Effect of Freezing Stress on Lipid Peroxidation and Antioxidant Enzyme Activities of Olive cvs. 'Fishomi' and 'Roughani'

Abuzar HASHEMPOUR [1]
Mahmood GHASEMNEZHAD [1] (✉)
Reza FOTOUHI GHAZVINI [1]
Mohammad MEHDI SOHANI [2]

Summary

Changes in freezing injury percentage, lipid peroxidation (malonaldehyde formation), antioxidant enzymes activity and proline content were monitored in the leaves of olive cvs. 'Fishomi' and 'Roughani' under different freezing temperatures (-5, -10, -15 and -20°C for 10 h). The results showed that freezing injury (determined by electrolyte leakage analysis) and malonaldehyde (MDA) content of cv. 'Fishomi' were significantly lower than of cv. 'Roughani' ones. The activities of peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and polyphenol oxidase (PPO) enzymes of cv. 'Fishomi' were significantly higher than those of cv. 'Roughani'. However, superoxide dismutase (SOD) activity of cv. 'Roughani' was higher than of cv. 'Fishomi'. The proline accumulated in leaves of cv. 'Fishomi' was significantly higher than of cv. 'Roughani' during freezing stress. The results demonstrated that freezing injury percentage was positively correlated with ion leakage percentage and MDA content in both cultivars. In contrast, SOD, APX and CAT activities and also proline content were negatively correlated with freezing injury percentage. There was a significant negative correlation between PPO activity and freezing injury in cv. 'Fishomi'. It can be concluded that the lower freezing injury percentage, ion leakage, and MDA content followed by the higher antioxidant enzyme activates as well as proline content in cv. 'Fishomi' is a consequence of more effective protective mechanisms.

Key words

Olea europaea L., freezing stress, antioxidant enzymes, lipid peroxidation

[1] Department of Horticultural Science, Faculty of Agriculture, University of Guilan, Rasht, Iran
✉ e-mail: ghasemnezhad@guilan.ac.ir
[2] Department of Biotechnology, Faculty of Agriculture, University of Guilan, Rasht, Iran

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Introduction

Freezing temperature is one of the most important environmental factors limiting the productivity and distribution of plants (Levitt, 1980). Plant tolerance to freezing injury varies greatly between species and genotypes. Freezing-tolerant plants are thought to have cell structures and intracellular components required for tolerating both mechanical and osmotic stresses generated by freezing (Nagao et al., 2005). Mechanisms of freezing tolerance have also been proposed based on the biochemical and physiological changes related to freezing injury (Elstner, 1991). It has been reported that the major target of freezing injury is cell membranes (Levitt, 1980). This could increase the level of reactive oxygen species (ROS) and then result in severe oxidative injury, give rise to lipid peroxidation, membrane deterioration, protein degradation, nucleic acid damage, chlorophyll bleaching, and metabolic function disruption (Lin et al., 2005). Plants have evolved both enzymatic and non-enzymatic antioxidant systems to prevent or alleviate membrane damage caused by ROS. The degree of damage depends on the balance between the formation of ROS and their detoxification by the antioxidative scavenging system. Thus, a high level of protective enzymes and antioxidants is essential for the maintenance of the concentration of ROS at a relatively low level, which is required for the survival of plants under low temperature stress (Scebba et al., 1998). Previous studies showed that SOD, APX, CAT and POD are the major antioxidative enzymes that efficiently scavenge ROS, and which resulted in the enhancement of freezing resistance (Jin et al., 2003; Luo et al., 2007; Cansev et al., 2009). Furthermore, PPO activity is also important in the response of plants against freezing stress, because they can help to avoid serious oxidative damage induced by freezing (Ortega-García and Peragón, 2009). Indeed, tolerance of plant towards adverse environmental conditions is correlated with an increased capacity to scavenge or detoxify ROS (Guo et al., 2006).

Olive (*Olea europaea* L.) is one of many tropical and subtropical crops which are often grown close to climatic limits of their cold-tolerance (Bartolozzi et al., 1999); these plants significantly lose their productivity due to an untimely frost or extremely cold winter temperatures (Yoshida and Uemura, 1990). In the last few years, the demand for olive oil has increased and, as a result, olive tree cultivation has spread outside of the traditional areas (Gómez-del-Campo and Barranco, 2005). In some cases higher quality oil is sought in areas with cold autumns where the post-ripening period is longer (Palliotti and Bongi, 1996). However, olive tree lives in warm temperatures showing low tolerance to frost (D'Angeli and Altamura, 2007). These trees can not survive below -12°C (Gómez-del-Campo and Barranco, 2005) and are damaged by frost below -7°C, reducing productivity (Palliotti and Bongi, 1996).

