Antioxidants
Oxidative Stress Management in Plants

About the Book

When plants are subjected to environmental stress conditions such as high light intensity, temperature extremes, drought, high salinity, herbicide treatment, or mineral deficiencies, the balance between the production of reactive oxygen species and the quenching activity of the antioxidants is upset, often resulting in oxidative damage. Oxidative damage to plants effects the overall production of the crop. To understand the effect of different environmental stresses it is essential to learn about the generation and quenching mechanism of the ROS. This volume gives the in-depth knowledge about how ROS is generated, what are its effects on biomolecules and how the crop productivity is affected and what are the mechanism of defense.

This book also shows how transgenic approaches help us to protect the plants from environmental stresses without any effect on the crop productivity. Phytohormones which are very essential for the growth and development of the plant have also been shown to minimize the oxidative stress in plants. How the stress is sensed by the plant is also described in this volume as signalling pathways during salt stress. Heavy metals are easily taken up by plants and enters the food chain, causing serious health disorders in humans. This volume also covers the effect of different heavy metals on plants and their ability to sustain in such an environment. The volume also considers the role of mycorrhizal associations in alleviating the oxidative stress. Role of potassium in abating moisture stress is also highlighted in this volume.

This volume provides invaluable information about the role of antioxidants in alleviating oxidative stress and will be very useful for biologists working on stress physiology. The volume is very much beneficial for the students, research scholars, plant physiologists and phytochemists of different research institutes and universities in India and abroad.

Studium Press (India) Pvt. Ltd.
4735/22, 2nd Floor Prakash Deep Building (Near PNB), Ansari Road, Darya Ganj, New Delhi-110002
Phone: +91-11-23240257, 43240200 (15 lines)
Fax: +91-11-23240273
E-mail: studiumpress@gmail.com
Website: http://www.studiumpress.in

ISBN 978-93-80012-38-4
9 789380 012384

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Antioxidant Regulation During Drought Stress in Mediterranean Fruit Crops

Adriano Sofo*, Bartolomeo Dichio, Cristos Xiloyannis

ABSTRACT

*This study provides an overview of the biochemical antioxidant mechanisms developed by olive (*Olea europaea *L.) and* Prunus spp. *trees subjected to water limited conditions. Two-year old olive plants (cv. 'Coratina') were subjected to a controlled water deficit and, after 20 days without irrigation, mean pre-dawn leaf water potential (Ψ_w) fell from –0.37 to –5.37 MPa. In a similar experiment on plants of four interspecific* Prunus *hybrids, mean Ψ_w fell from –0.34 to –3.30 MPa after 70 days of water shortage. In both olive and* Prunus *plants, a marked decrease in gas exchange occurred during the drought period. Olive trees showed higher photosynthetic rates during water deficit if compared to* Prunus *plants with the same values of Ψ_w. An up-regulation of some AOS-scavenging enzymes (superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase, indoleacetate oxidase, monodehydro-ascorbate reductase, dehydroascorbate reductase and glutathione reductase) as plants enter water deficit conditions was observed. The levels of ascorbate, glutathione and H_2O_2 observed in* Prunus *hybrids were directly related to the increase of drought stress. The increases of lipoxygenase activity and malondialdehyde levels during the progression of water deficit in olive plants highlighted that the cell membrane's damage is a direct consequence of water deficit. The results underline the important role of some antioxidant enzymes and compounds in protecting cellular apparatus during drought stress. They may be*

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important for a more complete understanding of the physiological behaviour of olive and Prunus *plants grown in semi-arid regions and for the selection for drought resistant cultivars and rootstocks.*

Keywords : AOS-scavenging enzymes, biochemical mechanism, mediterraneau summer, drought stress, *Olea europea*, *Prunus* spp

INTRODUCTION

The Mediterranean–type summer is characterised by low air humidity, high air temperatures and solar radiation, and consequently high rates of evapotranspiration. In these dry periods, water becomes a limiting factor producing prolonged and intense drought stress in plants. Mediterranean vegetation dealing with this peculiar climate has developed a number of physiological mechanisms to tolerate drought stress and grow under adverse climatic conditions (Lo Gullo and Salleo, 1988). Drought stress is the main cause of reduced plant growth and productivity in semi-arid regions and causes a complex of responses at molecular, cellular, physiological and developmental level (Ingram and Bartels, 1996; Nayyar and Gupta, 2006; Ozkur *et al.*, 2009). Severe drought stress predisposes the photosynthetic system of olive leaves to photoinhibition, resulting in a light-dependent inactivation of the primary photochemistry associated with photosystem II, which persists after rewatering (Angelopoulos *et al.*, 1996; Logan, 2005).

