt in the growth medium. Moreover, increase in proline contents was much higher due to exogenous foliar application of proline than in their corresponding salt treatment (Fig. 7A). Proline cytosolic concentration, evaluated by supposing that cytoplasm volume represents 10% of that of total cell volume (Flowers *et al.*, 1977) increased markedly with proline supply and it contribution to the "osmotic pool" attend 30% in leaf plants treated by 400 mM NaCl (Fig. 8).

Total soluble carbohydrates (TSC): Leaf accumulation of TSC increased in salt stressed plants grown at 200-400 mM NaCl. Moreover, exogenous application of proline supply also enhanced endogenous level of TSC in salt stressed plants than in control plants (100 mM NaCl) (Fig. 7B).

Discussion

The present study is aimed to assess up to what extent exogenously applied proline modulate the physiological responses of *Cakile maritima* to moderate and high salinity. The effect of exogenous proline was evaluated at three levels: ion and water relations, photosynthesis and antioxidant responses.

Moderate salt stress improved plant water status due to the accumulation of Na$^+$ and Cl$^-$ indicated that *Cakile maritima* is salt includer. However, contribution of Na$^+$ in osmotic adjustment (OA) is 50% which allowed plant growth at 200 and 400 mM NaCl (Fig. 8.2). In halophytes, contribution of sodium ion in osmotic adjustment is well documented in the literature. For example, Martinez *et al.* (2004) reported that accumulation of sodium ion contributed 15% to the total osmotic adjustment in leaves for *Atriplex halimus*. However, in *Sesuvium portulacastrum* the role of Na$^+$ accumulation in total OA is much higher and reached up to 47% at 100 mmol l^{-1} NaCl and mannitol (Slama *et al.*, 2007). In our study, the high contribution of

Na⁺ for the OA in the leaves of plants cultivated at 200 mM NaCl did not increase further when exogenous proline was supplied, suggesting the involvement of other solutes such as K, proline and carbohydrates in the decrease of the osmotic potential.

In the present study, salt stress caused a significant reduction in K^+ uptake in plants of *C. maritima*. However, exogenous foliar application of proline enhanced K^+ uptake and accumulation under saline conditions. These results are similar to those found in olive plants (Ben Ahmed *et al.*, 2011) and in *Miscanthus giganteus* (Płażek *et al.*, 2014). It was suggested that proline helped roots in preferential uptake of potassium ion while discriminating sodium ions. Athar *et al.* (2009) suggested that proline improved the accumulation of potassium by protecting activity of potassium transporters. However, the contribution of K^+ in osmotic adjustment was maximally up to 14% at 400 mM NaCl (Fig. 8. 2 Please check). To assess the degree at which the levels of these inorganic solutes are regulated with salinity to maintain growth before and after proline supply, we examined the correlation between Na⁺ and K⁺ concentrations and OP (Fig. 8. 1 Check). We found that Na was highly correlated with