Although protective enzymatic activities under low temperatures stress have been compared in different species of olive plants (Cansev et al., 2009; Ortega-García and Peragón, 2009; Cansev et al., 2011; Hashempour et al., 2014), these studies have examined single or few of protective defense enzymes. Moreover, little is known about the changes in the freezing injury level and MDA contents, the increased level of defense enzymes such as SOD, POD, CAT, APX and PPO as well as proline content in Iranian olive cultivars. In the present study, the changes in the contents of freezing injury level, MDA, the activities of protective enzymes and as well as proline content in 'Fishomi' and 'Roughani' cultivars were investigated in detail.

Material and methods

Plant material

One-year-old shoots of two local olive cultivars (cvs. 'Fishomi' and 'Roughani') were collected randomly from 40-year-old trees in the Roudbar Olive Research Institute in Guilan province, Iran, in winter (in February, 2012). Mean of minimum and maximum daily temperatures during experiment recorded in Roudbar region (from March 2011 to February 2012) are shown in Figure 1.

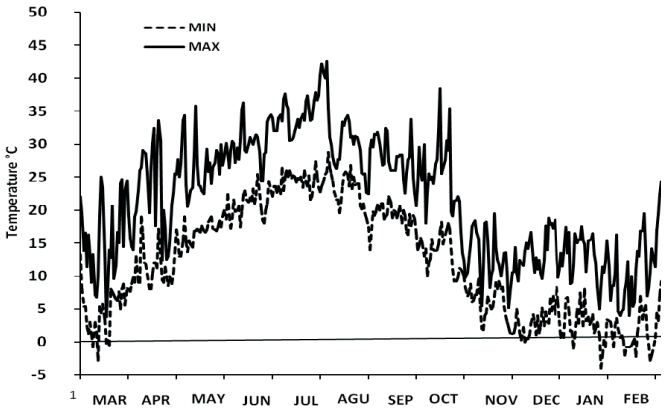

Figure 1. Mean of minimum and maximum daily temperatures during experimental recorded in Roudbar region (from March 2011 to February 2012).

Determination of freezing injury

Ion leakage of leaves was used to assess freezing injury percentage in two olive cultivars by exposing shoots to different freezing temperatures. Twenty cm long shoots were cut from the each cultivar and put in polythene bags, which were closed after the content had been given a spray of distilled water. Five shoots from each cultivar were included in each bag and than polythene bags were placed into a programmable test chamber (KATO, Japan). The programmable chamber temperature was decreased stepwise from 1.5°C/h to -5°C and thereafter 5°C/h until -20°C. Leaf samples were exposed to freezing temperatures (-5°C, -10°C, -15°C and -20°C) for 10 h. The control treatment consisted of samples kept at 4°C in the dark for 24 h (unfrozen samples). After exposing samples to each freezing temperature, recovery was performed by rising the temperature at the same rate until reaching again the temperature of 4°C for slow thawing. In the next step, samples of leaves removed from the third node from the top were used to determine freezing injury. A part of leaves samples were frozen in liquid nitrogen and kept at -80°C until further biochemical analysis.

Ion leakage of leaves was measured as described by Deshmukh et al. (1991) with some modification. Samples were cut into equal pieces (10 mm in diameter), placed in the test-tube containing 10 mL of distilled water, and kept at 45°C for 30 min in a water bath. The initial conductivity of the solution was measured using a Mi 306 EC/TDS Meter conductivity meter ("Milwaukee Instruments", Hungary). The tubes were then kept in a boiling water bath for 10 min, and their conductivity was measured once again after cooling to room temperature. Percentage of ion leakage for each treatment was converted to percentage of injury as:

$$\text{Percentage of injury} = [\%\ \text{IL (t)} - \%\ \text{IL (c)} / 100 - \%\ \text{IL (c)}] \times 100$$

where % IL (t) and % IL (c) are measurements of percentage of IL from the respective freeze-treatment temperature and the unfrozen control, respectively. LT_{50}, a measure of freezing tolerance, was derived for two olive cultivars by determining the freeze test temperature at which 50% injury (midpoint of maximum and minimum percentage of injury) occurred, as explained in Lim et al. (1998).

