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ARTICLE

Saline Water Irrigation Effects on Antioxidant Defense System and Proline Accumulation in Leaves and Roots of Field-Grown Olive

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Field-grown olive trees (*Olea europaea* L. cv. Chemlali) were used over two growing seasons to determine the effects of different saline water irrigation levels on levels of proline and chlorophyll contents and activities of superoxide dismutase (SOD), polyphenol oxidase (PPO), ascorbate peroxidase (APX), and catalase (CAT). The plants were irrigated with fresh water (FW; ECe = 1.2 dS m^{-1}) and saline water (SW; ECe = 7.5 dS m^{-1}). Leaf water relations (relative water content, water potential), photosynthetic activity, and leaf chlorophyll content decreased under irrigation with saline water. In spring 2005, net photosynthesis of young leaves was 24.5 and 14.9 μmol m^{-2} s^{-1} in FW- and SW-treated plants, respectively. In old leaves, these rates were 20.2 and 12.2 μmol m^{-2} s^{-1}, respectively. The relative reduction of net photosynthesis in SW-treated plants varied from 39 to 46% and from 39 to 61%, compared to FW-treated plants during the first and second crop seasons, respectively. The relative reduction of leaf chlorophyll (a + b) content under high water salinity level exceeds 50%, compared to FW-treated plants. However, proline content and activities of SOD, CAT, and APX increased under saline water irrigation. The increase of proline content was more important in leaves than in roots. In young leaves, the increment of antioxidant activities in SW-treated plants was 2.67, 3.61, and 1.85 times, respectively, for SOD, APX, and CAT, compared to FW-treated plants. From these results, interaction between antioxidant defense system and proline contents seems to be involved in the salt tolerance mechanisms of Chemlali olive tree.

KEYWORDS: *Olea europaea* L.; saline water; antioxidant status; photosynthetic performances; proline accumulation; leaf chlorophyll content

INTRODUCTION

The maintenance of an appropriate plant water status during water deficit, resulting from saline water irrigated conditions or a prolonged drought period, is essential for continued growth of the plant. This process can be achieved by stomatal regulation (*1*) and accumulation of compatible solutes in either leaves or roots (*2*, *3*).

Most studies focusing on olive tree response to salt stress conditions focused particularly on some photosynthetic and growth aspects (*4*, *5*). More recently, it has been shown that the accumulation of osmolytes as proline in either leaves or roots is a well-known adaptive mechanism against stressed conditions (*2*, *3*, *6*). It also has been shown that proline acts as an osmolyte facilitating the retention of water in the cytoplasm, and its accumulation can serve as a selection criterion for most tolerant species to stressed conditions (*7*). Moreover, proline has a protective action that prevents membrane damage and protein denaturation during severe stress (*8*). However, the improvement of stress tolerance due to proline accumulation is species-dependent (*6*).

One of the important biochemical changes occurring in plants subjected to biotic or abiotic stresses is the production of reactive oxygen species (ROS) (*2*, *9*). Under salinity conditions, the decrease of photosynthesis in olive plants is attributed mainly to stomatal closure and salt ion accumulation in either leaves or roots (*5*). However, as the stress progresses, photosynthetic CO_2 fixation may be limited more directly by biochemical constraints. To avoid photoinhibition resulting from limited water availability circumstances, electron transfer, along the photosynthetic chain, is directed to oxygen acceptors other than water (*6*, *10*). Consequently, the excess reducing power determines a redirection of photon energy into processes that favor the production of ROS. Chloroplasts, mitochondria, and peroxisomes are the major intracellular generators of ROS. In these organelles, ROS can be generated by direct transfer of excitation energy from chlorophyll to produce singlet oxygen or by univalent oxygen reduction of photosystem I (*7*, *9*, *10*).

The involvement of antioxidants in protection against oxidative stress has been demonstrated with transgenic plants subjected

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to enhanced levels of some antioxidative enzymes (*11*). According to Smirnoff (*12*), low water availability often is associated with increased levels of ROS such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), and singlet oxygen ($^1O_2$). ROS are highly reactive, and they can seriously disrupt the normal metabolism of the plant in the absence of any protective mechanism.

Plants use enzymatic and nonenzymatic antioxidative defense mechanisms to scavenge ROS. According to Kuwabara and Katoh (*13*), among antioxidant enzymes, superoxide dismutase (SOD; EC 1.15.1.1) is the major scavenger of superoxide. With catalase (CAT; EC 1.11.1.6), SOD catalyzes the dismutation of superoxide to H_2O_2 and O_2. The ascorbate peroxidases (APX; EC 1.11.1.11) detoxify the H_2O_2 produced. Polyphenol oxidase (PPO; EC 1.30.3.1), involved in the metabolism of phenols, oxidizes orthodiphenolic substrates to *o*-quinones.

