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

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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, stomal 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 H_2O 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).

1.

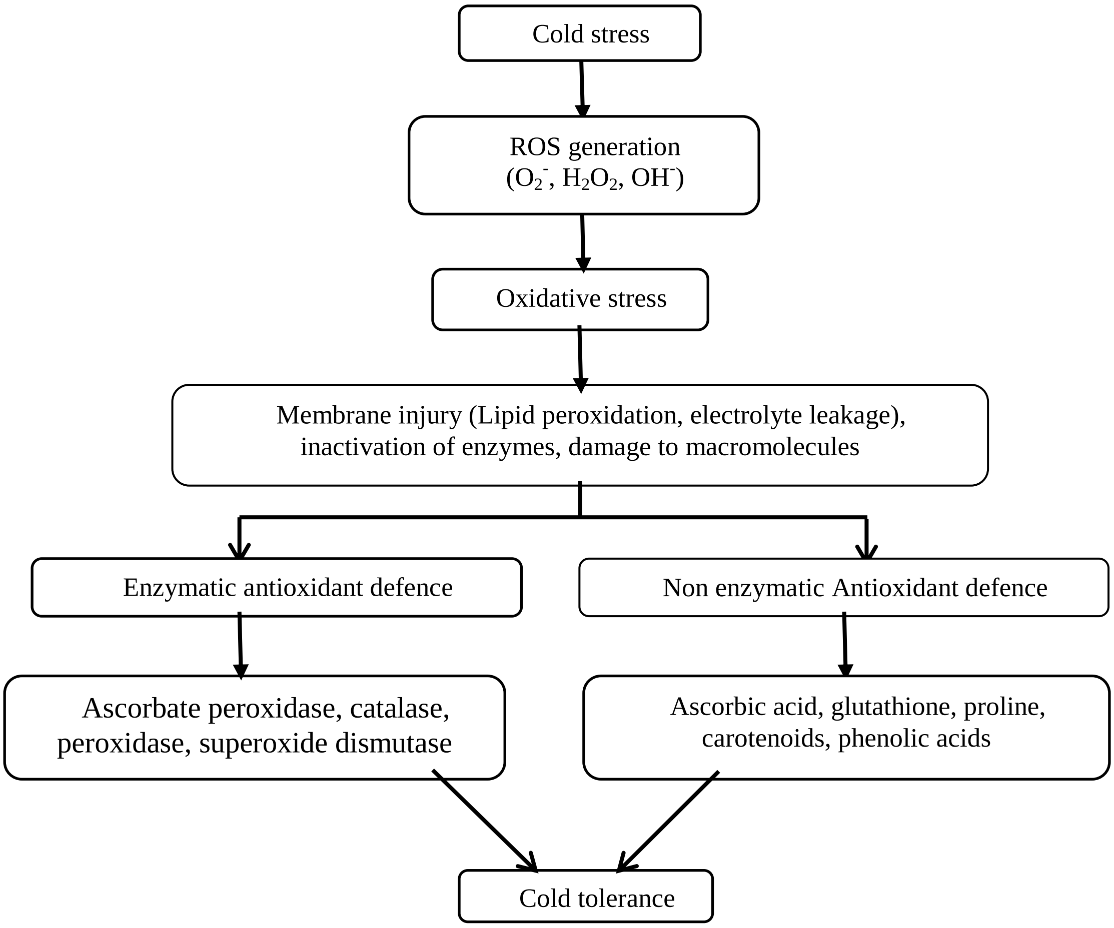

Figure 1. A schematic representation of cold stress consequences and antioxidant defence system of cold stress response in plants.

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).

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 (LT_{50}) 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 5×6 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