Lipid peroxidation (MDA formation). The level of membrane damage was measured by the determination of MDA as the end product of membrane lipid peroxidation (Heath and Parker, 1968). Leaves were homogenized in the solution containing 10% TCA and then centrifuged at 10 000 *g* for 10 min. To 1.5 mL of the supernatant aliquot, 1.5 mL of 20% (w/v) TCA containing 0.5% (w/v) TBA were added. The mixture was heated at 95°C for 60 min, cooled to room temperature, and centrifuged at 10 000 *g* for 10 min. The absorbance of the supernatant was read at 532 and 600 nm against TCA solution as a blank reagent. The content of MDA was determined using the extinction coefficient of 1.55/ (M cm) and expressed in nmol MDA per g fr wt.

Enzyme activities. Olive leaves (0.25 g) were homogenized in 1 mL of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM of EDTA in the presence of PVP. The homogenate was centrifuged at 15 000 *g* for 15 min at 4°C. The supernatant was used to measure the activities of SOD, POD, APX, CAT, and PPO and to determine total protein content. All assays were done at 25°C using a spectrophotometer (T80, "PG Instrument", UK).

SOD (EC 1.15.1.1) activity was determined by measuring its ability to inhibit the photoreduction of nitro blue tetrazolium (NBT) according to the methods of Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 200 mM methionine, 1.125 mM NBT, 1.5 mM EDTA, 75 µM riboflavin, and 0–50 µL of the enzyme extract. Riboflavin was added as the last component. Reaction was carried out in test-tubes at 25°C under illumination supplied by two fluorescent lamps (20 W). The reaction was initiated by switching on the light and allowing to run for 15 min, and light switching off stopped the reaction. The tubes were then immediately covered with aluminum foil in order to stop the reaction, and absorbance of the mixture was then read at 560 nm. SOD activity of the extract was expressed as activity unit/g fr wt.

POD (EC 1.11.1.7) activity in leaves was assayed by the oxidation of guaiacol in the presence of H_2O_2. The increase in absorbance was recorded at 470 nm (Chance and Maehly, 1955). The reaction mixture contained 100 µL of crude enzyme extract, 500 µL of 5 mM H_2O_2, 500 µL of 28 mM guaiacol, and 1900 µL of 50 mM potassium phosphate buffer (pH 7.0). POD activity of the extract was expressed as activity unit/(g fr wt min).

CAT (EC 1.11.1.6) activity was assayed according the method of Beers and Sizer (1952). The decomposition of H_2O_2 was monitored by the decrease in absorbance at 240 nm. The assay mixture contained 2.6 mL of 50 mM potassium phosphate buffer (pH 7.0), 400 µL of 15 mM H_2O_2, and 40 µL of enzyme extract. The CAT activity of the extract was expressed as activity unit/(g fr wt min).

APX (EC 1.11.1.11) activity was measured according to Nakano and Asada (1980). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbate, 1.0 mM H_2O_2, and 100 µL of the enzymes extract. H_2O_2-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm. The APX activity of the extract was expressed as activity unit/(g fr wt min).

PPO (EC 1.10. 3. 1) activity was assayed with 4-methylcatechol as a substrate as described in (Luh and Phithakpol, 1972) with some modifications. The assay of the enzyme activity was performed using 2 mL of 0.1 mM sodium phosphate buffer (pH 6.8), 0.5 mL of 100 mM 4-methylcatechol, and 0.5 mL of the enzyme solution. The increase in absorbance at 420 nm was recorded. The PPO activity was expressed as activity unit/100 g fr wt min).

Proline content. Proline content was determined spectrophotometrically by adopting the ninhydrin method of Bates et al. (1973). Three hundred mg of fresh leaf samples were homogenized in sulfosalicylic acid; then 2 mL of each acid ninhydrin and glacial acetic acid were added. The samples were heated at 100°C for 60 min. The mixture was extracted with toluene, free toluene was quantified at 520 nm using L-proline as a standard, and its content was expressed as µmol/g fr wt.

Statistical Analysis. The experiment was conducted in completely randomized design in factorial arrangement. Values presented in the text indicate mean values ± SE of three replicates. Statistical analysis was carried out using SAS software (Version 9.1, SAS Instituted, Cary, NC, USA). Analysis of variance between treatment means was carried out with using LSD test at $p < 0.05$. The graphics were done using Excel software.

Results

Ion leakage and freezing damage

Ion leakage values in the leaves of both olive cultivars increased with declining freezing temperature (Figure 2). The results also showed that ion leakage values in the leaves varied significantly between both cultivars. cv. 'Roughani' showed higher ion leakage than cv. 'Fishomi' during exposing to all freezing temperatures (Figure 2). The minimum ion leakage was found at unfrozen control temperature (29.1 and 31.7% for cvs. 'Fishomi' and 'Roughani', respectively). In contrast, the highest ion leakage was observed in the leaves of both cultivars when exposed to -20°C (58.59 and 63.7% for cvs. 'Fishomi' and 'Roughani', respectively).

