Amino Acids (2011) 40:565–573
DOI 10.1007/s00726-010-0677-1

ORIGINAL ARTICLE

Exogenous proline effects on water relations and ions contents in leaves and roots of young olive

Ch. Ben Ahmed · S. Magdich · B. Ben Rouina · S. Sensoy · M. Boukhris · F. Ben Abdullah

Received: 5 April 2010 / Accepted: 24 June 2010 / Published online: 9 July 2010

Abstract The ability of exogenous compatible solutes, such as proline, to counteract salt inhibitory effects was investigated in 2-year-old olive trees (*Olea europaea* L. cv. Chemlali) subjected to different saline water irrigation levels supplied or not with exogenous proline. Leaf water relations [relative water content (RWC), water potential], photosynthetic activity, leaf chlorophyll content, and starch contents were measured in young and old leaves. Salt ions (Na^+, K^+, and Ca^{2+}), proline and soluble sugars contents were determined in leaf and root tissues. Supplementary proline significantly mitigated the adverse effects of salinity via the improvement of photosynthetic activity (Pn), RWC, chlorophyll and carotenoid, and starch contents. Pn of young leaves in the presence of 25 mM proline was at 1.18 and 1.38 times higher than the values recorded under moderate (SS1) and high salinity (SS2) treatments, respectively. Further, the proline supply seems to have a more important relaxing effect on the photosynthetic chain in young than in old leaves of salt-stressed olive plants. The differential pattern of proline content between young and old leaves suggests that there would be a difference between these tissues in distinguishing between the proline taken from the growing media and that produced as a result of salinity stress. Besides, the large reduction in Na^+ accumulation in leaves and roots in the presence of proline could be due to its interference in osmotic adjustment process and/or its dilution by proline supply. Moreover, the lower accumulation of Na^+ in proline-treated plants, compared to their corresponding salinity treatment, displayed the improved effect of proline on the ability of roots to exclude the salt ions from the xylem sap flowing to the shoot, and thus better growth rates.

Keywords *Olea europaea* L. · Salinity stress · Proline supplement · Photosynthetic activity · Ions contents

Abbreviations

Chl	Chlorophyll
CP	Control plants
Ψ_{Lw}	Leaf water potential
Pn	Net photosynthesis
RWC	Leaf relative water content
SS1	Moderate salinity, plants irrigated with water containing 100 mM NaCl
SS2	High salinity, plants irrigated with water containing 200 mM NaCl
SS1 + P1	Plants irrigated with water containing 100 mM NaCl plus 25 mM proline
SS1 + P2	Plants irrigated with water containing 100 mM NaCl plus 50 mM proline
SS2 + P1	Plants irrigated with water containing 200 mM NaCl plus 25 mM proline
SS2 + P2	Plants irrigated with water containing 200 mM NaCl plus 50 mM proline

Ch. Ben Ahmed (✉) · S. Magdich · M. Boukhris · F. Ben Abdullah
Laboratory of Plant Biology and Ecophysiology in Arid Area, Department of Life Sciences, Faculty of Sciences of Sfax, 3000 Sfax, Tunisia
e-mail: benahmed.c@gmail.com

B. Ben Rouina
Laboratory of Improvement of Olive and Fruit Trees' Productivity, Olive Tree Institute, Sfax, Tunisia

S. Sensoy
Technical University of Istanbul, Istanbul, Turkey

Introduction

One of the common responses of many plant species exposed to different abiotic stresses, particularly salinity, is the accumulation of compatible organic solutes such as proline, glycine betaine, choline, and *O*-sulfate (Heuer 2003; Phang et al. 2008; Lehmann et al. 2010). Proline accumulation shown in different abiotic-stressed species (Zhou et al. 2008; Ben Ahmed et al. 2009a, b) allows them to maintain continuous water adsorption and/or stabilization of proteins and membranes against destabilizing effects of abiotic stresses leading generally to cellular water depletion (Parida and Das 2005).

The protecting roles of proline in plants under salinity conditions have already been reported in several species (Khedr et al. 2003; Hoque et al. 2007; Kaya et al. 2007). For example, in melon plants, Kaya et al. (2007) have demonstrated that exogenous proline mitigated the detrimental effects of salt stress. Similarly, Okuma et al. (2004) have signalled that proline mitigated the inhibition of growth of tobacco cells and reduces the oxidation of lipid membranes under saline conditions. Likewise, Ben Ahmed et al. (2010) showed that exogenous proline supplement allowed the preservation of olive growth at better level than salt-stressed plants, and mitigated the detrimental effects of salt stress by improving the activities of some antioxidative enzymes. As well, Ashraf et al. (2008) and Munns and Tester (2008) reported that proline protects higher plants against salt/osmotic stresses by protecting the photosynthetic apparatus. Besides, exogenous proline application to barley embryo cultures under saline conditions caused a significant decrease in shoot Na^+ and Cl^- concentrations (Lone et al. 1987). The same authors suggested that the ameliorative effect of proline in barley was due to membrane stabilization as was further supported by Mansour (1998), while assessing the improved effect of exogenous proline application on salt-stressed onion (*Allium cepa*). In rice, Roy et al. (1993) showed that exogenous application of 30 mM proline counteracted the adverse effects of salinity on early seedling growth, and enhances the K^+/Na^+ ratio.

Salt tolerance in olive is associated with effective mechanisms of ion exclusion and retention of Na^+ and Cl^- at root level (Tattini et al. 1995; Chartzoulakis et al. 2002; Loreto et al. 2003; Chartzoulakis, 2005). Salt stress has a double effect. First, it limits water availability to plant tissues leading to water deficit and secondly, it induces the accumulation of salt ions in the rooting zone, roots themselves and in aerial parts (Gucci et al. 1997). Chartzoulakis et al. (2002) have stated that salt accumulation in root zone of olive tree imposes osmotic stress and disrupts cell ion homeostasis by inhibiting the uptake of essential nutrients, as has also been reported in wheat (Raza et al. 2007) and sunflower (Akram et al. 2007). Tester and Davenport (2003) have showed that mechanisms minimizing damage from high salinity vary among plants and several mechanisms must operate in a coordinated manner to manage Na^+. Plants grown under salt-stressed conditions need to maintain internal water potential below that of soil to maintain turgor and sufficient water for growth. Such process requires an increase in osmotica, either by uptake of solutes from the soil or by synthesis of metabolically compatible solutes (Tester and Davenport 2003).

