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Original Article

Photosynthetic and Growth Responses of Olive to Proline and Salicylic Acid under Salinity Condition

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Abstract

Salinity has a negative impact on growth and productivity of crops on agricultural lands. Since proline and salicylic acid are used by plants to cope with stress conditions, to test whether they can help olive plants to alleviate negative impacts of salt stress, an experiment was carried out by spraying proline (15 mM) and salicylic acid (0.25 mM) on the plants that subjected to two salinity levels (0 and 100 mM NaCl). Salinity caused an alteration in biomass partitioning; in such a way that shoot vegetative growth was more restricted by salinity than root vegetative growth. Root volume was increased in proline-sprayed plants while salinity caused a decline in the root volume. Salinity resulted in an increase in specific leaf area. Net photosynthesis, stomatal conductance and transpiration rate were decreased by salinity application in root medium. Peroxidase activity decreased in plants that subjected to salinity stress. However, application of proline resulted in improvement of vegetative growth in both control and salinity conditions. Increase in chlorophyll index was observed following proline application, while salinity caused a decline in chlorophyll index. In conclusion, salinity can cause deleterious effects on photosynthesis and vegetative growth of olive trees. Exogenous application of proline can help plants to cope with negative effects of salt stress on olive plants.

Keywords: olive, photosynthesis, proline, salicylic acid, salinity

Introduction

Salinity and sodicity can decrease plant growth and yield via disturbing metabolic processes in plants (Hernandez *et al*., 1995; Tarakcioglu and Inal, 2002). Salinization of lands has been received lots of attention due to its progressive impact on agricultural lands, especially on the region where water supply is limited (Plaut *et al*., 2013).

Olive is cultivated in many regions around the world ranging from temperate to subtropical climates. The olive tree is a hypostomatous species which well adapted to semiarid regions of Mediterranean climate, and is traditionally grown under drought conditions (Issaoui *et al*., 2010). In most Mediterranean coastal areas with high olive tree plantation, the raised need for good quality water for urban use restricts the use of fresh water for irrigation. On the other hand, in those areas, large quantities of low quality water (mostly saline) are available, which can be used for olive tree irrigation (Chartzoulakis, 2005). In order to use moderately salinized lands for olive trees, it is necessary to find an efficient approach to improve the tolerance of crops to salinity stress.

Plant responses to salt stress range from growth retardation and accelerated leaf senescence under moderate stress to permanent wilting of shoots with subsequence plant death, under extreme salt stress conditions (Maksimović *et al*., 2010; Cabot *et al*., 2014). Exposure to salt stress would result in a wide range of physiological changes in the plants; among those, the accumulation of low-molecular-weight solutes such as Proline (Pro) and betaines that commonly referred to them as compatible solutes (Ashraf and Foolad, 2007). Free Pro accumulation in the leaf is one of the most important plant adaptations during stress conditions. Pro functions as an osmoprotectant (Ashraf and Foolad, 2007; Kaya *et al*., 2007) and as a storage compound for reduced carbon and nitrogen in the case of stress conditions (Sarker *et al*., 2005). It may act as a substrate for respiration, which might provide energy required for recovery from stress. Pro can increase the capacity of plant to survive under disturbed water balance conditions. Pro also can function as an antioxidant to regulate redox potentials (Serraj and Sinclair, 2002; Verbruggen and Hermans, 2008), Pro accumulation has been shown to be a late adaptive response in plant

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tissues under salt stress (Delauney and Verma, 1993; Serraj and Sinclair, 2002).

Salicylic acid (SA) is considered as a hormone-like substance, which has an important role in the regulation of plant growth and development, glycolysis, seed germination, fruit yield, flowering and heat production in thermogenic plants (Vlot *et al*., 2009; Rivas-San Vicente and Plasencia, 2011). SA application can influence ion uptake and transport (Harper and Balke, 1981)and gas exchange between plant and environment (Khan *et al*., 2003). The role of SA in defence mechanisms under both biotic and abiotic stresses has been shown in many plant species (Vlot *et al*., 2009; Ashraf *et al*., 2010; Montillet and Hirt, 2013). According to Borsani *et al*. (2001), SA multiplies the ROS generation under stress conditions. Nevertheless, direct physiological effect of SA on alteration of antioxidant enzyme activities has been also reported (Ashraf *et al*., 2010; Khokon *et al*., 2011; Kalachova *et al*., 2013). Therefore, SA is one of the signals that plants used to cope with stresses conditions.