In the Mediterranean area, 16% of the total cultivable land is occupied by fruit orchards (Olesen and Bindi, 2002) and in Italy alone, olive and *Prunus* orchards cover an area of approximately 1.0×10^6 and 1.0×10^5 ha, respectively (ISTAT, 2000; Lombardo e Parlati, 2002). Olive tree (*Olea europaea* L.) is a woody species typically cultivated in the Mediterranean basin, where plants are often exposed to long periods of water deficit and high irradiance levels during the dry season (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 perennial drought stress conditions (Connor and Fereres, 2005; Bacelar *et al.*, 2007). In this species, a series of strategies act synergically against drought stress. The most relevant mechanisms are the regulation of stomata closure and transpiration (Moreno *et al.*, 1996; Nogués, and Baker, 2000), the regulation of gas exchange (Moriana *et al.*, 2002), a very developed osmotic adjustment (Chartzoulakis *et al.*, 1999), the regulation of the antioxidant system (Bacelar *et al.*, 2007), the appearance of leaf anatomical alterations (Chartzoulakis *et al.*, 1999), and the ability of extracting water from the soil due to a deep root system (Fernandez *et al.,* 1997; Aganchich *et al.*,

2009) and to a high water potential gradient between canopy and root system (Tombesi *et al.*, 1986). Olive trees are confirmed to be efficient soil water users, thanks to their xylem sap transport and the ability to maintain significant rates of gas exchange even during drought stress (Tognetti *et al.*, 2004). For these reasons, the olive tree can be defined as a model plant for drought tolerance in Mediterranean climates.

The genus *Prunus* comprises more than 400 species adapted to temperate areas and cultivated in the Mediterranean basin (Esparza *et al.*, 2001). In particular, stone fruit crops, such as peach (*Prunus persica* L.), plum (*Prunus cerasifera* L. and *Prunus domestica* L.), almond (*Prunus dulcis* L.), apricot (*Prunus armeniaca* L.) and cherry tree (*Prunus avium* L.), are typical and economically important cultures mainly localized in Mediterranean regions, where the spring-summer period is often characterized by high temperatures, high irradiance levels and lack of precipitation. In particular, the peach tree (*Prunus persica* L.) is one of the most common and economically important species of the Mediterranean basin, where drought periods are frequent and irrigation water is a limiting factor for productivity. Peach drought tolerance is mainly based on stomatal control (Arndt *et al.*, 2000) and morphological characteristics (Rieger *et al.*, 2003), together with some degree of osmotic adjustment (Escobar-Gutiérrez *et al.*, 1998; Arndt *et al.*, 2000). Researches in this species covered subjects from the physiological processes adopted to regulate water status under drought conditions (Arndt *et al.*, 2000; Rieger *et al.*, 2003) to the biochemistry underlying plant response to water deficit and oxidative stress (Escobar-Gutiérrez *et al.*, 1998; Sofo *et al.*, 2005b).

In tree plants, very low water contents, resulting from severe dehydration, are often associated with increased levels of activated oxygen species (AOS), such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$) and singlet oxygen ($^1O_2$), which in turn damage cellular structures and macromolecules (Shvaleva *et al.*, 2006; Sánchez-Díaz *et al.*, 2007; Guidi *et al.*, 2008; Haberer *et al.*, 2008; Wang *et al.*, 2008) or act as signal molecules that activate multiple defence responses (Van Breusegem *et al.*, 2001; Vranová *et al.*, 2002). Chloroplasts, mitochondria and peroxisomes are the major sources of AOS in plant cells (Asada, 1999). Plants use enzymatic and non-enzymatic antioxidant defence mechanisms to scavenge AOS (Foyer *et al.*, 1994; Tambussi *et al.*, 2002; Caravaca *et al.*, 2005). The antioxidant enzymatic system includes superoxide dismutases (SOD; EC 1.15.1.1), which catalyze the dismutation of superoxide radicals to H_2O_2 and O_2, together with catalase (CAT; EC 1.11.1.6), guaiacol-type peroxidases (POD; EC 1.11.1.7) and enzymes of the ascorbate-glutathione cycle, such as ascorbate peroxidase (APX; EC 1.11.1.11), which detoxify the H_2O_2 produced. Polyphenol oxidase (PPO; EC 1.30.3.1) isoenzymes, located mainly in thylakoid lumen, oxidize *o*-diphenolic substrates to

o-quinones (Kuwabara and Katoh, 1999), and are therefore involved in metabolism of phenols, which have a non-enzymatic antioxidant action (Rice-Evans *et al.,* 1997). SOD and APX exist in multiple isoforms within the chloroplasts and, together with other antioxidant enzymes, constitute the major defence system against AOS produced by the electron transport chain located in chloroplast (Asada, 1999). CATs, heme-containing enzymes particularly abundant in the glyoxysomes, destroy the H_2O_2 generated by oxidases involved in the β-oxidation of fatty acids, and in the peroxisomes of green leaves, where they scavenge the H_2O_2 arising from the oxidation of the photorespiratory-produced glycolate. PODs and APXs are involved in the detoxification of H_2O_2 both within the cell and in the apoplast. PODs are less specific and can use a broad range of substrates as electron donors (Shinshi and Noguchi, 1975), whereas APXs are more specific and use ascorbate as electron donor, but, to a lesser extent, can also use guaiacol or other substrates (Mehlhorn *et al.*, 1996; Bartoli *et al.*, 2009). The antioxidant enzymatic system also includes other enzymes of the so-called ascorbate-glutathione cycle, that operates both in the chloroplasts and in the cytosol: monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2).