OP and the correlation coefficient attained 0.986. Potassium is known to be involved in lowering the OP. In the present study, potassium accumulation in the leaves is positively correlated with osmotic potential before and after application of proline (r = 0.938***; 0.98***). These results confirmed that potassium plays a central role in osmotic adjustment and stomatal regulation under normal or stress conditions. Similarly, sodium accumulation contributed in osmotic adjustment but its toxic impact is counteracted by biosynthesis and accumulation of compatible organic solutes which includes proline, glycinebetaine, trehalose etc. In the present study, all compatible solutes were not measured except the proline. From the results of the present study, it is clear that salt stress enhanced the endogenous level of proline. Moreover, foliar application of proline caused a further increase in accumulation of proline a common phenomenon in plants occurred under abiotic stress conditions. Under saline stress conditions, increase in endogenous level of proline might have been due to increase in biosynthesis of proline or decrease in proline degradation by inhibition of pyrroline-5-carboxylate dehydrogenase (Szabados & Savouré, 2010; Slama *et al.*, 2015).
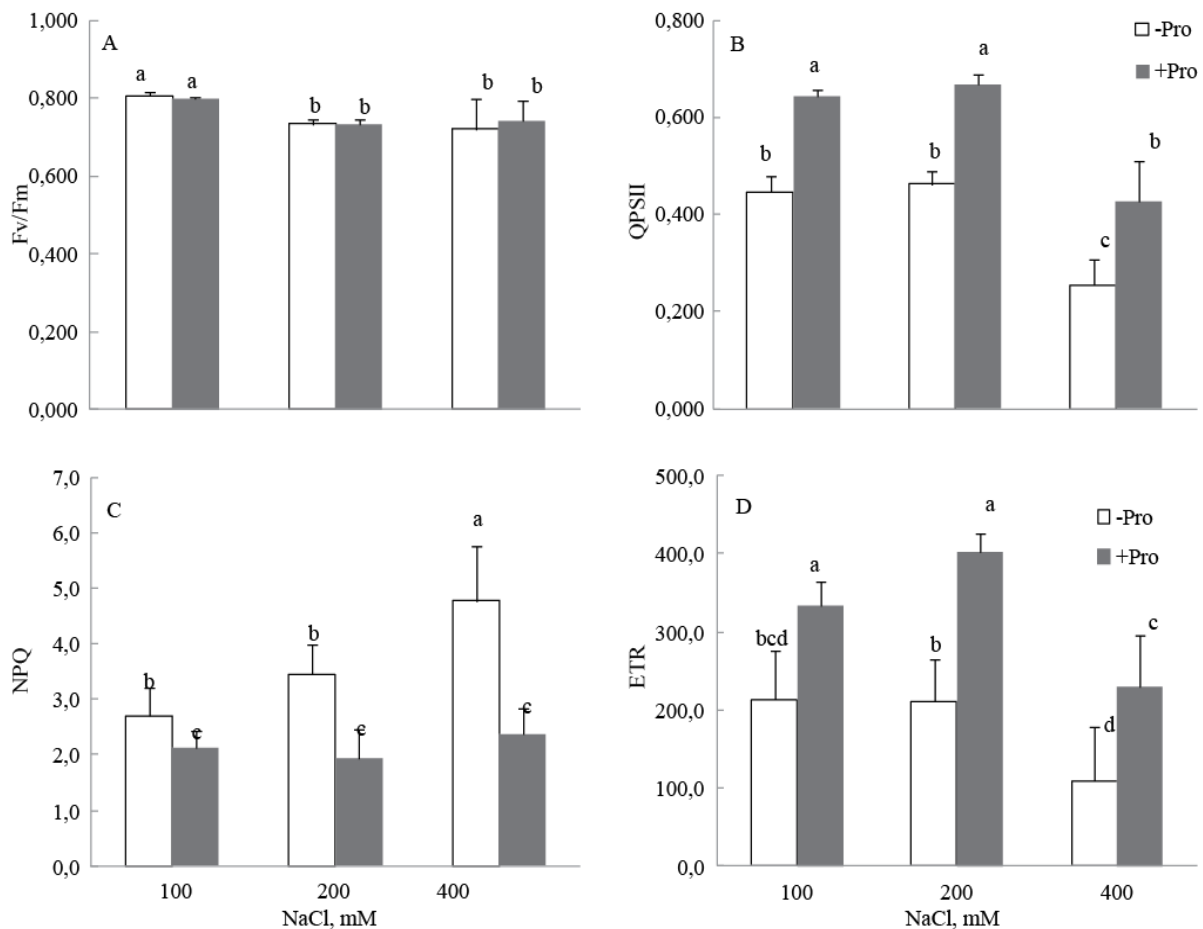

