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Presence of proline in salinized nutrient solution re-enforces the role of this amino acid in osmoregulation and protects lipid membrane peroxidation in *Arabidopsis thaliana*

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Abstract

Very little is known about the effect of proline addition on the accumulation of inorganic solutes (Na^+, K^+, Mg^{2+} and Ca^{2+}) and soluble sugars in the model plant *Arabidopsis thaliana*. Therefore, the aim of the present study was to assess the effect of 10 mM proline (P) supply in the culture medium on water status and solute accumulation of *Arabidopsis thaliana* seedlings exposed to 50 mM NaCl (S). The decrease of leaf osmotic potential was more pronounced in P+S as compared to S plants, indicating that former plants were able to accumulate more compounds involved in the osmotic adjustment process. Leaf potassium concentration was reduced by 15, 21 and 25% in P, S and P+S plants respectively, as compared to the control. When compared to S or P treatments, leaf proline and soluble sugar were more accumulated under P+S treatment. Under saline conditions, exogenous proline increased leaf Na^+, Ca^{2+} and Mg^{2+} concentrations by 27, 281 and 252%, respectively, as compared to the control. Interestingly, proline addition mitigated significantly the deleterious effects of salt on lipid membrane peroxidation. Regarding the contribution of soluble sugars to osmotic adjustment (OA), it amounted to 6% in S or P+S, plants. For proline, its contribution to OA did not exceed 3.4% under salinity (S), whereas in (P+S) treatment, it increased to 14.7%. As a whole, the positive effect of proline exogenous application under saline conditions could be partly explained by the enhanced role of this organic compound in osmoregulation and its likely protective effect against membrane lipid peroxidation.

Keywords: Malondialdehyde, nutrient status, osmotic adjustment, salt, soluble sugars.
Abbreviations*:* DW, dry weight; FW, fresh weight; LWC, leaf water content; MDA, malondialdehyde; OA, osmotic adjustment; P, proline; ROS, reactive oxygen species; S, salt; TBA, thiobarbituric acid; TCA, thrichloro acetic acid; Ψs, osmotic potential.

Introduction

Salinity is one of the most important environmental factors limiting plant growth and yield worldwide (Munns and Tester, 2008). To survive against this stress, plants respond and adapt using complex morphological, physiological and biochemical mechanisms. *Arabidopsis thaliana* is a glycophyte, sensitive to salt even at low doses (Bressan et al., 2001). According to Ghars et al. (2008), three-week-old plants of *A. thaliana* treated with 50 mM NaCl showed about 40% growth restriction as compared to the control. The adverse effect on plant growth under saline conditions was correlated with significant alteration of water relations, nutrient status and the induction of membrane lipid peroxidation (Ghars et al., 2008; M'rah et al., 2007). Reactive oxygen species (ROS) are generated as a normal byproduct of plant cellular metabolism. In some species, salinity leads to excessive ROS production causing progressive oxidative damage and ultimately cell death (Sharma et al., 2012). Whether ROS would serve as signaling molecules or could cause oxidative damage to the tissues depends on the delicate equilibrium between ROS production and their scavenging. The efficient scavenging of ROS produced by various environmental stress factors requires defensive mechanisms involving several non-enzymatic as well as enzymatic antioxidants present in tissues (Sharma et al., 2012). When exposed to stressful conditions, the biochemical defense system includes proline accumulation. This amino acid has been considered as constituent of proteins, and plays an important role in plant metabolism and development. It plays a highly beneficial role in plants exposed to various stress conditions. Besides acting as an excellent osmolyte, proline plays three major roles during stress as a metal chelator, an antioxidative defense molecule and a signaling molecule (Hayat et al., 2012; Szabados and Savouré, 2010). The production of ROS during environmental stresses including salinity may threaten cells through the induction of lipid peroxidation, protein oxidation, nucleic acid damage, enzyme inhibition, and programmed cell death activation (Sharma et al., 2012). The final product of lipid peroxidation (malondialdehyde) can be used as effective indicator parameter to evaluate damage causes by ROS (Molinari et al., 2007). In glycophyte species, proline and soluble sugars accumulate in response to salt stress (Dubey and Singh 1999; Krasensky and Jonak, 2012). Exogenous application of proline has been suggested to be an