The antioxidant non-enzymatic system includes ascorbate and glutathione, two constituents of the antioxidative ascorbate-glutathione cycle which detoxify H_2O_2 in the chloroplasts (Foyer *et al.*, 2006; Bartoli *et al.*, 2009) and are located both within the cell and in the apoplast (Foyer and Mulllineaux, 1998; Vranovà *et al.*, 2002). Ascorbate (AsA) is a major primary antioxidant synthesized on the inner membrane of the mitochondria which reacts chemically with $^1O_2$, $O_2^{\cdot -}$, $HO^{\cdot}$ and thiyl radical (Layne *et al.*, 1993), and acts as the natural substrate of many plant peroxidases (Scebba *et al.*, 2001). Moreover, AsA is involved in other functions such as plant growth, gene regulation, modulation of some enzymes and redox regulation of membrane-bound antioxidant compounds (Layne *et al.*, 1993; Foyer and Mulllineaux, 1998). Glutathione (GSH) is a tripeptide synthesized in the cytosol and the chloroplast which scavenges $^1O_2$ and H_2O_2 and is oxidized to glutathione disulfide (GSSG) when acts as an antioxidant and redox regulator (Van Breusegem *et al.*, 2001; Sofo *et al.*, 2004; Foyer *et al.*, 2006). GSH is the substrate of glutathione S-transferases (GSTs), which have a protective role in the detoxification of xenobiotics, and dehydroascorbate reductase (DHAR) (Vranovà *et al.*, 2002). Finally, GSH is a precursor of phytochelatins, which regulate cellular heavy metals levels, and is involved in gene expression (Layne *et al.*, 1993). LOX (EC 1.13.11.12) isoenzymes are nearly ubiquitous in the plant kingdom and are involved in many physiological processes such as flowering (Ye *et al.*, 2000), seed germination (Suzuki and Matsukura 1997), pigment bleaching (Pastore *et al.*, 2000), formation of flavour and aroma in plant products (Williams *et al.*, 2000) and plant growth and development

(Hildebrand *et al.*, 1991). LOXs catalyse the dioxygenation of polyunsaturated fatty acids containing a *cis, cis*-1,4-pentadiene backbone, producing hydroperoxy fatty acids, which are highly reactive compounds that are toxic to cells. These fatty acids are rapidly degraded into metabolites which lead to the production of jasmonates, conjugate dienoic acids and volatile aldehydes, such as malondialdehyde (MDA) (Bird and Draper 1984, Siedow 1991). Very often MDA levels have been utilised as a suitable marker for membrane lipid peroxidation (Masia 2003). Nonetheless, lipids are not the only targets for MDA activity. In fact MDA can react with DNA to form adducts to deoxyguanosine and deoxyadenosine (Marnett 1999).

The involvement and the role of antioxidants in protection against oxidative stress have been demonstrated using transgenic plants with enhanced levels of some antioxidant enzymes (Allen *et al.*, 1997; Sen *et al.*, 2000). Changes of expression and activities of antioxidant enzymes have been detected in many species of plants in response to adverse environmental conditions, such as water deficit and other abiotic, biotic and developmental stimuli (Smirnoff, 2005).

A better knowledge of the effects of water deficit on tree crops has a primary importance for improved management practices (Girona *et al.*, 2002), breeding programmes (Rieger *et al.*, 2003) and for predicting fruit growth and quality (Besset *et al.*, 2001). Understanding the mechanisms by which olive and peach plants face drought stress under extreme environmental condition is essential for selecting more drought-tolerant cultivars and hence for saving water resources in semi-arid environments. Unfortunately, the response of olive and peach to drought stress is a well documented process but most studies have focused on the physiological aspects of these tree crops (Xiloyannis *et al.*, 1988; Angelopoulos *et al.*, 1996; Fernández *et al.*, 1997; Besset *et al.*, 2001; Klein *et al.*, 2001; Girona *et al.*, 2002; Moriana *et al.*, 2002; Xiloyannis *et al.*, 2003). The relationships among drought stress and variations in the activity of photosynthetic apparatus and water related parameters in these two tree crops are sufficiently clear but very little is known, however, about the linkages between drought stress and antioxidant compounds.

For these reasons, the aim of this work was to study the changes of antioxidant enzyme activities and of the levels of some antioxidant non-enzymatic compounds (ascorbate and glutathione pools, and H_2O_2) in olive plants and in four *Prunus* interspecific hybrids grown under water shortage followed by a rewatering phase. The paper includes the study of the physiological parameters of olive and *Prunus* plants grown in environmental conditions characterized by high temperature and irradiance levels and with an imposed, progressive water shortage.

MATERIALS AND METHODS

Study Site and Experimental Design

The experiment was carried out in Southern Italy, Basilicata Region (N 40°24′, E 16°48′) using approximately 60 own-rooted two-year-old olive plants (*Olea europaea* L., cv. 'Coratina') four one-year-old interspecific *Prunus* hybrids named 'P3605' (*Prunus amygdalus* L. 'Garfi' x *Prunus persica* L. 'Nemared'), '8-9' (*Prunus cerasifera* L. 'P2980' x 'P3605'), '7-7' (*Prunus cerasifera* L. 'P2175' x *Prunus davidiana* L.) and '6-5' (*Prunus cerasifera* L. 'P2175' x *Prunus amygdalus* L. 'Garfi'). Olive plants were grown uniformly outdoors in 0.016 m^3 pots containing loamy sand (73.2% sand, 13.3% silt and 13.5% clay) whereas *Prunus* hybrids grew uniformly outdoors in 5.0 m^3 containers filled with a silty-clay loam. Trees were irrigated with drip emitters per plant discharging 3 L h^{-1}. Plant containers of SP were covered with plastic film in order to avoid rainfall infiltrations and evaporation from the soil surface. All plants were weighed each evening in order to calculate the amount of water transpired. Soil water content was maintained at a constant value of around 85% of soil water holding capacity by integrating, every evening, the amount of water lost through transpiration during the day.