Nowadays, the controlled use of low-quality water (saline water, treated wastewater) in irrigation is becoming important, particularly under conditions of limited water resources and rainfall scarcity in arid regions. For olive trees, there is some evidence that this species is tolerant to salinity conditions and that this tolerance level is cultivar and plant-age dependent (*7*, *14*). However, most of the investigations have been carried out on young plants, grown in pots and under controlled conditions. To our best of knowledge, there is no study on the effects of saline water on proline accumulation and the antioxidant defense system of adult olive tree grown under field conditions, and very little is known about the linkage between olive salt stress tolerance and antioxidative enzymes activities or between proline level and antioxidants in the olive tree under saline conditions, on the other hand. The objectives of this study were to determine the mechanisms of proline accumulation and to evaluate some antioxidative enzyme activities in adult, field-grown olive trees subjected to irrigation with saline water. We hypothesize that there would be correlations between the different parameters under stressed conditions. It is possible that both proline accumulation and antioxidative enzyme activities could be used as indices of salt tolerance in olive trees. Our experimental approach allows us to improve the understanding of the interaction between different mechanisms developed by field-grown Chemlali olive trees to resist saline water irrigation in the arid region in Tunisia.

MATERIALS AND METHODS

Plant Material, Treatments, and Climatic Conditions. Olive trees (*Olea europaea* L. cv. Chemlali), planted in 1992 in a sandy soil at a density of 625 trees ha^{-1} at Sfax, Tunisia (34° 43′ N, 10° 41′ E), were used over two successive crop seasons (2005 and 2006). The sandy soil of the experimental orchard (90.5% sand, 4.5% clay, and 5% silt) was characterized by an organic matter of 1.1%, 13.4% $CaCO_3$, 1.3% N, pH of 7.6, a field capacity (measured at 33 KPa) of 11.8% by weight, and a wilting point (measured at 1500 kPa) of 5.9%.

In 2004, 10 trees from two adjacent rows (total 20 trees per treatment), with four replications of 5 trees each, were selected to be similar in potential yield and canopy. The Chemlali olive trees were subjected to the following treatments: irrigation with fresh water, 1.2 dS m^{-1} ECe (FW), and high-saline water, 7.5 dS m^{-1} ECe (SW). The water used was either that supplied by the Tunisian National Water Carrier (FW) or saline water (SW) from the local reservoir situated in the area of the Olive Tree Institute in Sfax. The fresh and saline waters used were characterized by 145 and 600 mg L^{-1} Na^+, 326 and 1169 mg L^{-1} Cl^-, 280 and 520 mg L^{-1} K^+, 94 and 261 mg L^{-1} Ca^{2+}, and 57 and 102 mg L^{-1} Mg^{2+}, respectively.

The amount of water supplied to olive trees was estimated according to the Penman−Monteith FAO equation (*15*) as described by Ben Ahmed et al. (*1*). The irrigation was delivered with a drip system with four drip nozzles (two per side) of 4 L h^{-1} per tree set in a line along the rows (at 0.5 m from the trunk). Total water supplied to mature olive trees (without taking rainfall into account) was 4000 m^3 ha^{-1} $year^{-1}$. The plants were subjected to the same olive cultivation practices in the area.

Table 1. Principal Seasonal[a] Climatic Conditions (Air Temperature (*T*), Precipitation (*P*), and Photosynthetic Active Radiations (PAR)) of the Experimental Site during the Trial Period

year		spring	summer	autumn	winter
2004/2005	*T* (°C)	28 ± 2	36 ± 2	30 ± 2	23 ± 3
	P (mm)	32 ± 3	1	56 ± 4	73 ± 5
	PAR (μmol m^{-2} s^{-1})	1013 ± 56	1418 ± 59	970 ± 28	746 ± 47
2005/2006	*T* (°C)	28 ± 1	36 ± 2	29 ± 2	23 ± 2
	P (mm)	48 ± 5	1	61 ± 5	58 ± 3
	PAR (μmol m^{-2} s^{-1})	945 ± 45	1414 ± 67	1076 ± 44	776 ± 37

[a] Values of each season are means of averages of the three respective months ± SD. In Tunisia, spring, March−May; summer, June−August; autumn, September−November; and winter, December−February.

The region is characterized by an arid climate of Mediterranean type. The annual rainfall and temperature averages over a 52 years were 250 mm and 23 °C, respectively. In both crop seasons, precipitation was virtually absent during summer, and it was 218.5 and 285.5 mm, respectively, in the first and second crop seasons. The mean temperatures were 25.6 and 25.1 °C, respectively, and maximum temperatures were, respectively, 38 and 37 °C. The evapotranspiration rates were 1413 and 1271 mm in the 2004 and 2005 crop seasons, respectively. Climatic conditions of the experimental site during the trial period are summarized in **Table 1**.

Physiological Parameters. The measurements of different physiological parameters were carried out on young leaves (YL, the fully expanded leaves developed soon after the onset of the different saline water treatments) and old leaves (OL, the fully expanded leaves developed soon before the imposition of different saline water treatments) separately. For transpiration rates and stomatal conductance, results are the mean of values recorded in young or old leaves of each treatment. Leaf relative water content (RWC) was determined as

$$\mathrm{RWC}\ (\%) = [(\mathrm{fw} - \mathrm{dw})/(\mathrm{tw} - \mathrm{dw})] \times 100$$

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.