The specific involvement of proline in tolerance to stress, the inconsistency of the response to its exogenous application, the fact that it was mainly tested on bacteria, calli or isolated cell lines or from foliar application, and the socio-economic importance of cultivated olives were the leading decisive factors to carry out this research. In fact, very little is known about the linkage between proline level and physiological and nutritional indicators in olive tree under saline conditions. Thus, the purpose of this study was to provide additional information on exogenous proline effects on some physiological (water relations, photosynthetic activity), biochemical (sugars, proline, starch, pigments contents) and nutritional (nutrients status) characteristics in salt-stressed young Chemlali olive tree, and to assess the effectiveness of supplemental proline to counteract the deleterious effects of salinity stress on the most extended crop in arid region in Tunisia.

Materials and methods

Plant material and treatments

Trials were conducted at the Olive Tree Institute of Sfax, Tunisia (34°43 N; 10°41 E), from September 2008 to May 2009. Uniform 2-year-old self-rooted olive trees (*Olea europaea* L. cv Chemlali) were transplanted into 10-L pots filled with sand and perlite (3:1; v/v). The pots were kept under ambient environmental conditions with natural sunlight and temperature and were covered with plastic film and aluminum foil to reduce evaporation from the soil surface and to minimize temperature increases inside the containers. During the first 6 months of the trial period (September 2008–February 2009), all olive plants were irrigated with half-strength Hoagland solution. When plants developed shoots of 15–25 cm length, they were subjected to the following treatments: (1) control (CP), plants receiving nutrient solution; (2) moderate salinity (SS1), plants receiving nutrient solution plus 100 mM NaCl; (3) moderate salinity plus 25 mM proline (SS1 + P1); (4) moderate salinity plus 50 mM proline (SS1 + P2); (5) high salinity (SS2), plants receiving nutrient solution plus 200 mM NaCl; (6) high salinity plus

25 mM proline (SS2 + P1); and (7) high salinity plus 50 mM proline (SS2 + P2). The amount of water used for irrigation daily during the experimental period for the different treatments was equal to the amount lost by transpiration and determined as described by Ben Ahmed et al. (2009a). Each treatment consisted of three plots (groups) of four plants each (12 similar plants per treatment; 84 plants in total). The different measurements and analysis were taken on young leaves (fully expanded leaves that developed soon after the onset of the different treatments), old leaves (fully expanded leaves that developed soon before the imposition of different treatments), "thin roots" (roots with a diameter <3 mm), and "medium roots" (roots with a diameter between 3 and 8 mm).

Leaf water relations and gas exchange measurements

At the end of the experiment, measurements of leaf relative water content (RWC) were determined using the equation

$$\mathrm{RWC}(\%) = 100 \times (\mathrm{FW} - \mathrm{DW})/(\mathrm{TW} - \mathrm{DW});$$

where FW is the fresh weight, DW the dry weight, and TW the turgid weight of leaf samples. Leaves were excised before dawn, weighed fresh (FW), and placed in distilled water to rehydrate in the dark for 24 h. The following morning, leaf turgid weight (TW) was measured, and then leaves were dried at 80°C for 48 h and dry weight (DW) was determined.

Using a portable gas exchange system (Li-Cor Inc. 6200, Lincoln, NE, USA), gas exchange measurements were taken from 10:00 to 11:00 a.m. on both young and old leaves from three plants per treatment. At the end of the experiment, leaf water potential (ΨLW) was measured on the same leaves used for gas exchange parameters by a Scholander pressure chamber (pms-1000, Corvallis, OR, USA).

Proline, soluble sugars and starch content determinations

Leaf and root samples used for proline content determination were frozen immediately in liquid nitrogen. Free proline was determined according to the method of Bates et al. (1973). Soluble sugars content was determined by the method of Robyt and White (1987); and starch content was determined according to the method of Nilson (1943), as described by Ben Ahmed et al. (2009a).

Total chlorophyll and carotenoid concentrations

For the photosynthetic pigments contents determinations, leaf disks for each treatment were taken from young and old leaves of three plants with comparable leaf water potentials per treatment. Leaf sections were ground in 80% acetone. Total chlorophyll Chl ($a + b$) and carotenoid concentrations were determined according to the method of Arnon (1949).

Mineral ions contents determinations

Leaves and roots, served for ions determinations, were washed with distilled water to eliminate the dust, oven dried at 70°C for 72 h and then ground to a fine powder. One gram of the powder was placed in an oven at 250°C for 3 h and then transferred to 100 ml of dilute nitric acid. Concentrations of Na^+, K^+ and Ca^{2+} were determined in these digests using a flame photometer (Jenway, PEP-7).

Statistical analysis

Statistical analyses were performed using the statistical software package SPSS 10. Windows, and treatment means were compared using Least Significant Difference (LSD) test at $p < 0.05$, and plant tissues means were compared using Tukey's test calculated at $p \leq 0.05$ level.

Results

Leaf water relations and photosynthetic performance characteristics

Table 1 shows changes in RWC and leaf water potential in both young and old leaves of Chemlali olive plants subjected to the different NaCl and NaCl plus proline medium treatments. The irrigation with saline water at both salinity levels resulted in a decrease of leaf water relations characteristics (RWC and Ψ_{LW}). The higher the water salinity level was, the lower the RWC and leaf water potential were.