Successively, plants were divided in two groups: drought-stressed plants (SP) and control plants (CP). CP were maintained in an optimal soil water conditions during the whole experimental period, whereas SP subjected to drought stress applying a gradual and controlled water reduction for ten days, and after which irrigation ceased. Drought stress levels were defined on the basis of the values of leaf water potential (Ψ_w) measured pre-dawn (at 04.00 h) using a pressure chamber (PMS Instrument Co. Corvallis, OR, USA), according to Turner (1981).

The measurements of gas exchange were carried out on fully expanded leaves taken along the median segment of new-growth shoots using the portable photosynthesis system LCA-4 (Analytical Development Company, Hoddesdon, UK) operated at 500 µmol m^{-2} s^{-1} flow. Chlorophyll pigments were extracted in 10 ml of *N,N*-dimethylformammide from leaf discs (8 mm diameter) taken from the central part of the leaves and spectrophotometrically analysed (Metertech Inc., Taiwan; spectrophotometer model SP8001) at 647 and 664 nm. Chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) contents were calculated by the formulae reported by Moran (1982).

Antioxidant Compounds Determination

Leaves of olive and *Prunus* plants were collected at regular intervals from the beginning to the end of the drought stress period. In order to collect olive roots, plants were destructively harvested and only the roots with a

diameter between 1 and 5 mm were used for the following analyses, according to Sofo *et al.* (2005a). Leaf and roots samples were washed with distilled water, dried with filter paper, temporarily covered with an aluminium foil and put in a plastic envelope, and then immediately used for extraction and determination of the different antioxidant compounds.

Total SOD activity was measured according to Madamanchi *et al.* (1994) by measuring the capacity of the enzyme extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) to blue formazan. CAT activity was assayed according to Aebi (1984). The decomposition of H_2O_2 was followed spectrophotometrically by the decrease in A_{240}. One unit of CAT activity corresponded to the amount of enzyme that decomposes 1 µmol of H_2O_2 per minute, according to Havir and McHale (1987). POD activity was measured according to Chance and Maehly (1955). The activity of the mixture was determined spectrophotometrically at 470 nm after 10 min at 20°C. PPO activity was assayed according to Cañal *et al.* (1988) by reading absorbance at 420 nm. APX activity was measured spectrophotometrically by recording the decrease in ascorbate content at 290 nm, according to Ushimaru *et al.* (1997). MDHAR activity was tested after the method of Hossain *et al.* (1984). DHAR activity was determined by monitoring the increase in absorbance at 265 nm due to AsA production, according to Hossain and Asada (1984). GR activity was measured by following the decrease in absorbance at 340 nm due to NADPH oxidation after the method of Carlberg and Mannervik (1985). Ascorbic acid was measured spectrophotometrically by reading absorbance at 265 nm due to ascorbate oxidation by ascorbate oxidase, according to Foyer *et al.* (1983) and the concentration of dehydroascorbate (DHA) was calculated as the difference between total ascorbate and AsA. The levels of glutathione were measured spectrophotometrically by monitoring the reduction of 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 412 nm, after the method of Griffith (1980) and the concentration of reduced glutathione (GSH) was calculated as the difference between total glutathione and GSSG. The determination of H_2O_2 was carried out according to Lee and Lee (2000). Malondialdehyde content was measured spectrophotometrically, according to Du and Bramlage (1992). The total soluble proteins were determined according to Smith *et al.* (1985), using bovine serum albumine as calibration standard.

RESULTS AND DISCUSSION

Olive

In olive plants, morphological and anatomical features such as microphyllia, the thick leaf cuticle with large amounts of waxy substances, the hairiness of the leaf abaxial surface and the high specific weight of the leaves (sclerophylly) are means developed by this species to reduce water loss. Transpiration rates in olive are higher than in most other fruit tree species

under both well-watered and drought conditions, and the various tissues can withstand very negative values of water potential (Xiloyannis *et al.*, 2003). It is noteworthy that during the drought period, Chl *a* and Chl *b* content remained stable and Chl *a*/*b* ratio did not significantly change (Fig. 16.2). This physiological response suggests that the observed decrease of photosynthetic rate (Fig. 16.1A) is due to the inactivation of PS II enzymes or to stomatal factors, and not a decline of photosynthetic pigments, as chlorophyll synthesis keeps pace with its own degradation.

