Agricultural Journal 6 (6): 378-391, 2011
ISSN: 1816-9155

Possible Involvement of Proline and the Antioxidant Defense Systems in the Drought Tolerance of Three Olive Cultivars Grown under Increasing Water Deficit Regimes

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Abstract: The possible involvement organic metabolites and the antioxidative defence system in water drought tolerance were investigated in three olive cultivars. About 2 years old olive trees, grown in pots in greenhouse were subjected to increasing Water Deficit (WD) regime (5, 10, 20 and 40 days). After 40 days without irrigation, the biomass production, osmotic potential (Ψ_s) and turgor Potential (P) were decreased in the three olive cultivars and the lower reduction was observed in Zalmati. Under WD conditions, Chemlali and Zalmati showed a greater accumulation of proline associated with the better maintenance of plant water status and biomass production. Different cultivars developed certain antioxidative defense mechanisms including the accumulation of phenolic compounds and increased antioxidative enzymes activities. However, differences were observed between the three cultivars for these enzymatic and non-enzymatic antioxidants. Zalmati leaves accumulated the larger quantities of phenolic compounds followed by Chemlali which can improve their antioxidant responses. Superoxide Dismutase (SOD) activity was more enhanced in Zalmati than in Chemlali under water stress but was decreased in Chetoui. Catalase (CAT) activity was more enhanced in Chetoui under water stress. The activity of Peroxidase (POD) and Ascorbate Peroxidase (APX) increased under water stress and the higher levels were reached in Zalmati followed by Chemlali. In contrast, Polyphenol Oxidases (PPO) activity decreased under WD conditions in Chemlali and Zalmati, however this reduction was slightly in Chetoui. The lower increase in malondialdehyde content and H_2O_2 generation suggest that Zalmati was more able to maintain leaf cell integrity than Chemlali, especially Chetoui under severe water stress because of the more efficient osmoprotection and antioxidant defense systems. Thus, the great accumulation of phenolic compounds, the increased activity of SOD and the higher POD, especially APX activity might be linked to the better performance of the antioxidant defense in Zalmati olive cultivar under severe water deficit.

Key words: Organic metabolites, lipid peroxidation, phenolic compounds, antioxidant enzymes, drought tolerance, *Olea europaea* L.

INTRODUCTION

The evergreen olive tree (*Olea europaea* L.) is one of the most characteristic tree crops from the Mediterranean basin where plants are frequently subjected to prolonged drought periods (Connor and Fereres, 2005). If compared to other fruit tree species, olive tree is able to tolerate the low availability of water in the soil by means of morphological and physiological adaptations acquired in reply to cope with drought stress (Connor and Fereres, 2005; Bacelar *et al*., 2007). One of the first responses to drought stress is a reduction in plant growth. The adaptation mechanisms in olive also lead to changes in leaf water status and carbon assimilation (Wang *et al*., 2003). The maintenance of appropriate plant water status during water deficit is essential for continued growth and this process can be achieved by stomatal regulation (Ben Ahmed *et al*., 2007) and the accumulation of compatible solutes (Sofo *et al*., 2004; Ashraf and Foolad, 2007). The accumulation of proline is one of the most common responses of olive tree to water deficit (Sofo *et al*., 2004) playing a key role in osmotic adjustment by reducing the osmotic potential of plants such olive tree facilitating water flow into their roots and leaves thus maintaining cell turgor (Chartzoulakis *et al*., 1999; Ennajeh *et al*., 2009). Moreover in many studies, it has been suggested that proline can be used as a selection criterion for the tolerance of most species to stressed

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conditions (Parida and Das, 2005; Ashraf and Foolad, 2007). Water stress causes stomatal closure which reduces the CO_2/O_2 ratio in leaves and inhibits photosynthesis (Moussa, 2006) witch led to increasing the rate of Reactive Oxygen Species (ROS) like superoxide radical ($O_2^{-}\downarrow$) hydrogen peroxide (H_2O_2), hydroxyl radical (OH^{-}) and singlet oxygen ($^1O_2$) particularly in chloroplast and mitochondria (Mittler, 2002; Moller *et al*., 2007), via enhanced leakage of electrons to oxygen. These ROS are highly reactive and can alter the normal cellular metabolism through oxidative damage to proteins and nucleic acids as well as causing peroxidation of membrane lipids (Moller *et al*., 2007). Several parameters can be used to assess the extent of oxidative damage. Malondialdehyde (MDA), a product of lipid peroxidation and the H_2O_2 generation have been considered as biochemical indicator of oxidative damage and tend to show greater accumulation under water stress (Sofo *et al*., 2004; Bacelar *et al*., 2006).

In order to prevent oxidative damages, plants have evolved a complex antioxidant system which includes both enzymatic and non-enzymatic components differentially found in cell compartments (Mittler, 2002; Moller *et al*., 2007), responsible for maintaining the levels of ROS under tight control. Non-enzymatic antioxidants such as polyphenols, ascorbic acid, glutathione and carotenoids are employed by plants to eliminate ROS. Plant phenolic compounds are secondary metabolites their beneficial effects are related to their antioxidant activity, particularly their ability to scavenge free radicals to donate hydrogen atoms or electrons or to chelate metal cations (Ksouri *et al*., 2008). The antioxidative enzymes are the most important components in the scavenging system of reactive oxygen species (Dat *et al*., 2000). Thus, theses enzymes such as Superoxide Dismutase (SOD), Catalase (CAT), Peroxidase (POD) and Ascorbate Peroxidase (APX) are essential components of the plant's antioxidant defence system. Superoxide Dismutase (SOD) catalyzes the 1st step of the enzymatic defence mechanism, the conversion of superoxide radicals to yield molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). The hydrogen peroxide produced is then scavenged by catalase and a variety of peroxidases. Catalase which is apparently absent in the chloroplast, eliminate H_2O_2 by breaking it down to H_2O and O_2 whereas peroxidases which are located in cytosol, vacuole as well as in extracellular space, decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Meloni *et al*., 2003). APX plays an important role in protecting cells against oxidative damage which convert H_2O_2 to H_2O using ascorbate as an electron donor occur, they are localized in cytosol and various organelles (Madhusudhan *et al*., 2003). The balance between ROS production and activities of antioxidative enzymes determines whether oxidative signalling and/or damage will occur (Moller *et al*., 2007). Moreover, the ability to increase antioxidant capacity has been correlated with drought tolerance in a large number of plants such as *Olea europaea* (Ennajeh *et al*., 2009; Ben Ahmed *et al*., 2009), *Triticum aestivum* (Nikolaeva *et al*., 2010) and *Brassica napus* (Abedi and Pakniyat, 2010). Previous studies on olive tree report on osmolytes compounds and antioxidant system generated by water drought stress conditions (Bacelar *et al*., 2007; Ennajeh *et al*., 2009; Ben Ahmed *et al*., 2009) are little. Moreover, the antioxidant modulation in the tissues of Zalmati cultivar under water stress conditions is unknown. The aim of this study was to test the hypothesis that antioxidant defense systems (enzymatic and non-enzymatic antioxidant) contribute to different genotypic drought tolerance levels in olive cultivars. On the other hand, researchers test the hypothesis of the implication of proline in osmoregulation and its use as an index of drought tolerance in olive trees. For this, researchers have examined the effects of drought on several physiological and biochemical parameters such as water relationship, biomass production, proline content, phenolic compounds, malondialdehyde and H_2O_2 content as well as and the activity of antioxidant enzymes; SOD, CAT, POD, APX and PPO. Another objective of this study was to clarify the response to drought of Zalmati and to compare with that of the famous Tunisians olive cultivars Chetoui and Chemlali in order to better understand their differences on water stress tolerance.