The results also showed that freezing injury (expressed by reference to controls) gradually increased when freezing temperature declined and reached to maximum level at -20°C (Figure 3). The maximum values were 40.72 and 47.39% for cvs. 'Fishomi' and

'Roughani', respectively. The percentage of freezing injury varied significantly between two olive cultivars (Figure 3). Roughani cultivar showed higher freezing injury values than cv. 'Fishomi'.

Lipid peroxidation

The levels of lipid peroxidation as measured by the concentration of MDA are shown in Figure 4. The MDA content in the leaves of the two olive cultivars continuously increased during freezing stress and reached its maximum level at -20°C (Figure 4). MDA content of cv. 'Fishomi' was significantly higher than cv. 'Roughani'. The results also showed that at -5, -10 and -15°C, MDA levels were significantly greater than control plants in both 'Fishomi' and 'Roughani'. The highest MDA content (21.76 nmol/g fr wt) was observed in cv. 'Roughani' at -20°C.

Enzymes activity

The enzymatic activities of SOD, POD, APX, CAT and PPO in leaves of two olive cultivars were detected under different freezing temperatures.

The effect of freezing temperatures on activity of SOD in leaves of Fishomi and Roughani cultivars were shown in Figure 5. The SOD activity in cv. 'Fishomi' did not change at first, but thereafter, its activity gradually decreased to –20°C. The highest SOD activity content (84.59 U/g fr wt) was observed in cv. 'Roughani' at -10°C. SOD activity at -10°C was significantly greater (21.55%) than control samples in cv. 'Roughani'. Freezing stress at the level of -10°C caused significant decrease (31.66%) in SOD activity of cv. 'Fishomi' leaves when compared with control samples and thereafter sharply decreased to –20°C.

POD activity of both olive cultivars continuously increased during temperature declining, and reached the maximum level at -10 °C, thereafter gradually decreased to –20 °C (Figure 6). The results also showed that POD activity varied significantly between cvs. 'Fishomi' and 'Roughani' (Figure 6). Cv. 'Fishomi' showed higher POD activity than cv. 'Roughani'. In general, POD activity in the leaves was 39.16 and 1.77- fold higher than control samples for cvs. 'Roughani' and 'Fishomi' at -10 °C, respectively.

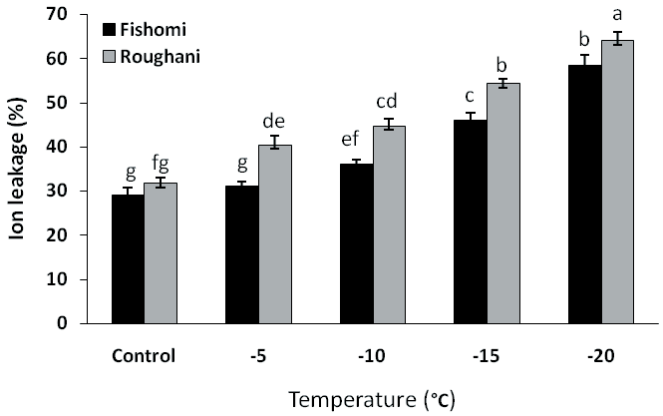

Figure 2. Changes in ion leakage in the leaves of cvs. 'Fishomi' and 'Roughani' under control and freezing temperatures. Data are presented as means ± SE (n = 3).

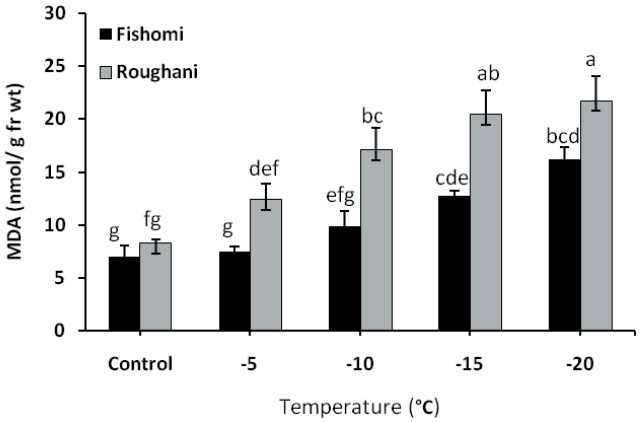

Figure 4. Changes in MDA content in the leaves of cvs. 'Fishomi' and 'Roughani' under control and freezing temperatures. Data are presented as means ± SE (n = 3)