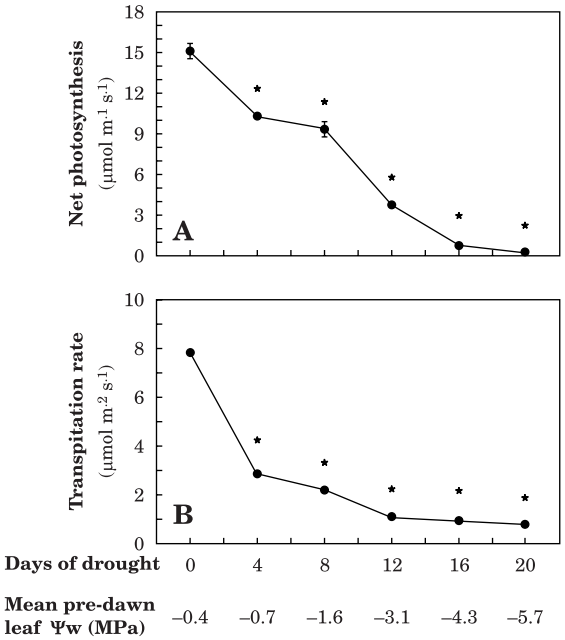

Fig. 16.1. Net photosynthesis (A) and transpiration rate (B) in olive plants at different levels of drought stress The values represent the mean of three measurements (±SE) on each of three selected plants having the same pre-dawn Ψ_w. Stars refer to differences between well-watered and drought-stressed plants at $P \leq 0.05$, according to Duncan's Multiple Range Test

The results show that the transpiration occurs at the maximum rate as long as soil moisture is sufficient to permit the normal course of plant physiological processes but, when Ψ_w becomes lower than 0.5 MPa, the transpiration starts to be inhibited (Fig. 16.1B). At a Ψ_w value of 0.65 MPa, daily transpiration per unit of leaf area is reduced by about 55% while photosynthesis decreases approximately of 30% if compared to well-irrigated plants (Fig. 16.1). However, unlike in other species, leaves continue to function even at Ψ_w of –6.0 MPa (Sofo *et al.*, 2008). In dry condition, olive leaves can use for transpiration about 60% of their water reserves without irreversible damage, and this water contributes to sustain the

demands of transpiration as stress increases, up to Ψ_w values of –7.0 MPa, when relative water content of the plant reaches 40% (Sofo *et al.*, 2009). This value is considerable if compared with that of other fruit species such as kiwifruit, which uses for transpiration a limited amount of water (about 9%) from its reserves under conditions of severe water deficit (Nuzzo *et al.*, 1997). In olive leaf, drought stress induces the inactivation of the primary photochemistry associated with photosystem II (PS II) (Angelopoulos *et al.*, 1996) and the 'down regulation' of PS II electron transport (Nogués and Baker, 2000). This could explain the decrease in net photosynthetic rates found in SP (Fig. 16.1A).

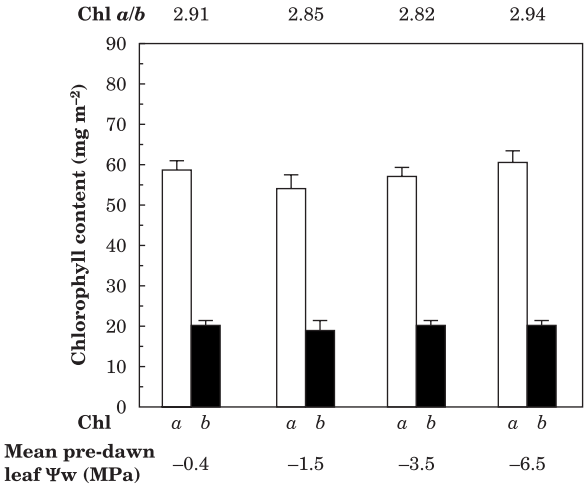

Fig. 16.2. Chlorophyll *a* (white columns) and *b* (black columns) contents and Chl *a*/*b* ratios in leaves of olive plants at different levels of drought stress. Statistics like in Fig. 16.1

Drought stress is often associated with increased cellular AOS levels and plants can increase the activity of antioxidant enzymes to remove them (Smirnoff, 1993). The results show that olive plants are able to up-regulate the enzymatic antioxidant system as plants enter water deficit conditions (Table 16.1). This response protects cellular apparatus and limits cellular damage caused by AOS. In fact, the activities of ascorbate peroxidase (APX) and catalase (CAT) showed a significant increase in leaves of drought-stressed plants, reaching values of 13.77 ± 0.55 units mg^{-1} DW and 11.78 ± 0.18 units mg^{-1} DW, respectively. The considerable increase in APX activity observed in leaves can protect chloroplasts, which under stress conditions present sustained electron flows and are the main producers and targets of AOS action (Asada, 1999). Ascorbate can then be regenerated by the ascorbate-glutathione cycle (Foyer and Mullineaux, 1998; Smirnoff, 2000). The maintenance of CAT activity in leaves of SP likely allowed the removal

of photorespiratory H_2O_2 produced during drought stress, according to Noctor *et al.* (2000). In fact, in these conditions photorespiration works as an energy sink preventing the over-reduction of the photosynthetic electron transport chain and photoinhibition (Wingler *et al.*, 2000). Moreover, photorespiration produces glycine which is involved in stress protection mechanisms and is necessary for the synthesis of reduced glutathione (Wingler *et al.*, 2000).

