Cold Tolerance in Olive Leaves of Three Cultivars Related to Some Physiological Parameters during Cold Acclimation and De-Acclimation Stages

S. Saadati¹, B. Baninasab¹*, M. Mobli¹, and M. Gholami¹

ABSTRACT

This research studied changes in antioxidant enzymes activity, Total Soluble Proteins (TSPs), Malondialdehyde (MDA), and proline content in the leaves of three olive (Olea europaea L.) cultivars (Amphisis, Gorgan, and Manzanilla) at five different dates, and investigated their relationship with cold tolerance. The results revealed that cold-acclimation dramatically increased cold tolerance. Furthermore, antioxidant enzymes activity, MDA, TSP, and proline content increased throughout the acclimation stage, whereas they declined in the de-acclimation stage. The ascorbate peroxidase, catalase, peroxidase, and superoxide dismutase activities in the leaves tissues correlated with the alterations in cold tolerance. Higher TSP, greater antioxidant enzyme activities, and more proline content together with lower MDA content in Amphisis cultivar led to relative improvement in cold tolerance capacity of this cultivar. Our results showed antioxidant enzymes activities, TSP and proline content could be useful indices to screen cold tolerance in olive cultivars.

Keywords: Antioxidant defense enzymes, Lipid peroxidation, Olea europaea L.

INTRODUCTION

Cold and frost are important environmental factors limiting geographic distribution of plants and crop yields worldwide (Korn et al., 2008; Wang et al., 2018). In temperate woody perennial plants, a period of exposure to low temperatures and shortening day length enables them to adapt to cold and freezing stress. This process is called Cold Acclimation (CA), which involves several biochemical, physiological, and molecular alterations (Vitasse et al., 2014), such as changes in membrane composition, accumulation of sugar or other compounds, photosynthetic efficiency, stomatal closure, and alterations in the antioxidant enzyme activities and their gene expressions (Beck et al., 2007).

The cold-induced stomatal closure, increases Reactive Oxygen Species (ROS), creating disturbances in biochemical pathways (Suzuki and Mittler, 2006). Therefore, oxidative stress causes lipid peroxidation and damages other important biomolecules. Membranes are the main places for cold-induced damages to organelles and cells, since ROS is capable of reacting with unsaturated fatty acids to cause membrane lipid peroxidation in intracellular organelles or plasmalemma peroxidation (Karabal et al., 2003). To survive cold stress, plants have antioxidant mechanisms that are divided into two components: non-enzymatic antioxidants and enzymatic antioxidant systems to scavenge ROS and mitigate their toxic effects (Ahmad et al., 2010; Kasote et al., 2015). The extent of

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damage is dependent on the equilibrium between ROS formation and their detoxification by antioxidant scavenging system. By scavenging superoxide radicals and promoting their conversion into hydrogen and oxygen peroxide, Superoxide Dismutase (SOD) performs a constructive role in preserving plants against the deleterious effects of oxidative stress. Then, Peroxidase (POD) and Catalase (CAT) convert hydrogen peroxide into H₂O and oxygen. The peroxide and surplus active oxygen species can be removed and lipid peroxidation can be inhibited by those enzymes working together (Das and Roychoudhury, 2014) (Figure1).

One of the most typical evergreen species in the Mediterranean Basin, Olive (*Olea europaea* L.) grows between the latitudes of approximately 30° and 45° in both northern and southern hemispheres. Nevertheless, possession of high oil nutritional value and tolerance to environmental stresses has recently extended the cultivation area of this crop into the various regions in Iran (Saadati et al., 2013). Olive is partly freezing-tolerant, however, when temperature falls below a certain threshold of -7°C, the plant may be hurt (Palliotti and Bongi, 1996), while at -12°C the damage can be so serious that the tree life is threatened (Gomez-del Campo and Barranco, 2005).

Figure 1. A schematic representation of cold stress consequences and antioxidant defense system of cold stress response in plants.