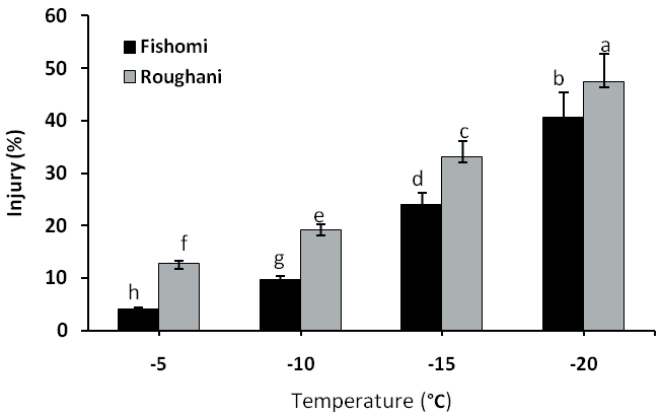

Figure 3. Changes in injury percentage in the leaves of cvs. 'Fishomi' and 'Roughani' under control and freezing temperatures. Data are presented as means ± SE (n = 3).

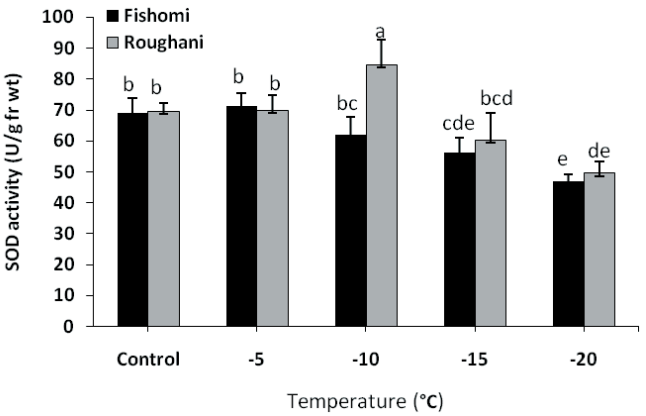

Figure 5. Changes in SOD activity in the leaves of cvs. 'Fishomi' and 'Roughani' under control and freezing temperatures. Data are presented as means ± SE (n = 3).

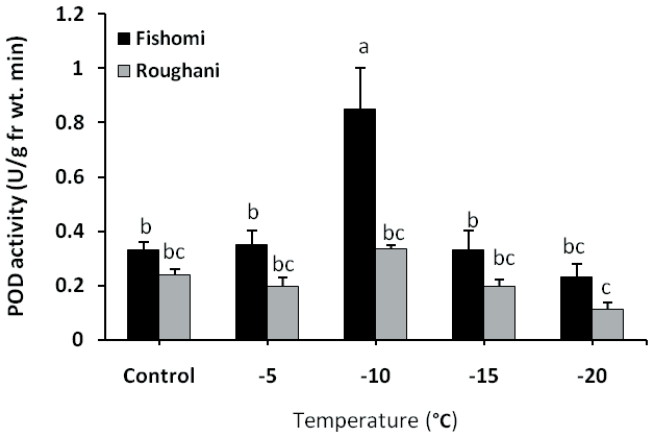

Figure 6. Changes in POD activity in the leaves of cvs. 'Fishomi' and 'Roughani' under control and freezing temperatures. Data are presented as means ± SE (n = 3).

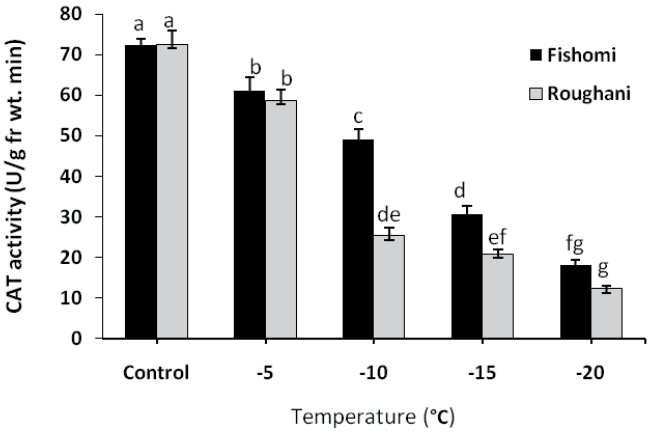

Figure 8. Changes in CAT activity in the leaves of cvs. 'Fishomi' and 'Roughani' under control and freezing temperatures. Data are presented as means ± SE (n = 3).