Table 16.1. Activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (POD), polyphenol oxidase (PPO) and lipoxygenase (LOX) in leaves and roots of drought-stressed and well-watered control olive plants. Each value represents the mean of three measurements (±SE) from three plants having a similar value of pre-dawn leaf water potential (Ψ_w). Stars refer to differences between well-watered and drought-stressed plants at $P \leq 0.05$, according to Duncan's Multiple Range Test

Tissue	Pre-dawn leaf water potential (MPa)	Enzyme activity (units mg^{-1} protein)				
		SOD	APX	CAT	POD	PPO
Leaves	–0.4 (control)	5.07 ± 0.27	1.29 ± 0.07	1.51 ± 0.06	9.74 ± 0.41	11.16 ± 0.29
	–1.6	10.24 ± 0.30*	2.45 ± 0.02*	2.27 ± 0.02	13.02 ± 0.66*	8.85 ± 0.28*
	–4.3	10.56 ± 0.63*	4.04 ± 0.09*	3.81 ± 0.25*	16.06 ± 0.67*	8.04 ± 0.45*
	–5.7	8.39 ± 0.35*	4.59 ± 0.18*	3.93 ± 0.06*	13.55 ± 0.24*	6.79 ± 0.30*
Roots	–0.4 (control)	2.66 ± 0.07	0.12 ± 0.00	0.69 ± 0.01	7.96 ± 0.11*	12.73 ± 0.33
	–1.6	3.73 ± 0.09*	0.14 ± 0.00	1.03 ± 0.02*	13.02 ± 0.52*	11.88 ± 0.25
	–4.3	5.31 ± 0.20*	0.19 ± 0.01*	0.91 ± 0.03*	15.92 ± 0.70*	7.14 ± 0.14*
	–5.7	5.73 ± 0.47*	0.17 ± 0.01*	0.86 ± 0.02*	14.85 ± 0.42*	6.35 ± 0.11*

The activities of superoxide dismutase (SOD) and peroxidase (POD) increased both in leaves and roots (Table 16.1). This can be an important protection mechanism of olive tree against the excessive increase of AOS during drought stress. In fact, SOD isoforms play a key role in AOS-scavenging by regulating the levels of $O_2^{\cdot -}$ produced in chloroplasts, mitochondria and cytosol (Bowler *et al.*, 1992). Moreover, SOD activity reduces the possibility of $HO^{\cdot}$ formation, a very strong oxidant with a very high affinity for biological molecules, via Haber-Weiss and/or the metal-catalysed Fenton reaction (Smirnoff, 1993). The role of POD isoenzymes is based mainly on their involvement in lignin biosynthesis (Bacon *et al.*, 1997) and IAA oxidation (Shinshi and Noguchi, 1975). PODs participate in the modulation of cell wall properties during plant growth partly through catalysing the formation of covalent cross-links after oxidation of ester- and ether-bound phenolic acids and partly through the oxidative coupling of cinnamoyl alcohol moieties to generate lignin (Bacon *et al.*, 1997).

Therefore, the observed increases in POD activity in olive tissues (Table 16.1) reflects the changed mechanical properties of the cell wall, which in turn, can be correlated with drought adaptation.

PPO isoenzymes are copper-containing monooxygenases catalysing the *o*-hydroxylation of phenols and the oxidation of *o*-diphenols to the corresponding *o*-quinones, at the expense of molecular oxygen (Kuwabara and Katoh, 1999). As phenolics are physiologically active secondary compounds with a non-enzymatic antioxidant action, the regulative action of PPO plays an important role in the physiological aspects of plants subjected to water deficit conditions (Grace *et al.*, 2005). In olive tree, drought stress can improve the antioxidant action of phenols by inhibiting PPO activity (Table 16.1) and consequently by maintaining the phenol compounds pool in the reduced state. Moreover, the proteolytic activity of PPO (Kuwabara and Katoh, 1999) suggests that the enzyme could be involved in removing the proteins damaged by AOS.

Significant increases of lipoxygenase (LOX) activity and malondialdehyde (MDA) content, two markers of oxidative stress, were also observed during the progressive increment of drought stress in both leaf and root tissues of olive plants (Table 16.2), thus indicating a higher lipid peroxidation in SP in comparison to CP. This hypothesis was supported by MDA accumulation and high LOX activities determined in other drought stressed species (Lima *et al.*, 2002; Maccarone 2006). The observed high degree of lipid peroxidation could produce lipid-derivatives acting as secondary messengers capable to activate some drought stress-associated genes by means of specific transcription factors, in such a way starting the response of plant to desiccation (Ingram and Bartels 1996, Shinozaki and Yamaguchi-Shinozaki 1997).