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Cold stress

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ROS generation

(O₂⁻, H₂O₂, OH⁻)

Oxidative stress

Membrane injury (Lipid peroxidation, electrolyte leakage), inactivation of enzymes, damage to macromolecules

Enzymatic antioxidant defense

Ascorbate peroxidase, catalase, peroxidase, superoxide dismutase

Non enzymatic Antioxidant defense

Ascorbic acid, glutathione, proline, carotenoids, phenolic acids

Cold tolerance
There are many studies on the reactions of olive trees to cold temperature and relationship between some antioxidant defense enzyme activities with cold tolerance of olive leaves during the process of cold acclimation and de-acclimation stages (Cansev et al., 2009; Cansev et al., 2011; Hashempour et al., 2014a and b). However, more detailed physiological data are needed on the role of antioxidant defense enzymes, protein, MDA, and proline contents during cold acclimation stage to understand the mechanisms of cold tolerance in olive cultivars.

Therefore, in this study, we aimed to study the seasonal patterns of these parameters and to compare them in various periods of acclimation and de-acclimation cycle, ranging from mid-autumn to the early spring and their correlation with the values of Lethal freezing Temperature (LT50) in leaves. Eventually, comparing the cold tolerance-related physiological responses in Amphisis, Gorgan, and Manzanilla cultivars could serve as appropriate markers for breeding and selecting cultivars and provide a deeper insight into the molecular mechanisms of tolerance against cold-induced oxidative stress.

**MATERIALS AND METHODS**

**Plant Material**

The research was carried out in 2016 and 2017 on 15-year-old olive trees, in a research orchard in Isfahan University of Technology, Iran (latitude 41° 0' N, longitude 51° 53' E, alt. 1595 m). The study used three cultivars of olive, namely a cold-tolerant (Amphisis), an intermediate variety (Gorgan), and a cold sensitive cultivar (Manzanilla) (Simkeshzadeh et al., 2011; Hashempour, et al., 2014a; Saadati et al., 2019). All cultivars were off-year trees, subject to similar agricultural practices. The olives were grown on their own root under a drip irrigation system, spaced 5x6 m, pruned, and fertilized almost every year. The soil texture was clay loam, consisting of 26% sand, 34% clay, and 40% silt with 0.96% organic matter content. The soil pH was 6.9, with electrical conductivity of 4.3 dS m⁻¹.

The means of minimum and maximum air temperatures during the experiment were recorded in Isfahan region, as shown in Figure 2. Although this region has relatively long and warm summers, the winter temperature may fall below -10°C or even
lower, causing serious damage to olive plants (Figure 2). The average temperatures were 18.15°C (range 10.1–26.2°C), 9.8°C (range 3.1–16.6°C), 6.3°C (range -1.2–13.8°C), 5.6°C (range -0.8–11.9°C) and 10.9°C (range 4.6–17.1°C) in different sampling stages: November, December, January, February, and April, respectively (Figure 2).

For every cultivar, a total number of nine trees were randomly selected, from which two-year-old shoots (30 cm long) were gathered (Cansev et al., 2009). It was tried to collect at least three cuttings from each tree during two periods of acclimation and de-acclimation at five regular intervals: 5 November and 3 December 2016, as well as 1 January, 4 February and 3 April 2017. Samples were immediately wrapped in wet paper, sealed in plastic bags, preserved in ice boxes and taken to the laboratory. The leaves, which were uniformly sized and fully expanded, were removed from the mid-part of the collected shoots (Eris et al., 2007). Then, they were divided into two groups: one group was subjected to different freezing temperatures to determine the LT50 values, and samples in the other group were frozen in liquid nitrogen and stored at −80°C to be later used for physiological parameters analysis. For all physiological parameters, the leaf samples of each replication (n= 3) were collected from three trees and pooled for determination of parameters.