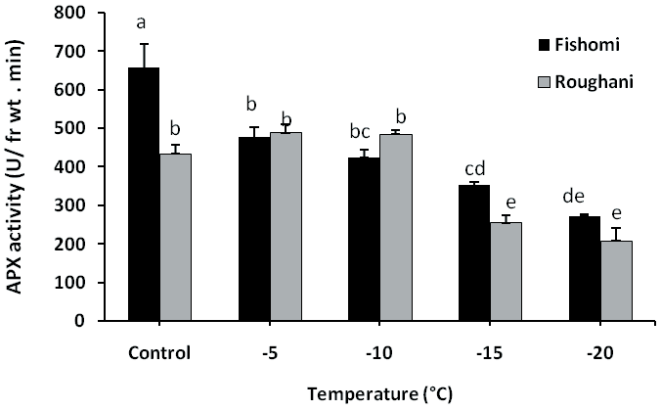

Figure 7. Changes in APX activity in the leaves of cvs. 'Fishomi' and 'Roughani' under control and freezing temperatures. Data are presented as means ± SE (n = 3).

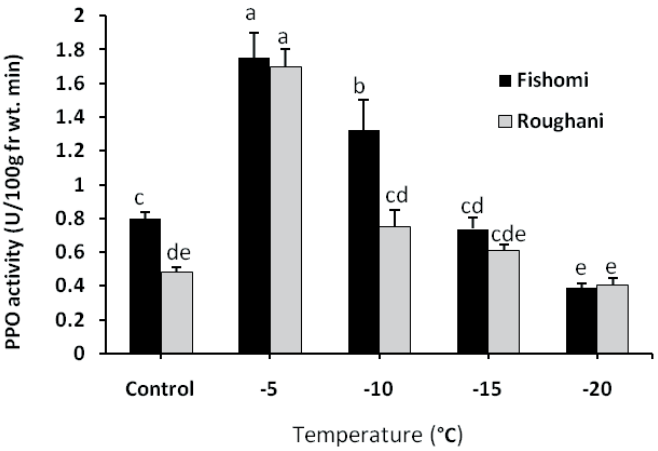

Figure 9. Changes in PPO activity in the leaves of cvs. 'Fishomi' and 'Roughani' under control and freezing temperatures. Data are presented as means ± SE (n = 3).

As it is shown in the Figure 7, APX activity significantly decreased in cv. 'Fishomi' when the freezing temperatures declined, in comparison with control samples. The APX activity didn't change at -5 and -10°C in cv. 'Roghani' but significantly decreased at -15 and –20°C. However, cv. 'Fishomi' showed higher APX activity values than cv. 'Roghani' in control sample, -15 and -20°C. In cv. 'Roghani', freezing stress caused a significant decrease in APX activities at -15 and -20°C in comparison with control samples. In cv. 'Fishomi', freezing temperatures at -5, -10, -15 and -20°C caused 27.27, 32.26, 46.32 and 58.51% decreases in APX activity in comparison with control samples. It must be noted that the enzyme activity showed no significant differences between -5 and -10°C in both cultivars (Figure 7).

As shown in Figure 8, the activity of CAT changed in leaves of both olive cultivars during freezing stress. In general, CAT activity was the highest in control samples and its activity decreased in parallel to declining of the temperature until -20°C. The percentage of CAT activity was declining at -5, -10, -15 and -20°C, compared to the control, and it was 15.34, 32.16, 57.53 and 4.83%, respectively for cv. 'Fishomi' and 19.05, 65.15, 71.35 and 83.14%, respectively for cv. 'Roughani'.

The effect of freezing temperatures on activity of PPO in leaves of cvs. 'Fishomi' and 'Roughani' is shown in Figure 4. The PPO activity in the leaves of both cultivars considerably increased at -5°C, and reached its maximum level but thereafter gradually decreased to –20°C (Figure 9). The PPO activity value in cv. 'Fishomi' was 76% higher than in cv. 'Roughani' at -10 °C. The results also showed that PPO activity varied significantly between cultivars. Cv. 'Fishomi' showed higher PPO activity than cv. 'Roughani' (Figure 9).