Table 16.2. Lipoxygenase (LOX) activity and malondialdehyde (MDA) level in leaves and roots of drought-stressed and well-watered control olive plants. Each value represents the mean of three measurements (± SE) from three plants having a similar value of pre-dawn leaf water potential (Ψ_w). Stars refer to differences between well-watered and drought-stressed plants at P de 0.05, according to Duncan's Multiple Range Test

Tissue	Pre-dawn leaf water potential (MPa)	MDA levels (nmol g^{-1} fresh weight)	LOX activity (units mg^{-1} protein)
Leaves	–0.4 (control)	14.87 ± 1.06	49.98 ± 2.45
	–1.6	23.99 ± 1.15*	80.03 ± 3.04*
	–4.3	35.39 ± 1.84*	153.78 ± 9.40*
	–5.7	51.21 ± 2.65*	164.14 ± 11.43*
Roots	–0.4 (control)	6.66 ± 0.13	16.94 ± 0.91
	–1.6	13.14 ± 0.42*	21.41 ± 2.46*
	–4.3	15.46 ± 0.46*	24.89 ± 2.52*
	–5.7	18.53 ± 0.70*	26.50 ± 3.28*

Prunus

In *Prunus* trees, the water shortage is paralleled by a significant decrease in transpiration rate and net photosynthetic rate in all the hybrids starting from mean Ψ_w values lower than –1.8 MPa (Fig. 16.3AB). In SP, the values of gas exchange decreased throughout the drought period, reaching the lowest values at severe water deficit conditions, between –3.0 and –4.0 MPa (Fig. 16.3).

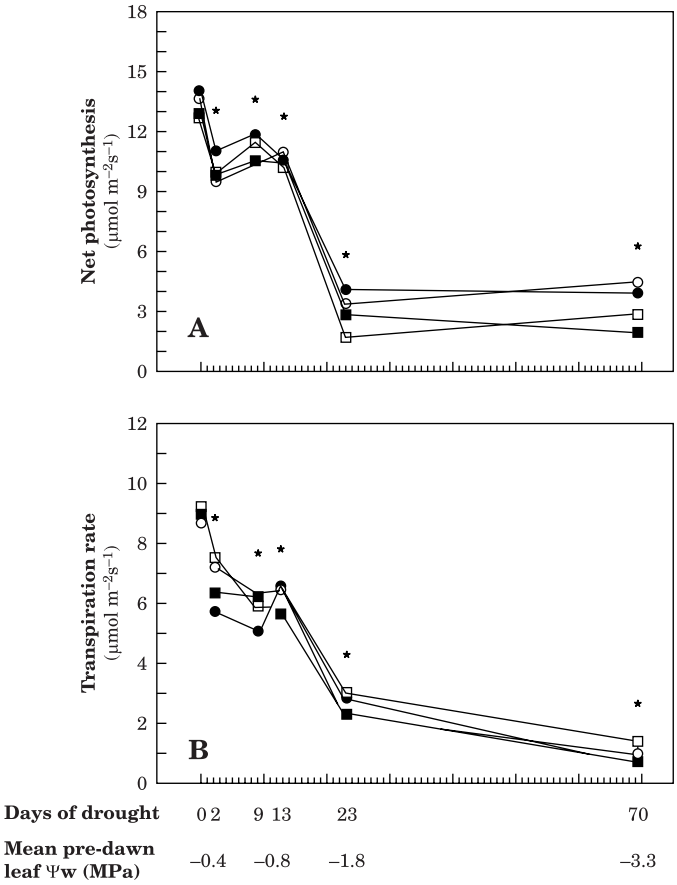

Fig. 16.3. Net photosynthesis (A) and transpiration rate (B) in *Prunus* hybrids at different levels of drought stress. Hybrids: P3605 (■), 6–5 (•), 7–7 (□), 8–9 (○). Statistics like in Fig. 16.1.

APX and MDHAR activities showed an increase starting from Ψ_w values lower than –1.8 MPa, decreasing slightly only at the end of the drought phase (Fig. 16.4). DHAR activity was directly related to drought stress levels, showing a gradual increase starting from relatively high mean values of Ψ_w (–0.4 MPa), whereas GR activity showed a sharp increase only at severe levels of drought stress (Fig. 16.4). AsA and DHA content

increased in relation to the duration of drought stress in all the hybrids examined, with a slight decrease at the highest level of water deficit for DHA (Fig. 16.5). Drought stress caused marked increases in GSH content in P3605 and 8–9 and, in a lesser extent, 7–7 and 6–5 (Fig. 16.5). GSSG levels showed an increase during the progressive water shortage followed by a decrease at the highest values of Ψ_w (Fig. 16.5). In all the hybrids studies, the levels of H_2O_2 were directly related to Ψ_w, presenting a continuous increase during all the drought phase (Fig. 16.5E).