Freezing Procedure and Cold Tolerance Determination

Samples of each cultivar were tested in a programmable freezing chamber (Eyela, Tokyo, Japan) to assess their freezing tolerance. For every freezing temperature, five leaves were first washed in distilled-deionized water, then wrapped in a moist paper towel and subsequently with aluminium foil, and then transferred to the programmable freezing chamber. The temperature was decreased stepwise at 1.5°C h⁻¹ to 0°C, and thereafter at 5°C h⁻¹ down to −25°C. Samples were exposed to low temperatures at 0, −5, −10, −15, −20, or −25°C for 12 hours (Cansev et al., 2009). Then, samples were removed from each low temperature treatment, recovery was done by increasing the temperature at the same rate until 4°C for slow thawing (Hashempour et al., 2014a). The electrolyte leakage of leaf samples was quantified based on Arora et al. (1992). Briefly, leaf discs (1 cm in diameter) were cut from the leaves and placed in test tubes containing 10 mL of deionized water and kept at room temperature for 24 hours in a shaker. Then, an electrical conductivity meter (CC-501, Elmetron, Zabrze, Poland) was employed to measure the solution Electric Conductivity (EC1). The tubes were then transferred into a boiling water bath at 100°C for about 10 minutes and their conductivity was measured once again after cooling to room temperature (EC2). Finally, the electrolyte leakage was measured as EC1/EC2 and presented as a percentage. Cold tolerance was calculated and expressed as LT50 (which is a Lethal Temperature at which 50% of the total ion leakage occurs). LT50 was calculated by fitting response curves using the following logistic sigmoid function:

\[ R = \frac{a}{1 + e^{b(x-c)}} + d \] (1)

Where, R represents Relative electrolyte leakage percentage, based on LT50 estimation method; x denotes treatment temperature; b indicates the slope of the function at inflection point c, c indicates the temperature at the inflection point (LT50) and a and d are the upper and lower asymptotes of the function, respectively (Fiorino and Mancuso, 2000).

Antioxidant Enzyme Activity

APX, CAT, POD, and SOD activities were determined in leaf samples (0.1 g) which were collected and immediately frozen at −80°C. The APX (EC 1.11.1.11) activity was determined according to the methods of
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Nakano and Asada (1980). The method of Chance and Maehly (1955) was adopted to estimate the CAT (EC 1.11.1.6) activity. The POD (EC 1.11.1.7) activity was measured according to the method of Chance and Maehly (1955). Beauchamp and Fridovich’s (1971) method was used to determine the SOD (EC 1.15.1.1) activity.

Total Soluble Protein Content

Adopting Bradford (1976) and employing Bovine Serum Albumin (BSA) as a standard, protein content in 0.1 g of leaf samples was determined.

MDA Content

The ultimate product of membrane lipid peroxidation, the MDA was measured in leaf samples (0.1 g) to determine the membrane damage level as estimated by the method of Heath and Parker (1968).

Proline Content

Proline content was determined in leaf samples (0.5 g), as described by Bates et al. (1973).

Statistical Analysis

The experiment was arranged in a randomized complete block design, with three replications; each replication contained three trees, for a total of nine trees per cultivar. Statistical analysis was carried out using SAS software (Version 9.1, SAS Institute, Cary, NC, USA). Means comparisons were carried out adopting LSD test at P≤ 0.05. Correlation analysis between physiological traits and LT_{50} were performed using Pearson’s correlation coefficient.

RESULTS

Cold Tolerance

Cold tolerance expressed as the LT_{50} was significantly affected by sampling dates, cultivars, and the interaction of sampling dates and cultivars (Table 1). The results showed that LT_{50} decreased in the acclimation dates from November to January, but increased in February and reached the highest in April (Figure 3-A). Based on LT_{50} values, differences in cold tolerance of three olive cultivars (by lowering LT_{50}) were observed. Amphisis showed the highest cold tolerance, while Manzanilla was the least cold tolerance cultivar (Figure 3-A). Cold-acclimation raised cold hardiness in all cultivars, however, cultivars did not show simulant acclimation rate. Cold hardiness in Manzanilla and Gorgan increased 2.9 and 5.7 °C, respectively, from November to January (Figure 3-A). An enormous difference in cold tolerance was observed.