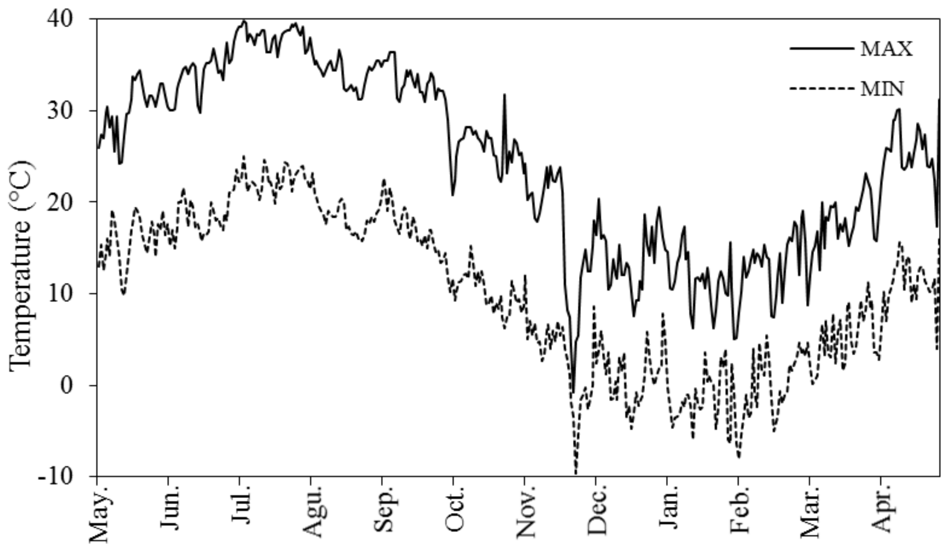

Figure 2. Daily minimum and maximum air temperatures recorded in Isfahan region (May 2016–April 2017).

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 LT_{50} 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 (EC_1). 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 (EC_2). Finally, the electrolyte leakage was measured as EC_1/EC_2 and presented as a percentage. Cold tolerance was calculated and expressed as LT_{50} (which is a Lethal Temperature at which 50% of the total ion leakage occurs). LT_{50} was calculated by fitting response curves using the following logistic sigmoid function:

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

Where, R represents Relative electrolyte leakage percentage, based on LT_{50} 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 (LT_{50}) 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 Instituted, Cary, NC, USA). Means comparisons were carried out adopting LSD test at $P \leq 0.05$. 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

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

Source of variance	F values								
	df	LT_{50}	APX	CAT	POD	SOD	TSP	MDA	PC
S	4	70.66^{**}	34.04^{**}	170.14^{**}	93.97^{**}	60.79^{**}	21.33^{**}	21.82^{**}	46.79^{**}
Cv	2	20.89^{**}	23.34^{**}	7.06^{**}	8.79^{**}	10.13^{**}	7.89^{**}	68.86^{**}	29.83^{**}
S×Cv	8	6.01^{**}	2.51^{*}	6.19^{**}	1.55^{ns}	0.33^{ns}	1.09^{ns}	0.83^{ns}	1.33^{ns}
Error	24	-	-	-	-	-	-	-	-

[a] 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 $P \leq 0.05$ and $P \leq 0.01$, respectively.

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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 (-15.11ºC), whereas, the least hardy cultivar, Manzanilla, reached 50% mortality at -7.55ºC (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 hardiness 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

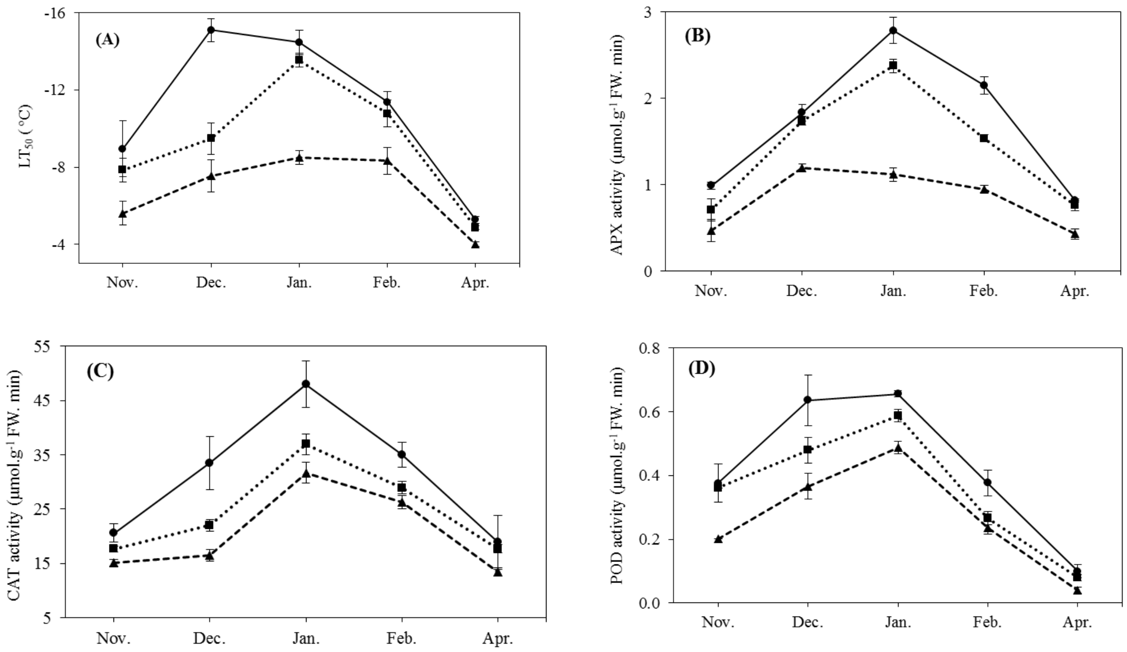

Figure 3. Changes in LT_{50} (A), APX activity (B), CAT activity (C) and POD activity (D) in olive leaves of three cultivars of Amphisis (●), Gorgan (■), and Manzanilla (▲) at five dates of November, December, January, February, and April. Values are means of three replicates±SE.