Proline content

The proline content in the leaves of the two olive cultivars gradually decreased during freezing stress, and reached its

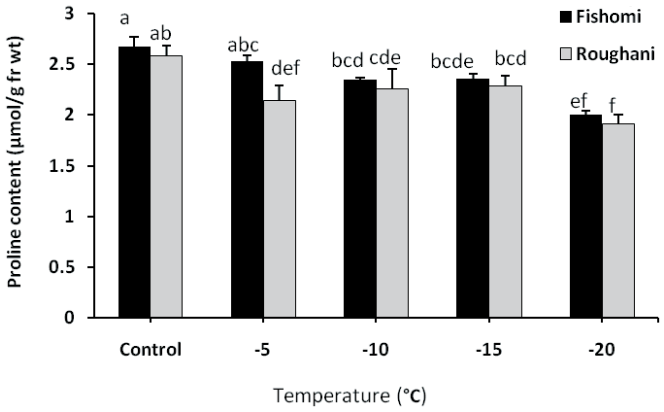

Figure 10. Changes in proline content in the leaves of cvs. 'Fishomi' and 'Roughani' under control and freezing temperatures. Data are presented as means ± SE (n = 3).

minimum levels at -20°C (Figure 10). Proline content in control and at -5°C was significantly higher in cv. 'Fishomi' than in cv. 'Roughani'. Maximum proline content was 2.4 and 2.6 nmol / g FW for cvs. 'Fishomi' and 'Roughani', respectively. However, proline content at -15 and -20°C was higher in cv. 'Fishomi' than in cv. 'Roughani'. It must be noted that its content showed no significant differences in both cultivars (Figure 10).

Correlation between freezing injury percentage and freezing stress-related biochemical parameters

In order to evaluate the relationship between freezing injury percent and freezing stress-related biochemical parameters, Pearson correlation coefficients between freezing injury percent and these parameters were calculated (Table 1). The freezing injury percent was positively correlated with ion leakage percent (r= 0.979** and r= 0.981** for cvs. 'Fishomi' and 'Roughani', respectively) and MDA content (r= 0.922** and r= 0. 815** for cvs. 'Fishomi' and 'Roughani', respectively) (Table 1). In contrast, SOD (r= 0.874** and r= 0.589* for cvs. 'Fishomi' and 'Roughani', respectively), APX (r= 0.843** and r= 0.815* for cvs. 'Fishomi' and 'Roughani', respectively), and CAT (r= 0.924** and r= 0.798** for cvs. 'Fishomi' and 'Roughani', respectively) activities negatively correlated with freezing injury percentage. Furtheremore, a negative significant correlation was found between freezing injury percentage and proline content. Although considerable levels of POD activity increased in the leaves of the two cultivars during beginning of freezing stress, they had no significant correlation with freezing injury percentage. Finally, PPO activity in the leaves of cv. 'Fishomi' had significant correlation with freezing injury but in cv. 'Roughani' had no significant correlation with freezing injury (Table 1).

Table 1. Pearson correlation coefficients between injury percentage and the levels of various biochemical parameters in the leaves of cvs. 'Fishomi' and 'Roughani

Variable	Correlation coefficient (r)	
	Fishomi	Roughani
Ion leakage %	0.979**	0.981**
MDA content	0.922**	0.815**
SOD activity	–0.874**	–0.589*
POD activity	–0.176ns	0.262ns
APX activity	–0.843**	–0.815**
CAT activity	–0.914**	–0.798**
PPO activity	–0.665**	–0.112ns
Proline content	0.692**	–0.727**

Note: ns, *, ** Not significant or significant at P < 0.05 or 0.01

Discussion

Freezing temperature is one of the most important environmental factors that limits the productivity and distribution of plants (Levitt, 1980). Under stress conditions caused by low temperature, oxidative damage can occur, triggering overproduction of ROS (Okuda et al., 1991). ROS has been proposed to be responsible for cold-induced injury because they are produced at higher concentration during low temperature stress, and may initiate degradative reactions, causing lipid peroxidation, membrane deterioration and protein degradation (Elstner, 1991). Generally, tissue viability is evaluated after a freeze-thaw cycle by measuring the consequences of primary injuries in plant membranes (Mahajan and Tuteja, 2005). We investigated the degree of cell membrane damage by ion leakage and lipid peroxidation in order to determine the leaves response of two olive cultivars to various freezing temperatures (–5, –10, –15 and –20°C). Freezing temperatures caused a significant increase in ion leakage (Figure 2), freezing injury (Figure 3) and MDA content (Figure 4) in both cultivars, indicating that freezing stress could cause damages to the integrity of the cellular membranes and to cellular components, such as lipids. Cv. 'Fishomi' exhibited a significantly lower freezing injury, ion leakage and MDA content than cv. 'Roughani' when exposed to different freezing temperatures. The freezing injury percentage was positively correlated with ion leakage percent and MDA content in both cultivars (Table 1). These results are in agreement with previous study in *Eupatorium adenophorum* by Lu et al. (2008), who reported that MDA was increasing with lowering temperature. Cansev et al. (2011) also indicated that freezing injury percentage in olive (cv. 'Gemlik') increases with lowering temperature. The cultivars difference freezing tolerance may be associated with their genetic background and capacity of metabolic defense responses (Zhang and Ervin, 2008). The scavenging enzymes are a key protein fraction in the acquisition of freezing tolerance in plants (Lee and Chen, 1992). To cope with oxidative damage under extremely adverse conditions, plants have developed an antioxidant defense system that includes the antioxidant enzymes SOD, APX, POD, CAT (Foyer and Noctor, 2005). It is clear that the response of antioxidant system to freezing stress depends on the severity of stress and on the cultivars. Tolerant plant species generally have a better capacity to protect themselves against freezing-induced oxidative stress, which can also be achieved via the enhancement of the activities of antioxidant enzymes (Luo et al., 2007; Cansev et al., 2009; Cansev et al., 2011).