Predawn and midday leaf water potentials (LPWP and LMWP) were measured on leaves taken from the median part of the same shoots used for leaf relative water content determination by a Scholander pressure chamber (pms-1000, Corvallis, OR) (*4*). To minimize water loss during the transfer of the leaf to the chamber, leaves were enclosed immediately, after excision, in a black plastic bag.

Gas exchange measurements were taken from 10:00 a.m. to 1:00 p.m. on well-exposed leaves from three plants per treatment, using a portable gas exchange system (Li-CorInc. 6200, Lincoln, NE) (*16*).

Proline, Total Chlorophyll, and Carotenoid Contents. Leaf (collected from the median part of selected shoots) and root samples for proline content determination were frozen immediately in liquid nitrogen. Free proline was determined according to the method of Bates et al. (*17*). A 0.5 g portion of frozen powder was mixed with 5.0 mL of 3% (w/v) sulfosalicylic acid in glass tubes covered at the top and boiled for 5 min in a water bath at 100 °C. After cooling, the mixture was centrifuged at 2000*g* for 5 min at 25 °C. A 200 μL portion of the extract was mixed with 400 μL of distilled water and 20 mL of the reagent mixture (30 mL of glacial acetic acid, 20 mL of distilled water, and 0.5 g of ninhydrin) and boiled at 100 °C for 1 h. After cooling the mixture, we added 6.0 mL of toluene. The products were extracted by vortex mixing; the upper phase (toluene) was separated, and absorption at 520 nm was read, using toluene as a blank. Proline concentration was calculated using L-proline for the standard curve (0−50 μg/mL).

Leaf disks for pigment (chlorophyll and carotenoid) determinations were taken from three fully expanded leaves of plants with comparable leaf water potentials for each treatment. Leaf disks were weighed and ground in 5 mL of 80% acetone/water solution (v/v), using a pestle and mortar.

After filtration, the extraction was repeated with another 5 mL of acetone solution (80%). The combined filtrates were made up to 50 mL with 80% acetone solution, and the total chlorophyll Chl ($a + b$) and carotenoid contents were determined spectrophotometrically according to the method of Arnon (*18*).

Enzyme Activities. Leaf and root samples collected for enzyme activity determinations were arranged as young and old leaves as described above, and roots were divided into two groups, thin roots (TR), with a diameter < 3 mm, and medium roots (MR), with 3 < diameter < 8 mm. Frozen leaf and root samples were ground to a fine powder in liquid nitrogen, using a mortar and pestle previously chilled with liquid nitrogen, and frozen powder served for enzyme extraction. For SOD, APX, and CAT, 1.0 g of frozen powder was added to 10 mL of absolute ethanol for 30 min. The mixture was centrifuged at 4 °C for 30 min at 20000*g*; the supernatant was discarded, and the extracted pellet was used for enzyme activities.

The total SOD activity was determined according to the method described by Giannopolitis and Ries (*19*). To a 3 mL reaction mixture containing 50 mM sodium−potassium phosphate buffer (1.0 mL NaKPi), pH 7.8, 13 mM methionine (0.4 mL), 75 μM nitroblue tetrazolium (0.4 mL), 0.1 μM EDTA (1.0−0.2 mL), and 20−100 μL of enzyme extract was added 2 μM riboflavin. The tubes were shaken and illuminated with a 15 W fluorescent tube placed 50 cm from the reaction mixture. The reaction was operated for 10 min, after which the light was eliminated and the absorbance was read at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of nitroblue tetrazolium chloride reduction.

For APX, the extracted pellet was suspended in 5.0 mL of cold 50 $NaKP_i$, pH 7.8, 100 μM EDTA, 500 μM ascorbate, and 0.1% (W/V) polyvinylpolypyrrolidone (PVPP) prepared and stored at 4 °C. After 30 min, the mixture was centrifuged at 10000*g* for 30 min, and the supernatant was used for the enzyme activity assay. The APX activity was assayed by recording spectrophotometrically the decrease of ascorbate absorption at 290 nm, according to the method of Ushimaru et al. (*20*). APX activity was estimated by excluding the contribution of peroxide dismutase activity (POD) in the extract to the oxidation of ascorbate. Hydroxylamine, a selective inhibitor of APX, was used to determine the POD activity. One unit of APX activity was defined as the amount of enzyme that oxidizes 1 μmol of ascorbate per minute. An absorption coefficient of 2.47 mM^{-1} cm^{-1} was used for calculations.

For the CAT activity determination, the extracted pellet was resuspended in 10.0 mL of 10.0 mM cold $NaKP_i$, pH 7.0, and 0.1% (w/v) PVPP, mixed, and stored at 4 °C. After 30 min of vortex agitation, the mixture was centrifuged at 15000*g* for 15 min. CAT activity was assayed according to the method of Aebi (*21*). The decomposition of H_2O_2 was followed spectrophotometrically by the decrease in A_{240}. One unit of CAT activity corresponds to the amount of enzyme that decomposes 1 μmol of H_2O_2 per minute.