Table 1. Analyses Of Variance (ANOVA) of Sampling dates (S), Cultivars (Cv), and their interactions on cold tolerance (LT_{50}) and some physiological parameters. *

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>df</th>
<th>LT_{50}</th>
<th>APX</th>
<th>CAT</th>
<th>POD</th>
<th>SOD</th>
<th>TSP</th>
<th>MDA</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>4</td>
<td>70.66**</td>
<td>34.04**</td>
<td>170.14**</td>
<td>93.97**</td>
<td>60.79**</td>
<td>21.33**</td>
<td>21.82**</td>
<td>46.79**</td>
</tr>
<tr>
<td>Cv</td>
<td>2</td>
<td>20.89**</td>
<td>23.34**</td>
<td>7.06**</td>
<td>8.79**</td>
<td>10.13**</td>
<td>7.89**</td>
<td>68.86**</td>
<td>29.83**</td>
</tr>
<tr>
<td>SxCv</td>
<td>8</td>
<td>6.01**</td>
<td>2.51**</td>
<td>6.19**</td>
<td>1.55ns</td>
<td>0.33ns</td>
<td>1.09ns</td>
<td>0.83ns</td>
<td>1.33ns</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* LT_{50}: Lethal Temperature at which 50% of the total ion leakage occurs, APX: Ascorbate Peroxidase Activity; CAT: Catalase activity; POD: Peroxidase activity; SOD: Superoxide Dismutase activity; TSP: Total Soluble Protein content; MDA: Malondialdehyde content, PC: Proline Content. ns, *, and **: Non significant, significant at P≤ 0.05 and P≤ 0.01, respectively.
hardiness was detected among cultivars during cold acclimation period in December. Based on the $LT_{50}$ values, the highest cold-hardiness in this date was associated with Amphisis (-15.11°C), whereas, the least hardy cultivar, Manzanilla, reached 50% mortality at -7.55°C (Figure 3-A). Cold tolerance of all cultivars diminished noticeably in April, and they showed a similar ranking in cold-hardiness. Overall, a fairly low variation in cold hardness was observed in cultivars in November and April, in comparison with other sampling dates (Figure 3-A).

**Antioxidant Enzymes Activity**

APX activity was significantly affected by sampling dates, cultivars, and their interaction (Table 1). As shown in Table 2, APX activity rose during CA from November to January, then dropped during February, and reached the lowest activity in April. The highest and the lowest APX activities were detected in Amphisis and Manzanilla cultivars, respectively (Figure 3-B). From November to January, APX activity increased by 1.81 times in Amphisis and more than twice in Gorgan and Manzanilla cultivars. However, in January, maximum activities were detected in Amphisis and Gorgan and minimum activity was observed in Manzanilla (Figure 3-B). APX activity had a dramatic drop in April and no difference was detected among Amphisis and Gorgan cultivars on this date (Figure 3-B). A negative correlation between $LT_{50}$ and APX activity was found in all sampling dates (Table 3).

CAT activity was significantly affected by sampling dates, cultivars, and their interaction (Table 1). During cold acclimation, an enhancement was seen in CAT activity from November to January, then, a decrease occurred in February, which reached its lowest activity in April (Figure 3-C). The highest and the lowest CAT activities were detected in Amphisis and Manzanilla, respectively (Figure 3-C). Changes in leaf CAT activity showed the same trend in cultivars during all sampling dates. The highest variation in CAT activity

![Figure 3](image_url)

**Figure 3.** Changes in $LT_{50}$ (A), APX activity (B), CAT activity (C) and POD activity (D) in olive leaves of three cultivars of Amphisis (●), Gorgan (■), and Manzanilla (▲) at five dates of November, December, January, February, and April. Values are means of three replicates±SE.
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of cultivars was shown in January compared to the other sampling dates (Figure 3-C). However, in this date, maximum activity of CAT was detected in Amphisis, which was 51 and 30% higher than Manzanilla and Gorgan cultivars, respectively (Figure 3-C). A high correlation was observed between LT_{50} and CAT activity in all sampling dates. (Table 3).