Since Pro and SA have many positive roles on plant functions especially under stress conditions, therefore, the hypothesis of the present study was to investigate the possibility of decreasing deleterious effects of salinity stress by exogenous application of Pro and SA on olive trees. Furthermore, the individual effects of chemical substances and salinity on photosynthetic parameters, proxidase activity and vegetative growth of olive trees were investigated. The aims of this study were to investigate: (i) the effects of salinity on growth and photosynthesis of olive trees; (ii) the effects of exogenous Pro and SA applications on vegetative and photosynthetic characteristics of olive trees; and consequently (iii) the improving effects of exogenous applications of Pro and SA on salinity tolerance of olive trees.

Materials and Methods

Plant growth conditions and treatments

One-year-old own rooted olives plants (cv. 'Zard') were transplanted into 12l pots containing perlite:sand:vermiculite (50:25:25, v:v) for hydroponic culture. During the experiment, the pots were kept into the glasshouse with a temperature of 30±3 during the day and 20±3 °C at night. The experiment was conducted by spraying 15 mM proline (Pro) (Ashraf and Foolad, 2007), 0.25 mM salicylic acid (SA) (Arfan *et al*., 2007) and distilled water (control) on the plants that treated with two salinity levels (0 and 100 mM NaCl) on their root medium. Salt concentrations were added to half strength of Hoagland solution (Hoagland and Arnon, 1950). The plants were irrigated daily for one month with a half-strength of Hoagland then were pruned to a single shoot per plant. Salinity treatment was continuously imposed one week after pruning. The electrical conductivity (EC) of the nutrient solution without NaCl was within the rage of 2.7-2.8 ds m^{-1}, while in the nutrient solution with 100 mM NaCl it increased to 13.2 ds m^{-1}. pH of the nutrient solution was adjusted to 6.5 by adding H_2SO_4. Pro and SA were applied to plant leaves 1.5 and 2 months (two times) after pruning. The glasshouse experiment lasted for six months. At the end of the sixth month, plants were removed from the substrate. The root removed from the plants and shoot and root weights were recorded. After weighing, the roots were placed into a gradient cylinder filled with water. After placing the roots into the cylinder the amount of increased water level was indicative of root volume. Finally roots and shoots were dried at 80 °C in an air forced oven for 48 h.

Photosynthetic parameters

Leaf photosynthesis (P_n), stomatal conductance (g_s) and transpiration rate (E) of the mid-lamina portion of the youngest fully expanded leaves were measured using a portable photosynthesis system (Walz, Model Da-1010, Germany). The rate of P_n were measured at 450 μmol CO_2 and PAR was set to 800 μmol m^{-2} S^{-1}. Reference CO_2 concentration was set to the inside of glasshouse. The leaf chamber temperature was adjusted to 28 °C and relative humidity was ranged from 70 to 80%. The time of measurement was between 9:00 and 14:00 o'clock. The chlorophyll index was measured using a chlorophyll-meter (SPAD-502, Tokyo, Minolta, Japan). At the end of experiment leaves removed in order to measure the leaf area using a leaf area-meter (Li-Cor, Model Li-1300, USA). Specific leaf area (SLA) was calculated according to formula:

SLA=LA/LDwt

LA: leaf area

LDwt: leaf dry weight

Peroxidase extraction and assay

For enzyme extraction 0.5 g of leaf samples were homogenized in 5 ml 10 mM potassium phosphate buffer (pH 7.0) containing 4% polyvinylpyrrolidon. The homogenate was centrifuged at 15000 × g for 20 min and obtained supernatant was used as enzyme extract. All steps in the preparation of the enzyme extract were carried out at 0-4 °C. The activity of peroxidase (POX; EC 1.11.1.7) was assayed according to the method of Chaparzadeh et al. (2004) with some modifications. The following reaction mixture was used: 10 mM potassium phosphate buffer, pH 7.0, 70 μl enzyme extract and 350 μl guaiacol 1% aqueous solution. The reaction was started by adding 0.2 ml H_2O_2. Optical density at 470 nm was recorded in a spectrophotometer Shimadzu against an identical mixture to which no H_2O_2 was added. Peroxidase activity was calculated as ΔA_{470} g^{-1} Fwt min^{-1}.

