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Original Article

The Effect of Zinc Nutrition on Two Olive (*Olea europaea* L.) Cultivars Components and Alleviate Oxidative Damage in Salinity Conditions

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Abstract

The role of zinc (Zn) in enhancing defense capacity of several plants against salinity has been demonstrated but there is limited information on the impact of Zn nutrition on alleviating salinity-induced oxidative damage in olive. One-year-old seedlings of two varieties of olive (*Olea europaea* L. cvs. Frontoio and Conservolea) supplied with three Zn levels (0, 1 and 5 mM in the form of $ZnSO_4.7H_2O$) were exposed to four salinity levels (0, 40, 80 and 120 mM NaCl). The increase in plasma membrane permeability and elevated leakage of potassium (K) and Zn from the olive roots were considered as indices of oxidative damage caused by salinity on root cells. In contrast, root membrane permeability and leakage of Zn and K ions in plants supplied with Zn was less than those non-supplied with Zn. Addition of Zn resulted in higher activity of CAT and APX. Higher salt-tolerance of Frontoio cultivar was associated with higher concentration of sulfhydryl (-SH) groups and lower membrane permeability of its roots in comparison with Conservolea cultivar. Based on the results obtained, addition of Zn improved plant enzymatic defense system and partly alleviated oxidative injuries induced by salinity on the olive.

Keywords: Ionic leakage, Environmental stress, Catalase, Ascorbate peroxidase, Sulfhydryl groups

Introduction

Olive is an important horticultural crop widely cultivated in arid and semiarid regions worldwide, particularly in salt-affected soils of Iran [1]. Despite moderate tolerance of olive to salinity stress [2], it is important to find out modern approaches to improve growth and yield of this strategic plant in salt-affected soils.

Soil salinity is an important environmental stress aggravated by intensive agricultural practices and the elevated irrigation requirements. Salt-affected soils make about 20% of the cultivated areas and nearly half of all irrigated lands worldwide [3].
Lower crop productivity in saline soils might be resulted from either lower water potential or the toxic effects of Na^+ and Cl^- ions on metabolism [4]. Osmotic stress, ion toxicity, and imbalance of the nutrients are consequences of salinity that cause

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in plant growth reduction [5-7]. Excess levels of Na and Cl can be toxic for plants and reduce absorption of essential nutrients such as K and Ca [8,9].

Oxidative damages in plant cells can also be induced by salinity stress [10-11]. Salinity reduces gas exchange and CO_2 supply to the leaves [12,13] and thereby causes the over-reduction of the photosynthetic electron transport chain [14]. The reactive oxygen species (ROS), such as singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radical are generated under saline conditions [15,16]. The ROS damages macromolecules in cells such as proteins, nucleic acids, and lipids [17].

On the other hand, zinc (Zn) is an essential micronutrient [18-19] that its role in alleviating injuries induced by ROS on plant cells has been documented [20]. Zinc is required for detoxification of ROS including superoxide radical and H_2O_2 [7] and plays role in improving anti-oxidative defense capacity of plants against environmental stresses [20].

The effect of Zn nutrition on growth and yield of several plants exposed to salinity has been reported by different researchers [21-23]; however, the roles of Zn nutrition in all eviating salt-induced oxidative damages in olive are still poorly understood. Therefore, this study was aimed to investigate how Zn nutrition affects anti-oxidative response of olive to salt stress.

Materials and Methods

Plant Growth

The one-year-old olive seedlings with the similar height (50 cm) and other growth characteristics were prepared from Qom Fadak field in central Iran. The roots were washed with distilled water and then the seedlings were transferred into10-liter plastic pots containing sand and perlite at a ratio of 1:1. The pots were placed in Fadak research greenhouse with 85% relative humidity. The day and night temperature of greenhouse varied between 25-30 and 18-20 °C, respectively. Plants were daily irrigated with a half Hoagland nutrient solution. Three Zn rates (0, 1, and 5 µM Zn in the form of $ZnSO_4.7H_2O$) and four salinity levels (0, 40, 80 and 120 mM NaCl) were used.

Twenty days after transferring the seedling into the pots, salinity treatments were started by using irrigation water with adding desired levels of 4 mM NaCl. To avoid osmotic stress, salinity was applied in gradual daily increments (12 mM NaCl per day) and the final concentrations of NaCl in the nutrient solution were achieved after 10 days. After 60 days, the seedlings were harvested and separated into roots and shoots. Plant materials were washed with deionized water and dried at 70 ^{0}C for 48 h. leaf and root dry mass was determined for each replicate.