Fig. 3. Effect of proline supply (20 mM) in PSII functioning of *C. maritima* grown under different concentrations of salinity (100, 200, 400 mM NaCl) during four weeks. (A) Fv/Fm, the maximum quantum yield of PSII photochemistry, (B) ΦPSII, quantum efficiency of PSII photochemistry, (C) NPQ, the non-photochemical quenching and (D) ETR, the relative PSII electron transport rate. The values showed means 4 replicates ± SE (standard error) per treatment. For each parameter, means followed by at least one same letter were not significantly different at p<0.05.
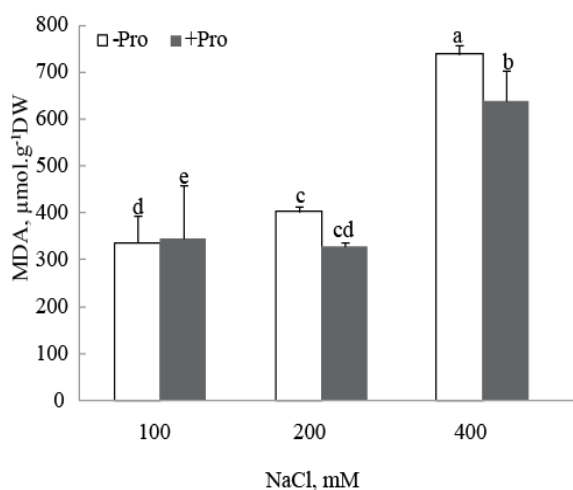
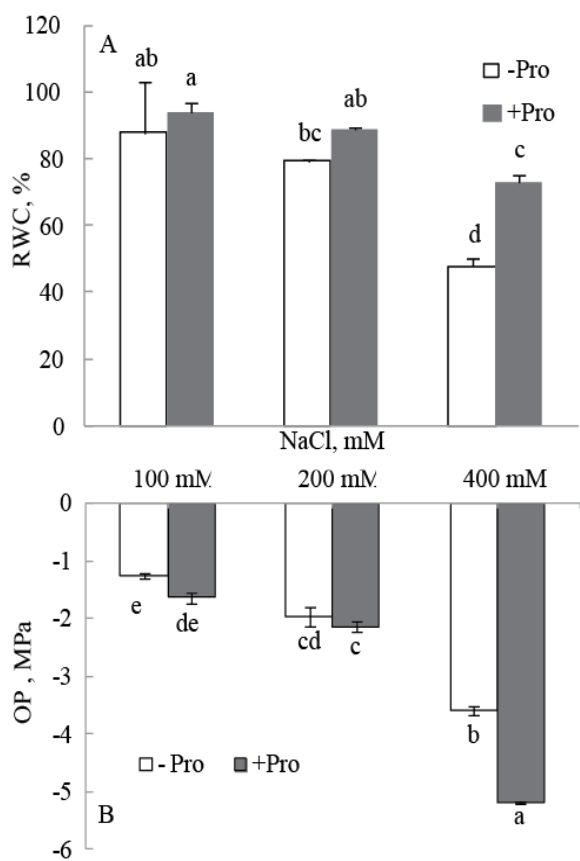Fig. 4. Effect of proline supply (20 mM) on MDA content in leaves of *C. maritima* grown under different concentrations of salinity (100, 200, 400 mM NaCl) during four weeks. The values showed means 4 replicates ± SE (standard error) per treatment. For each parameter, means followed by at least one same letter were not significantly different at p<0.05.
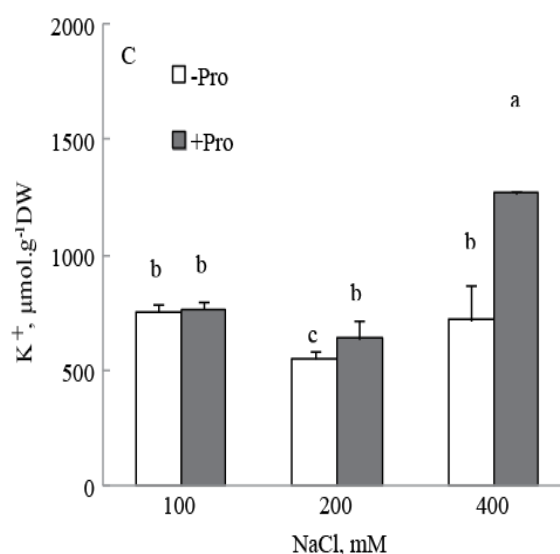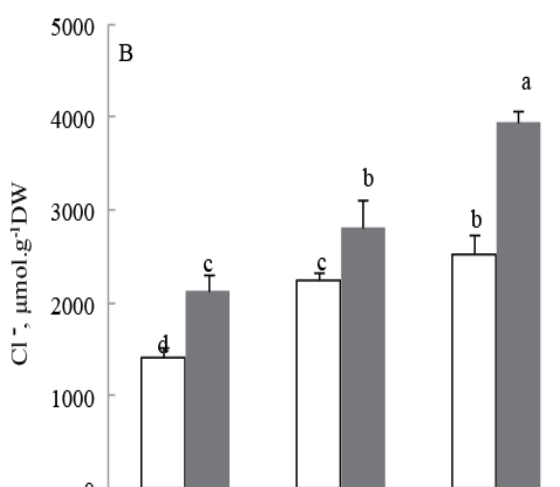