Statistical analyses

The data analysis was made using analysis of variance (ANOVA) in the SAS 8.2 software and treatment means were compared using least significant difference (LSD) test. Four plants per each treatment were used as four independent observations.

Results

Growth parameters

The results of analysis of variance for chemical substances, NaCl and their interactions (chemical substances × NaCl) for all of the assessed parameters are given in Table 1. Shoot fresh and dry weights ($P < 0.05$) and root dry weight ($P < 0.01$) were significantly affected by interaction between chemical substances and salinity (Table 1). While, no significant differences were found for the interactions between chemical substances and salinity for root volume, root fresh weight, fresh and dry weights of shoot/root ratio, leaf area, peroxidase activity and photosynthetic parameters (Table 1).

In control condition, SA treatment led to significant reductions in both shoot fresh and dry weights (Table 2). While, in saline condition, the highest and lowest shoot fresh and dry weights were observed in Pro and control plants, respectively (Table 2). Highest and lowest root dry weights in non-saline condition were observed in Pro and SA-sprayed plants, respectively (Table 2). However, in saline condition the highest and lowest root dry weights were recorded for Pro and control plants, respectively (Table 2). The root volume in Pro and SA-sprayed plants was higher than its volume in control plants (Fig. 1). However root volume in salt stressed-plants was reduced by approximately 10% when compared to root volume of control plants (Fig. 2).

The shoot/root ratios (for both aspects of fresh and dry weights) were considerably reduced by NaCl treatment in root medium (Fig. 3). The fresh and dry weight aspects of shoot/root ratios in salt treated-plants were approximately one-half of shoot/root ratios in control plants. Leaf area in salt-treated plants was reduced by 60% compared with the leaf area in control plants (Fig. 4).

Photosynthetic parameters

P_n was severely influenced by NaCl treatment in root zone (Table 3). P_n of control plants was approximately two times higher than P_n in salt treated-plants. E was also influenced by salinity treatment in root medium (Table 3). E in salt-treated plants was one-half of the E on the leaf of control plants. Moreover, g_s in control plant was two times higher than its value in salt treated-plants (Table 3).

Salinity stress in root medium significantly increased SLA of olive leaves (Fig. 4); Salinity led to an increase (9%) in SLA compared with the SLA in the leaves of the plants that grown in non-saline condition. In other words, salinity caused production of thinner leaves in olive trees.

Highest and lowest chlorophyll indices were recorded for Pro and control plants, respectively (Fig. 5). The chlorophyll

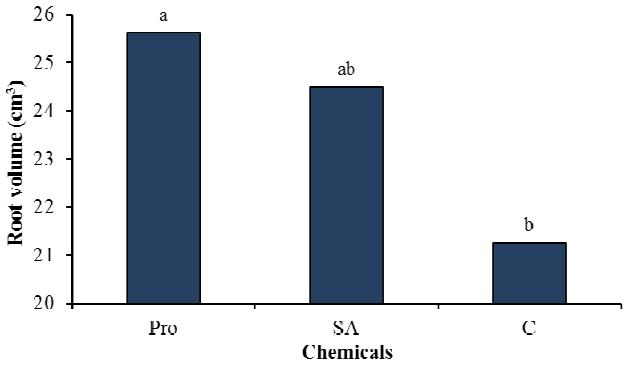

Fig. 1. Effect of chemical substances on root volume of olive plants (cv. 'Zard'). Column not followed by the same letter present statistical difference as determined by least significant difference (LSD). The treatments included: 15 mM Proline (Pro), 0.25 mM salicylic acid (SA) and distilled water (C). Each value represents mean of four independent observations