alternative approach to genetic engineering to improve crop productivity under stress conditions (Heuer, 2003). Exposure to exogenous proline can effectively enhance plant stress tolerance (Gomes et al., 2010). For example, the addition of 20-33 mM proline to cell cultures mitigated the adverse effects of NaCl stress in Mung bean (*Vigna radiate*; Kumar and Sharma 1989). In alfalfa (*Medicago sativa*) callus cultures, 10 mM exogenous proline was effective in alleviating the effects of salt stress (Ehsanpour and Fatahian, 2003). In rice, exogenous proline counteracted the adverse effects of salinity on early seedling growth (Roy et al., 1993). Besides, the addition of proline to the halophyte species *Allenrolfea occidentalis* restored plant growth and lowered ethylene production due to salt or drought stress (Chrominski et al., 1989). Exogenous proline application also improved tobacco behaviour under salinity by stimulating antioxidant enzyme activities (Hoque et al., 2007). Despite its protective role under a variety of stress conditions, external supply with proline was reported as toxic in some plant species such as tomato (Heuer, 2003), though this toxicity was dose-dependent (Ashraf and Foolad, 2007). The response of *A. thaliana* to exogenous proline application has been studied on the basis of some parameters such as growth, root length and chloroplast and mitochondrion ultrastructure either under salt-free conditions (Kant et al., 2006, Hellmann et al., 2000; Hare et al., 2002) or under drought stress (Moustakas et al., 2011). However, no information is available in the literature about the effects of proline supply on inorganic solute (Na^+, K^+, Mg^{2+} and Ca^{2+}) and soluble sugar accumulation in this model plant when salt-challenged. The determination of solute concentration in plants is very important. Indeed, it has been reported by Flower and Colmer (2008) that ionic solute accumulation and compartmentalization (especially Na^+) in plant tissues was found to be very informative indicator to salt tolerance. Sodium accumulation in cytosol affects metabolic reactions and enzyme activities. Besides Calcium plays a major role during stress as a signaling molecule. In addition, before starting biochemical studies on the antioxidant system and on enzyme activities linked to carbohydrate and proline metabolism, it is necessary to determine first, and this is our objective, the effects of exogenous proline on nutrient status, sugar and proline accumulation and the degree of membrane lipid peroxidation in salt treated *A. thaliana* seedlings.

Results

Leaf water content and osmotic potential

As compared to control plants (C), leaf water content (LWC) decreased significantly by 12, 15 and 22% in plants subjected to proline (P), salt (S), and their combination (P+S), respectively (Fig. 1A). Proline addition (P) to the culture medium had no significant effect on leaf osmotic potential which was comparable to that of the control (-0.68 MPa), whereas salt treatment resulted in a significant decrease (-17%) (Fig. 1B). This trend was more pronounced (-54%) under combined effects of proline and salt (P+S).

Proline and soluble sugar accumulation

Under control conditions, proline was low accumulated in leaves (0.1 µmol. g^{-1} FW) (Fig. 2). Salt and/or proline addition led to a 5- and 15-fold increase in leaf proline concentration, respectively. A much higher (54-fold) additional accumulation was recorded in plants subjected to salt in the presence of 10 mM proline (P+S). A slight but significant increase of soluble sugar concentration was observed in plants grown either with salt or proline as compared to control (Fig. 3). Salt-treated plants supplied with 10 mM proline showed the highest leaf soluble sugar concentration (+84% as compared to the control).

Sodium and potassium concentrations

Under control conditions, proline addition had no significant effect on leaf Na^+ concentration (Fig. 4A), whereas this parameter showed a 10-fold increase under saline conditions. A much higher increase was found under combined effects of salinity and proline supply, leaf Na^+ concentration reaching 2500 µmol. g^{-1} DW. Leaf potassium concentration (Fig. 4B) was reduced in P, S and P+S treatments by 15, 21 and 25%, respectively. However, no significant differences were observed in this parameter between S and P+S treatments.