Fig. 5. Effect of proline supply (20 mM) on leaf relative water content (RWC %) (A) and leaf osmotic potential (OP, MPa) (B) in *C. maritima* grown under different concentrations of salinity (100, 200, 400 mM NaCl) during four weeks. The values showed means 4 replicates ± SE (standard error) per treatment. For each parameter, means followed by at least one same letter were not significantly different at p<0.05.
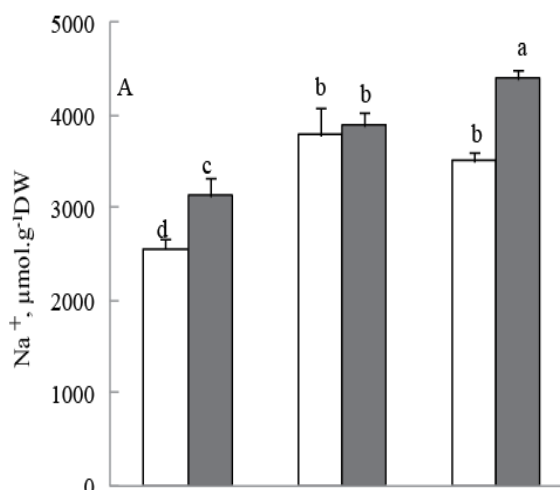

Fig. 6. Effect of proline supply (20 mM) on leaf Na$^+$ (A), Cl$^-$ (B), K$^+$ (C) concentration in *C. maritima* grown under different concentrations of salinity (100, 200, 400 mM NaCl) during four weeks. The values showed means 4 replicates ± SE (standard error) per treatment. For each parameter, means followed by at least one same letter were not significantly different at p<0.05.
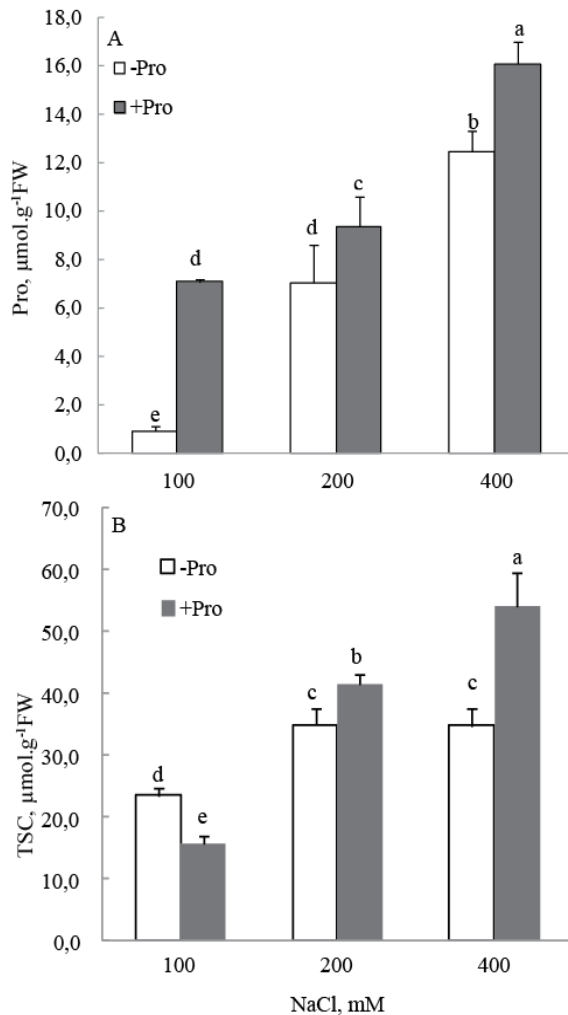
Fig. 7. Effect of proline supply (20 mM) (A) on leaf proline concentration (B) on leaf total soluble carbohydrates (TSC) in *C. maritima* grown under different concentrations of salinity (100, 200, 400 mM NaCl) during four weeks. The values showed means 4 replicates ± SE (standard error) per treatment. For each parameter, means followed by at least one same letter were not significantly different at p<0.05.