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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 Novuary 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

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Table 2. Mean comparisons of the sampling dates and cultivars on cold tolerance (LT_{50}) and some physiological parameters.[a]

	LT_{50} (ºC)	APX (µmol g^{-1} FW min)	CAT (µmol g^{-1} FW min)	POD (µmol g^{-1} FW min)	SOD (µmol g^{-1} FW)	TSP (mg g^{-1} FW)	MDA (nmol g^{-1} FW)	PC (µmol g^{-1} FW)
Sampling dates								
November	-7.47 ± 1.31^{c}	0.72 ± 0.27^{c}	17.78 ± 4.08^{d}	0.31 ± 0.07^{b}	7.91 ± 0.83^{b}	4.21 ± 0.68^{c}	10.66 ± 1.29^{c}	2.98 ± 0.78^{b}
December	-10.71 ± 0.81^{b}	1.58 ± 0.20^{b}	24.04 ± 3.06^{c}	0.49 ± 0.05^{a}	9.96 ± 0.62^{a}	4.70 ± 0.44^{c}	20.02 ± 1.68^{a}	5.87 ± 0.35^{a}
January	-12.18 ± 0.66^{a}	2.09 ± 0.11^{a}	38.89 ± 2.41^{a}	0.58 ± 0.06^{a}	6.67 ± 0.82^{c}	7.97 ± 0.43^{a}	18.14 ± 1.71^{a}	6.44 ± 0.44^{a}
February	-10.16 ± 1.22^{b}	1.54 ± 0.28^{b}	30.08 ± 2.84^{b}	0.30 ± 0.08^{b}	5.35 ± 0.88^{d}	5.72 ± 0.61^{b}	14.86 ± 1.50^{b}	3.16 ± 0.68^{b}
April	-4.71 ± 1.33^{d}	0.66 ± 0.27^{c}	16.68 ± 2.86^{d}	0.07 ± 0.07^{c}	3.85 ± 0.38^{e}	4.33 ± 0.63^{c}	12.64 ± 1.25^{bc}	2.42 ± 0.66^{b}
Cultivars								
Amphisis	-11.03 ± 1.02^{a}	1.72 ± 0.21^{a}	31.20 ± 3.16^{a}	0.43 ± 0.06^{a}	7.37 ± 0.64^{a}	6.11 ± 0.52^{a}	12.81 ± 1.00^{c}	5.25 ± 0.60^{a}
Gorgan	-9.31 ± 0.81^{b}	1.42 ± 0.19^{b}	24.66 ± 2.00^{b}	0.36 ± 0.05^{ab}	6.75 ± 0.57^{ab}	5.23 ± 0.45^{b}	15.08 ± 1.19^{b}	4.06 ± 0.47^{b}
Manzanilla	-6.80 ± 0.51^{c}	0.83 ± 0.09^{c}	20.62 ± 1.95^{c}	0.27 ± 0.04^{b}	6.13 ± 0.58^{b}	4.82 ± 0.37^{b}	17.89 ± 1.05^{a}	3.20 ± 0.35^{b}

[a] Symbols are defined under Table 1 and main text. [a-c] Values are means of three replicates±SE. Means followed by the similar letters in each column are not statistically different ($P \leq 0.05$) as compared by LSD test.

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 Nov. from 2.48 to 3.80 µmol g^{-1} FW and April from 1.81 to 2.93 µmol g^{-1} FW (Figure 4-D). 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 4-D). Proline content noticeably decreased in April, whereas no difference was monitored among cultivars on this date (Figure 4-D). 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 is low temperature (Macek *et al*., 2012). Prolonged exposure to cold stress exerted particularly common effects on higher plants due to ROS accumulation. 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 hardiness of the 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 an earlier development of cold tolerance than other cultivars. In addition, the highest enhancement of cold