Analysis of Zn

Root and leaf dry samples (1 g) were placed into ceramic vessels and combusted in a muffle furnace at 500 °C for 8 h. Ashed samples were removed from the muffle furnace, cooled, and the ash dissolved in 2 M HCl. Analyses of Zn were carried out with an atomic absorption spectrophotometer (Varian, spectra Model 220).

Ion leakage

Sub-samples of seedlings were transferred to 2 L of aerated 0.5 mM $CaSO_4$ plus 0.01 mM H_3BO_3 for 15 min to remove nutrient solution adhering to root surfaces. Subsequently, seedling roots were rinsed in water and transferred to polyethylene pots (400 mL) containing ion leakage solution (0.5 mM $CaSO_4$ and 0.01 mM H_3BO_3). The seedling roots were completely immersed in an aerated solution. After 4 h, the seedlings were removed and ion leakage solutions were collected. Zinc concentration of leakage solutions was measured by AAS. Concentration of K in the leakage was measured by flame photometer (Model G405, Electric Fater Company).

Antioxidant Enzymes Assay

The leaf enzymatic extract was prepared according to the Dyndsa method [24]. A crude enzyme extract was prepared by homogenizing 500 mg of leaf tissue in a pestle and mortar with 100 mM TRIS-HCl buffer (pH 8) containing 2 mM EDTA, 5 mM DL-dithiothreitol, 10% glycerol, 100 mM sodium borate, 4% (w/v) insoluble polyvinylpyrrolidone (PVP), and 1 mM phenyl methyl sulphonyl fluoride (PMSF). The homogenate was filtered through four layers of muslin cloth and centrifuged at 12000×g for 40 min at 4 °C. The supernatant was subsequently used for the enzymatic assays [25].

Catalase (CAT) activity of the leaves was measured according to Cakmak and Marschner (1992) [26]. The CAT assay mixture (3 mL) contained 25 mM sodium phosphate buffer (pH 7.0) plus 10 mM H_2O_2 and 0.1 mL leaf extract. The reaction was

initiated by the addition of leaf extract and the enzyme activity was determined by measuring the initial rate of disappearance of H_2O_2 at 240 nm (ε = 39.4 $mM^{-1}cm^{-1}$) for 70 s.

Ascorbate peroxidase (APX) activity was determined according to Nakano and Asada (1981) [27]. The reaction mixture, with a total volume of 3 mL, consisted of 25 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbate, 1.0 mM H_2O_2, and 100 µL of the leaf extract. H_2O_2-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (ε= 2.8 $mM^{-1}cm^{-1}$).

Reactive sulfhydryl group assay

Roots of olive seedlings were analyzed for –SH groups by the method described by Sedlak and Lindsay (1968) [28] using sulfhydryl-reactive reagent DTNB [5,5'-dithio-bis (2-nitrobenzoic acid)]. Fresh roots were immersed in 75 ml of sulfhydryl reaction buffer (0.2 M Tris-HCl plus 0.02 M Na-EDTA adjusted to pH 8.2 with NaOH). A 1.0-mL of 10 mM DTNB, dissolved in absolute methanol, was added to the reaction buffer at time zero. After 15 min, the roots were removed from the reaction buffer. Reactive sulfhydryl groups in 2.0-mL aliquots of sulfhydryl buffer solution were immediately assayed after collection through spectrophotometry at 412 nm wavelength. Sulfhydryl concentrations were calculated from standard curves prepared from cystein standards made immediately before the assay.

Root cell membrane permeability

Cell membrane permeability of olive roots was assessed by modified method described by Yan *et al*. (1996) [29]. The 1-cm washed root segments were placed in a 250-mL beaker containing 10 mL deionized water. The root samples were immersed at 30 °C for 3 h, and then the conductivity of the solution was measured. The samples were boiled for 2 min, cooled to room temperature (25 °C) and then, their electrical conductivity (EC) was measured. The electrolyte leakage was calculated as follows:

EC (%) = (C1/C2) ×100

where C1 and C2 are the EC measured before and after boiling, respectively.

Statistical analysis

The experiment was set up in a completely randomized factorial design; each treatment contained three replicates and 5 pots for each replication. Analysis of variance procedures were conducted with MSTATC procedure. Mean separations were performed using Fisher's protected Duncan method at $P < 0.05$.

Results

Leaf Dry Mass

with increasing salinity, Leaf dry mass decreased, while with increasing zinc level of zero to 1 and 5 micro molar, leaf dry mass increased at all salinity levels, that this increase was significant up to 80 mM sodium chloride level (Fig.1).