MATERIALS AND METHODS

Plant material and growth conditions: About 2 years old, own-rooted cuttings of olive cultivars Chetoui, Chemlali and Zalmati were transplanted in 10 dm^3 pots filled with a mixture of sandy soil and peat (2:1, v/v) in a glasshouse at the Agronomic Institute of Chott-Meriam (Tunisia, 35 490N, 10 402E). Throughout the experimental period, greenhouse temperature was 21-33°C, relative humidity was 65-85% and Photosynthetic Photon Flux (PPF) was about 1200 μmol m^{-2} sec^{-1}. The plants of the three cultivars were homogeneous and had a height of about 120 cm. All of the pots were covered with plastic film and aluminium foil to reduce evaporation from the soil surface and to minimize solar heating of the pot. Plants were watered weekly to field capacity with Hoagland's solution for 2 months before the experiment. After this period, the three olive cultivars were exposed to different water regimes. Ten plants of each cultivar were used as

controls (well watered) and irrigated every 2 days to maintain soil water content close to field capacity. An additional fifty plants (ten plants for treatment) from the three cultivars were stressed by withholding water during increasing periods (5, 10, 20, 40 days). Soil water potential (Ψsoil) in the pots in the drought treatment was about -2.5 MPa at the end of the experiment. During these drought treatments, growth and water relations, osmolytes and phenolic compounds, MDA conetent, H_2O_2 amount and antioxidant enzymes activities were measured on five plants of each cultivar under withholding water period (control, 5, 10, 20 and 40 days).

Growth activity and plant water status: The Dry Mass (DM) was measured after the Fresh Material (FM) was dried at 70°C for 48 h. For the fresh mass at full turgor (TM), it was determined by immersing the leaf petioles in demineralised water for 48 h in the dark at 4°C. The osmotic potential (Ψ_s) of the sap was measured using a vapour pressure osmometer (model 5500, Wescor). Leaf water potential (Ψ_{LW}) was measured using five fully expanded leaves of similar age and position in the canopy for each treatment. The values of Ψ_{LW} were carried between 8:00 and 10:00 h using a Sholander pressure chamber (Skye Instruments, Powys, UK). Turgor Potential (P) and Leaf relative Water Content (LWC) was calculated in five plants per treatment as follows:

$$P = \Psi_{LW} - \Psi_s$$

$$LWC = (FM_L - DM_L)/(TM_L - DM_L)$$

Where:
DM = The total dry mass
FM_L = Leaf fresh mass
DM_L = Leaf dry mass
TM_L = Leaf fresh mass at full turgor

Determination of proline: Proline was also determined spectrophotometrically following the Ninhydrin method described by Bates *et al*. (1973). Approximately, 300 mg of dry tissue was homogenized in 10 mL of 3% aqueous sulphosalicylic acid and filtered. To 2 mL of the filtrate, 2 mL of acid ninhydrin were added followed by the addition of 2 mL of glacial acetic acid and boiling for 60 min. The mixture was extracted with toluene and the free proline was quantified spectrophotometrically at 520 nm from the organic phase using toluene as a blank.

Total phenolic compounds: The extraction procedure was determined using the method described by Waterman and Mole (1994) with some modifications. Briefly, lyophilised leaf samples (5 g) were extracted twice with 100 mL of 70% methanol thereafter with 100 mL of 70% acetone at the temperature of 4°C. Phenolic content was assayed using the Folin-Ciocalteu reagent following Singleton's method slightly modified (Dewanto *et al*., 2002). An aliquot (0.125 mL) of appropriately diluted sample extract was added to 0.5 mL of distilled water and 0.125 mL of the Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min before adding 1.25 mL of 7% Na_2CO_3 solution. The solution was then diluted with deionized water to a final volume of 3 mL and mixed thoroughly. After incubation for 90 min at 23°C in dark, the absorbance was measured at 765 nm by using a UV-Vis spectrophotometer (UV-2500, Shimadzu Corp., Kyoto, Japan). Total phenolic content of leaves was expressed as mg Gallic Acid Equivalents (GAE) per gram of dry weight (mg GAE/g DM) through the calibration curve with gallic acid.

Hydrogen peroxide (H_2O_2) estimation: The H_2O_2 content was determined as described by Velikova *et al*. (2000). Fresh leaf tissue (0.5 g) was homogenized with 5 mL of 0.1% (w/v) Trichloroacetic Acid (TCA) inapre-chilled pestle and mortar. This homogenate was then centrifuged at 12,000 g for 15 min. To the 0.5 mL of the supernatant 0.5 mL of potassium phosphate buffer (pH 7.0) and 1 mL of potassium iodide were added. The mixture was vortexed and its absorbance was read at 390 nm using a UV-visibles spectrophotometer (UV-2500, Shimadzu Corp., Kyoto, Japan) and the H_2O_2 concentration was calculated according to the standard curve.

Lipid peroxidation: Lipid peroxidation was estimated by determining the Malonyldialdehyde (MDA) content in the leaves according to the method of Cakmak and Horst (1991). Fresh leaf samples (0.5 g) were ground in 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) at 4°C. The homogenate was centrifuged at 12,000×g for 5 min. A 1 mL aliquot of the supernatant was mixed to 4 mL of 0.5% (w/v) Thiobarbituric Acid (TBA) prepared in 20% (w/v) TCA and incubated at 90°C for 30 min. Thereafter, the reaction was stopped in ice bath. Centrifugation of the samples was performed at 10,000×g for 5 min and absorbance of the supernatant was measured at 532 nm on a spectrophotometer (UV-2500, Shimadzu Corp., Kyoto, Japan). After subtracting the non-specific absorbance at 600 nm, the malondialdehyde content was calculated using its absorption coefficient (ε) and expressed as nmol malondialdehyde g^{-1} fresh mass following the equation:

$$MDA\ (nmol\ g^{-1}\ FM) = [(A532 - A600) \times V \times 1000)/\varepsilon] \times W$$

Where:
ε = The specific extinction coefficient (155 mM cm^{-1})

V = The volume of crushing medium
W = The fresh weight of leaf
A600 = The absorbance at 600 nm wavelength
A532 = The absorbance at 532 nm wavelength

Determination of enzymatic activities: For the enzyme assays, 0.3 g leaves were ground with 2 mL ice-cold 25 mM HEPES buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM ascorbate and 2% Polyvinyl Polypyrrolidone (PVPP). The homogenates were centrifuged at 4°C for 20 min at 12,000×g and the resulting supernatants were used for the determination of enzymatic activity and protein content assays (Zhu *et al*., 2000). All steps in the preparation of the enzyme extract were carried out at 4°C. All spectrophotometric analyses were conducted on a Shimadzu UV-2500 spectrophotometer. Each measurement of antioxidant enzymes was made four replicates in four plants. Protein concentration was determined using a Coomassie brilliant blue with bovine serum albumin as the standard (Bradford, 1976).