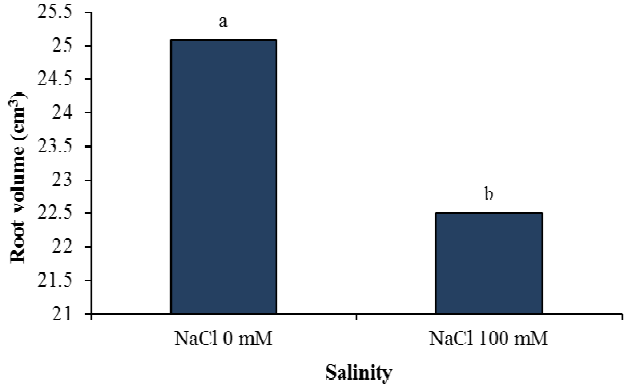

Fig. 2. Effect of salinity (0 and 100 mM NaCl) on root volume of olive plants (cv. 'Zard'). Each value represents mean of four independent observations. Column not followed by the same letter present statistical difference as determined by least significant difference (LSD)

Table 1. Analysis of variance (*F* values) for assessed parameters for olive (cv. 'Zard') plants grown in mixture of perlite, sand and vermiculite and irrigated with half strength of Hoagland solution under saline (0 mM NaCl) and non-saline (100 mM NaCl) conditions and sprayed with 15 mM Proline, 0.25 mM salicylic acid and distilled water

Dependent variable	Independent variables		
	Chemical substances	NaCl	Chemical substances × NaCl
Shoot fresh weight	2.08 ^{ns}	60.70 ***	4.09 *
Shoot dry weight	2.75 ^{ns}	54.66 ***	3.23 *
Root volume	3.29 *	3.19 *	2.90 ^{ns}
Root fresh weight	2.80 ^{ns}	1.02 ^{ns}	2.43 ^{ns}
Root dry weight	7.08 **	7.71 **	11.16 **
Fresh weight of shoot/root ratio	0.411 ^{ns}	60.75 ***	3.0 ^{ns}
Dry weight of shoot/root ratio	0.75 ^{ns}	30.19 ***	1.88 ^{ns}
Leaf area	0.51 ^{ns}	83.15 ***	2.26 ^{ns}
Net photosynthesis	2.1 ^{ns}	6.26 *	0.98 ^{ns}
Stomatal conductance	0.66 ^{ns}	6.33 *	2.99 ^{ns}
Transpiration rate	0.29 ^{ns}	4.91 *	0.78 ^{ns}
Chlorophyll index	8.07 **	34.84 **	1.78 ^{ns}
Specific leaf area	2.14 ^{ns}	4.69 *	2.72 ^{ns}
Peroxidase activity	0.85 ^{ns}	14.31 **	1.20 ^{ns}

ns: Non significance. * Significance at 0.05 probability level. ** Significance at 0.01 probability level. *** Significance at 0.001 probability level according to least significance difference (LSD) test.

Table 2. The effects of proline, salicylic acid and salinity stress on vegetative characteristics of olive plants (cv. 'Zard')

Chemicals × salinity	Shoot fresh weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})
Pro × $NaCl_0$	26.91 ab	10.93 ab	9.41 a
SA × $NaCl_0$	23.54 bc	9.06 bc	6.87 c
C × $NaCl_0$	29.58 a	11.36 a	8.69 b
Pro × $NaCl_{100}$	19.05 c	7.51 c	8.19 b
SA × $NaCl_{100}$	15.56 cd	6.16 cd	8.14 b
C × $NaCl_{100}$	13.33 d	5.19 d	5.28 d

Means within each column not followed by the same letter present statistical difference as determined by least significant difference (LSD). The plants were grown in a mixture of perlite, sand and vermiculite and irrigated with half strength of Hoagland solution under saline (0 mM NaCl) and non-saline (100 mM NaCl) conditions and sprayed with 15 mM Proline (Pro), 0.25 mM salicylic acid (SA) and distilled water (C)

Table 3. The effects of salinity stress (0 and 100 Mm NaCl) on photosynthetic parameters of olive plants (cv. 'Zard')