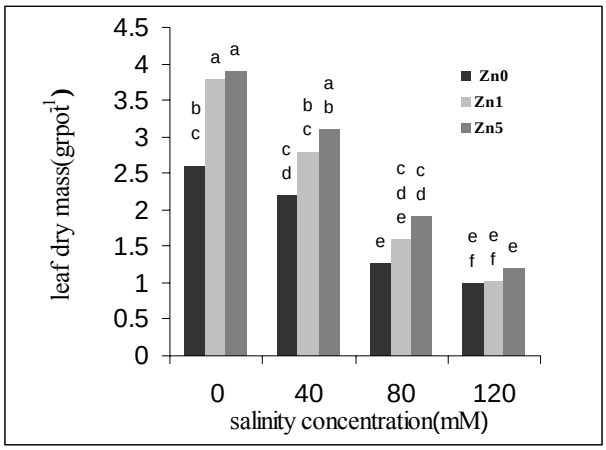


Fig. 1 The effect of salinity and zinc nutrition on leaf dry mass of two olive cultivars. Bars with the same letter are not significantly different at P < 0.05 according to Duncan's test.

Root Dry Mass

Increasing salinity up to 40 mM had no significant effect on the root dry mass of Frontoio cultivar while it significantly reduced root dry mass of Conservolea cultivar (Fig.2). Significant reductions in root dry mass of both olive cultivars were found at 80 and 120 mM salinity levels. Addition of Zn resulted in higher root dry mass of both cultivars, although magnitude of this increase was greater for Frontoio cultivar than Conservolea cultivar (Fig. 2).

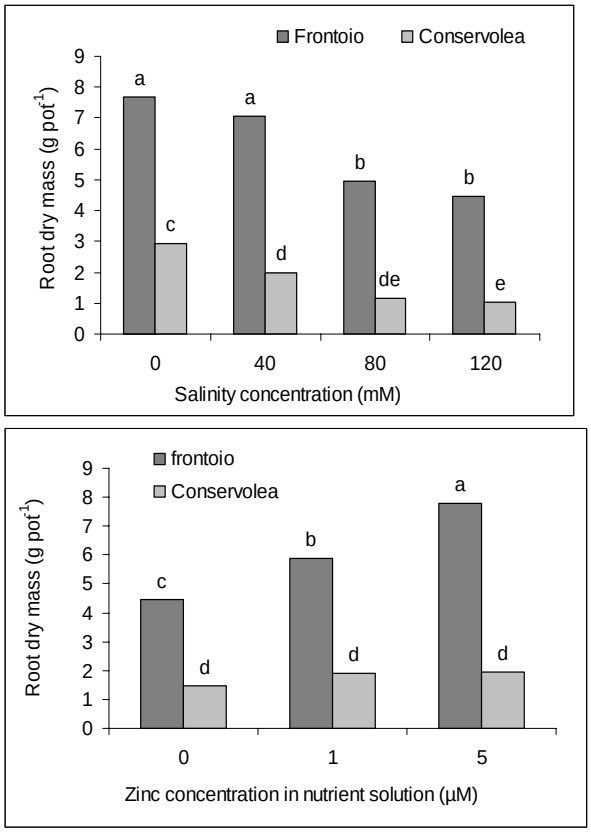


Fig. 2 The effect of salinity (a) and zinc (b) on root dry mass of two olive cultivars. Bars with the same letter are not significantly different at P<0.05 according to Duncan's test.