The largest reduction in RWC was recorded in SS2-treated plants, being of 16 and 19% of CP values for young and old leaves, respectively (Table 1). Differences in RWC and Ψ_{Lw} between both salt stress treatments were statistically significant ($p = 0.05$). At the end of the experiment, Ψ_{Lw} values of young leaves in SS1- and SS2-treated plants were at 2 and 2.36 times lower, respectively, in comparison to CP. The levels recorded in old leaves were at 1.75 and 2 times lower, respectively. Either leaf water relations characteristics (RWC and Ψ_{Lw}) were significantly improved in the presence of proline but at different extent among treatments and leaf tissues. The increment rate of RWC in young leaves in the presence of 25 and 50 mM proline was 1.04 and 1.06 times, respectively, if compared to values recorded in SS1-treated plants. In SS2 plus proline-treated plants, this increase was at 1.05 and 1.09 times

Table 1 Leaf relative water content (RWC), and leaf water potential (Ψ_{LW}) of young (YL) and old (OL) leaves from Chemlali olive plants subjected to different NaCl and NaCl plus proline treatments

Treatments	RWC (%)		Ψ_{LW} (MPa)	
	YL	OL	YL	OL
CP	96.2 ± 3.1^{a}	92.1 ± 3.3^{a}	-1.1 ± 0.2^{a}	-1.6 ± 0.2^{a}
SS1	88.4 ± 2.6^{b}	$82.3 \pm 3.2^{b*}$	-2.2 ± 0.4^{bd}	-2.8 ± 0.4^{b}
SS1 + P1	92.2 ± 2.4^{ad}	$89.6 \pm 3.8^{c*}$	-3.0 ± 0.4^{cd}	-3.3 ± 0.2^{c}
SS1 + P2	94.6 ± 3.1^{a}	91.1 ± 3.6^{a}	-3.3 ± 0.5^{ce}	-3.8 ± 0.5^{cd}
SS2	81.5 ± 3.5^{c}	$75.4 \pm 3.3^{d*}$	-2.6 ± 0.5^{d}	$-3.2 \pm 0.3^{c*}$
SS2 + P1	85.6 ± 3.2^{bc}	$80.7 \pm 3.1^{b*}$	-3.2 ± 0.5^{ce}	-3.7 ± 0.3^{c}
SS2 + P2	89.4 ± 3.4^{bd}	$83.6 \pm 3.4^{b*}$	-3.8 ± 0.4^{e}	$-4.4 \pm 0.3^{d*}$

Values represent means of six replications per treatment ± SE. Different letters (a, e) indicate significant differences ($p = 0.05$) between treatments within each leaf type treated separately. An asterisk indicates significant difference between young and old leaves within each treatment treated separately ($p \leq 0.05$, Tukey's test)

higher than those recorded in severe salt stress treatment (SS2), respectively. Nevertheless, these values remained lower than those registered in unstressed control plants.

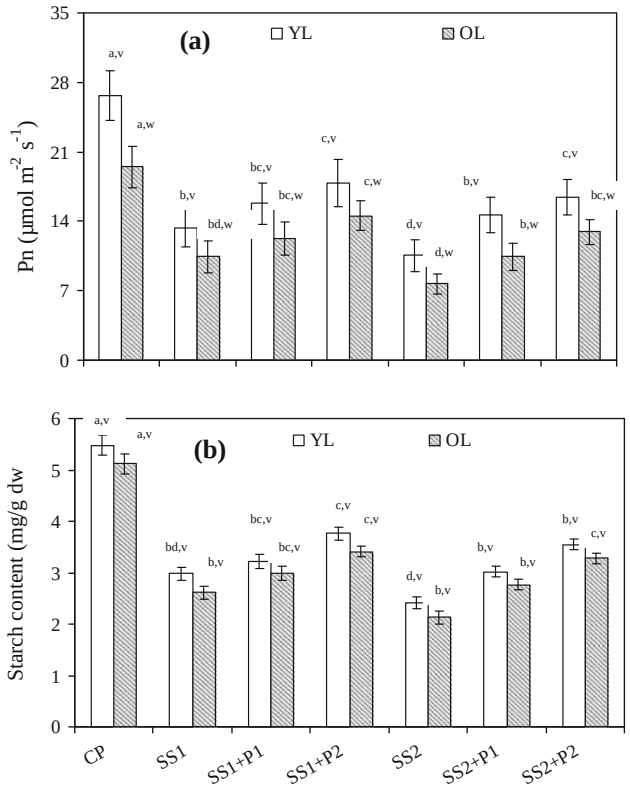

Fig. 1 Net photosynthesis (**a**) and starch contents (**b**) in young and old leaves from Chemlali olive plants subjected to different NaCl and NaCl plus proline treatments. Values represent means of ten replications (for net photosynthesis measurements) and three replications (for starch content determination) per treatment ± SE. Different letters (*a*, *d*) indicate significant differences ($p = 0.05$) between treatments within each leaf type treated separately. Different letters (*v*, *w*) indicate significant differences between young and old leaves within each treatment treated separately

Similarly, the increment of proline medium supplement was accompanied with a decrease of Ψ_{Lw} values. For the different NaCl and NaCl plus proline treatments, young leaves showed statistically higher RWC values than old ones ($p = 0.05$), except for the (SS1 + P2)-treated plants. Under both proline mediums, leaf water potential of old leaves showed lower values than those of young leaves.

The decrease of leaf water relations characteristics under salinity conditions was accompanied with that of photosynthetic activity (Fig. 1a). However, either externally supplied proline medium increased the above-mentioned parameters compared to non-treated stressed plants. Nevertheless, net photosynthesis (Pn) of young and old leaves was still much lower than that of control plants. Further, in all leaf tissues, the higher the proline medium was, the more important the net photosynthesis was, and the improved photosynthetic activity in both proline mediums was different among them. Pn of young leaves in the presence of 25 mM proline was at 1.18 and 1.38 times higher than the values recorded under SS1 and SS2 treatments, respectively. In the presence of 50 mM proline, these increments rates were at 1.33 and 1.55 times for the respective treatments. Besides, under the different treatments, young leaves showed higher photosynthetic activity than old ones.