Salinity	Pn (μmol m^{-2} s^{-1})	Tr (mmol m^{-2} s^{-1})	gs (mmol m^{-2} s^{-1})	Chlorophyll (index)
$NaCl_0$	2.27	0.28	13.39	70.77
$NaCl_{100}$	1.36	0.13	6.73	51.09

Means determined by least significant difference (LSD). Abbreviations are: net photosynthesis (Pn), transpiration rate (Tr), stomatal conductance (gs). The plants were grown in a mixture of perlite, sand and vermiculite and irrigated with half strength of Hoagland solution under saline (0 mM NaCl) and non-saline (100 mM NaCl) conditions.

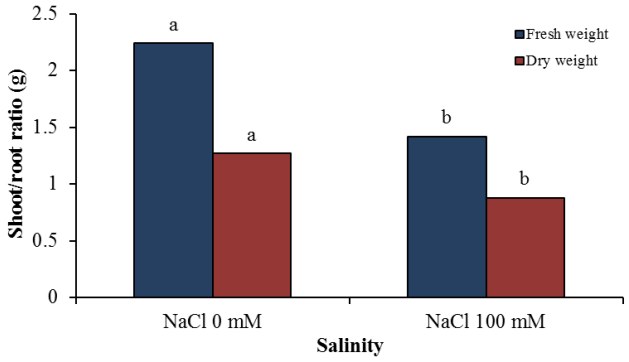

Fig. 3. Effect of salinity (0 and 100 mM NaCl) on shoot/root ratio (fresh and dry weight) of olive plants (cv. 'Zard'). Each value represents mean of four independent observations. Column not followed by the same letter present statistical difference as determined by least significant difference (LSD)

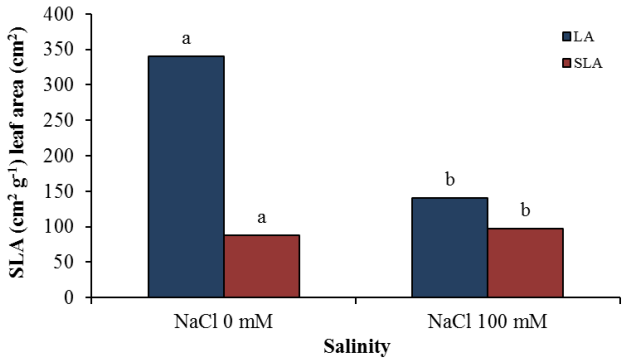

Fig. 4. Effect of salinity (0 and 100 mM NaCl) on leaf area and specific leaf area of olive plants (cv. 'Zard'). Each value represents mean of four independent observations Column not followed by the same letter present statistical difference as determined by least significant difference (LSD)

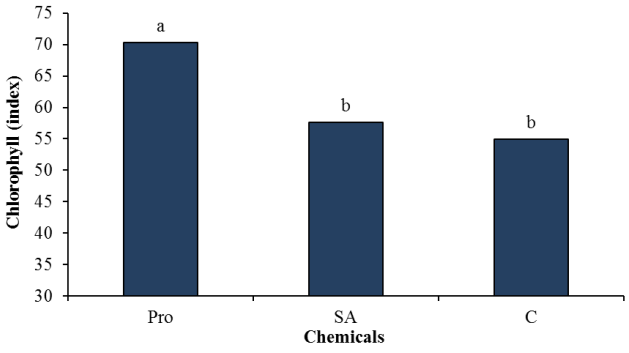

Fig. 5. Effect of chemical substances on chlorophyll index of olive plants (cv. 'Zard'). The chemical treatments included: 15 mM Proline (Pro), 0.25 mM salicylic acid (SA) and distilled water (C). Each value represents mean of four independent observations. Column not followed by the same letter present statistical difference as determined by least significant difference (LSD)