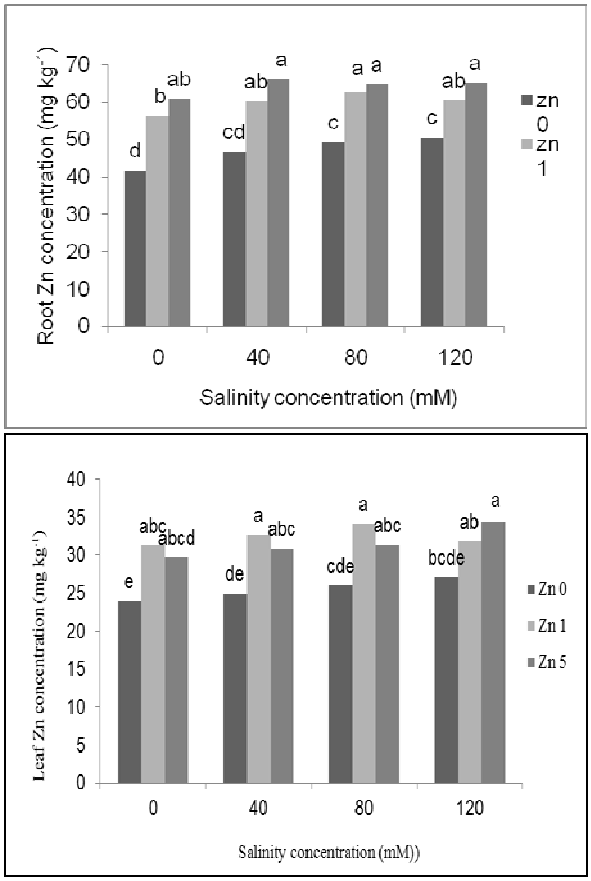


Fig. 3 The effect of salinity and zinc nutrition on root (a) and leaf zinc concentration (b) of two olive cultivars. Bars with the same letter are not significantly different at P<0.05 according to Duncan's test.

Zinc Concentration and Content

With increasing zinc level of the nutrient solution, concentration of Zn in plant root and leaf significantly increased (Fig. 3). The Conservolea cultivar accumulated higher levels of Zn in its roots and leaves in comparison with the Frontoio cultivar. At all salinity levels, leaf and root Zn concentration was increased by application of 1 µM Zn in the nutrient solution and then it remained unchanged with increasing Zn concentration to 5 µM (Fig. 3).

For both cultivars, increasing salinity up to the 120 mM NaCl concentration resulted in lower contents of Zn in the root and leaf (Fig. 4). Regardless of salinity and Zn treatments, the Frontoio cultivar had higher Zn contents in its roots in comparison with the Conservolea cultivar.

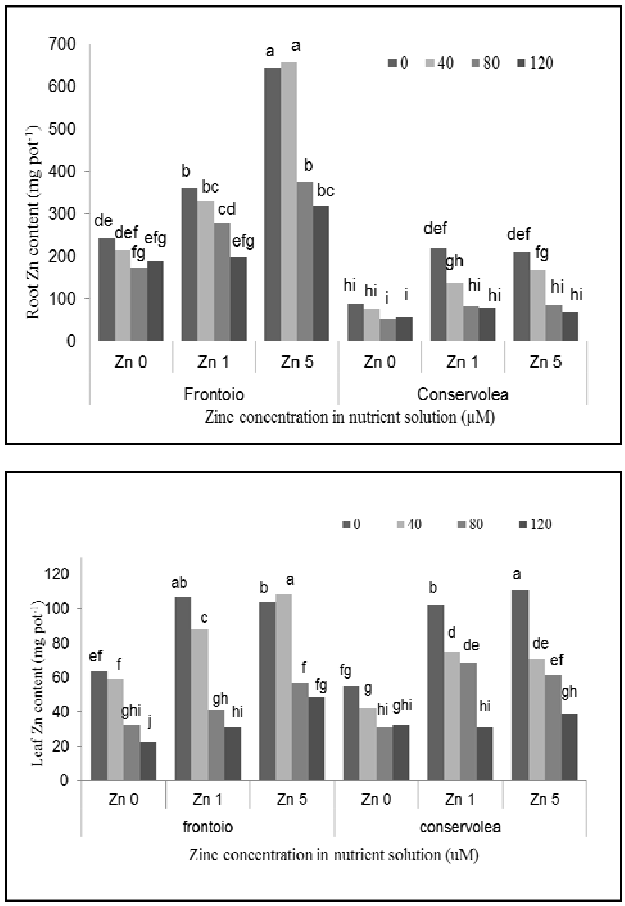


Fig 4 The effect of salinity and zinc nutrition on root (a) and leaf zinc content (b) of two olive cultivars. Bars with the same letter are not significantly different at P < 0.05 according to Duncan's test.

Relative Translocation of Zn

With increasing salinity, the relative translocation of Zn significantly decreased in both olive cultivars (Fig. 5). In both olive cultivars, the relative translocation of Zn was ineffective by addition of Zn into the nutrient solution (Fig. 5). The relative translocation of Zn from roots to shoots was higher in Conservolea cultivar than in Frontoio cultivar.