For PPO activity estimation (units mg^{-1} of dw), 1.0 g of leaf or root frozen powder was added to 10.0 mL of cold 200 μM Napa, pH 7.0, 5 mM Na_2EDTA, 0.1% (w/v) PVPP, 3 mM dithiothreitol, 15 mM β-mercaptoethanol, and 10 mM sodium metabisulfite, mixed, and stored at 4 °C. After 30 min of vortex agitation, the mixture was centrifuged at 15000*g* for 30 min, and the absorbance was read at 420 nm. PPO activity was assayed according to the method of Canal et al. (*22*).

For the different measurements, at least three replicates were used for each field and laboratory test.

Statistical Analysis. Statistical analyses were performed using the statistical package SPSS 10 for Windows, and treatment means were compared using the least significant difference (LSD) test at $p < 0.05$.

RESULTS

Physiological Parameters. Irrigation for two successive crop seasons resulted in a significant decrease of RWC levels of young or old leaves of SW-treated plants. During both crop seasons, RWC of FW-treated plants varied from 84.58 to 94.83%. In SW-treated plants, these values varied from 70.78 to 88.27%. In SW-treated plants, the highest values of RWC were recorded during autumn and winter seasons in coincidence with more favorable climatic conditions (high precipitation, low temperature, and low photosynthetic active radiations). For both treatments, the lowest values of RWC were registered during the summer season. During the experimental period, young leaves (YL) of both treatments maintained higher values of RWC than old ones (OL) (**Table 2**), yet differences between RWC values of leaf tissues (YL or OL) of SW treatment were higher than those recorded in leaves of FW-treated plants. Regardless of the treatment, RWC values were not maintained at stable levels in the experimental period.

Table 2. Relative Water Content (RWC) in Young and Old Leaves from Fresh Water (FW) and Saline Water (SW) Irrigated Field-Grown Chemlali Olive Plants[a]

	young leaves		old leaves	
season	FW	SW	FW	SW
		2005		
spring	91.24 ± 3.19 av	85.53 ± 2.52 bv	90.33 ± 3.19 av	81.36 ± 2.93 bv
summer	85.45 ± 3.65 av	77.08 ± 3.27 bv	84.58 ± 3.65 av	70.78 ± 2.53 bw
autumn	92.25 ± 2.87 av	82.32 ± 3.26 bv	89.62 ± 3.14 av	75.52 ± 2.73 bw
winter	93.02 ± 3.74 av	87.44 ± 3.16 bv	90.88 ± 3.19 av	80.24 ± 2.92 bw
		2006		
spring	91.52 ± 3.54 av	81.81 ± 3.16 bv	90.13 ± 2.82 av	77.13 ± 3.03 bv
summer	87.06 ± 3.20 av	76.35 ± 3.10 bv	86.44 ± 3.04 av	70.92 ± 3.06 bw
autumn	92.43 ± 3.03 av	81.07 ± 3.05 bv	89.43 ± 3.53 av	77.07 ± 3.14 bv
winter	94.83 ± 2.44 av	88.27 ± 3.12 bv	91.45 ± 3.44 av	82.57 ± 2.78 bw

[a] Values represent means of five measurements (± SE). Different letters (a, b) 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.

Table 3. Leaf Predawn and Midday Water Potentials of Fresh Water (FW) and Saline Water (SW) Irrigated Field-Grown Chemlali Olive Plants[a]

	LPWP (MPa)		LMWP (MPa)	
season	FW	SW	FW	SW
		2005		
spring	−0.95 ± 0.25 av	−1.84 ± 0.18 b	−1.44 ± 0.21 a	−2.67 ± 0.32 b
summer	−1.19 ± 0.26 a	−2.02 ± 0.24 b	−1.93 ± 0.22 a	−3.32 ± 0.28 b
autumn	−0.69 ± 0.24 a	−1.27 ± 0.19 b	−1.75 ± 0.19 a	−3.34 ± 0.27 b
winter	−0.75 ± 0.24 a	−1.58 ± 0.28 b	−1.45 ± 0.25 a	−2.54 ± 0.32 b
		2006		
spring	−0.92 ± 0.19 a	−1.69 ± 0.27 b	−1.25 ± 0.19 a	−2.72 ± 0.31 b
summer	−1.08 ± 0.25 a	−2.45 ± 0.22 b	−1.84 ± 0.29 a	−4.07 ± 0.35 b
autumn	−0.91 ± 0.26 a	−1.96 ± 0.23 b	−1.78 ± 0.30 a	−3.53 ± 0.37 b
winter	−0.75 ± 0.19 a	−1.86 ± 0.25 b	−1.42 ± 0.29 a	−2.86 ± 0.33 b

[a] Values represent means of five measurements (± SE). Different letters (a, b) indicate significant differences ($p < 0.05$) between treatments.