Proline, soluble sugars and starch contents

The irrigation with saline water at different levels resulted in the increase of either proline and/or soluble sugars contents in leaves and roots of olive plants, if compared to the unstressed control plants, but at different extent among treatments and plant organelles (Table 2). Further, leaves (young and old) of both salt- stress treatments (SS1 + SS2) accumulate higher proline and soluble sugars than roots. Further, old leaves accumulate proline and sugars at higher

Table 2 Proline and soluble sugars contents in young (YL) and old (OL) leaves and thin (TR) and medium (MR) roots from Chemlali olive plants subjected to different NaCl and NaCl plus proline treatments

Treatments	Proline (μmol mg^{-1} FW)				Soluble sugars (μmol mg^{-1} FW)			
	Leaves		Roots		Leaves		Roots	
	YL	OL	TR	MR	YL	OL	TR	MR
CP	0.52 ± 0.09^{a}	0.72 ± 0.18^{a}	0.41 ± 0.12^{a}	0.51 ± 0.08^{a}	0.68 ± 0.18^{a}	1.02 ± 0.09^{a}	0.21 ± 0.06^{a}	0.31 ± 0.05^{a}
SS1	1.85 ± 0.13^{b}	$2.72 \pm 0.26^{b*}$	1.26 ± 0.13^{b}	1.58 ± 0.19^{b}	1.83 ± 0.31^{b}	$2.17 \pm 0.29^{b*}$	0.55 ± 0.11^{b}	0.71 ± 0.09^{bc}
SS1 + P1	2.25 ± 0.19^{c}	$3.12 \pm 0.28^{bc*}$	1.41 ± 0.16^{b}	1.95 ± 0.17^{bc}	1.47 ± 0.27^{b}	1.94 ± 0.29^{bc}	0.42 ± 0.09^{b}	0.61 ± 0.07^{bc}
SS1 + P2	2.55 ± 0.12^{cd}	$3.69 \pm 0.31^{cd*}$	1.85 ± 0.13^{bc}	2.26 ± 0.21^{c}	1.11 ± 0.29^{b}	1.51 ± 0.19^{c}	0.30 ± 0.11^{a}	0.44 ± 0.06^{ab}
SS2	2.18 ± 0.22^{bc}	$3.43 \pm 0.31^{cd*}$	1.62 ± 0.14^{b}	1.92 ± 0.25^{b}	2.98 ± 0.36^{c}	3.36 ± 0.34^{d}	0.81 ± 0.12^{b}	0.95 ± 0.12^{c}
SS2 + P1	3.24 ± 0.25^{d}	$4.14 \pm 0.32^{d*}$	2.24 ± 0.16^{c}	2.69 ± 0.27^{c}	2.37 ± 0.31^{c}	$2.82 \pm 0.27^{d*}$	0.72 ± 0.14^{b}	0.89 ± 0.15^{c}
SS2 + P2	4.19 ± 0.21^{e}	$5.26 \pm 0.31^{e*}$	2.81 ± 0.19^{c}	$3.79 \pm 0.27^{d*}$	1.46 ± 0.33^{b}	$2.15 \pm 0.26^{b*}$	0.59 ± 0.13^{b}	0.68 ± 0.09^{b}

Values represent means of 3 replications per treatment ± SE. Different letters (a, e) indicate significant differences ($p = 0.05$) between treatments within each leaf and root type treated separately. An asterisk indicates significant difference between young and old leaves and thin and medium roots within each treatment treated separately ($p \leq 0.05$, Tukey's test)

amounts than the young ones, and differences were significant ($p = 0.05$), except for control treatment. Similarly, medium roots showed higher levels of these solutes than thin roots, but differences among them were not significant, except for the (SS2 + P2)-treated plants.

As expected, the externally supplied proline improved the proline contents in leaves and roots of salt-stressed-treated plants, and the higher the proline medium was, the more important the proline content was. In young leaves, the increment rate of proline content, in the presence of 25 mM proline, was at 1.21 and 1.48 times, if compared to SS1 and SS2-treated plants, respectively. In the presence of 50 mM proline, these increment rates were at 1.37 and 1.92 times, respectively. In old leaves, these rates were at 1.14 and 1.21 times in the presence of 25 mM proline, and 1.35 and 1.53 times in the presence of 50 mM proline, respectively, for the various treatments.

On the other hand, the supplement of proline medium at both levels has led to the decrease of soluble sugars contents in the different analyzed tissues, but at different extent among treatments and organelles (Table 2). Under stressed conditions, the lowest values of sugars contents for the different analyzed tissues were registered in (SS1 + P2)-treated plants. For the various treatments, old leaves showed higher sugars contents than young leaves. Nevertheless, differences among sugars contents in leaf tissues (young and old) were significant for only SS1, (SS2 + P1), and (SS2 + P2) treatments. Likewise, medium roots showed higher amounts of soluble sugars than thin roots, but differences among them were not significant.

Data shown in Fig. 1b demonstrated that starch content in young and old leaves of olive plants were significantly reduced by both water salinity treatments, if compared to the unstressed control plants. For both leaf tissues, the lowest values of starch contents were recorded in SS2-treated plants. Further, the externally supplied proline increased the above-mentioned parameter, but values were still lower than those of control plants. Under stressed conditions, the highest amounts of starch content in both leaf tissues were recorded in (SS1 + P2)-treated plants. Moreover, for the various treatments, differences in starch content between young and old leaves were not significant.

Photosynthetic pigments

The data in Table 3 show that 100 and 200 mM NaCl treatments caused a significant decrease of both chlorophyll ($a + b$) and carotenoid contents, in comparison to control plants, as well in young as in old leaves, with higher amounts in young than in old leaves, and the extent of differences between them varied among treatments. Proline-treated olive plants showed higher photosynthetic pigments contents compared to the non-treated stressed ones, but at different levels among the proline mediums and leaves (Table 3). Yet, the improved levels were still lower than those of control plants. The highest levels of chlorophyll and carotenoid contents were recorded in young leaves of (SS1 + P2)-treated plants. These values were 10.12 and 5.12 mg g^{-1} dw, respectively, for chlorophyll ($a + b$) and carotenoid contents.