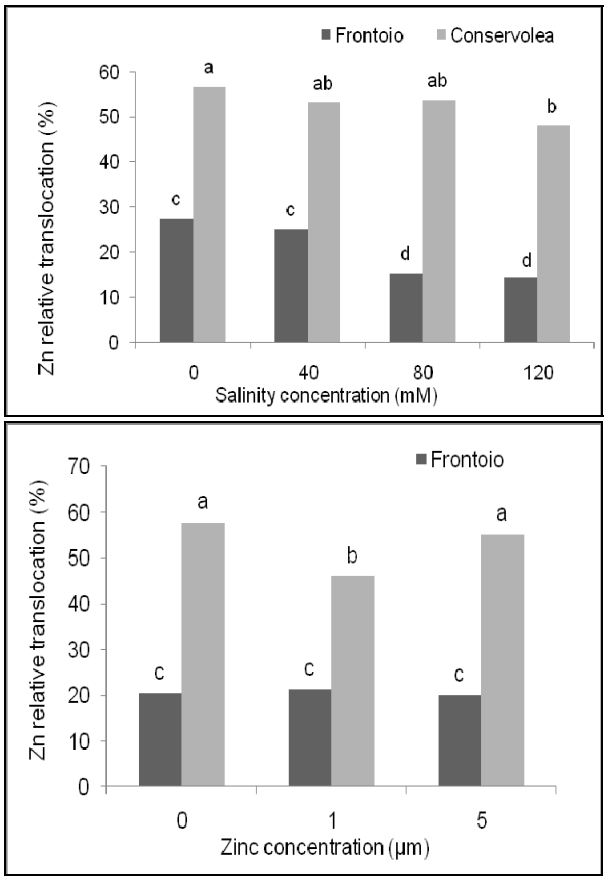


Fig 5 The effect of salinity (a) and zinc nutrition (b) on relative translocation of Zn from root to shoots of two olive cultivars. Bars with the same letter are not significantly different at $P < 0.05$ according to Duncan's test.

Root reactive -SH Group Concentration

With increasing salinity, concentration of -SH groups in the roots of Frontoio cultivar was unchanged while in Conservolea cultivar significantly reduced (Fig. 6). Concentration of -SH groups was higher in the Frontoio cultivar than Conservolea cultivar. With Addition of Zn in nutrient solution in all salinity levels increased the root –SH groups concentration, but was not statisticaly significant (Fig.6).

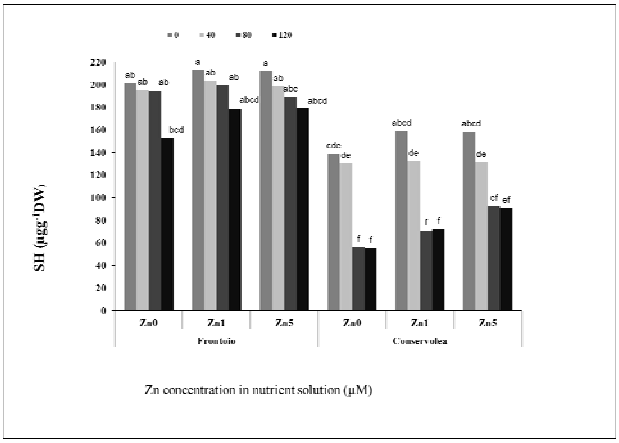


Fig. 6 The effect of salinity and zinc nutrition on root – SH concentration of two olive cultivars. Bars with the same letter are not significantly different at $P < 0.05$ according to Duncan's test.

Root Membrane Permeability

Higher root membrane permeability was found in the olive plants exposed to salinity stress than those unexposed to the salinity (Fig. 7). On the other hand, addition of Zn was ineffective in reducing permeability of root membrane in Frontoio cultivar, while root membrane permeability in Conservolea cultivar was reduced by application of 1 µM Zn in the nutrient solution and then it remained unchanged with increasing Zn concentration to 5 µM. The root membrane permeability was higher in the Conservolea cultivar than the Frontoio cultivar (Fig. 7).

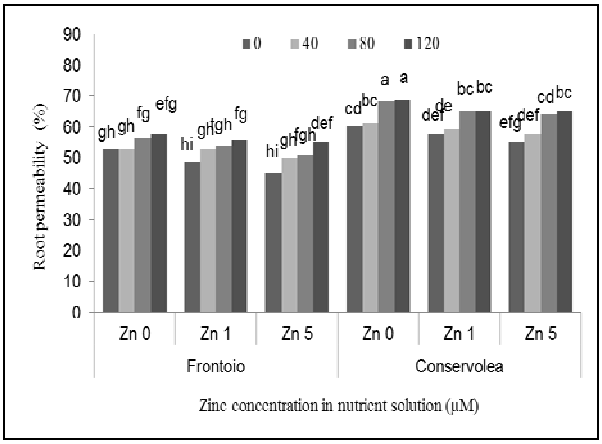


Fig. 7 The effect of salinity and Zn nutrition on root permeability of two olive cultivars. Bars with the same letter are not significantly different at $P < 0.05$ according to Duncan's test.