Based on assumption that cytosol of cells is 10% of total cell volume (Flowers *et al.*, 1977), our findings suggest that 400 mM NaCl salinity caused proline accumulation up to 350 mM and in proline applied salt stressed plants its concentration exceeded to 450 mM. Therefore, the contribution of proline in cytosolic osmotic adjustment is 20% under saline conditions and 30% in salt stressed plants treated with proline (Fig. 8.2 Check). Accumulation of proline is also positively correlated with leaf osmotic potential (r = 0.953***; 0.983***; Fig. 8.1 Check).

In addition of its function as an efficient osmolyte, proline led to a slight enhancement of growth at 200 mM NaCl. Exogenous foliar application of proline increased net CO_2 assimilation rate (*A*), stomatal conductance (g_s) and transpiration (*E*) in salt stressed plants. During osmotic stress, proline biosynthesis is augmented from glutamate. This accumulation is localized in the chloroplasts and controlled by P5CS1 and generate $NADP^+$ that can be used in supporting a number of growth and developmental processes (Kruger & von Schaewen, 2003). This later represents also the terminal electron acceptor of the photosynthetic electron transport chain (Szabados & Savouré, 2010). In the present study, it was found that exogenously proline applied salt stressed plants of *C. maritima* had higher ΦPSII and ETR values associated with lower NPQ suggested that proline protected chloroplast structures from toxic effects of salt stress. While working with isolated thylakoid membranes from the leaves of *Brassica juncea* seedlings, Alia *et al.* (1991) reported that greater endogenous proline concentration increased the photosynthetic electron transport by protecting PSII from salt stress. Similarly, it was also reported that reduction in PSII electron transport activity due to salt stress improved due to stabilizing effect of non toxic osmolyte glycinebetaine on PSII (Allakhverdiev *et al.*, 2003).

In addition to enhancement in endogenous level of proline, foliarly applied proline also increased total carbohydrates and soluble sugars under saline conditions which indicate enhanced net CO_2 assimilation while maintaining chloroplastic redox homoestasis. This was further confirmed by lower membrane damage due to oxidative stress. These results from the present study suggested that proline played a major role in protecting PSII from salt-induced osmotic and oxidative stress (Miller *et al.*, 2010). Soluble sugars and other carbohydrates have also capacity to scavenge chloroplastic ROS directly or indirectly by activating antioxidative defense systems (Van den Ende & Valluru, 2009). A number of published reports are available in which it is documented that phenolic compounds have a strong antioxidant potential to scavenge chloroplastic reactive oxygen species (ROS) generated when abiotic stresses damaged photosynthetic metabolism (Sreenivasulu *et al.*, 2000). Our results showed that proline treatment increased both polyphenol contents and antioxidant activities in salt stressed plants of *Cakile maritima*. These results from the present study are similar to findings of Karjalainen *et al.* (2002) who also reported that exogenous application of compatible solutes enhanced the phenolic compounds in maize and strawberry under water stress conditions. Similarly, Djeridane *et al.* (2006) suggested that proline stimulated the biosynthesis of those phenolic compounds witch exhibit a better antioxidant activity.