POD activity was significantly influenced by sampling dates and cultivars (Table 1). POD activity continuously increased during acclimation period from November to January, thereafter sharply decreased until April (Table 2). The highest and the lowest POD activities were detected in Amphisis and Manzanilla, respectively (Table 2). POD activities in leaf showed a similar trend in cultivars during all sampling dates. The highest POD activity in Amphisis, Gorgan, and Manzanilla cultivars was observed in January (Figure 3-D). A high correlation between LT_{50} and POD activity was detected in November, December, January and April (Table 3).

SOD activity was significantly affected by sampling dates and cultivars (Table 1). An increase in SOD activity was observed during cold acclimation period from November to December, then a decrease occurred in January, and the lowest activity was discerned in April (Table 2). An increase in SOD activity was observed in all cultivars, particularly in Amphisis in December (Figure 4-A). SOD activity gradually dropped in January and no difference was found among Gorgan and Manzanilla cultivars on this date. In February and April, the highest SOD activity was discerned in Amphisis and Gorgan cultivars (Figure 4-A). LT_{50} was highly correlated with SOD activity in December, February, and April (Table 3).

**Total Soluble Protein (TSP)**

TSP content was significantly influenced by sampling dates and cultivars (Table 1). Leaf TSP increased from November through January, thereafter decreased in February, and reached the lowest in April (Table 2). The highest and the lowest TSP contents were detected in Amphisis and Manzanilla, respectively (Table 2). In December and January, the largest differences among cultivars were observed in the TSP contents (Figure 4-B). From November to January, TSP contents increased from 82 to 95% in all cultivars. TSP content considerably dropped in February and no difference was observed among cultivars at this date (Figure 4-B). There were correlations between cold hardiness (by lowering LT_{50}) and TSP in January and April, whereas no correlation was seen between TSP and cold hardiness in November, December, and February (Table 3).

**MDA Content**

MDA content was significantly affected by sampling dates and cultivars (Table 1). The MDA content in the leaves of the olive cultivars enhanced with the onset of cold temperatures in November, reached their apex in December, and tended to decline from January to April (Table 2). The highest and the lowest MDA contents were detected in Manzanilla and Amphisis, respectively (Table 2). From November to December, MDA content showed considerable increase in all three cultivars, which was concomitant with the LT_{50} decrease (Figures 3-A and 4-C). In December, maximum amounts of MDA were detected in Manzanilla, while the minimum content was found in Amphisis (Figure 4-C). A noticeable reduction was found in MDA content in April, compared to December, which was consistent with LT_{50} profile on this sampling date (Figures 3-A and 4-C). High correlations between LT_{50} and MDA were found in December and April (Table 3).

**Proline Content (PC)**

Proline content was significantly affected by sampling dates and cultivars (Table 1). An increase in proline content was detected during cold acclimation period from
November to January. Then, a decrease was observed during deaclimation period until it reached its lowest content in April (Table 2). The highest and the lowest proline contents were detected in Amphisis and Manzanilla, respectively (Table 2). Proline content had a low range of variation among cultivars in November from 2.48 to 3.80 µmol g\(^{-1}\)FW and April from 1.81 to 2.93 µmol g\(^{-1}\)FW (Figure 4D). During acclimation period, proline content showed an increase, particularly in Amphisis. Proline showed more accumulation in January than the other sampling dates in all cultivars (Figure 4D). Proline content noticeably decreased in April, whereas no difference was monitored among cultivars on this date (Figure 4D). LT\(_{50}\) revealed a correlation with the amount of leaf proline in December, January and February; however, no correlation was observed between LT\(_{50}\) and proline content in November and April (Table 3).

**DISCUSSION**

Of the major environmental factors that pose limits on plants distribution and their productivity, low temperature (Macek et al., 2012) is particularly common. It is reported that ROS accounts for cold-induced damage as it is generated at higher content throughout low-temperature stress, leading to protein degradation, lipid peroxidation, and membrane deterioration (Suzuki and Mittler, 2006). In this study, we investigated the severity of cell membrane injury by MDA and ion leakage to ascertain the cold hardiness of three olive cultivars during cold acclimation and de-acclimation. Cold acclimation and de-acclimation of olive cultivars varied at each sampling date and cultivars did not display a similar trend during cold acclimation. Based on LT\(_{50}\) estimations, Amphisis exhibited earlier development of cold tolerance than other cultivars in winter. The highest enhancement of cold hardiness of these olive cultivars during cold acclimation was reported that ROS accounts for cold-induced damage as it is generated at higher content throughout low-temperature stress, leading to protein degradation, lipid peroxidation, and membrane deterioration (Suzuki and Mittler, 2006).