The Superoxide Dismutase (SOD) activity was estimated by measuring its ability to inhibit the photochemical reduction of Nitroblue Tetrazolium (NBT) (Rao and Sresty, 2000). The reaction mixture (3 mL) contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT nitroblue tetrazolium, 2 μM riboflavin, 0.1 mM EDTA and 0.05 mL of enzyme extract. The reaction was started by adding 2 μM riboflavin and placing the tubes under 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme which gave the maximal color served as control. The reaction was stopped by switching off the light and then the tubes were covered with a black cloth. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm and 1 Unit enzyme activity (U) was defined as the quantity of SOD required to produce a 50% inhibition of reduction of Nitroblue Tetrazolium (NBT) and the specific enzyme activity was expressed as units/mg protein. POD activity was measured by monitoring the increase in absorbance at 470 nm in 50 mM of phosphate buffer (pH 5.5) containing 1 mM of guaiacol, 0.5 mM of H_2O_2 and 0.1 mL of enzyme extract. One unit of POD activity is defined by the increase in absorbance at 470 nm for 1 min due to guaiacol oxidation. Catalase (CAT) activity was estimated according to Chakraborty and Tongden (2005) which measures the initial rate of disappearance of H_2O_2 at 240 nm. The CAT reaction mixture contained 50 mM of Na-phosphate buffer pH 7.0, 15 mM of H_2O_2 and 0.1 mL of enzyme extract. Changes in the absorbance of the reaction solution at 240 nm were recorded after every 20 sec. One unit of CAT activity is defined by the decrease at 240 nm for 1 min due to H_2O_2 consumption. The activity of APX was assayed according to the method described by Nakano and Asada (1981) using ascorbic acid as a substrate.

The oxidation of ascorbate was initiated by H_2O_2 and the decrease at 290 nm was monitored for 1.5 min. One unit of APX was defined as the amount of enzyme required to oxidize 1 mM of ascorbate. The Polyphenol Oxidase activity (PPO) was estimated according to Canal *et al*. (1988) which the absorbance was read at 420 nm. The PPO reaction mixture contained 200 μM NaPi (pH 7.0), 5 mM Na2EDTA, 0.1% (w/v) PVPP, 3 mM dithiothreitol, 15 mM β-mercaptoethanol and 10 mM sodium metabisulfite and 0.1 mL of enzyme extract.

Statistical analysis: The data were subjected to Analysis of Variance (ANOVA) and comparisons between the mean values of treatments were made by the Duncan's multiple range test ($p<0.05$). Statistical analyses were performed using the SPSS statistical package (SPSS 13).

RESULTS

Biomass production and leaf water relations: Shoot Dry Mass (SDM) decreased significantly ($p<0.05$) with increasing water stress in the three olive cultivars and the higher reduction was shown in Chetoui (Table 1). At the end of the experiment, SDM decreased by 49.3, 35.1 and 27% in Chetoui, Chemlali and Zalmati, respectively in comparison to their respective plants under well-watered conditions. Similarly, Root Dry Mass (RDM) decreased significantly with water drought periods in Chetoui and

Table 1: Effects of Water Deficit (WD) periods on shoot and root dry mass and the root/shoot ratio in Chetoui, Chemlali and Zalmati olive cultivars

	Shoot dry mass (g plant^{-1})			Root dry mass (g plant^{-1})			Root/shoot		
WD (days)	Chetoui	Chemlali	Zalmati	Chetoui	Chemlali	Zalmati	Chetoui	Chemlali	Zalmati
Control	162.2±3.2^{a}	158.4±4.2^{a}	157.1±04.5^{a}	59.5±1.9^{a}	55.4±2.1^{a}	53.2±1.5^{a}	0.367±0.02^{b}	0.349±0.01^{a}	0.338±0.01^{a}
5	153.8±3.4^{b}	156.4±3.7^{b}	156.2±4.7^{b}	56.3±1.9^{b}	54.0±2.2^{b}	52.4±1.6^{ab}	0.368±0.02^{b}	0.345±0.01^{a}	0.335±0.01^{a}
10	137.8±2.7^{c}	145.1±3.1^{c}	153.8±5.1^{c}	50.8±1.8^{c}	49.2±1.7^{c}	51.2±1.4^{b}	0.369±0.02^{b}	0.339±0.01^{a}	0.332±0.01^{a}
20	113.8±2.2^{d}	127.4±2.6^{d}	136.1±3.5^{d}	43.1±1.5^{d}	41.4±1.6^{d}	43.4±1.5^{c}	0.377±0.01^{b}	0.325±0.01^{b}	0.319±0.01^{b}
40	82.2±1.7^{e}	102.8±2.3^{e}	114.6±3.1^{e}	33.2±1.3^{e}	33.6±1.7^{e}	36.3±1.2^{d}	0.404±0.02^{a}	0.326±0.01^{b}	0.317±0.01^{b}

Data are means values±SE of 5 measurements; values in each column with the same letter are not significantly different (p = 0.05) as described by Duncan's test

Chemlali cultivars. However for Zalmati cultivar, the significant drop in RDM was observed only at moderate to severe water stress (20-40 days) (Table 1). At severe drought stress (40 days), RDM decreased by 44.2, 39.3 and 31.7% in Chetoui, Chemlali and Zalmati, respectively in comparison to the control. The root-to-shoot ratio was increased at severe water stress in Chetoui, however it decreased at moderate to severe water stress in Chemlali and Zalmati. For both cultivars tested, leaf water relations characteristics (Ψ_s and P) were affected by water deficit treatment. The turgor potential (P) decreased significantly with increasing water stress in both olive cultivars and the higher reduction was shown in Chetoui (Fig. 1a). Thus at severe drought stress, P decreased by 84.7, 63.2 and 48% in Chetoui, Chemlali and Zalmati, respectively in comparison to plants under control conditions. Osmotic potential (Ψ_s) in water stressed plants declined during the stress cycle in both cultivars (Fig. 1b). The cultivar with the highest turgor potential under drought conditions (Zalmati) showed the highest osmotic potential (-4.2 MPa) compared to Chemlali (-5.1 MPa) and Chetoui (-5.9 MPa).