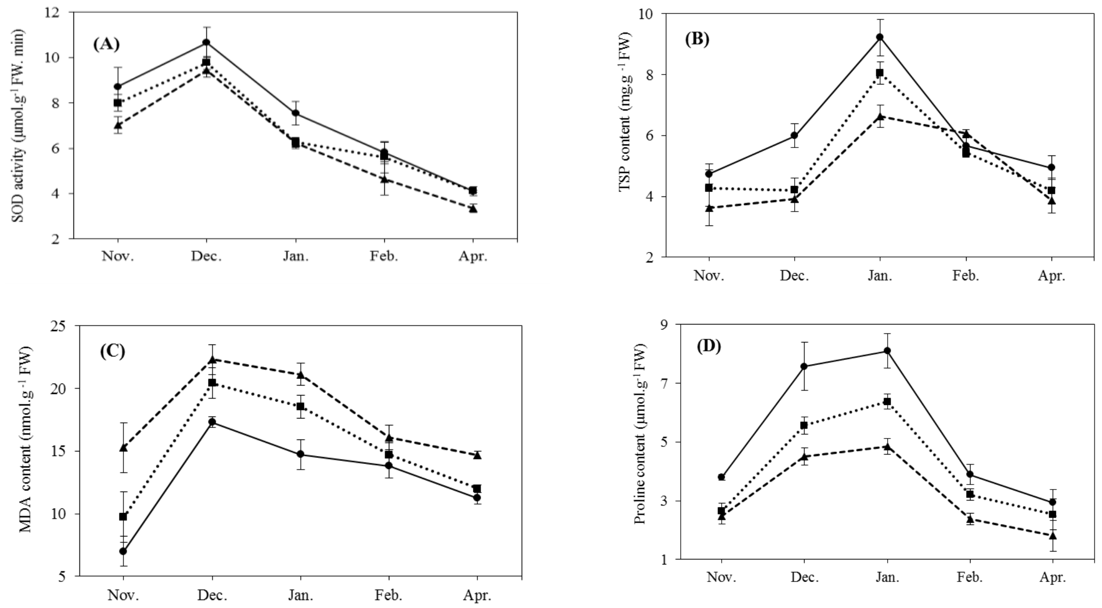

Figure 4. Changes in SOD activity (A), TSP content (B), MDA content (C) and proline content (D) in olive leaves of three cultivars of Amphisis (●), Gorgan (■), and Manzanilla (▲)at five dates of November, December, January, February, and April. Values are means of three replicates±SE.

Table 3. Pearson correlation coefficients between LT_{50} and some physiological parameters in leaf of three olive cultivars at five dates of November, December, January, February, and April.

Variables[a]	November	December	January	February	April
APX	-0.63*	-0.70*	-0.72*	-0.67*	-0.78*
POD	-0.66*	-0.80**	-0.90**	ns	-0.96**
SOD	ns	-0.66*	ns	-0.66*	-0.82**
CAT	-0.65*	-0.83**	-0.73*	-0.73*	-0.71*
TSP	ns	ns	-0.57*	ns	-0.56*
MDA	ns	0.74**	ns	ns	0.83**
PC	ns	-0.87**	-0.76*	-0.74*	ns

[a] Symbols are defined under Table 1 and main text.$^{ns,\ *,\ and\ **}$: Non significant, significant at P≤ 0.05 and P≤ 0.01, respectively.

tolerance was observed in Amphisis from November to December, by 6.16ºC 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

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ROS-scavenging mechanisms in various plants (Eris, *et al*., 2007; Luo *et al*., 2007).

As a fundamental scavenger of H_2O_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_2O_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_2O_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_2O_2 into O_2 and H_2O (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_2O_2 (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.

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تحمل به سرمای برگ سه رقم زیتون در رابطه با برخی صفات فیزیولوژیکی در طی دوره سازگاری و عدم سازگاری به سرما

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چکیده

در این پژوهش به بررسی تغییرات فعالیت آنزیم‌های آنتی اکسیدان، پروتئین‌های محلول کل (TSP)، مالون دی آلدئید(MDA) ، و میزان پرولین در برگ سه رقم زیتون آمفی سیس، گرگان و مانزانیلا در پنج زمان مختلف پرداخته و همبستگی بین این صفات فیزیولوژیک با تحمل به سرما بررسی شد. نتایج نشان داد که سازگاری به سرما به طور چشمگیری باعث افزایش تحمل سرما شد. علاوه بر این، فعالیت آنزیم‌های آنتی اکسیدان، MDA، TSPو محتوای پرولین در طی مرحله سازگاری به سرما افزایش و در مرحله عدم سازگاری به سرما کاهش یافت. فعالیت آنزیم¬های آنتی اکسیدان آسکوربات پراکسیداز، کاتالاز، پراکسیداز و سوپر اکسید دیسموتاز در بافت‌های برگ با میزان تحمل به سرما همبستگی داشت. مقادیر بالای پروتئین¬های محلول، فعالیت آنزیم¬های آنتی اکسیدان و پرولین همراه با مقادیر کم MDA در رقم متحمل به سرمای آمفی سیس موجب بهبود نسبی ظرفیت تحمل به سرما در این رقم شد. نتایج ما نشان داد که فعالیت آنزیم‌های آنتی اکسیدان، TSP و پرولین شاخص مناسبی برای غربالگری ارقام زیتون از نظر تحمل به سرما می‌باشند.

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