### Table 2. Mean comparisons of the sampling dates and cultivars on cold tolerance (LT\(_{50}\)) and some physiological parameters.

<table>
<thead>
<tr>
<th>Sampling dates</th>
<th>LT(_{50}) (°C)</th>
<th>APX (µmol g(^{-1})FW min)</th>
<th>CAT (µmol g(^{-1})FW min)</th>
<th>POD (µmol g(^{-1})FW min)</th>
<th>SOD (µmol g(^{-1})FW)</th>
<th>TSP (mg g(^{-1})FW)</th>
<th>MDA (µmol g(^{-1})FW)</th>
<th>PC (µmol g(^{-1})FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>November</td>
<td>-7.47 ± 1.31(^b)</td>
<td>0.72 ± 0.27(^a)</td>
<td>17.78 ± 4.08(^b)</td>
<td>0.31 ± 0.07(^b)</td>
<td>7.91 ± 0.83(^b)</td>
<td>4.21 ± 0.68(^b)</td>
<td>10.66 ± 1.29(^b)</td>
<td>2.98 ± 0.78(^b)</td>
</tr>
<tr>
<td>December</td>
<td>-10.71 ± 0.81(^b)</td>
<td>1.58 ± 0.20(^a)</td>
<td>24.04 ± 3.06(^b)</td>
<td>0.49 ± 0.05(^a)</td>
<td>9.96 ± 0.62(^b)</td>
<td>4.70 ± 0.44(^a)</td>
<td>20.02 ± 1.68(^b)</td>
<td>5.87 ± 0.35(^b)</td>
</tr>
<tr>
<td>January</td>
<td>-12.18 ± 0.66(^a)</td>
<td>2.09 ± 0.11(^a)</td>
<td>38.89 ± 2.41(^b)</td>
<td>0.58 ± 0.06(^a)</td>
<td>6.67 ± 0.82(^b)</td>
<td>7.97 ± 0.43(^b)</td>
<td>18.14 ± 1.71(^b)</td>
<td>6.44 ± 0.44(^a)</td>
</tr>
<tr>
<td>February</td>
<td>-10.16 ± 1.22(^b)</td>
<td>1.54 ± 0.28(^a)</td>
<td>30.08 ± 2.84(^b)</td>
<td>0.30 ± 0.06(^b)</td>
<td>5.35 ± 0.88(^b)</td>
<td>5.72 ± 0.61(^b)</td>
<td>14.86 ± 1.50(^b)</td>
<td>3.16 ± 0.68(^b)</td>
</tr>
<tr>
<td>April</td>
<td>-4.71 ± 1.33(^a)</td>
<td>0.66 ± 0.27(^a)</td>
<td>16.68 ± 2.86(^b)</td>
<td>0.07 ± 0.07(^b)</td>
<td>3.85 ± 0.38(^b)</td>
<td>4.33 ± 0.63(^b)</td>
<td>12.64 ± 1.25(^b)</td>
<td>2.42 ± 0.66(^a)</td>
</tr>
</tbody>
</table>

\(^a\) Symbols are defined under Table 1 and main text. \(^b\) Values are means of three replicates±SE. Means followed by the similar letters in each column are not statistically different (P ≤ 0.05) as compared by LSD test.
tolerance was observed in Amphisis from November to December, by 6.16°C on average, compared with other cultivars. There have been previous reports of analogous patterns of cold tolerance in different olive cultivars (Barranco et al., 2005; Eris et al., 2007). Cold tolerance rises were recorded during cold acclimation in olive (Cansev et al., 2009; Hashempour et al., 2014a), along with similar reports in a large number of plant species such as grapevine (Ershadi et al., 2016) and pomegranate (Ghasemi Soloklui et al., 2012).