Proline and total phenolic contents: In the beginning of experiment, the contents of proline in leaves of control plants were higher in Chemlali and Zalmati cultivars as compared with Chetoui (Table 2). Under WD treatment, leaf proline contents increased and this raise was more enhanced at severe water stress. After 40 days of water drought, proline accumulation showed large increases, representing 3.4, 6.4 and 6.6 fold the control in Chetoui, Chemlali and Zalmati, respectively indicating that the largest increase in proline content was observed in leaves of Zalmati cultivar. Total Phenolic (TP) content increased in the leaves of Chemlali and Zalmati cultivars when water drought intensified (Table 2). This increase was higher in Zalmati leaves thus at 40 days of water drought, total phenolic accumulation increased by 217.7 and 166.6% in Zalmati and Chemlali, respectively in comparison to their respective plants under well-watered conditions. In contrast, the amount of total phenols in leaves of Chetoui remained unchanged with water availability thus, only a slightly increase at severe water stress (40 days) was occurred (Fig. 2).

Oxidative stress evaluation: The extent of lipid peroxidation upon drought periods of various durations was estimated from MDA content (Fig. 3a). MDA content increased significantly ($p<0.05$) with increasing water stress in Chetoui cultivar. In contrast for Chemlali and Zalmati cultivars the significant raise in MDA content was observed only at moderate to severe water stress (20-40 days). However, this increase was more acute in Chetoui leaves. Thus at the end of the experiment,

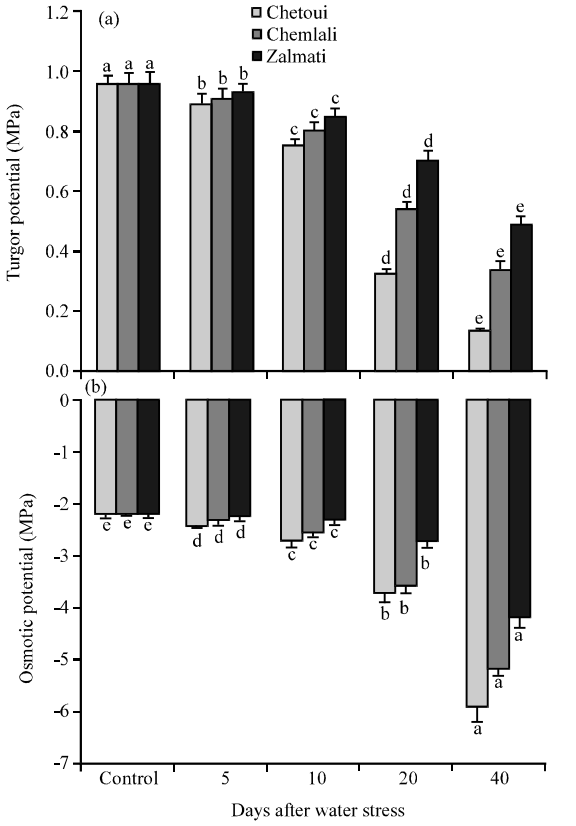

Fig. 1: a) Turgor Potential (P) and (b) osmotic potential (Ψ_s) in Chetoui, Chemlali and Zalmati olive cultivars treated with increasing drought stress. Bars followed by the same letter do not differ statistically at $p<0.05$ (Duncan's multiple range test). Averages of 5 repetitions are presented with bars indicating SE

Table 2: Effects of Water Deficit (WD) periods on proline, total soluble sugars and total phenols accumulation in leaves of Chetoui, Chemlali and Zalmati cultivars

	Proline (μmol g^{-1} DM)			Total soluble sugars (μmol g^{-1} DM)			Total phenols (mg GAE g^{-1} DM)		
WD (days)	Chetoui	Chemlali	Zalmati	Chetoui	Chemlali	Zalmati	Chetoui	Chemlali	Zalmati
0	1.26±0.08^{e}	1.59±0.09^{d}	1.45±0.08^{d}	215.2±4.1^{e}	180.6±5.7^{b}	187.2±4.7^{a}	18.8±0.57^{bc}	21.7±1.09^{d}	20.8±0.73^{e}
5	1.57±0.12^{d}	1.95±0.11^{d}	1.60±0.08^{d}	239.1±4.5^{d}	185.1±6.4^{b}	181.1±3.5^{b}	19.2±0.75^{ab}	22.5±0.78^{cd}	22.7±0.65^{d}
10	2.16±0.21^{c}	4.56±0.40^{c}	3.52±0.30^{c}	261.8±6.9^{b}	181.2±5.5^{b}	189.8±5.2^{a}	18.1±0.74^{c}	23.5±0.83^{c}	25.9±0.94^{c}
20	3.51±0.18^{b}	6.50±0.25^{b}	5.86±0.24^{b}	287.7±7.3^{a}	186.3±4.8^{b}	186.4±4.5^{ab}	18.8±0.68^{bc}	27.8±1.12^{b}	34.8±1.25^{b}
40	4.32±0.24^{a}	10.28±0.64^{a}	9.78±0.58^{a}	246.5±5.7^{c}	212.6±5.6^{a}	186.6±4.2^{ab}	19.7±0.77^{a}	36.1±1.28^{a}	45.2±1.93^{a}

Data are means values±SE of 5 measurements; values in each column with the same letter are not significantly different (p = 0.05) as described by Duncan's test

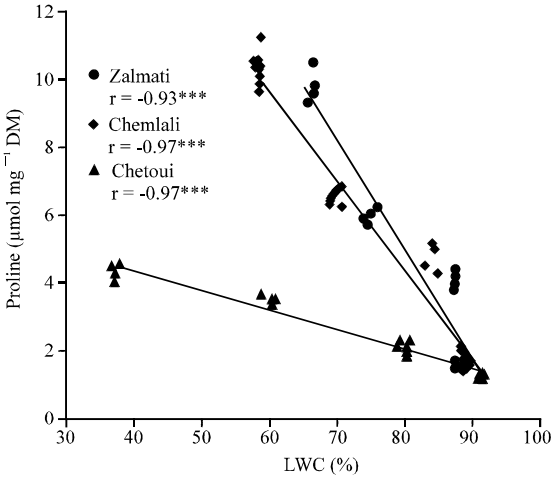

Fig. 2: Correlation between proline accumulation and the relative Leaf Water Content (LWC) contents in Chetoui, Chemlali and Zalmati olive cultivars cultivated under water deficit. An average of 5 repetitions and confidence interval was calculated at the threshold of 95%

MDA accumulation increased by 97.9, 42.4 and 29.9% in Chetoui, Chemlali and Zalmati, respectively in comparison to their respective plants under well-watered conditions. A similar trend was observed in H_2O_2 generation, this parameter increased in all drought treatments in Chetoui cultivar and only at moderate to severe water stress in Chemlali and Zalmati cultivars and this increase was always significantly higher in Chetoui as compared to that in Chemlali and Zalmati (Fig. 3b). In comparison with the control plants, H_2O_2 generation increased by 287.8% in Chetoui, 195.7% in Chemlali and only 161.8% in Zalmati leaves under 40 days of water stress.