Mineral ions contents

Na^+, K^+ and Ca^{2+} contents and Na^+/K^+ and Na^+/Ca^{2+} ratios in young and old leaves of Chemlali olive plants grown under the different treatments are shown in Table 4. The results show that Na^+ contents in young and old leaves increased significantly in water salinity-treated plants (SS1 + SS2), compared to values recorded in control plants, with higher values in old than in young leaves. This

Table 3 Total chlorophyll ($a + b$) and carotenoid contents and chlorophyll/carotenoid ratio and of young (YL) and old (OL) leaves from Chemlali olive plants subjected to different NaCl and NaCl plus proline treatments

Treatments	Photosynthetic pigments					
	Chl ($a + b$) (mg g^{-1} dw)		Car (mg g^{-1} dw)		Chl/Car	
	YL	OL	YL	OL	YL	OL
CP	11.37 ± 0.23^{a}	$9.12 \pm 0.19^{a*}$	6.78 ± 0.13^{a}	$5.41 \pm 0.11^{a*}$	1.67	1.68
SS1	8.45 ± 0.20^{b}	$6.37 \pm 0.14^{bc*}$	4.22 ± 0.11^{bd}	$3.18 \pm 0.11^{b*}$	2.00	2.01
SS1 + P1	9.28 ± 0.19^{b}	$7.63 \pm 0.14^{b*}$	4.89 ± 0.11^{bd}	$3.91 \pm 0.11^{bc*}$	1.89	1.95
SS1 + P2	10.12 ± 0.22^{ab}	9.21 ± 0.21^{a}	5.12 ± 0.11^{bd}	4.56 ± 0.13^{c}	1.97	2.01
SS2	7.09 ± 0.11^{c}	$5.42 \pm 0.15^{c*}$	3.27 ± 0.11^{c}	2.67 ± 0.11^{d}	2.16	2.02
SS2 + P1	8.94 ± 0.15^{b}	$7.21 \pm 0.13^{b*}$	3.98 ± 0.11^{cd}	3.37 ± 0.11^{b}	2.24	2.13
SS2 + P2	9.15 ± 0.18^{b}	8.58 ± 0.15^{a}	4.71 ± 0.11^{d}	$3.95 \pm 0.11^{bc*}$	1.94	2.17

Values represent means of three replications per treatment ± SE. Different letters (a, d) indicate significant differences ($p = 0.05$) between treatments within each leaf type treated separately. An asterisk indicates significant difference between young and old leaves within each treatment treated separately ($p \leq 0.05$, Tukey's test)

Table 4 Na^+, K^+ and Ca^{2+} contents (% dry matter) in young and old leaves of young Chemlali olive plants subjected to different NaCl and NaCl plus proline treatments

Treatments	Young leaves					Old leaves				
	Na^+	K^+	Na^+/K^+	Ca^{2+}	Na^+/Ca^{2+}	Na^+	K^+	Na^+/K^+	Ca^{2+}	Na^+/Ca^{2+}
CP	0.37 ± 0.06^{a}	1.34 ± 0.09^{a}	0.27^{a}	4.39 ± 0.36^{a}	0.08^{a}	0.56 ± 0.16^{a}	1.21 ± 0.19^{a}	0.46^{a}	$3.86 \pm 0.39^{a*}$	0.14^{a}
SS1	0.82 ± 0.11^{b}	0.99 ± 0.15^{bc}	0.82^{b}	3.10 ± 0.29^{b}	0.26^{bc}	$1.05 \pm 0.18^{b*}$	0.84 ± 0.09^{b}	1.25^{bd*}	2.81 ± 0.29^{b}	0.37^{b}
SS1 + P1	0.61 ± 0.09^{bc}	1.19 ± 0.14^{ab}	0.51^{c}	3.42 ± 0.27^{b}	0.17^{b}	$0.92 \pm 0.15^{b*}$	$0.97 \pm 0.08^{ab*}$	0.94^{b*}	3.15 ± 0.26^{c}	0.29^{b}
SS1 + P2	0.50 ± 0.08^{ac}	1.12 ± 0.14^{ab}	0.44^{ac}	3.96 ± 0.27^{a}	0.12^{b}	0.65 ± 0.16^{c}	1.03 ± 0.07^{ab}	0.63^{a}	$3.37 \pm 0.31^{c*}$	0.19^{a}
SS2	1.06 ± 0.12^{d}	0.88 ± 0.09^{c}	1.21^{d}	2.65 ± 0.22^{c}	0.40^{c}	$1.46 \pm 0.18^{d*}$	$0.65 \pm 0.06^{b*}$	2.24^{c*}	$2.05 \pm 0.26^{d*}$	0.71^{c*}
SS2 + P1	0.91 ± 0.13^{bd}	1.02 ± 0.11^{bc}	0.89^{b}	2.99 ± 0.24^{c}	0.30^{b}	1.11 ± 0.21^{b}	$0.81 \pm 0.06^{b*}$	1.37^{d*}	2.63 ± 0.29^{b}	0.42^{b}
SS2 + P2	0.78 ± 0.14^{b}	1.09 ± 0.12^{bc}	0.71^{b}	3.34 ± 0.33^{b}	0.23^{b}	$1.03 \pm 0.22^{b*}$	0.94 ± 0.08^{ab}	1.09^{b*}	3.11 ± 0.33^{c}	0.33^{b}

Values represent means of three replications per treatment ± SE. Different letters (a, d) indicate significant differences ($p = 0.05$) between treatments. An asterisk indicates significant difference between young and old leaves within each treatment treated separately ($p \leq 0.05$, Tukey's test)

increase was accompanied with a significant decrease ($p = 0.05$) of K^+ and Ca^{2+} contents for all leaf tissues of both salt stress treatments. Such patterns resulted in a significant reduction in Na^+/K^+ and Na^+/Ca^{2+} ratios for all analyzed leaf tissues. The increase of Na^+ contents and Na^+/K^+ and Na^+/Ca^{2+} ratios under salinity conditions was affected by exogenous proline supplement. Indeed, in both leaf tissues, the mentioned-parameters decreased in proline-treated plants, compared to salt stressed ones, but at different extent among treatments and leaf tissues. In young leaves grown under (SS1 + P1)-treated plants, the proline- induced decrease was significant for Na^+/K^+ ratio, compared to SS1-treated plants. In (SS1 + P2) treatment, this decrease was significant for Na^+ content and Na^+/K^+ ratio. Compared to SS2-treated plants, the decrease was significant for Na^+/K^+ and Na^+/Ca^{2+} ratio of (SS2 + P1)-treated plants. In (SS2 + P2)-treated olive plants, the decrease was significant for the different parameters. For old leaves, the decrease was significant for the various parameters in (SS1 + P2)-treated plants, compared to SS1-treated olive plants. Compared to values registered in SS2 treatment, both proline medium levels induced a significant decrease of these parameters.