Leaf predawn (LPWP) and midday (LMWP) water potentials of either treatment displayed patterns similar to those of RWC values with significantly lower values in SW-treated plants than in FW-treated ones (**Table 3**). Leaf predawn water potential values in FW-treated plants varied from −1.19 to −0.75 MPa, whereas those in SW-treated ones ranged from −2.45 to −1.27 MPa. For either treatment, the highest values of LPWP were recorded during autumn and winter seasons. Leaf midday water potential in SW-treated plants was approximately half that of FW ones. The extent of this decrease was not maintained at the same level during the two successive crop seasons. During 2004−2005, the lowest values of LMWP were −1.93 and −3.34 MPa, respectively

Table 4. Net Photosynthesis (Pn) in Young and Old Leaves of Fresh Water (FW) and Saline Water (SW) Irrigated Field-Grown Chemlali Olive Plants[a]

	Pn (μmol of CO_2 m^{-2} s^{-1})			
	young leaves		old leaves	
season	FW	SW	FW	SW
		2005		
spring	24.5 ± 2.6 av	14.9 ± 3.7 bv	20.2 ± 2.4 aw	12.2 ± 1.7 bv
summer	9.6 ± 2.1 av	5.5 ± 0.9 bv	11.3 ± 2.1 av	4.3 ± 0.9 bv
autumn	12.8 ± 1.8 av	7.4 ± 1.2 bv	10.9 ± 1.9 av	5.4 ± 0.8 bv
winter	7.3 ± 1.5 av	4.3 ± 0.9 bv	6.2 ± 1.0 av	3.8 ± 0.9 bv
		2006		
spring	26.1 ± 3.4 av	14.3 ± 2.1 bv	22.6 ± 3.0 aw	10.5 ± 1.2 bw
summer	7.6 ± 1.0 av	4.1 ± 0.9 bv	5.7 ± 0.9 av	2.7 ± 0.8 bv
autumn	14.2 ± 2.1 av	8.0 ± 1.5 bv	11.5 ± 1.8 av	6.1 ± 0.9 bv
winter	9.7 ± 1.0 av	5.4 ± 0.9 bv	8.7 ± 0.9 av	3.7 ± 0.8 bv

[a] Values represent means of 20 measurements (± SE). Different letters (a, b) 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.

Table 5. Stomatal Conductance (Gs) and Transpiration Rate (E Rate) of Fresh Water (FW) and Saline Water (SW) Irrigated Field-Grown Chemlali Olive Plants[a]

	Gs (mmol of H_2O m^{-2} s^{-1})		E rate (mmol of H_2O m^{-2} s^{-1})	
season	FW	SW	FW	SW
		2005		
spring	146.5 ± 4.5 a	100.1 ± 2.4 b	6.9 ± 1.1 a	3.8 ± 0.6 b
summer	98.7 ± 3.3 a	65.0 ± 2.2 b	3.9 ± 0.7 a	1.7 ± 0.4 b
autumn	103.1 ± 3.1 a	84.2 ± 2.2 b	6.3 ± 0.7 a	3.7 ± 0.4 b
winter	98.8 ± 2.7 a	77.6 ± 2.5 b	3.9 ± 0.5 a	2.5 ± 0.4 a
		2006		
spring	165.7 ± 2.5 a	88.9 ± 2.4 b	8.6 ± 0.8 a	3.5 ± 0.5 b
summer	83.6 ± 2.1 a	47.9 ± 2.3 b	3.7 ± 0.6 a	1.1 ± 0.5 b
autumn	108.3 ± 3.4 a	74.4 ± 2.6 b	6.8 ± 0.7 a	3.1 ± 0.5 b
winter	93.2 ± 2.9 a	72.6 ± 2.5 b	3.1 ± 0.6 a	1.9 ± 0.4 a

[a] Values represent means of 20 measurements (± SE). Different letters (a, b) indicate significant differences ($p < 0.05$) between treatments.

in FW- and SW-treated plants. In the second crop season, these minimums were −1.84 and −4.07 MPa, respectively.

SW-treated plants showed a significant decrease of net photosynthesis (Pn) in young or old leaves, compared with FW-treated olive trees (**Table 4**). For either treatment, young leaves presented higher photosynthetic rates than the old ones. During both crop seasons, the highest net photosynthetic rates of young leaves from all plants were recorded during the spring season. These rates were 24.5 and 26.1 μmol of CO_2 m^{-2} s^{-1} in FW-treated plants and 14.9 and 14.3 μmol m^{-2} s^{-1} in SW-treated plants, respectively, during the 2004−2005 and 2005−2006 crop seasons. In old leaves, carbon dioxide assimilation averages were 20.2 and 22.5 μmol of CO_2 m^{-2} s^{-1} in FW-treated plants and 12.2 and 10.5 μmol of CO_2 m^{-2} s^{-1} in SW-treated ones, respectively, for the two seasons. In either treatment, the lowest values of photosynthetic rates were recorded during the summer season. The relative reduction of Pn in SW-treated plants varied from 39 to 46% and from 39 to 61%, compared to FW-treated plants during the first and second crop seasons, respectively. Transpiration rates (E rates) and stomatal conductance (Gs) patterns in SW-treated plants exhibited a similar pattern (**Table 5**), as both displayed a significant decrease in comparison to FW-treated plants. Stomatal conductance values varied between 84 and 166 mmol of H_2O m^{-2} s^{-1} and from 47 to 100 mmol of H_2O m^{-2} s^{-1} in FW- and SW-treated plants, respectively. Similarly, the seasonal average of transpiration rates of SW-treated plants did not exceed 4 mmol of H_2O m^{-2} s^{-1} during the two crop seasons. However, in FW-treated plants, these averages reached 8.66 mmol of H_2O m^{-2} s^{-1} in spring 2006.