Activities of antioxidative enzymes: Significant differences were found in the activity of SOD among cultivars and watering regimes (Fig. 4a). The activity of SOD in Chetoui leaves was unchanged at lower water drought periods (5-10 days) however, it decreased by 7.7 and 17.9% at moderate (20 days) and severe (40 days) drought periods, respectively. In contrast, the activity of SOD in leaves of Chemlali increased significantly in the range of 5-20 days of water drought, reaching a maximal value (176.6% of the control) at 10 days of water stress. At severe water stress, the SOD activity of Chemlali cv. was reduced but it remained significantly higher than that in control. In Zalmati cultivar, the SOD activity was increased significantly when water drought intensified and the highest value (248.7% of the control) was measured at 20 days of water drought. In the beginning

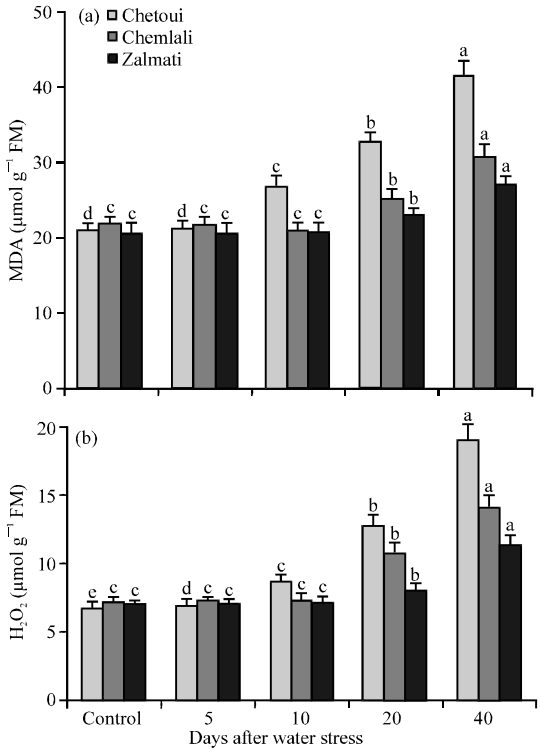

Fig. 3: Effects of water deficit treatments (control 5, 10, 20 and 40 days) on Malondialdehyde (MDA) accumulation (a) and hydrogen peroxide (H_2O_2) contents (b) in Chetoui, Chemlali and Zalmati olive cultivars. Bars followed by the same letter are not statistically different at $p<0.05$ (Duncan's multiple range test). Averages of 5 repetitions are presented with bars indicating SE

of experiment, the activity of POD in leaves of control plants was higher in Chemali and Zalmati cultivars as compared with Chetoui (Fig. 4b). Under WS treatment, leaf POD activity increased with intensifying water drought in Chetoui and Zalmati cultivars and only at severe water stress in Chemlali cultivar. After 40 days of water drought, the highest activity of POD was recorded Zalmati (17.6 U mg^{-1} protein) and Chemlali cultivars (14.1 U mg^{-1} protein) as compared with Chetoui. Initial CAT activity was similar in the leaves of both cultivars (Fig. 4c). The activity of CAT increased with water drought periods in Chetoui leaves and only at severe water stress (40 days) in Chemlali and Zalmati cultivars.

The highest activity of CAT was observed in Chetoui leaves, thus in leaves subjected to 40 days of water stress, the activity of POD reached 291.7% of control in Chetoui and only 185.3-158% of the control in Chemlali and Zalmati cultivars, respectively. Under the no drought condition, the highest APX activity was registered in Zalmati cultivar (Fig. 4d). In the both cultivars, the activity

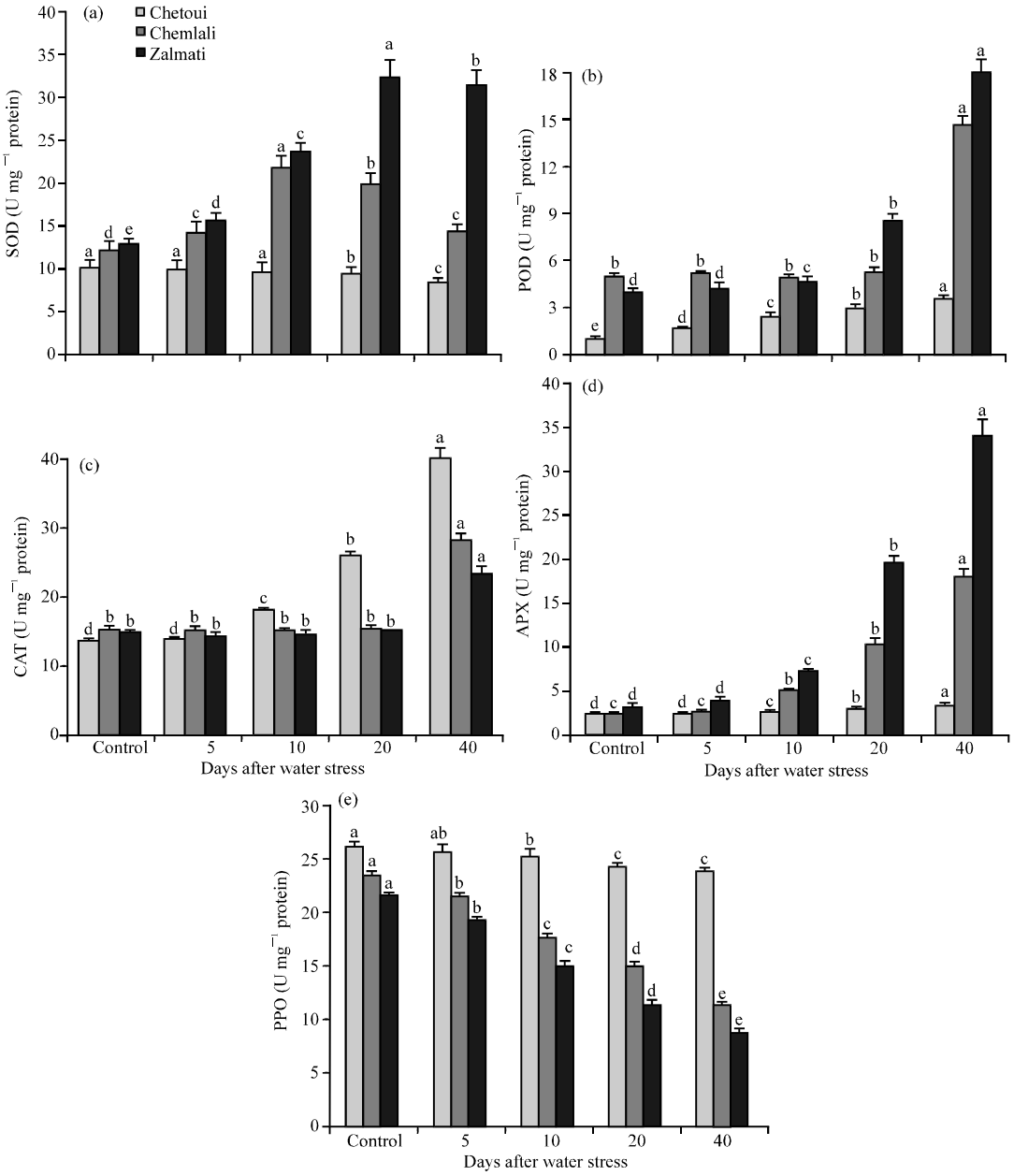

Fig. 4: Effects of water deficit treatments (control, 5, 10, 20 and 40 days) on the contents of: a) superoxide dismutase (SOD); b) peroxidase (POD); c) catalase (CAT); d) ascorbate peroxidase (APX) and e) Polyphenol Oxidases (PPO) in the leaves of Chetoui, Chemlali and Zalmati olive cultivars; bars followed by the same letter are not statistically different at $p<0.05$ (Duncan's multiple range test). Averages of 5 repetitions are presented with bars indicating SE