Calcium and magnesium concentrations

Proline exogenous application (P) enhanced leaf Ca^{2+} (+88%) and Mg^{2+} (+80%) concentrations (Fig. 5A, B), whereas salt treatment (S) led to a significant decrease in leaf Ca^{2+} and Mg^{2+} concentrations. (P+S) plants accumulated respectively 4 and 3 times more calcium and magnesium than those of (S) treatment.

Membrane lipid peroxidation

The lipid peroxidation level in *Arabidopsis thaliana* leaves was assessed as the concentration of MDA (Fig. 6). Under control conditions, leaf MDA concentration was about 25 nmol g^{-1} FW. Proline supply to the culture medium had no effect on this parameter whereas plants grown at 50 mM NaCl had significantly higher significantly MDA leaf concentration (+56% a compared to the control). Under saline conditions, proline supply significantly decreased leaf MDA concentration by 43%.

Discussion

Our data showed that salinity reduced leaf water content whether proline was applied or not. These data further confirm the sensitivity of *A. thaliana* to salt, as reported by Ghars et al., (2008) and M'rah et al., (2007). Salinity is known to induce not only osmotic stress by limiting water uptake from soil but also ionic stress resulting from high concentrations of potentially toxic salt ions within plant cells. In this study, proline addition under non saline conditions had no effect on leaf sodium concentration. However, the presence of proline in salinized culture medium led to a significant increase of this parameter. In halophytic species, sodium could be involved in osmotic adjustment as observed in *Sesuvium portulacastrum* and *Atriplex halimus* (Slama et al., 2007; Martinez et al., 2005). However in a glycophyte, the accumulation of sodium in leaves is often associated with plant salt sensitivity. Our data showed that potassium concentration was significantly affected in plants subjected to exogenous proline and/or 50 mM NaCl. Potassium is not involved in osmotic adjustment in *A. thaliana*. The decrease in leaf potassium concentration has been also reported by Ghars et al. (2008) in *A. thaliana* plants subjected to salt and by Heuer (2003) in tomato (*Lycopersicon esculentum* L) when salt and/or proline were added to the culture medium.
Proline supply to the culture medium induced a significant increase in leaf calcium concentration for control and salt treatments. This tendency was significantly more pronounced in (P+S) plants. Kaya et al. (2007) reported that in melon

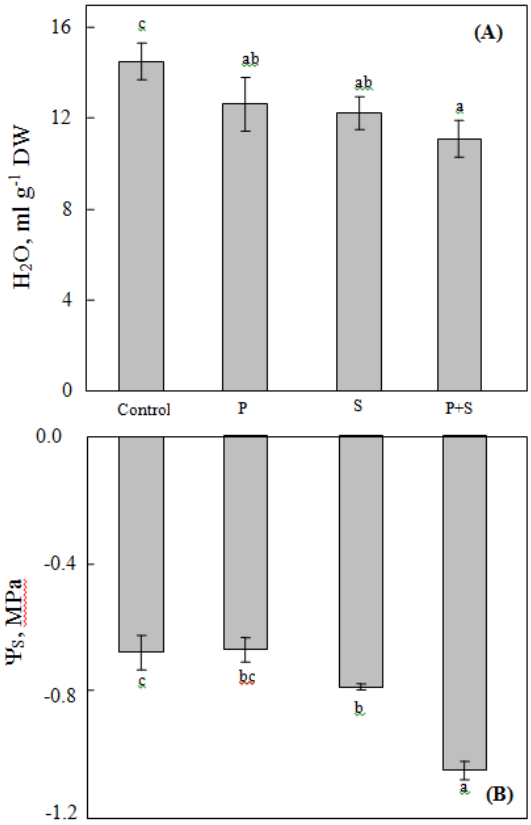

Fig 1. Changes in leaf water content (A) and osmotic potential (B) of *Arabidopsis thaliana* plants grown with 10 mM proline (P), 50 mM NaCl (S) or under their combined application (P+S). Values are means of five replicates, vertical bars are SE. Values sharing a common latter are not significantly different at P<0.05%.