Leakage of Zn and K from the Roots

With increasing salinity, K leakage increased from the root of both cultivars (Fig. 8). In contrast, addition of Zn in both levels resulted in lower leakage of K from plant roots. Regardless of plant

cultivar and salinity level, K leakage from the root of Conservolea cultivar was higher than the Frontoio cultivar.

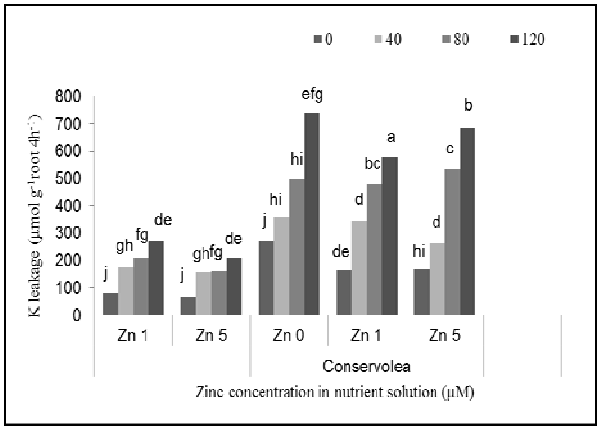
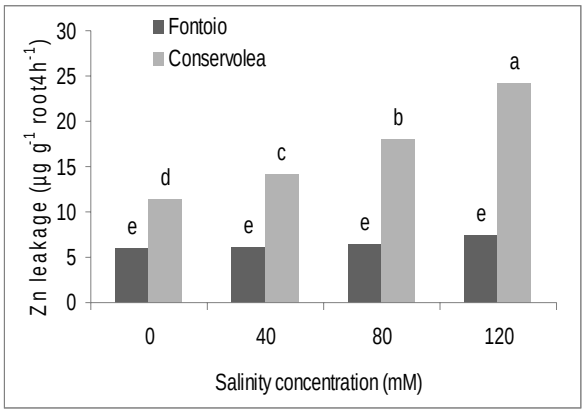


Fig. 8 The effect of salinity and Zn nutrition on K leakage from roots of two olive cultivars.
Bars with the same letter are not significantly different at $P < 0.05$ according to Duncan's test.



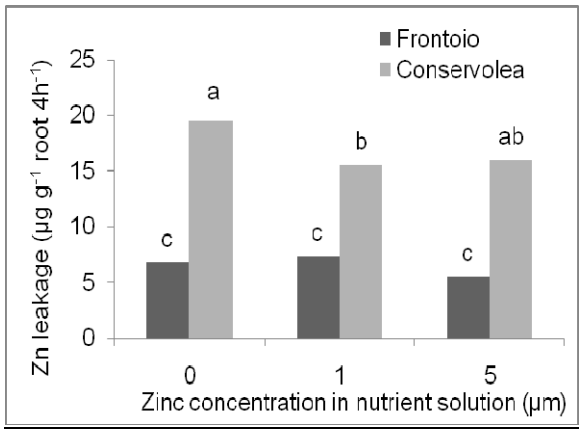
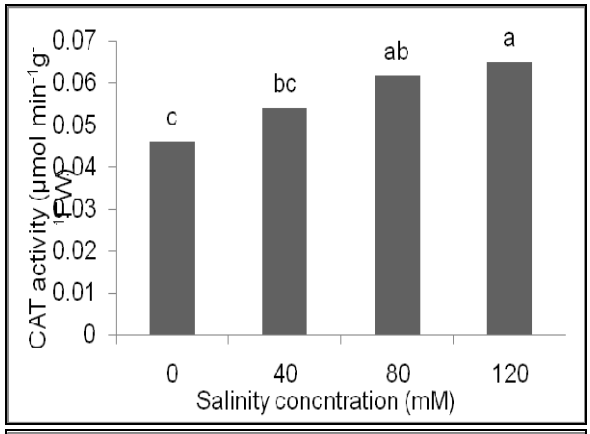


Fig. 9 The effect of salinity (a) and Zn nutrition (b) on Zn leakage from roots of two olive cultivars. Bars with the same letter are not significantly different at P<0.05 according to Duncan's test.

The effect of salinity on Zn leakage from the roots was different depending on the plant cultivar (Fig. 9). Salinity resulted in higher leakage of Zn from the roots of Conservolea cultivar but it had no effect on Zn leakage from the root of Frontoio cultivar (Fig. 9). The effect of Zn nutrition on Zn leakage from the olive roots varied upon the cultivar. For Frontoio cultivar, addition of Zn had no significant effect on the Zn leakage from the roots, while it significantly reduced the leakage of Zn from the roots of Conservolea cultivar. The Zn leakage from roots of Conservolea cultivar was higher than Frontoio cultivar.