of APX was significantly increased in the range of drought periods between 5 and 40 days and the higher increase was recorded Zalmati followed by Chemlali. For example in leaves subjected to severe water stress (40 days), the activity of APX reached 10.2 and 7.5 fold of the control in Zalmati and Chemlali, respectively and only 1.3 fold of the control in Chetoui cultivar. The activity of PPO decreased significantly with increasing water stress in Chemlali and Zalmati cultivars (Fig. 4e). At the end of the experiment, PPO decreased by 51.1 and 59.3% in Chemlali and Zalmati, respectively in comparison to plants under control conditions. However, only a slightly reduction (9.1%) in the activity of PPO was observed at moderate to severe water stress in Chetoui cultivar.

Table 3: Correlations coefficients (r) of various physiological and biochemical parameters with shoot biomass accumulation (SDM) and Malondialdehyde (MDA) content in the leaves of Chetoui, Chemlali and Zalmati cultivars

	Chetoui		Chemlali		Zalmati	
Parameters	SDM	MDA	SDM	MDA	SDM	MDA
SDM	1.00	-0.98***	1.00	-0.96***	1.00	-0.98***
Ψ_s	0.97***	-0.97***	0.98***	-0.94***	0.97***	-0.96***
P	0.98***	-0.98***	0.99***	-0.93***	0.97***	-0.96***
MDA	-0.99***	1.00	-0.94***	1.00	-0.97***	1.00
H_2O_2	-0.98***	0.97***	-0.97***	0.96***	-0.96***	0.96***
Pro	-0.97***	0.96***	-0.98***	0.90***	-0.94***	0.93***
TSS	-0.49*	0.47*	-0.82**	0.82**	0.20^{NS}	0.10^{NS}
TP	0.31^{NS}	0.29^{NS}	-0.97***	0.95***	-0.96***	0.94***
SOD	0.89***	-0.88***	0.14^{NS}	0.12^{NS}	-0.78**	0.70**
POD	-0.95***	0.93***	-0.85***	0.91***	-0.98***	0.98***
CAT	-0.98***	0.97***	-0.84***	0.90***	-0.88***	0.92***
APX	-0.95***	0.94***	-0.99***	0.95***	-0.99***	0.96***
PPO	0.81**	-0.82**	0.96***	-0.85***	0.98***	-0.78**

Shoot Dry Mass (SDM), Osmotic potential (Ψ_s), turgor Potential (P), Malondialdehyde content (MDA), Hydrogen peroxide generation (H_2O_2), Proline content (Pro), Total Soluble Sugars content (TSS), Total Phenolic compounds (TP), Superoxide Dismutase activity (SOD), Peroxidase activity (POD), Catalase Activity (CAT), Ascorbate Peroxidise activity (APX) and Polyphenol Oxidase activity (PPO). The probabilities are shown as *p<0.05, **p<0.01, ***p<0.001 and Not Significant (NS)

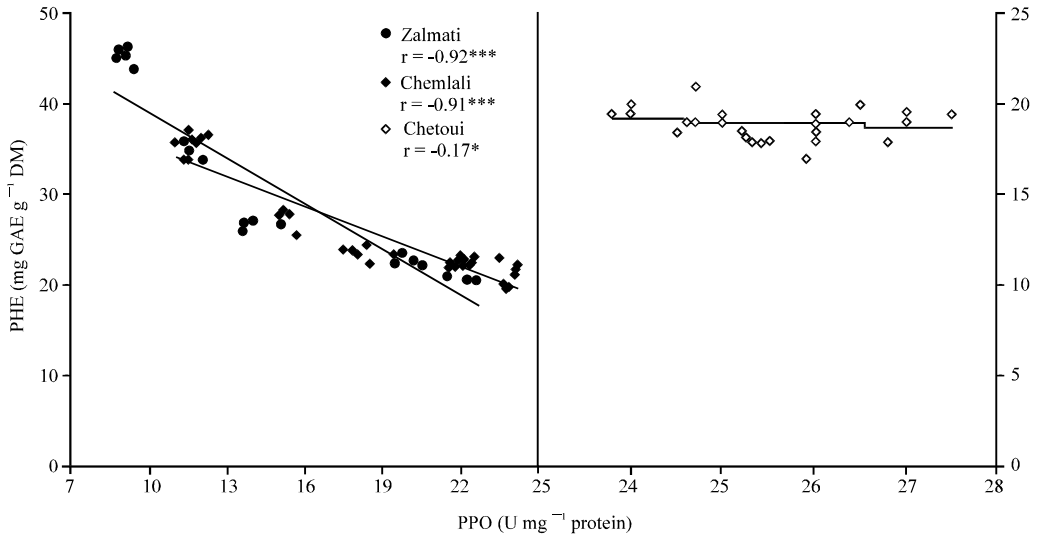

Fig. 5: Correlation between phenolic compounds accumulation (PHE) and the activity of Polyphenol oxidases (PPO) in Chetoui, Chemlali and Zalmati olive cultivars cultivated under water deficit. An average of 5 repetitions and confidence interval was calculated at the threshold of 95%.

Correlation analysis: In both cultivars, the Shoot Dry Mass (SDM) was positively correlated with osmotic potential (Ψ_s), turgor potential (P) and the PPO activity but was negatively associated with MDA, H_2O_2, proline and the majority of antioxidant enzymes activities (POD, CAT and APX) (Table 3). The correlation between SDM and total phenolic compounds was negative in Chemlali and Zalmati whereas this correlation was non significant in Chetoui.

The correlation between SDM and SOD activity was positive in Chetoui and negative in Zalmati whereas, this correlation was non significant in Chemlali. Conversely, in both cultivars, MDA content was negatively correlated with osmotic potential (Ψ_s), turgor Potential (P) and the PPO activity but was positively associated with H_2O_2, proline and the antioxidant enzymes activities (POD, CAT and APX) (Table 3). The relationship between MDA and total phenolic compounds was positive in Chemlali and Zalmati whereas, this correlation was non significant in Chetoui. The relationship between MDA content and SOD activity was negative in Chetoui and positive in Zalmati whereas, this correlation was non significant in Chemlali.

On the other hand, a highly negative relationship was observed between proline accumulation and leaf water content in both olive cultivars (Fig. 3). A highly negative correlation was observed between phenolic compounds accumulation and PPO activity in the leaves of Chemlali and Zalmati however this correlation was minor in Chetoui (Fig. 5).