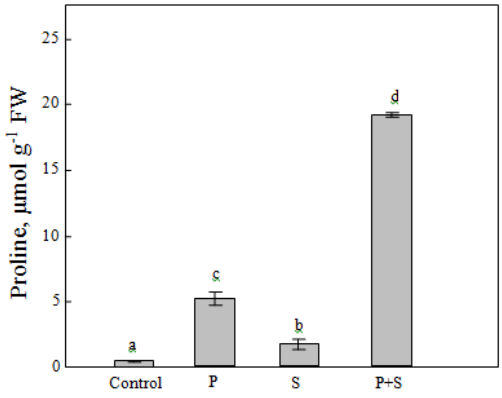

Fig 2. Changes in leaf proline concentration of *Arabidopsis thaliana* plants grown with 10 mM proline (P), 50 mM NaCl (S) or under their combined application (P+S). Values are means of five replicates, vertical bars are SE. Values sharing a common latter are not significantly different at P<0.05%.

plants (*Cucumis melo* L.), exogenous proline counteracted the deleterious effect of salt and improved calcium uptake under salinity. Calcium ion plays an important role in coupling physiological responses to external and developmental signals in plants. Transient changes in calcium concentration in cytosol have been observed during growth, development and under stress conditions. Our data showed a significant increase in leaf calcium concentration in (P+S) plants, suggesting that its accumulation may contribute to salt stress defense mechanisms. Such a contribution, including calcium signaling in the abscisic acid (ABA) regulation system and calcium sensing in stomatal closure in salt-treated plants was suggested by Suriyan et al. (2012) after a high accumulation of Ca^{2+} in *Oryza sativa*. Exogenous application of compatible solutes offers a valuable tool for studying mechanisms of salt tolerance. One of these mechanisms depends on the capacity not only in water uptake and ion accumulation and compartmentalization but also in osmotic adjustment, which allows growth to continue under saline conditions (Gomes et al., 2010). Among compatible solutes, proline has an important role in osmoregulation and intracellular homeostasis, including redox balance and energy status. (Szabados et al., 2011). Our results showed that proline addition in the culture medium induced an increase in leaf proline content under saline conditions and to a lesser extent under non saline conditions. This higher accumulation of proline may result from an increased endogenous production, a decreased metabolism or a direct effect of salinity on the uptake and translocation of this amino acid. Our results are in accordance with those of Heuer (2003), who noted a high increase in proline accumulation in tomato plants (*Solanum esculentum*) treated with 135 mM NaCl in the presence of 1 mM proline. In addition, stimulative effects of exogenous proline on *in vitro* shoot organogenesis from *A. thaliana* hypocotyl explants are consistent with its important role in the interconversions of this osmoticum and its precursors in regulating cell division and differentiation (Hare et al., 2002). Proline application under non saline conditions did not affect on leaf osmotic potential. When plants were subjected to salt only, a decrease in this parameter was however detected, together with higher proline accumulation in leaves. Yet, the contribution of proline to the total osmotic adjustment did not exceed 3.4%. In (P+S) plants, a marked decrease in leaf osmotic potential was observed, the contribution of this osmoticum in the total osmotic adjustment amounting to 14.7%. Hence, proline contribution to osmotic adjustment was treatment-dependant; the combined effects strengthening proline role in osmoregulation. In the same way, plants subjected simultaneously to 50 mM NaCl and 10 mM proline showed the highest leaf soluble sugar concentration (Fig. 5). Soluble sugars may be involved in osmotic adjustment under saline conditions (Dubey and Singh, 1999). Our results showed that their contribution to osmotic adjustment in (S) and (P+S) treatments was low (6%). Although soluble sugars could be involved in osmotic adjustment, their accumulation is not considered as a salt- and water deficit-tolerance criterion since these constraints impair carbohydrate transport from source to sink organs and to disturb their metabolism. Regarding ROS damage, which was estimated by the lipid peroxidation as leaf MDA concentration, our findings indicate that proline supply under saline conditions (50 mM NaCl) led to a significant decrease of leaf MDA concentration, strongly suggesting that this compound mitigates the drastic effects of salt on membrane structural integrity. In the same context, Hoque et al. (2007) reported the reduction in catalase and peroxidase activities but not superoxide dismutase activity under salt stress following proline exogenous application. The latter also improved cell metabolism and reduced the peroxidation of lipid membranes in cultivated tobacco cells under saline conditions (Okuma et al., 2004). Soluble sugars can be involved in, or related to, ROS-producing metabolic pathways. These compounds might either directly detoxify ROS in chloroplasts and