Either proline medium supplement led to an increase of both K^+ and Ca^{2+} contents in young and old leaves, compared to salt stressed ones, but at different extent among leaf tissues and treatments. In young leaves, this increase was significant for Ca^{2+} content of salt stressed plants treated with 50 mM proline ((SS1 + P2) and (SS2 + P2) treatments). In old leaves, this increase was significant for the different salt stressed plants treated at either 25 or 50 mM proline. However, for the K^+ content, the increase was not significant in all leaves.

The distribution of these nutrients in thin and medium roots from plants of all treatments showed the same pattern

Table 5 Na^+, K^+ and Ca^{2+} contents (% dry matter) and Na^+/K^+ and Na^+/Ca^{2+} ratios in thin and medium roots of young Chemlali olive plants subjected to different NaCl and NaCl plus proline treatments

Treatments	Thin roots					Medium roots				
	Na^+	K^+	Na^+/K^+	Ca^{2+}	Na^+/Ca^{2+}	Na^+	K^+	Na^+/K^+	Ca^{2+}	Na^+/Ca^{2+}
CP	0.68 ± 0.06^{a}	1.02 ± 0.09^{a}	0.66^{a}	3.09 ± 0.36^{a}	0.22^{a}	0.84 ± 0.16^{a}	0.93 ± 0.19^{a}	0.91^{a}	2.76 ± 0.39^{a}	0.30^{a}
SS1	1.48 ± 0.11^{b}	0.65 ± 0.15^{b}	2.27^{b}	2.10 ± 0.29^{be}	0.70^{b}	1.75 ± 0.18^{b}	0.54 ± 0.09^{b}	3.24^{b*}	$1.41 \pm 0.29^{b*}$	1.24^{be*}
SS1 + P1	1.15 ± 0.09^{ce}	0.88 ± 0.14^{ab}	1.33^{c}	2.32 ± 0.27^{b}	0.49^{ab}	1.32 ± 0.15^{c}	0.61 ± 0.08^{b}	2.16^{c*}	$1.55 \pm 0.26^{bc*}$	0.85^{c*}
SS1 + P2	1.02 ± 0.08^{e}	0.91 ± 0.14^{a}	1.12^{c}	2.86 ± 0.27^{c}	0.35^{a}	1.12 ± 0.16^{ac}	0.78 ± 0.07^{ab}	1.43^{d}	$1.87 \pm 0.31^{c*}$	0.59^{ac}
SS2	1.76 ± 0.12^{d}	0.58 ± 0.09^{b}	3.03^{d}	1.45 ± 0.22^{d}	1.21^{c}	$2.96 \pm 0.18^{d*}$	0.45 ± 0.06^{b}	6.57^{e*}	$1.05 \pm 0.26^{d*}$	2.81^{d*}
SS2 + P1	1.38 ± 0.13^{bc}	0.67 ± 0.11^{b}	2.05^{b}	1.96 ± 0.24^{e}	0.70^{b}	$1.77 \pm 0.21^{b*}$	0.58 ± 0.06^{b}	3.05^{b*}	$1.23 \pm 0.29^{bd*}$	1.43^{e*}
SS2 + P2	1.12 ± 0.14^{c}	0.86 ± 0.12^{ab}	1.31^{c}	2.14 ± 0.33^{be}	0.52^{ab}	1.43 ± 0.22^{c}	0.71 ± 0.08^{ab}	2.01^{c*}	$1.51 \pm 0.33^{bc*}$	0.94^{bc*}

Values represent means of three replications per treatment ± SE. Means within each column followed by different letters are significantly different ($p = 0.05$). An asterisk indicates significant difference between thin and medium roots within each treatment treated separately ($p \leq 0.05$, Tukey's test)

as in young and old leaves, as both displayed an increase in Na^+ content, Na^+/K^+ and Na^+ Ca^{2+} ratios, and a decrease of K^+ and Ca^{2+} contents under either salinity levels, but at different extent among them (Table 5). The proline supplement at either medium level resulted in an increase of K^+ and Ca^{2+} contents, and a decrease of Na^+ content, Na^+/K^+ and Na^+/Ca^{2+} ratios as well in thin as in medium roots. For the various treatments, medium roots displayed higher values of Na^+ content, Na^+/K^+ and Na/Ca^{2+} ratios than thin roots. These latter, in contrary, had higher levels of both K^+ and Ca^{2+} contents.

Discussion

Salinity stress altered leaf water status, chlorophyll and carotenoid contents, and photosynthetic activity of olive plants used in this experiment. The highest reduction of the above-mentioned parameters was observed in old leaves. As suggested by Ashraf and Foolad (2007) and Munns and Tester (2008), suppression of plant growth under saline conditions may be due to osmotic reduction in water availability or to excessive ion (particularly Na^+) accumulation in plant tissues. The differential pattern of water status and photosynthetic activity between young and old leaves of stressed plants resulted from the higher RWC in young tissues and the high salt ions accumulation in the old ones. Further, this tendency displayed the important protective role played by old leaves of olive plants, in such a way to preserve better water status and activity of young leaves, photo-synthetically more active than old ones, and per consequent the maintenance of plant growth at suitable levels. Such adaptive mechanisms prevented the appearance of toxicity symptoms (leaf shedding, necrosis, chlorosis, …) in the olive plants used in this experiment as well under moderate (SS1) as under high water salinity (SS2) level. On the other hand, the reduction of K^+ and Ca^{2+} contents in salinized olive plants could be due to the interference of Na^+ with phloem loading inhibiting thus the translocation of assimilated reserves, independent on leaf water potential and RWC as reported by Kaya et al. (2007) in melon plants.