Activity of CAT and APX

Activity of CAT in plants exposed to the salinity stress was higher than those unexposed to salinity (Fig. 10). Addition of Zn resulted in higher activity of CAT.



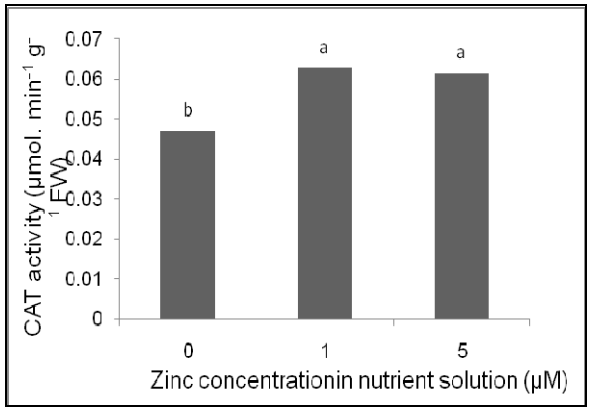


Fig. 10 The effect of salinity (a) and Zn nutrition (b) on leaf CAT activity of two olive cultivars. Bars with the same letter are not significantly different at P<0.05 according to Duncan's test.

Salinity caused in a significant increase of leaf APX activity for both olive cultivars (Fig. 11). Effect of Zn nutrition on activity of APX differed dependent on the plant cultivars. In Conservolea cultivar, addition of Zn in both applied concentrations increased APX activity while it had no effect on APX activity in Frontoio cultivar (Fig. 11).

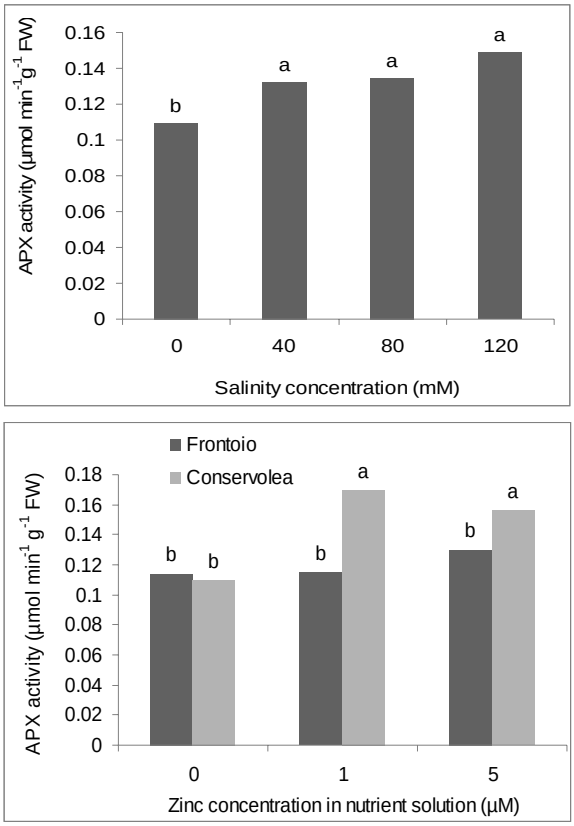


Fig. 11 The effect of salinity (a) and Zn nutrition (b) on leaf APX activity of two olive cultivars. Bars with the same letter are not significantly different at P<0.05 according to Duncan's test.

Discussion

In the present experiment, significant reductions in root and leaf dry mass of both olive cultivars by salinity was observed, although the magnitude of this reduction was greater for Conservolea cultivar. Zinc nutrition partly alleviated deterimental effect of salinity on the root and leaf dry mass of both cultivars. Salt tolerance improvement and reduced negative effects of salinity on wheat by Zn nutrition has also been reported by Daneshbakhs *et al*. (2012) [21].

A possible reason for plant growth inhibition under salt stress conditions is induced oxidative stress and impaired cell membrane integrity [30]. In this study, permeability of root membrane and ion leakage was used as indices for severity of oxidative damages on cell membranes by ROS. The activated oxygen species are produced when plants are subjected to environmental stresses such as salinity [31]. These reactive oxygen species can seriously induce oxidative damage and impair membrane integrity [32,33]. Greater root dry mass reduction under saline conditions was associated with higher root membrane permeability of Conservolea cultivar than Frontoio cultivar.