In conclusion, exogenous application of proline improved the tolerance of *C. maritima* to moderate salinity, which is associated with the capacity of proline in osmotic adjustment, and to protect photosystems II activity against oxidative damages. The increased polyphenol contents and the antioxidant activities under proline supply suggest an important role of proline as antioxidant molecule beside the osmo-protectant function. The molecular mechanisms by which proline can play these roles remain to be elucidated in the future.
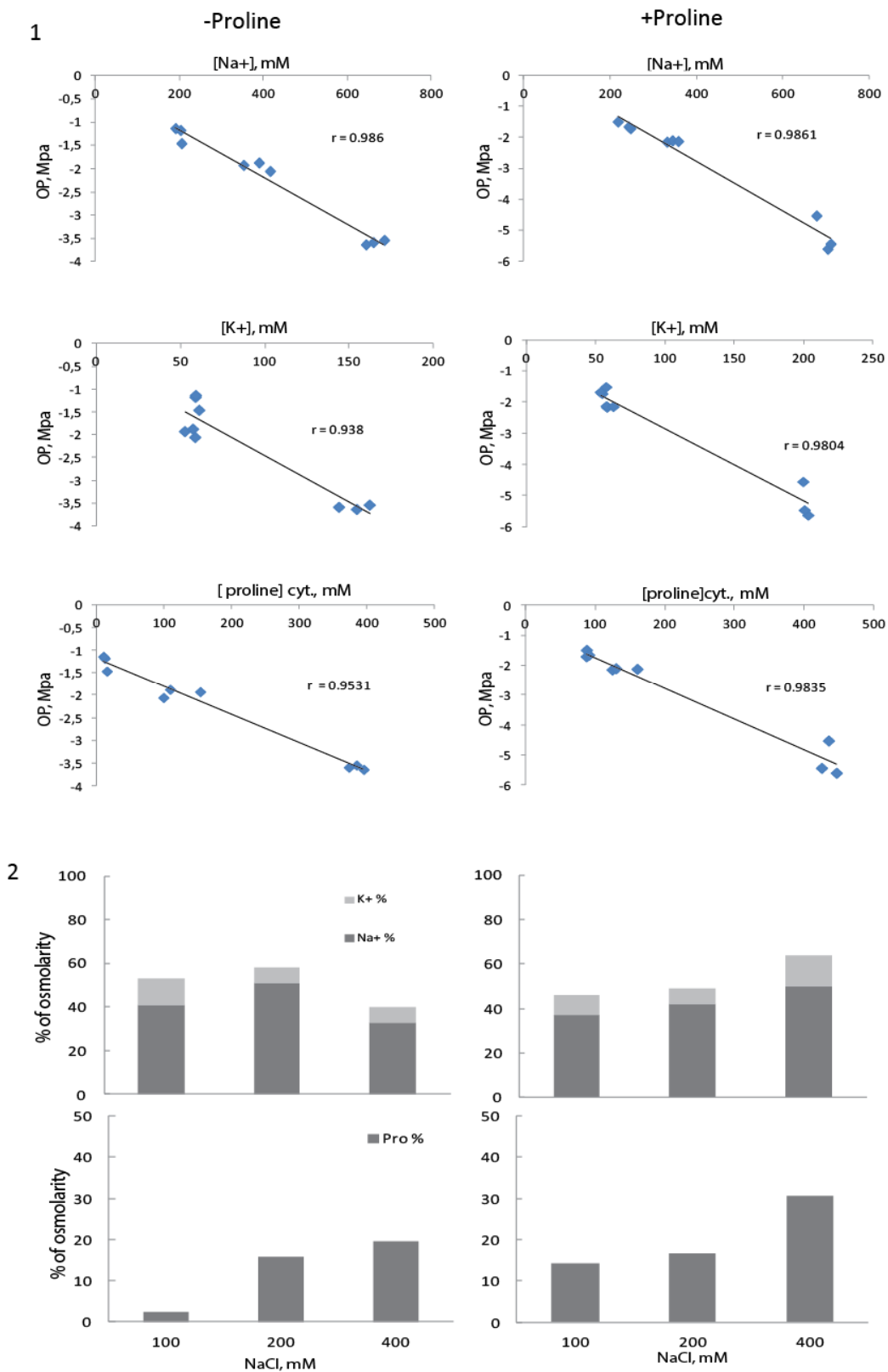
Fig. 8. (1) Correlation between Na^+, K^+ and proline cytoplasmic concentrations and OP in *C. maritima*. Plants grown under different concentrations of salinity (100, 200, 400 mM NaCl) during four weeks, in the absence or presence of 20 mM proline. (2) The contribution of individual solutes to the osmotic potential (OP) in leaves of *C. maritima*. Plants grown under different concentrations of salinity (100, 200, 400 mM NaCl) during four weeks, in the absence or presence of 20 mM proline.

Acknowledgements

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