To preserve homeostasis and avoid oxidative stress and damage by ROS, plants have developed a complicated defence mechanism of non-enzymatic scavengers and a series of antioxidant enzymes (Figure 1) (Ahmad et al., 2010). Tolerance towards adverse environmental conditions is correlated with an increased capacity to scavenge or detoxify ROS. Indeed, previous studies showed enhanced tolerance to coldness, attainable by the increment of
ROS-scavenging mechanisms in various plants (Eris, et al., 2007; Luo et al., 2007).

As a fundamental scavenger of H$_2$O$_2$ in the plant cell, APX plays a key role in the AsA-GSH pathway. APX converts hydrogen peroxide to water using ascorbate as the reductant (Sharma et al., 2012). With the progression of cold stress, APX activity increased in our study reaching its maximum in cold conditions in January, and then gradually decreasing during the de-acclimation stage in April (Figure 3-B). The results also showed a significant negative correlation between the LT$_{50}$ and APX under cold acclimation and de-acclimation stages. This finding is in agreement with the results of Luo et al. (2007) who reported that increasing APX enzyme activity improved cold tolerance in poplar tree.

Mostly located in peroxisomes and glyoxysomes, CAT enzyme converts H$_2$O$_2$ into water and oxygen (Racchi, 2013). CAT activity was substantially enhanced in all the olive cultivars throughout cold acclimation, implying a more highly efficient H$_2$O$_2$ scavenging that may lead to more effective preservation against peroxidation (Cansev et al., 2009). CAT activity was found to be the highest in the cold-hardy cultivar Amphisis during cold acclimation, whereas the lowest activity was detected in the cold-sensitive cultivar Manzanilla cultivar (Figure 3-C). A negative correlation was found between LT$_{50}$ and CAT activity in all sampling stages. This finding showed that CAT plays an important role in protecting olive plants against freezing stress. These results fully agree with the reports of CAT enzyme activity in cold acclimation of olive (Cansev et al., 2009; Hashempour et al., 2014a), wheat leaves (Baek and Skinner, 2003) and populus (Luo et al., 2007).

The POD is another antioxidant enzyme that causes the conversion of H$_2$O$_2$ into O$_2$ and H$_2$O (Sudhakar et al., 2001). There was a close relationship between POD activity and cold hardiness of olive leaves in November, December, January and April; however, this correlation was relatively weaker in November and non-significant in February (Table 3). The higher activity of POD in cold-tolerant cultivars expressed the larger ROS-scavenging capacity, which eventually led to the lower injury in the lipids of the plasma membrane in cold stress conditions (Hashempour et al., 2014a). With a high level of cold tolerance, Amphisis cultivar displayed the highest POD activity during all sampling dates in comparison with other cultivars. Previous studies revealed that POD is associated with cross linking cell-wall components (Passardi et al., 2004) and enhancing cellular tolerance against cold stresses (Turhan et al., 2012).

SOD is a metalloenzyme that scavenges the toxic superoxide radicals and catalyzes the conversion of two superoxide anions into O$_2$ and H$_2$O (Seppanen and Fagerstedt, 2000). SOD activity was revealed to display considerable increase in olive cultivars throughout cold acclimation, with a decline in de-acclimation stage (Figure 4-A). The results also showed a significant negative correlation between the LT$_{50}$ and SOD. These findings are acknowledged by recent studies which have reported that SOD activity precisely corresponds with freezing tolerance (Seppanen and Fagerstedt, 2000; Hashempour et al., 2014a).

The modification of cell membranes permeability, induced by dehydration throughout the generation of extracellular ice, can be avoided by the help of an accumulation of soluble and specific proteins (Guy, 1990). In this work, TSP content gradually increased till January; and thereafter markedly decreased until April. However, a similar rate was not reported in olive cultivars in cold acclimation and de-acclimation stages. The increase in TSP content in January may be associated with synthesis of soluble and specific proteins, such as antifreeze proteins and expression of specific genes (Guy, 1990). In addition, the drop in TSP content is probably because of the widespread damage inflicted on protein synthesizing system or synthesis and/or activating considerable quantities of proteolytic enzymes as protease (Krishna et al., 2000). A negative correlation was found
between LT_{50} and TSP content in January and February stages. Similar to this study, Cansev et al. (2009) found that the larger overall protein content was related to the higher cold tolerance of the olive cultivars.