On the other hand, addition of Zn was effective in reducing root membrane permeability of both olive cultivars exposed to salt stress conditions. The positive role of Zn in lowering root cell membrane oxidation of wheat genotypes differing in Zn-deficiency tolerance has been documented [21, 34]. This result can partly explain improved growth of olive cultivars supplied with Zn under saline conditions. It seems that the destructive effect of salinity on plant growth is partly related with impaired cell membrane integrity. On the other hand, the beneficial role of Zn in alleviating salt-induced damages could be related to lower permeability of cell membranes in the presence of Zn.

Another evidence for impaired root cell membrane integrity of olive under salinity conditions is elevated leakage of K and Zn. Higher permeability of cell membrane as a result of salinity stress leads to increase of ion leakage from the roots [30]. Higher leakage of K from roots of Zn-deficient plants under saline conditions compared with Zn-supplied plants is in accordance with the data on root membrane permeability. These results highlight the role of zinc in protecting plant cell wall integrity. Sufficient concentration of Zn is necessary to protect cells against oxidative damage induced by salinity [20]. Welch *et al*. (1982) reported impairment in membrane integrity and increased membrane permeability to ions in Zn-deficient wheat plants [34]. In regard with greater leakage of P and Cl from roots of Zn-deficient plants than Zn-sufficient plants, they concluded that Zn has a direct effect on the structural integrity of biomembranes. In the present study, the effect of Zn on membrane permeability of root cells was dependent on the olive cultivar and salinity level.

In this experiment, the root membrane permeability and consequently K and Zn leakage in Conservolea cultivar was higher than Frontoio cultivar. The possible reason for higher root membrane permeability in Conservolea cultivar is that the concentration of root Sulfhydryl groups (-SH) in this cultivar was lower than Frontoio cultivar. It has been reported that concentration of -SH groups in roots of salt-tolerant cultivars is often higher than salt-sensitive cultivars [20,21]. The -SH groups represent an important component of antioxidant capacity of plant cells. Most of the non-protein -SH groups in plants represent glutathione which is an

important antioxidant in plant cells and play role in detoxification of ROS [35-36].

A decrease in the -SH groups content in association with an increase in the leaf activity of CAT and APX in olive cultivars exposed to salt stress indicate the anti-oxidative response of olive to salinity. Increasing salinity resulted in decrease of the -SH groups content and increased leaf activity of CAT and APX in both studied olive cultivars. In line with this result, Daneshbakhsh *et al*. (2012) reported a decrease in the root -SH groups content and a increase in activity of CAT in wheat [21]. These results suggest that under saline conditions, anti-oxidative defense mechanisms were activated to protect root membrane structure against oxidation. Zinc nutrition increased concentration of -SH groups in both olive cultivars. This result is in agreement with the findings of Cakmak (2000) and Daneshbakhsh *et al*. (2012) that Zn ion can partially prevent the loss in –SH groups under stress conditions [20-21]. Zinc ions may prevent oxidation of sulfhydryl groups by physically capping them. Welch and Norvell (1993) suggested that Zn can protect -SH groups in certain membrane proteins against oxidation and formation of disulfides. In fact, Zn may protect membrane protein sulfhydryl groups from oxidative damage [37]. Welch and Norvell (1993) reported that -SH groups concentration in root cell plasma membrane proteins was decreased by Zn deficiency in barley [37].

Plants have enzymatic and non-enzymatic defense systems for scavenging active oxygen species produced under saline conditions to protect cells from oxidative damages [38]. Antioxidant enzymes have basic role in the defense system of plant against salt-induced oxidative stress. In the present study, a significant increase in activity of CAT and APX was found by increasing salinity level. Similar to this result, elevated activity of CAT, glutathione reductase (GR) and APX in rice [39], wheat [21], and cotton [16] under salt-stress conditions has been reported. Rapid and sustained increase in CAT activity shows that this enzyme can play an important role in detoxification of hydrogen peroxide in plant cells exposed to salt stress [40].

Conclusion

The results showed that addition of Zn could partly alleviate oxidative damages of salt stress on olive. Zinc nutrition increased plant resistance to salt stress by stabilizing root cell membranes, as indicated by significant reduction in the root membrane permeability and reduced ion leakage from roots. Significant increase in activity of antioxidant enzymes CAT and APX by Zn may play a crucial role in preventing plant cells from salt injury. Lower permeability of root cell membrane and lower leakage of K and Zn from roots of plants supplied with Zn under salinity stress suggest the protective role of Zn against oxidative stress induced by salinity. Further research is needed to fully understand the role of CAT and APX in salt tolerance of olive.

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