DISCUSSION

Drought through its osmotic effect induces growth inhibition as shown by the reduced biomass production in the shoots and roots of the three olive cultivars (Table 1). Generally when water is not restricting growth, plants invest a considerable fraction of photoassimilates in the expansion of photosynthetic tissues, maximizing light interception and as a consequence, growth (Dale, 1988). In addition, the maintenance of growth rate during drought can be considered a general measure of drought tolerance (Tschaplinski *et al*., 2006). In this fact, the shoot dry mass decreased relatively slightly for Zalmati as compared to Chemlali and Chetoui in severe drought stress suggesting a better maintenance of biomass production under water deficit stress in this cultivar. On the other hand, genetic variability has been found for the dry matter partitioning thus, researchers noted an increase in root-to shoot ratio in Chetoui in agreement with the findings of Aydi *et al*. (2008) on *Phaseolus vulgaris* plants witch seems to be associated with decreased drought stress tolerance in this cultivar since this parameter was increased in Chemlali and Zalmati (Table 1). It is possible that under osmotic stress the plant spends more photosynthetic energy on root production in search of water and/or reducing water loss (Kafkafi, 1991). The olive trees water status has been put in evidence by osmotic potential (Ψ_s) and turgor Potential (P) showing a close positive correlation with shoot biomass production and negative relationship with lipid peroxidation (Table 3).

Turgor potential (Fig. 2) and leaf water content (Fig. 3) decreases early in Chetoui and Chemlali cultivars while for Zalmati, the effect of the drought stress appeared during the moderate and severe stress. In addition, the comparison of the leaf water status between the three cultivars showed that osmotic potential of Chetoui (-5.9 MPa) decreased more under water deficit than did Chemlali and Zalmati. Previous researches also reported that drought-tolerant cultivars exhibited higher LWC compared to drought-sensitive cultivars in several species including olive (Boussadia *et al*., 2008; Ennajeh *et al*., 2009) and wheat (Nikolaeva *et al*., 2010). It is well known that excessive accumulation of Reactive Oxygen Species (ROS) in plants is one of the major damage induced by water drought stress which induces the lipid peroxidation of biomembranes. The product of lipid peroxidation (content of MDA) and the generation of hydrogen peroxide have been considered as indicators of oxidative damage (Meloni *et al*., 2003). In the present study, the H_2O_2 accumulation resulted in a marked increase in MDA content. The high positive correlation between H_2O_2 generation and MDA amount (Table 3), confirmed the hypothesis that H_2O_2 brings about lipid peroxidation leading to membrane damages (Hichem *et al*., 2009). Furthermore, a negative correlation was observed between Shoot Dry Mass (SDM) and MDA contents in the three cultivars (Table 3) indicating that higher lipid peroxidation resulted in reduced biomass production. This increase in lipid peroxidation may be due to the incapability of antioxidants to scavenger reactive oxygen species results from water deficit stress. On the other hand, the research showed that Zalamti had the lower values of MDA and H_2O_2 indicating that at cellular level this cultivar is better equipped with efficient antioxidative defense system that offers more protection against oxidative damage. This confirms that Zalmati is the more drought-tolerant followed by Chemlali. Similar results were observed on olive (Sofo *et al*., 2004; Bacelar *et al*., 2006) and other plants such as common bean (Turkan *et al*., 2004) and Wheat (Nikolaeva *et al*., 2010) who indicated that drought-tolerant cultivars or genotypes showed a the lower lipid peroxidation level than non-tolerant ones.

The accumulation of proline protects the cell under stress by balancing the osmotic strength of the cytosol with that of the vacuole and the external environment (Gadallah, 1999). In the present study when the intensity of drought stress increased the levels of proline significantly increased in both cultivars which could be linked to their ability to perform tissue osmotic adjustment to lower the osmotic potential and protect plants from damages of dehydration. The higher accumulation of proline in Chemlali and Zalmati cultivars than in Chetoui is in accordance with previous data (Jaleel *et al*., 2007; Ben Ahmed *et al*., 2009) which suggests to be associated with drought tolerance. A direct consequence of the higher proline accumulation and other eventual osmolytes in Chemlali and Zalmati cultivars is the relatively maintenance of the turgor potential and the relative water content in the leaves (Fig. 3) witch caused a lower lipid peroxidation and H_2O_2 generation. Nevertheless, the close correlation between shoot dry mass and proline content (Table 3) on the one hand and between proline content and MDA content on the other hand reinforces the involvement of proline accumulation in drought tolerance mechanisms. The great reduction of turgor potential and LWC at severe water drought stress detected in Chetoui show that the contribution of proline was not sufficient to provide osmotic adjustment and to maintain water balance of the plants under these conditions. Besides their role in osmotic adjustment these solute may act to protect specific macromolecules such as enzymes and to stabilize

membrane structures (Bartels and Sunkar, 2005). In addition, proline may be act as radical scavenger and protects cells against detoxification under drought conditions (Kuznetsov *et al*., 1999).

The excessive accumulation of Reactive Oxygen Species (ROS) in plants is one of the major damage induced by water drought stress. Multiple antioxidant systems and substrates are believed to overcome the catastrophic effect of induced oxidative stress by scavenging the ROS and thus protect cells from this damage (Singh and Rajini, 2004). The phenolic compounds produced through the phenyl propanoid pathway has been considered pertinent in oxidative stress tolerance (Sgherri *et al*., 2004) and their accumulation is critically dependent on the sensitivity of plants to water drought (Ennajeh *et al*., 2009). In the present study, the cultivars differed in their polyphenols accumulation induced by water drought stress. Thus, the content of these secondary compounds was significantly increased with salt stress in Chemlali and Zalmati leaves which is similar toother results reported by Bacelar *et al*. (2006) and Ennajeh *et al*. (2009). However, the amount of polyphenols in Zalmati leaves was higher as compared to Chemlali especially with increasing WD which can explain at least in part the better leaf cell integrity in the leaves of the former cultivar. The antioxidant activity of phenolic compounds can play an important role in neutralizing ROS (Zheng and Wang, 2001; Blokhina *et al*., 2003). Indeed, some phenolics compounds have the ability to alter peroxidation kinetics by modification of the lipid packing order and to decrease fluidity of the membranes (Arora *et al*., 2000). By contrast, the non-significant relationship between the polyphenols content and SDM and MDA content in Chetoui cv. suggests that theses secondary metabolites does not play a key role in the antioxidant system defense in this cultivar (Table 3). In agreement with the finding of Bacelar *et al*. (2007) indicating that the accumulation of total phenols was unaffected with drought stress and there is no correlation between total phenols amount and the radical scavenging capacity.