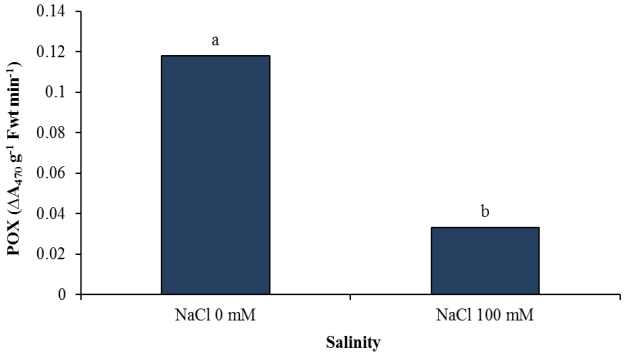

Fig. 6. Effect of salinity (0 and 100 mM NaCl) on peroxidase activity in olive leaves (cv. 'Zard'). Each value represents mean of four independent observations. Column not followed by the same letter present statistical difference as determined by least significant difference (LSD)

index in Pro-sprayed plants was increased by 18 and 21.8% compared with the chlorophyll indices of SA and control plants, respectively. However, chlorophyll index of olive trees was decreased by approximately 28% in response to salinity stress in root medium (Table 3).

Peroxidase activity was reduced by application of NaCl salinity in the root medium (Fig. 6), peroxidase activity in the leaf of the plants that grown in non-saline condition was approximately quadruplicated when compared to its activity in the leaf of salinized plants.

Discussion

Our data indicated that foliar application of Pro improved shoot vegetative characteristics in olive plants. In addition, root vegetative characteristics such as root dry weight and root volume were increased by Pro application in both saline and non-saline conditions. Increased activity of P5CR and Pro accumulation has been reported in *Chlorella* (Laliberté and Hellebust, 1989) and in NaCl-adapted cells of *Mesembryanthemum* (Treichel, 1986). Accumulation of Pro in the leaf helps plant to cope with abiotic stress conditions. Pro concentration is generally higher in salt tolerant plants (Ashraf and Foolad, 2007). Pro acts as an osmoprotectant during stress period (Molinari *et al*., 2007). The accumulation of Pro and other osmolytes may help to improve plant growth and cellular function under abiotic stress conditions (Delauney and Verma, 1993; Molinari *et al*., 2007). In many plant species under various abiotic stresses, the concentration of Pro can increase up to 80% of the amino acid pool (Matysik *et al*., 2002). In many occasions, a positive correlation between magnitude of free Pro accumulation and salt tolerance has been suggested as an index for determining salt tolerance potentials between cultivars (Ramanjulu and Sudhakar, 2000). In many plant species that growing under saline conditions exogenously-supplied Pro provided osmoprotection and facilitated growth (Ashraf and Foolad, 2007). In rice, exogenous application of 30 mM Pro counteracted the adverse effects of salinity on early seedling growth, while higher concentration resulted in reduced growth (Roy *et al*., 1993). In the present study we found that salinity decreases chlorophyll index and exogenous Pro application kept higher chlorophyll index compared with the chlorophyll index in control plants. Our results are in agreement with the earlier report by Kumar *et al*. (2003) who showed that chlorophyll stability decreased with increasing concentrations of NaCl in mulberry cultivars. In their study compared with salt-sensitive cultivar, the better chlorophyll stability was found in salt-tolerant cultivar (which had higher Pro content). Therefore, Pro can decrease chlorophyll degradation after exposure to abiotic stresses (Kumar *et al*., 2003).

The vegetative growth of olive plants was impaired by application of NaCl in the root zone. The primary effect of salinity on non-halophytes is reduction in growth and yield (Maas and Hoffman, 1977). It is believed that salinity reduces plant growth by water stress in the root zone and salt toxicity in the plant tissues (Munns and Tester, 2008). In current study biomass partitioning altered due to salinity in root medium; in a way that shoot/root ratio was significantly decreased by salinity stress. Since more allocation of dry and fresh matters direct towards roots under salinity and drought conditions, shoot/root ratio is considered as a criterion for adaptation to water stress in plants (Slama *et al*., 2006).

Photosynthesis is accounted as most important physiological process for biomass production in plants. Therefore, environmental stresses that negatively affect photosynthesis would lead to growth reduction (Tabatabaei, 2006). In accordance to our result, negative effects of salinity on olive fresh and dry weights have been previously reported (Chartzoulakis *et al*., 2002; Tabatabaei, 2006).