Table 6. Proline Contents in Leaves and Roots from Fresh Water (FW) and Saline Water (SW) Irrigated Field-Grown Chemlali Olive Plants[a]

	proline contents (μmol mg^{-1} of fw)			
	leaves		roots	
season	FW	SW	FW	SW
		2005		
spring	0.42 ± 0.09 a	0.88 ± 0.10 b	0.32 ± 0.10 a	0.67 ± 0.10 b
summer	0.47 ± 0.10 a	1.06 ± 0.11 b	0.37 ± 0.11 a	0.85 ± 0.11 b
autumn	0.49 ± 0.11 a	1.44 ± 0.10 b	0.43 ± 0.07 a	1.02 ± 0.11 b
winter	0.51 ± 0.09 a	1.86 ± 0.12 b	0.48 ± 0.09 a	1.36 ± 0.11 b
		2006		
spring	0.44 ± 0.11 a	2.17 ± 0.11 b	0.43 ± 0.09 a	1.51 ± 0.12 b
summer	0.47 ± 0.09 a	2.22 ± 0.16 b	0.42 ± 0.05 a	1.68 ± 0.14 b
autumn	0.44 ± 0.09 a	2.14 ± 0.19 b	0.36 ± 0.03 a	1.64 ± 0.13 b
winter	0.32 ± 0.11 a	2.05 ± 0.20 b	0.26 ± 0.02 a	1.39 ± 0.12 b

[a] Values represent means of three measurements (± SE). Different letters (a, b) indicate significant differences ($p < 0.05$) between treatments within each plant organelle treated separately.

Table 7. Total Chlorophyll ($a + b$) and Carotenoid Contents and Chlorophyll/Carotenoid Ratio from Fresh Water (FW) and Saline Water (SW) Irrigated Field-Grown Chemlali Olive Plants[a]

treatment	chl ($a + b$) (mg g^{-1} of dw)	carotenoid (mg g^{-1} of dw)	chl/carotenoid ratio
FW	16.82 ± 1.21 a	3.11 ± 0.56 a	5.4 a
SW	7.56 ± 1.14 b	2.16 ± 0.72 a	3.5 b

[a] Values represent the means of three samples (± SE). Different letters (a, b) indicate significant differences ($p < 0.05$) between treatments.

Proline, Chlorophyll, and Carotenoid Contents. During the first crop season, proline content in SW-treated plants increased significantly compared to FW-treated values, particularly in leaves (**Table 6**). During the first crop season, the seasonal average of proline content in leaves varied from 0.42 and 0.51 μmol mg^{-1} of fw and from 0.88 and 1.86 μmol mg^{-1} of fw in FW- and SW-treated plants, respectively. During the second crop, these averages varied from 0.32 and 0.47 μmol mg^{-1} of fw and from 2.05 and 2.22 μmol mg^{-1} of fw, respectively. During the experimental period, the seasonal averages of proline contents in roots were between 0.32 and 0.48 μmol mg^{-1} of fw and between 0.67 and 1.68 μmol mg^{-1} of fw in FW- and SW-treated plants, respectively. During 2005−2006, the increment of proline content in leaves or roots of SW-treated plants was superior to that recorded during the first crop season.

At the end of the experimental period, a significant decrease of total chlorophyll content occurred in SW-treated plants compared to FW-treated ones (**Table 7**). The relative reduction of chlorophyll (chl $a + b$) content under high water salinity level exceeds 50%, compared to FW-treated plants. Although not statistically significant, the carotenoid content decreased under SW conditions. The decrease of pigment contents resulted in a significant drop of chlorophyll/carotenoid ratio in the case of SW-treated plants.

Table 8. Antioxidative Enzyme Activities of Young and Old Leaves from Fresh Water (FW) and Saline Water (SW) Irrigated Field-Grown Chemlali Olive Plants[a]

	enzyme activity (units mg^{-1} of dw)							
	young leaves				old leaves			
	SOD	APX	CAT	PPO	SOD	APX	CAT	PPO
FW	12.56 ± 1.10 av	4.27 ± 0.55 av	6.45 ± 0.67 av	39.67 ± 2.50 av	14.32 ± 1.10 a,v	5.22 ± 0.34 av	6.41 ± 0.48 av	37.29 ± 1.30 av
SW	33.56 ± 2.89 bv	15.43 ± 1.03 bv	11.98 ± 1.04 bv	26.14 ± 1.20 bv	36.71 ± 2.10 bw	17.56 ± 0.80 bv	13.29 ± 0.51 bv	22.55 ± 1.31 bw

[a] Values represent the means of three measurements (± SE). Different letters (a, b) 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.