The scavenging system malfunction results in membrane lipid peroxidation, which can damage the main cellular components (Zhao et al., 2014). The MDA content is mostly used as an indicator of lipid peroxidation (Karabal et al., 2003; Gao et al., 2015). MDA showed an enhancement during cold acclimation from November to December, then, began to decline in January, reaching its lowest content in April. The results of this study were consistent with those achieved by Hashempour et al. (2014b), studying olive cultivars, and by Khazaei et al. (2015), investigating chickpea plants, indicating that an increase in MDA content was closely correlated with a decrease in temperature. Of the other cultivars, the highest MDA content was detected in the cold sensitive cultivar Manzanilla, corroborating the fact that freezing stress might inflict damage on the cellular membranes integrity and other cellular components, including lipids.

Proline content in higher plants is ascribed to intense abiotic stress like chilling stress (Hayat et al., 2012). Proline properly preserves key cellular macromolecules, notably, the lipid membranes and proteins such as enzymes (Verbruggen and Hermans, 2008). The highest and lowest proline accumulation was found here in the cold-tolerant cultivar Amphisis and cold-sensitive cultivar Manzanilla, respectively. In addition, correlation coefficient between LT_{50} and proline content during cold acclimation was statistically significant. Likewise, a positive correlation between the proline concentration and improved cold hardiness was found to exist in grapevine (Ershadi et al., 2016).

**CONCLUSIONS**

The results reported in this study provided further evidence regarding the important role of antioxidant enzyme activities, TSP, and proline content in cold acclimation or cold tolerance of olive leaf tissues. The activities of APX, CAT, POD, and SOD enzymes, followed by TSP and proline accumulation in leaf tissues had a negative correlation with LT_{50}. The highest production of antioxidants, and TSP, and the lowest MDA content in the leaf tissues were observed in Amphisis, the cold-hardy olive cultivar. Therefore, the considerable activity of APX, CAT, POD, and SOD enzymes, TSP, and proline content could be adopted as significant selection criteria in screening cold tolerant olive cultivars in cold climate regions.

**REFERENCES**


تحول به سرماهای برگ سه رقم زینوت در رابطه با برخی صفات فیزیولوژیکی در طی دوره سازگاری و عدم سازگاری به سرما

چکیده

در این پژوهش به بررسی تغییرات فعالیت آنزیم‌های آنتی اکسیدان، پروتئین‌های محلول کل (TSP)، مالون دی آلدید (MDA)، و میزان پرولین در برگ سه رقم زینوت آمیتیس، گرگان و مازندران در پنج زمان مختلف برداشت و همبستگی بین این صفات فیزیولوژیک با تحميل به سرما بررسی شد. نتایج نشان داد که سازگاری به سرما به طور چشمگیری باعث افزایش تحميل به سرما شد. علوا بر این، فعالیت آنزیم‌های آنتی اکسیدان، MDA، TSP و محتوای پرولین در طی مرحله سازگاری به سرما افزایش و در مرحله عدم سازگاری به سرما کاهش یافت. فعالیت آنزیم‌های آنتی اکسیدان آسکاربیت پراکسیداز، کاتالاز، پراکسیداز و سویا آکسید دیسمونتال در بافت‌های برگ با میزان تحميل به سرما همبستگی داشت. مقادیر بالای پروتئین‌های محلول، فعالیت آنزیم‌های آنتی اکسیدان و پرولین همراه با مقادیر کم MDA در رقم متحمل به سرماهای آمیتیس موجب بهبود نسبی ظرفیت تحميل به سرما در این رقم شد. نتایج مانند نشان داد که فعالیت آنزیم‌های آنتی اکسیدان، TSP و پرولین شاخص مناسب برای غرببلگری ارقام زینوت از نظر تحميل به سرما می‌باشد.