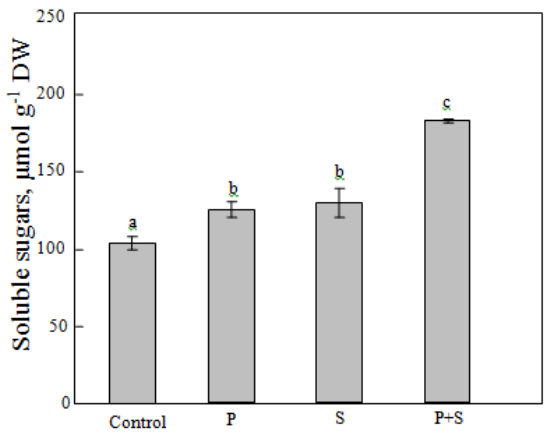

Fig 3. Changes in leaf soluble sugar concentration of *Arabidopsis thaliana* plants grown with 10 mM proline (P), 50 mM NaCl (S) or under their combined application (P+S). Values are means of five replicates, vertical bars are SE. Values sharing a common latter are not significantly different at P<0.05%.

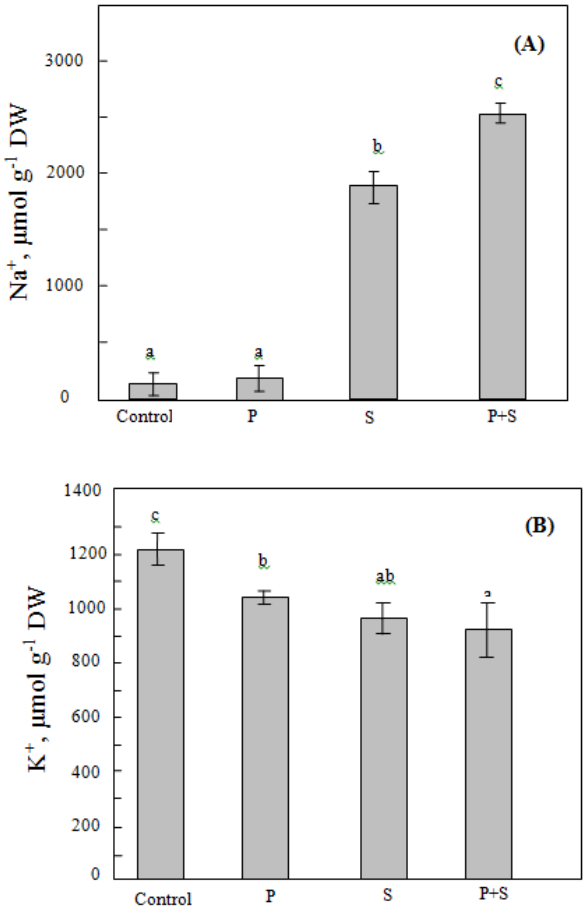

Fig 4. Changes in leaf Na^+ (A) and K^+ (B) concentrations of *Arabidopsis thaliana* plants grown with 10 mM proline (P), 50 mM NaCl (S) or under their combined application (P+S). Values are means of five replicates, vertical bars are SE. Values sharing a common latter are not significantly different at P<0.05%.