In addition to the antioxidants metabolites, plants have evolved ROS-scavenging enzymes such as SOD, CAT, POD and APX to protect cellular membranes and organelles from damaging effects of ROS in order to endure oxidative damage under unfavourable conditions (Gomez *et al*., 2004). The ability of plants to overcome oxidative stress partly relies on the induction of SOD activity and subsequently on the up regulation of other downstream antioxidant enzymes (Alscher *et al*., 2002). In the present study, the cultivars differed in their SOD activity induced by drought stress. Thus, a significant enhanced SOD activity was occurred in Chemlali and Zalmati leaves exposed to water stress (Fig. 4a), suggests that this enzyme may function as a ROS scavenger by converting O_2^- to H_2O_2 and the higher capacity for this conversion was occurred in Zalmati cv. which might lead to its higher protection against water deficit. The increase in the SOD activity may be attributed to the increased production of active oxygen species as substrate that lead to increased expression of genes encoding SOD (Abedi and Pakniyat, 2010). Similar increases in the activities of SOD in response to drought stress have been reported in Olea europaea (Bacelar *et al*., 2007; Ben Ahmed *et al*., 2009) and other plants such as *Brassica napus* (Abedi and Pakniyat, 2010). On the other hand, the relative reduction of SOD activity in Chemlali at severe water stress may be related to the impaired scavenging function of SOD, favouring O_2^- accumulation under severe water deficit. However, Chetoui cv. showed a significant decrease in SOD activity as water stress intensified (Fig. 4a). Furthermore, the positive relationship between SOD activity and SDM on the one hand and the negative correlation between SOD activity and MDA content on the other hand (Table 3), suggests that this antioxidant enzyme do not play a key role in the antioxidant system defense in Chetoui cv. Moreover, this decrease in the activity of SOD in Chetoui cv. was associated with the highest increase in H_2O_2 generation as compared with Chemlali and Zalmati cultivars witch suggest that SOD does not seem to be the only source of H_2O_2. In agreement with the findings of Tsai *et al*. (2005) suggested that source of H_2O_2 in NaCl-treated leaves of rice can be NADPH oxidase. One mode of ROS formation is closure of stomata as a result of drought and a consequent decrease in CO_2 concentration in leaf mesophyll tissue, resulting in accumulation of NADPH; oxygen acts as an alternative acceptor of electrons, leading to the formation of superoxide radicals (Cadenas, 1989). Therefore, the H_2O_2 produced by SOD to prevent cellular damage must be eliminated by conversion to water and oxygen in subsequent reactions involving POD, CAT and APX. Higher activity of CAT decrease H_2O_2 level in cell by breaking it down directly to form H_2O and O_2 and increase the stability of membranes and CO_2 fixation because several enzymes of the Calvin cycle within chloroplasts are extremely sensitive to H_2O_2 (Yamazaki *et al*., 2003). The data showed that CAT activity increased under water stress conditions in both tolerant and susceptible cultivars but this raise was earlier in Chetoui and only at severe drought stress in Chemlali and Zalmati (Fig. 4b). Similar results were found in olive plants under drought stress (Aganchich *et al*., 2007). However, the highest activity was occurred in the lesser

drought-tolerant Chetoui cultivar in disagreement with the findings of Ennajeh *et al*. (2009) showing that activity of CAT was more acute in the drought-tolerant olive cultivar. POD are involved in the detoxification of toxic compounds such as H_2O_2 produced in chloroplasts and thus help in ameliorating the adverse effects of oxidative damage (Chaparzadeh *et al*., 2004). APX is an important antioxidant enzymes involved in ascorbate-glutathione cycle which plays a key role in destroying the H_2O_2 (Foyer and Noctor, 2005). Improved expression of APX has been reported in cytosol as well as in cellular organelles under stressful conditions (Yoshimura *et al*., 2000). In the present study, drought stress led to a significant increase in POD and APX activity (Fig. 4c and d), especially at severe stress and this increase was higher in Zalmati followed by Chemlali, suggesting a higher capacity to decompose H_2O_2 under drought stress and may explain, at least in part, the better ability of Zalmati to maintain its cell membrane integrity. In addition, the study clearly indicate marked increase in APX activity in Zalmati cultivar, signifying a potential role of this enzyme in providing antioxidative defense mechanism of this olive cultivar against drought stress conditions. Indeed, the significant increase in APX activity suggests a key role of this enzyme in detoxification of H_2O_2 under conditions of severe drought stress and could be considered among the main mechanisms developed by olive trees for the protection of chloroplasts which under stress conditions present sustained electron flows and are the main producers and targets of ROS action (Asada, 1999). For the Polyphenol Oxidase (PPO), involved in the metabolism of phenols, oxidizes o-diphenolic substrates to o-quinones (Kuwabara and Katoh, 1999). The study showed a decreased activity of PPO with increasing drought periods especially in Chemlali and Zalmati whereas, only a slightly reduction was observed in Chetoui (Fig. 4e). Indeed, a the highly negative correlation between phenolic accumulation and PPO activity in Chemlali and Zalmati (Fig. 5) suggest that drought stress improve the antioxidant action of phenols by inhibiting polyphenol oxidase and consequently by maintaining phenolic compounds at acceptable levels. In contrast, the very slightly modification in phenolic accumulation in Chetoui cv. with the increase of water deficit stress may be caused by the maintenance of the activity of polyphenol oxidase at higher levels in this cultivar under severe water stress. On the other hand, the positive correlation between the higher proline accumulation and the higher activities of SOD, CAT, POD and APX observed in Zalmati olive cultivar under WD (data not shown) suggest that proline accumulation could activate the antioxidative defense mechanism in olive trees as has been suggested by Yan in salt-stressed soybean plants.

CONCLUSION

In this study, an intra-specific difference in the response of olive cultivars to water drought stress was occurred. Theses variation was observed in plant water relations, proline accumulation, MDA content, H_2O_2 generation, phenolic amount and antioxidative enzymes activities. From theses results, it can be concluded that olive cultivars respond to water stress by developing many physiological adaptations to reduce water loss at the leaf level.

The results obtained in the present study indicate a key role of proline in the osmoregulation in Chemlali and Zalmati cultivars. Based on the behaviour of Zalmati plants which showed a lower reduction in leaf water status, a lower increase in lipid peroxidation and H_2O_2 generation, reserchers believe that this cultivar can withstand water stress more effectively than Chemlali plants and may be promising for cultivation in arid areas like the South of Tunisia. This better tolerance of Zalmati to water deficit may be at least in part attributed to the higher accumulation of proline to make osmotic adjustments and osmopretection and to the better resistance to oxidative stress by enhancing their antioxidative capacity via increasing phenolic compounds and the activities of SOD, POD, CAT and APX. In other words, the great performance of the antioxidant system in Zalmati might be achieved particularly by the highest activity of SOD and APX and the highest accumulation of phenolic compounds.

In addition, these results confirmed that Chemlali exhibited higher drought tolerance associated with higher proline and phenolic compounds accumulation and higher antioxidative enzyme activity than Chetoui cultivar which should be considered as unsuitable for planting in dry areas. Finally, these results may be used as practical biochemical traits such as greater antioxidant activity and proline accumulation when selecting drought tolerant olive cultivars for breeding in arid regions.

ACKNOWLEDGEMENTS

Researchers gratefully acknowledge all the technical staff of the Superior Agricultural Institute (ISA) and the Superior Institute of Biotechnology (ISBM) for their help to conducting these experiments.

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