Cv. 'Fishomi' exhibited a significantly higher POD (Figure 6), APX (Figure 7), CAT (Figure 8) and PPO (Figure 9) activity than cv. 'Roughani' when exposed to different freezing temperatures. This might demonstrate gene-dependence in changes of antioxidant enzymes. However, it must be noted that the activity of SOD

showed no significant differences in both cultivars at freezing temperatures except in cv. 'Roughani' at -10°C, which showed higher SOD activity (Figure 5). SOD is a metallo-enzyme that scavenges the toxic superoxide radicals and catalyzes the conversion of two superoxide anions into oxygen and H_2O_2 (Miyake and Yakota, 2000). APX and POD catalyze the breakdown of H_2O_2. APX together with other reductases can detoxify H_2O_2 by using ascorbate as an electron donor through the Halliwell-Asada pathway (Halliwell, 1987). CAT dismutates H_2O_2 into water and oxygen; this enzyme is located mostly in peroxysomes and glyoxysomes (Scandalios et al., 1997). Furthermore, PPOs catalyse the oxidation of O-diphenols to O-diquinones, as well as the hydroxylation of monophenols at enzymatic browning reactions (Mayer, 2006). PPOs are also important in the response of plants against freezing stress and they can help avoid serious oxidative damage induced by freezing (Ortega-García and Peragón, 2009). Our results showed that SOD, APX and CAT activities negatively correlated with freezing injury percentage. Significantly negative correlations were also found between freezing injury percentage and proline content. Although considerable levels of POD activity increased in the leaves of the two cultivars during beginning of freezing stress, they had no significant correlation with freezing injury percentage. PPO activity in cv. 'Fishomi' had significant correlation with freezing injury percentage but in cv. 'Roughani' it had no significant correlation with freezing percentage. (Table 1). Higher activity of antioxidant enzymes in cv. 'Fishomi' under freezing stress suggests that the freezing-tolerant olive cultivar possesses a better O_2^- -scavenging ability. Similar results were reported in olive (Cansev et al., 2009) and in *Populus* (Luo et al., 2007) under freezing stress. Cansve et al. (2009) reported that 'Leccino' and 'Ascolona' cultivars, which had the highest freezing tolerance, had higher APX and CAT activity in their leaf tissues compared to freezing sensitive cultivars such as 'Meski' and 'Uslu'. Similar to our results, higher PPO activity was also reported in olive under low temperatures (Ortega-García and Peragón, 2009).

Proline may inhibit membrane lipid peroxidation in plant tissue by acting as an antioxidant to neutralize the chilling-induced free radicals (Xin and Li, 1993). In the present study, freezing stress has decreased proline content (Figure 10) and cv. 'Fishomi' exhibited significantly higher proline content than cv. 'Roughani' when exposed to different freezing temperatures. Heber et al. (1973) showed that proline is capable of preventing freezing-induced membrane damage.

Conclusion

The results demonstrated that olive cv. 'Fishomi' showed lower freezing injury and lipid peroxidation (MDA content) than cv. 'Roughani'. The activities of POD, CAT, APX and PPO enzymes in cv. 'Fishomi' were higher than those in cv. 'Roughani', under freezing stress. The activity of SOD, CAT and APX followed by proline accumulation in leaf tissues was negatively correlated with freezing injury percentage. The higher antioxidant enzymes activity and proline content in leaf tissues of cv. 'Fishomi' seems to protect and stabilize its cell membrane to make the hardier trees than those of cv. 'Roughani'. Overall, higher SOD, CAT and APX activity level and proline content could be used as important selection criteria in screening tolerant olive cultivars.

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