vacuoles or indirectly stimulate the classic antioxidant defense systems (Van den Ende and Valluru, 2009) and control the ROS balance (Couée et al., 2006). Our results showed that exogenous application of proline enhanced soluble sugar accumulation in *A. thaliana* subjected to salinity and that both functioned to alleviate the oxidative damage in plants caused by salinity. According to our findings, exogenous proline application under saline conditions led to an additional increase in soluble sugar, proline and calcium concentrations. This marked accumulation supports the hypothesis that these compounds could be involved in the protection of organelle integrity.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (ecotype Columbia, wide type) seeds were surface-sterilized with 0.2% (w/v) sodium hyperchlorite for 2 min and maintained for 10 days at 4°C to break seed dormancy and grown on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) in 14-cm-diameter Petri dishes (the total number of Petri dishes is 15 containing 18 plants each). After an overnight period at 4°C to break dormancy, seedlings were allowed to grow for 12 days at 22°C under continuous light with a luminosity of 60 µmol photons.m^{-2}.s^{-1}. Twelve-day-old seedlings were transferred into 5-cm–diameter Petri dishes containing 5 ml of half-strength MS liquid medium supplemented or not with 50 mM NaCl in the presence or in the absence of 10 mM proline. All experiments were repeated five times. After 24 hours of incubation, seedlings were collected, washed four times with distilled water and one part was immediately frozen in liquid nitrogen and stored at -80°C for further analysis. The other part was dried for 72 h at 65°C for ion and soluble sugar analysis.

Water relation and leaf water potential

Seedling fresh weight (FW), dry weight (DW) and water content (LWC) were determined. DW was obtained after oven drying samples at 60°C until a constant weight was reached. Leaf water content was determined according to the equation:

LWC (ml g^{-1}) = (FW − DW)/DW

For osmotic potential (Ψs), leaves were quickly collected, cut into small segments, then placed in Eppendorf tubes perforated with four small holes and immediately frozen in liquid nitrogen. After being individually transferred in a second intact Eppendorf tube, they were placed for 30 min and centrifuged at 15,000 x *g* for 15 min**.** Osmolality (C) was assessed with a vapour pressure osmometer (Wescor 5500).

Cation assay

Na^+, K^+, Ca^{2+} and Mg^{2+} were extracted by nitric acid HNO_3, 0.5 % at ambient temperature. Na^+ and K^+ were assayed by flame emission photometry. Ca^{2+} and Mg^{2+} concentrations were determined by atomic absorption spectrometry.

Proline and soluble sugar determination

Free proline was assayed spectrophotometrically using the method of Bates et al. (1973). Leaf samples (100 mg FW) were homogenized in 3% (w/v) aqueous sulfosalicylic acid and centrifuged for 30 min at 14,000 *g*. Then, 2 ml of acid ninhydrin and 2 ml of glacial acetic acid were added to the

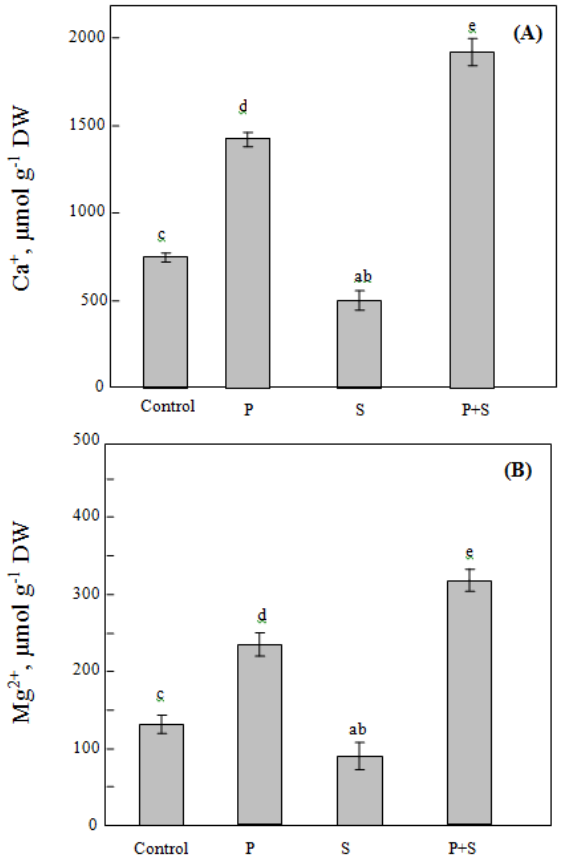

Fig 5. Changes in leaf Ca^{2+} (A) and Mg^{2+} (B) concentration of *Arabidopsis thaliana* plants grown with 10 mM proline (P), 50 mM NaCl (S) or under their combined application (P+S). Values are means of five replicates, vertical bars are SE. Values sharing a common latter are not significantly different at P<0.05%.