In the present study, application of Pro improved photosynthetic parameters in olive trees. Environmental conditions (e.g. light, VPD, water availability and salinity) can influence stomatal responses (Aliniaeifard *et al*., 2014; Aliniaeifard and van Meeteren, 2014; Merilo *et al*., 2014) and as a result they can have direct or indirect effects on photosynthesis. Under stress conditions, ions (e.g. K^+ and Cl^-) and water effluxes would result in loss of guard cell turgor and consequently stomatal closure, while in the absent of adverse environmental conditions, water as a result of K^+ and Cl^- accumulation in guard cells would enter to the guard cells lead to stomatal opening which would be favour for gas exchange, photosynthesis and growth (Blatt, 2000; MacRobbie, 2000; Schroeder *et al*., 2001a,b; Aliniaeifard and van Meeteren, 2013, 2014, 2016). Stomatal closure minimizes loss of water by transpiration and this affects chloroplast light harvesting for CO_2 assimilation. The extent to which stomatal closure influences photosynthetic capacity depends on the magnitude of partial pressure of CO_2 inside the leaf (Genty *et al*., 1989). Water and salt stress-altered stomatal responses have been widely documented (Tabatabaei, 2006; Aliniaeifard and van Meeteren, 2013; Aliniaeifard *et al*., 2014; Aliniaeifard and van Meeteren, 2014; Merilo *et al*., 2014). Under abiotic stress conditions, abscisic acid (ABA) usually acts as the main phytohormone for induction of stomatal closure (Luan, 2002; Davies *et al*., 2005; Aliniaeifard and van Meeteren, 2013; Aliniaeifard *et al*., 2014; van Meeteren and Aliniaeifard, 2016). Guard cell ABA signal transduction for stomatal closure has been also extensively documented (Luan, 2002; Joshi-Saha *et al*., 2011). Increase in ABA levels, when the only source of CO_2 for photosynthesis is stomata, would result in decrease in photosynthesis (Aliniaeifard *et al*., 2014; Aliniaeifard and van Meeteren, 2014). Therefore, increased ABA levels due to salinity stress can primarily decrease plant photosynthesis and growth (Chaves *et al*., 2009).

Salt stress decreases photosynthesis through stomatal and nonstomatal factors. Reduction in leaf chlorophyll content could be an important factor connected with photosynthesis under abiotic stress conditions. In the current study proline application improved chlorophyll index, while salinity decreased chlorophyll index in olive trees. Pro acts in the crossroad between carbon and nitrogen assimilation pathways in plant (Kumar *et al*., 2003). A positive correlation between foliar nitrogen level and chlorophyll content has been reported (Aliniaeifard and Tabatabaei, 2010). Previous researches reported that salinity could increase chlorophyllase activity (Rao and Rao, 1981), this may be caused by the inhibitory effect of salinity on the absorption of some ions, such as Mg and Fe, which are mediated in chloroplast formation or by oxidative stress (Hernandez *et al*., 1995).

In the present study, salinity caused an increase in specific leaf area. It has been found that olive plants undergo some anatomical alterations, especially in their leaves, which are the main organs for water loss in plant. Such alterations include changes in: cuticle thickness, density of stomata, non-glandular

scales, epidermal and mesophyll cells in order to save water under stress conditions (Bosabalidis and Kofidis, 2002). Munns (1993) proposed that decrease in leaf cellular turgor is not the main reason for the reduction in stomatal conductance, photosynthesis and limited leaf expansion in saline conditions. In our study a significant decrease was observed in the activity of peroxidase in salinity condition (Fig. 6), which is in agreement with the earlier report in *Calendula officinalis* (Chaparzadeh *et al*., 2004) and rice (Demiral and Türkan, 2005).

In conclusion, the results presented in this paper clearly indicate that exogenous application of Pro can improve shoot vegetative growth under saline condition and root vegetative growth under both saline and non-saline conditions. Pro alleviated the detrimental effects of salinity on the olive trees. On the other hand, salinity had harmful effects on vegetative and photosynthetic parameters, enzyme activity and allocation of fresh and dry matters in olive trees.

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