Face to the loss of turgor induced by salinity conditions, olive plants tend to accumulate osmolyte compounds such as proline and sugars as well in leaves as in roots. On the other hand, leaf tissues presented higher amounts of the above-mentioned parameters than roots. Besides, under both salinity treatments, the young leaves had better Pn levels, while the older ones had higher levels of proline and soluble sugars.

The higher accumulation of proline and sugars in old leaves, compared to young ones, could be due to their lower RWC and photosynthetic activity. In fact, as soon as the olive tree perceived water deficit induced by ions accumulation, it rapidly began accumulating osmolytes (proline) to facilitate water uptake to growing tissues. Further, in order to maintain the translocation of assimilates, such as sugars to the growing parts, the old leaves, with low photosynthetic activity, tend to synthesize more soluble sugars. On the other hand, the decrease in starch content in stressed plants could be due to starch degradation and/or to the increase in soluble sugar concentration under limited water availability circumstances. Likewise, Todaka et al. (2000) indicated that β-amylase activity increased under stressed conditions. Furthermore, alterations of photosynthetic performance under limited water availability may alter carbon assimilate partitioning between sucrose and starch. The lower concentrations of starch in leaves of salt-stressed plants in conjunction with low photosynthetic rates suggest that carbon was translocated out of the leaves.

A large body of evidence reported that exogenous application of compatible solutes offers a valuable tool for

studying mechanisms of salt tolerance. Among the main studied mechanisms, the osmotic adjustment which allows the preservation of growth under saline conditions (Heuer 2003). This process is mainly achieved by uptake of inorganic ions (particularly Na^+ and Cl^-), and partly by synthesis and accumulation of compatible compounds (Flowers et al. 1977; Serraj and Sinclair 2002). The increased levels of proline content in salinized plants supplied with exogenous proline were higher than that recorded in their corresponding salt treatments. This greater accumulation of proline may result from an increased endogenous production, from a decreased metabolism, or from a direct effect of salinity on the uptake and translocation of proline. Salt-stressed olive plants may have evolved a mechanism to coordinate synthesis, catabolism, and transport activities for the accumulation of proline which could be absorbed and metabolized in plant tissues. These processes seem to be more active in leaves than in roots.

More to the point, the decrease of soluble sugars contents in salted proline-treated plants revealed the important osmoprotectant effect played by the added proline in such a way to limit the need of salt-stressed plants for soluble sugars synthesis, and thus relaxing the pressure on the photosynthetic chain. In young and old leaves, the proline medium at 50 mM had a more important osmotic effect on the stressed plants than the 25 mM proline medium. On the other hand, the differential pattern in net photosynthetic activity among young and old leaves (with higher amounts in the young ones) could be explained by the fact that young leaves seem to be more able in adjusting the supplied proline than the old ones. Indeed, it appears that a difference exists among these tissues in distinguishing between the proline taken from the growing media and that produced by the leaves as a result of exposure to salinity. Such processes suggest that for young leaves, the added proline, which was metabolized, would to reduce its de novo formation in the cells, and thus less energy was dissipated for solutes synthesis allowing a preservation of higher activity. In contrast, for the old leaves, it seems that a lot of energy was preserved for such process, phenomenon that could alter consequently their photosynthetic activity.

According to the results of Kaya et al. (2007), the improvement of plant water status in salted olive plants supplied with proline might be attributed to the inhibition of water efflux via effects of this solute on membrane stability and reduced leaf water potential, and thus activation of water adsorption by the aerial parts, via its major role in osmotic adjustment. Similarly, Zhao et al. (1992) reported that proline provides protection against destabilization of proteins and membranes. The more chlorophyll and carotenoid contents recorded in proline-treated olive plants reinforce this idea and confirmed the experiment of Gadallah (1995) on cotton plants.

The large reduction in Na^+ accumulation in both leaves and roots in the presence of proline could be due to its interference in osmotic adjustment process and/or its dilution by proline supply. Besides, the lower accumulation of Na^+ in tissues of proline-treated plants, compared to their corresponding salinity treatment, displayed the improved effect of proline on the ability of roots to exclude the salt ions from the xylem sap flowing to the shoot, and thus better growth rates, as suggested by Heuer (2003). This behavior was justified by the higher photosynthetic activity of young leaves of salted proline-treated plants.

On the other hand, the application of supplied proline enhanced K^+ and Ca^{2+} uptake by olive plants. Such results suggest that proline application improved the capacity of olive plants in distinguishing between essential potassium and calcium and potentially toxic sodium ions under salinity conditions, and ameliorated the competition among these ions in favor of both potassium and calcium. Consequently, the Na^+/K^+ and Na^+/Ca^{2+} ratios were also different in the presence of 25 and 50 mM proline. According to Munns and Tester (2008), the higher improvement of K^+ uptake under salinity in the presence of 50 mM proline, than that recorded in the presence of 25 mM proline supply, could be explained by the fact that 50 mM proline supplement seems to be more effective in improving the ability of salted olive plants for selectivity between essential nutrient and potentially toxic ions.

Conclusion

From the results of this experiment, proline supply seems to counteract the deleterious effects of salinity stress on Chemlali olive plants used in this trial via the reduction of Na^+ accumulation, the improvement of Ca^{2+} and K^+ uptake under salinity and the amelioration of physiological activity and water relations characteristics, but at different extent among the two proline levels applied. More to the point, the relaxing effect of the proline supply on the photosynthetic chain, by limiting the need for soluble sugars synthesis under salinity conditions, was more advanced in the presence of 50 mM proline than that of 25 mM proline, but at different extent among leaf tissues. Such behavior makes it possible to recommend the treatments of olive plants grown under saline conditions with this chemical solute, at least under the described experimental conditions. To better manage such practice and improve olive extension in saline water irrigated lands, further studies focusing on the determination of the effective proline concentration range for olive cultivars grown under arid region in Tunisia and the proper olive plant developmental phase for external proline application are on the way.

Acknowledgments Financial support from the Ministry of Agriculture, Hydraulic Resources and Fisheries (IRESA, Tunisia) is highly appreciated. Authors express their debt to the editor and to two anonymous reviewers for their interest and comments to improve the manuscript quality. The Authors express their thanks to Mr Soua N. for his technical assistance.