Table 9. Antioxidative Enzyme Activities of Thin and Medium Roots from Fresh Water (FW) and Saline Water (SW) Irrigated Field-Grown Chemlali Olive Plants[a]

	enzyme activity (units mg^{-1} of dw)							
	thin roots				medium roots			
	SOD	APX	CAT	PPO	SOD	APX	CAT	PPO
FW	6.84 ± 0.47 av	1.04 ± 0.32 av	3.12 ± 0.71 av	31.27 ± 2.22 av	7.26 ± 0.83 av	1.12 ± 0.69 av	2.56 ± 0.43 av	39.27 ± 2.10 aw
SW	13.67 ± 1.30 bv	2.88 ± 0.61 av	8.39 ± 1.13 bv	14.26 ± 1.61 bv	18.35 ± 1.40 bw	2.81 ± 0.42 av	4.33 ± 0.81 bw	21.54 ± 1.31 bw

[a] Values represent means of three measurements (± SE). Different letters (a, b) indicate significant differences ($p < 0.05$) between treatments within each leaf type treated separately. Different letters (v, w) indicate significant differences between thin and medium roots within each treatment treated separately.

Activities of Antioxidative Enzymes. For all tissues tested in this experiment, SOD, APX, and CAT activities increased significantly in SW-treated plants, compared to their respective FW-treated ones. In young leaves, this increase was 2.67, 3.61, and 1.85 times, respectively for SOD, APX, and CAT enzymes in comparison to their respective activities in FW-treated plants (**Table 8**). In old leaves, this increase was 2.56, 3.36, and 2.07, respectively. Regardless of the salinity water level treatment, old leaves showed significantly higher SOD activity than young leaves.

In thin and medium roots of SW treatment, the SOD activity was, respectively, 1.99- and 2.52-fold higher than those of FW-treated plants (**Table 9**). For the APX activity, this increase was 2.76 and 2.5 times, respectively. Regardless of the water salinity treatment, medium roots presented significantly higher activity of SOD and lower activity of CAT than thin roots.

PPO was the only enzyme clearly down-regulated by high saline water level, and a significant decline of PPO activity was noted in all tissues of the SW treatment (**Tables 8** and **9**). The relative reductions of PPO activity in SW-treated plants, compared to FW-treated plants, were 34.1 and 39.5%, respectively, in young (YL) and old leaves (OL). In thin roots (TR) and medium roots (MR), the decline rates were 54 and 45%, respectively, compared to FW-treated plants. Regardless of the treatment, young leaves of SW-treated plants presented higher levels of PPO activity than the old ones. In root tissues, for either treatment, PPO activity of medium roots was significantly higher than that of thin roots.

DISCUSSION

High water salinity level may induce combined negative effects on plants including osmotic stress, ion toxicity, and oxidative stress. High water salinity level (SW) significantly affected relative water content, leaf water potentials and photosynthetic performances of plants.

The differential pattern of water status and photosynthetic activity between young and old leaves of SW-treated plants resulted from the higher RWC in young tissues and the high salt ion accumulation in the old ones (data not shown). These results confirmed previous findings (*3*, *5*, *23*) suggesting that old leaves seem to play a protective role for the young ones against salt ion damage.

Previous papers have correlated the decrease of net photosynthetic rate in salt-stressed plants mainly to lower Gs and to salt ion accumulation in the different plant tissues (*5*). Our data confirmed this hypothesis, whereas nonstomatal limitation on photosynthetic activity might have also occurred in leaves of high water salinity treated plants. For instance, the lowered leaf chlorophyll content in SW-treated plants might have contributed to the decrease of net photosynthesis.

The inhibition of photosynthetic activity by high water salinity level would induce oxidative stress resulting from the imbalance between light capture and its utilization (*24*). Changes in the phytochemistry of the chloroplasts in leaves of salt-stressed plants resulted in dissipation of excess energy, thus generating active oxygen species, which are potentially dangerous to plant health under stressed conditions. The decrease of leaf chlorophyll content and the increase of SOD, CAT, and APX activities in leaves or roots of SW-treated plants exhibits the oxidative stress induced by saline water irrigation and suggests that the antioxidant defense system would play an important role in the salt tolerance response of olive trees.

These results confirmed earlier findings suggesting that salt tolerance is related to the efficiency of antioxidative enzymes (*25*, *26*). The decrease of chlorophyll content has been considered to be a typical symptom of oxidative stress and may be the result of pigment photooxidation, chlorophyll degradation, or lack of chlorophyll synthesis (*12*). According to Büssis et al. (*27*), expanded leaves exposed to limited water availability degraded their photosynthetic apparatus, possibly to mobilize resources for the production of new acclimated leaves. Such a mechanism could explain the higher photosynthetic rates registered in young leaves. Either young or old leaves of SW-treated plants showed a considerable increase of APX activity with higher levels than those recorded in thin and medium roots. This tendency suggests that the APX activity could be attributed mainly to the chloroplast-located enzyme of leaf tissues. Indeed, the important increase of APX activity in leaves could be considered among the main mechanisms developed by olive trees for the protection of chloroplasts, which under stress conditions present sustained electron flows and are the main producers and targets of ROS action (*13*).