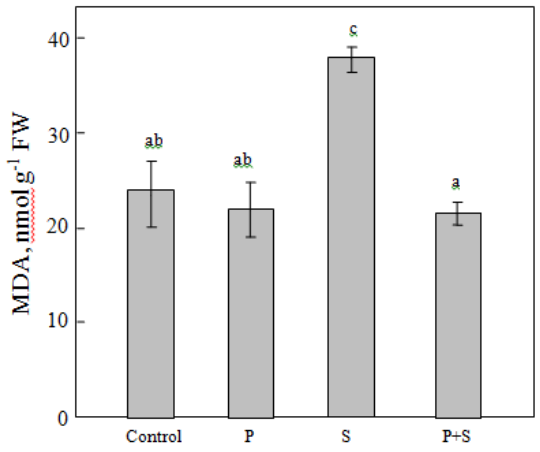

Fig 6. Changes in leaf MDA (malondialdehyde) concentration of *Arabidopsis thaliana* plants grown with 10 mM proline (P), 50 mM NaCl (S) or under their combined application (P+S). Values are means of five replicates, vertical bars are SE. Values sharing a common latter are not significantly different at P<0.05%.

supernatant. The mixture was then boiled for 1 h. After extraction with toluene, free proline was quantified at 520 nm from the organic phase using an Anthelie Advanced 2, SECOMAN spectrophotometer (Domont, France). Soluble sugars were determined by the anthrone reagent method according to Yemm and Willis (1954).

Lipid peroxidation

The extent of lipid peroxidation was estimated by determining the concentration of malondialdehyde (MDA) according to Draper and Hadley (1990). Leaf material (FW) was homogenized in 0.1% (w/v) TCA solution. The homogenate was centrifuged at 15,000 x *g* for 10 min and the obtained supernatant was added to 0.5% (w/v) TBA in 20% (w/v) TCA. The mixture was then incubated at 90°C for 30 min, and the reaction tubes were placed in an ice water bath. Samples were centrifuged at 10,000 x *g* for 5 min, and the absorbance of the supernatant was read at 532 nm.

Solute contributions to osmotic adjustment

The contribution of each solute to the total osmotic adjustment was determined according to Patakas et al. (2002), assuming that 40 µmol g^{-1} of symplastic water equals to 0.1 MPa. The contribution of each solute (s) to the total osmotic adjustment (OA*t*) was calculated using the formula : $OA_{(S)}$ in % =($[Solutes]_{stressed}$ - $[Solutes]_{control}$)x0:1x100)/(40x$OA_{t.}$) [Solutes] was determined as the following ratio: solute concentration (µmol g^{-1} DW)/water content (ml g^{-1} DW). The degree of total osmotic adjustment (OA*t*) was defined as the difference between the osmotic potential of control and stressed plants (Hesseni et al., 2008).

Statistical analysis

Analysis of variance (ANOVA) was performed using the AV1W MSUSTAT 4.12, program (Lund, 1989) with orthogonal contrasts and mean separation procedures were carried out using the multiple range tests with Fisher's least significant difference (LSD) (P < 0.05).

Conclusion

Under saline conditions exogenous, proline led to a marked increase in the accumulation of proline sugars, Mg^{2+} and Ca^{2+}. The presence of salt and proline in the culture medium reinforced proline contribution in the osmoregulation process. Finally, proline and soluble sugars may be involved not only in decreasing leaf water potential and serving as osmoregulators, but also as ROS scavengers as indicated by the decrease in lipid peroxidation level.

Acknowledgments

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