References

Akram MS, Athar HR, Ashraf M (2007) Improving growth and yield of sunflower (*Helianthus annuus* L.) by foliar application of potassium hydroxide (KOH) under salt stress. Pak J Bot 39:234–241

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant Physiol 24:1–15

Ashraf M, Foolad MR (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 59:206–216

Ashraf M, Athar HR, Harris PJC, Kwon TR (2008) Some prospective strategies for improving crop salt tolerance. Adv Agric 97:45–109

Bates LS, Waldren RP, Teare IK (1973) Rapid determination of free proline for water stress studies. Plant Soil 39:205–208

Ben Ahmed Ch, Ben Rouina B, Sensoy S, Boukhris M, Ben Abdallah F (2009a) Changes in gas exchange, proline accumulation and antioxidative enzyme activities in three olive cultivars under contrasting water availability regimes. Environ Exp Bot 67:345–352

Ben Ahmed Ch, Ben Rouina B, Sensoy S, Boukhris M, Ben Abdullah F (2009b) Saline water irrigation effects on antioxidant defense system and proline accumulation in leaves and roots of field-grown olive. J Agric Food Chem 57:11484–11490

Ben Ahmed Ch, Ben Rouina B, Sensoy S, Boukhris M, Ben Abdullah F (2010) Exogenous proline effects on photosynthetic performances and antioxidant defense system of young olive. J Agric Food Chem 58:4216–4222

Chartzoulakis K (2005) Salinity and olive: growth, salt tolerance, photosynthesis and yield. Agric Water Manag 78:108–121

Chartzoulakis K, Loupassaki M, Bertaki M, Androulakis I (2002) Effects of NaCl salinity on growth, ion content and CO_2 assimilation rate of six olive cultivars. Sci Hortic 96:235–247

Flowers TJ, Troke PF, Yeo AR (1977) The mechanism of salt tolerance in halophytes. Annu Rev Plant Physiol 28:875–884

Gadallah MAA (1995) Effects of water stress, abscissic acid and proline on cotton plants. J Arid Environ 30:315–325

Gucci R, Lombardini L, Tattini M (1997) Analysis of leaf water relations in leaves of two olive (*Olea europaea*) cultivars differing in tolerance to salinity. Tree Physiol 17:13–21

Heuer B (2003) Influence of exogenous application of proline and glycinebetaine on growth of salt stressed tomato plants. Plant Sci 165:693–699

Hoque MA, Okuma E, Banu MNA, Nakamara Y, Shimoishi Y, Murata Y (2007) Exogenous proline mitigates the detrimental effects of salt stress more than exogenous betaine by increasing antioxidant enzyme activities. J Plant Physiol 164:553–561

Kaya C, Levent Tuna A, Ashraf M, Altunlu H (2007) Improved salt tolerance of melon (*Cucumis melo* L.) by the addition of proline and potassium nitrate. Environ Exp Bot 60:397–403

Khedr AHA, Abbes MA, Wahid AAA, Quick WP, Abogadallah GM (2003) Proline induces the expression of salt stress responsive proteins and may improve the adaptation of *pancratium maritimum* L. to salt stress. J Exp Bot 54:2553–2562

Lehmann S, Funck D, Szabados LS, Rentsch D (2010) Proline transport and metabolism in plants. Amino Acids. doi:10.1007/s00726-010-0525-3

Lone MI, Kuech JSH, Wyn Jones RG, Bright SWJ (1987) Influence of proline and glycine betaine on salt tolerance of cultured barley embryos. J Exp Bot 38:479–490

Loreto F, Centritto M, Chartzoulakis K (2003) Photosynthetic limitations in olive cultivars with different sensitivity to salt stress. Plant Cell Environ 26:595–601

Mansour MMF (1998) Protection of plasma membrane on onion epidermal cells by glycine betaine and proline against NaCl stress. Plant Physiol Biochem 36:767–772

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681

Nilson JP (1943) Rapid determination of starch. Ind Eng Chem 15:3–7

Okuma E, Murakami Y, Shimoishi Y, Tada M, Murata Y (2004) Effects of exogenous application of proline and betaine on the growth of tobacco cultured cells under saline solutions. Soil Sci Plant Nutr 50:1301–1305

Parida AK, Das B (2005) Salt tolerance and salinity effects on plants. Ecotoxicol Environ Saf 60:324–349

Phang J, Donald S, Pandhare J, Liu Y (2008) The metabolism of proline, a stress substrate, modulates carcinogenic pathways. Amino Acids 35:681–690

Raza SH, Athar HR, Ashraf M, Hameed A (2007) GB-induced modulation of antioxidant enzymes activities and ion accumulation in two wheat cultivars differing in salt tolerance. Environ Exp Bot 59:206–216

Robyt JF, White BJ (1987) Biochemical technique: theory and practices. Waveland Press, Prospect Heights, pp 40–72

Roy D, Basu N, Bhunia A, Banerjee SK (1993) Counteraction of exogenous L-proline with NaCl in salt-sensitive cultivar of rice. Biol Plant 35:69–72

Serraj R, Sinclair TR (2002) Osmolyte accumulation: can it really help increase crop yield under drought conditions? Plant Cell Environ 25:331–341

Tattini M, Gucci R, Coradeschi MA, Ponzio C, Everard JD (1995) Growth, gas exchange and ion content in *Olea europaea* plants during salinity stress and subsequent relief. Physiol Plant 95:203–210

Tester M, Davenport R (2003) Na^+ tolerance and Na^+ transport in higher plants. Ann Bot 91:503–527

Todaka D, Matsushima H, Morohashi Y (2000) Water stress enhances beta-amylase activity in cucumber cotyledons. J Exp Bot 51:739–745

Zhao Y, Aspinall D, Paleg IG (1992) Protection of membrane integrity in *Medicago sativa* L. by glycinebetaine against the effects of freezing. J Plant Physiol 140:541–543

Zhou Y, Zhu W, Bellur PS, Rewinkel D, Becker DF (2008) Direct linking of metabolism and gene expression in the proline utilization A protein from *Escherichia coli*. Amino Acids 35:711–718