Nevertheless, the extent of the increase in antioxidant activities was different from that recorded in previous findings in Coratina olive grown under water-deficit conditions (*9*) and in salt-stressed *Crithmum maritimum* (*28*) species. Indeed, the extent to which the activities of antioxidative enzymes are elevated under

water-stressed or salt-stressed conditions is variable among plant species and even among cultivars of the same species as reported by Bartoli et al. (*29*). These differences could be also related to environmental conditions characterizing each environment.

Moreover, it has been reported that CAT and SOD participate in the elimination of hydrogen peroxide produced in plants dealing with an environmental stress (*25*). Similarly, Kim et al. (*30*) suggested that an antioxidant defense system is involved in eliminating H_2O_2 from salt-stressed roots. This observation was true in thin or medium roots of olive, whereas the system SOD−APX−CAT seemed responsible for the antioxidant defense system in the case of young and old leaves as reported by Ben Amor et al. (*28*). The catalase, localized in peroxisomes, decomposes hydrogen peroxide to water and molecular oxygen without consuming reductants and, thus, may provide plant cells with an energy-efficient mechanism to remove hydrogen peroxide (*6*, *10*).

The proline accumulation in either leaves or roots of SW-treated plants would be of importance for the activation of water transport and the maintenance of suitable water potential under conditions of reduced water availability as reported in previous papers (*7*, *9*). More to the point, the positive evolution of CAT, SOD, and APX activities and proline accumulation in leaves or roots of SW-treated plants reinforces the hypothesis developed by Khedr et al. (*31*) signaling that the increase of antioxidative enzyme activities under salinity conditions could be due to proline accumulation. The same authors suggested that proline can act as a free radical scavenger to alleviate salt stress. The same concept was developed by Kohler et al. (*26*), suggesting that proline can act as a scavenger of reactive oxygen species.

From our results, interaction between the antioxidant system and proline contents seems to be involved strongly in olive tree performance under saline conditions. Furthermore, the surroundings experimental conditions (sandy soil texture, drip irrigation system, and rainfall pattern) seem to play an important role in the maintenance of plant water status and photosynthetic performances during the experimental period at acceptable levels (*32*).

The protective role of proline has been reported to improve the tolerance of some plants facing environmental constraints such as heat, drought, or salinity (*3*, *7*). Although the role of proline in the regulation of plant stress response is still unclear, it has been suggested that proline may increase antioxidant enzyme activities and reduce cell membrane peroxidation of salt-stressed plants (*26*, *28*).

Furthermore, the high level of proline accumulated in salt-stressed olive plants may ensure the olive tree protection against salt or osmotic stresses, not only by increasing leaf water potential but also by stabilizing many functional units such as complex II electron transport, membranes and proteins, and enzymes such as rubisco as suggested by Mäkelä et al. (*33*). The pattern of proline accumulation in SW-treated plants during the trial period showed also its dependence on environmental conditions and the vegetative growth cycle of the olive tree. Indeed, the high levels of proline in both leaves and roots of SW-treated plants, particularly during the second crop season, were recorded during summer and autumn periods in coincidence with high temperature averages.

Polyphenol oxidase is among the major enzymes responsible for the oxidation of phenolic compounds (*34*). The decrease of PPO activity in SW-treated olive tissues could be developed by the olive tree to improve the antioxidative action of phenols. Indeed, the reduction of PPO activity allows the maintenance of phenol compound contents at acceptable levels. Indeed, PPO may participate in the degradation of natural phenols with complex structures (*34*). Besides, the proteolytic action of PPO implies that PPO could be involved in removing the proteins damaged by oxidative stress effects (*13*). The same authors have signaled that H_2O_2 accelerates the protein degradation and that the H_2O_2-induced degradation was inhibited by metal chelators as well as by a polyphenolic substrate of PPO. Polyphenol oxidases are copper-containing monooxygenases catalyzing the orthohydroxylation of phenols and the oxidation of *o*-diphenols to the corresponding *o*-quinones, at the expense of molecular oxygen (*13*). Similarly, Kuwabara and Katoh (*35*) have signaled that PPO activity led to the degradation of the 23 kDa protein of PSII.

In conclusion, it appears that olive tree response to saline water irrigation under field conditions is dependent on the different surroundings experimental conditions. The salt tolerance of olive trees resulted from the interaction between different mechanisms leading to the activation of water uptake. The positive evolution of antioxidative enzyme activities and proline content in SW-treated plants is further evidence of direct interaction between proline accumulation mechanism and antioxidant defense system for improving olive salt tolerance. To confirm better this hypothesis, further experiments focusing on the effects of exogenous application of proline on some antioxidative enzymes of different olive cultivars grown in Tunisia are underway.

ABBREVIATIONS USED

APX, ascorbate peroxidase; CAT, catalase; Chl, chlorophyll; FW, fresh water; LMWP, leaf midday water potential; LPWP, leaf predawn water potential; MR, medium roots ; OL, old leaves; PPO, polyphenol oxidase; ROS, reactive oxygen species; RWC, leaf relative water content; SOD, superoxide dismutase; SW, high saline water; TR, thin roots ; YL, young leaves.

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