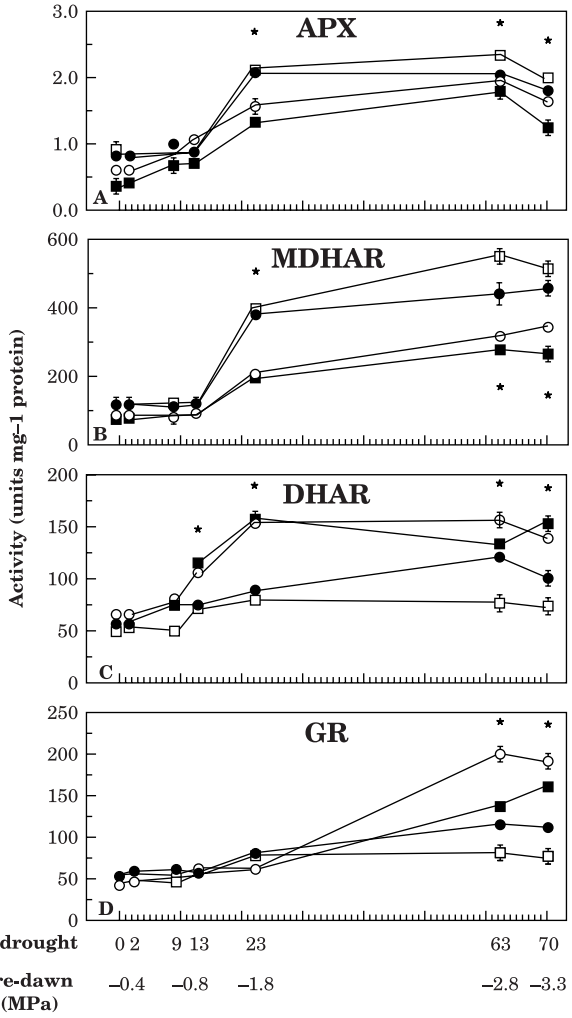

Fig. 16.4. Activities of ascorbate peroxidase (APX), monodehydroascorbate redictase (MDHAR), dehydroascroabate reductase (DHAR) and glutathione reductase (GR) in leaves of *Prunus* hybrids at different levels of drought stress. Hybrids: P3605 (■), 6–5 (•), 7–7 (□), 8–9 (○). Statistics like in Fig. 16.1

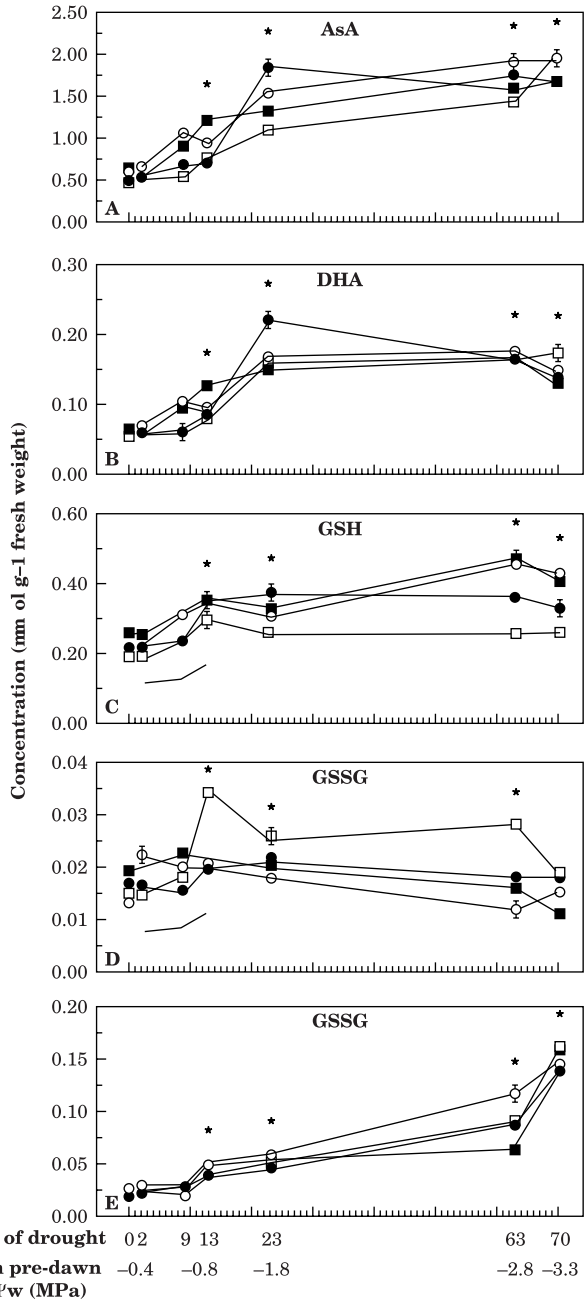

Fig. 16.5. Levels of ascorbate (AsA), dehydroascorbate (DHA), reduced glutathione (GSH), oxidized glutathione (GSSG) and hydrogen peroxide (H_2O_2) in leaves of *Prunus* hybrids at different levels of drought stress. Hybrids: P3605 (■), 6–5 (•), 7–7 (□), 8–9 (○). Statistics like in Fig. 16.1

Generally, the activities of antioxidant enzymes and the levels of the molecules involved in the ascorbate-glutathione cycle increased in all the hybrids examined in parallel to the severity of drought stress, and in particular when LWP reached values lower than about –2.0 MPa (Fig. 16.3; Tables 16.3 and 16.4).

Among the enzymes examined, it seems evident a linkage between APX and DHAR, which showed an increase of activity directly related to the degree of drought stress in all the tested tissues (Fig. 16.4). Monodehydroascorbate (MDHA), a free radical intermediate produced by APX catalysis, can disproportionate spontaneously to AsA and dehydroascorbate (DHA) or be enzymatically reduced to AsA by MDHAR, a FAD enzyme with an high specificity to MDHA which uses NAD(P)H as a reductant (Asada, 1999). SP of hybrids 6–5 and 7–7 showed high activities of APX and MDHA and low AsA level if compared to 8–9 and 3605 (Tables 16.3 and 16.4). This suggests that antioxidant protection in these hybrids could be attributed mainly to APX and DHAR, with a partial contribute of the other components of ascorbate-glutathione cycle (Fig. 16.4). In 6–5 and 7–7, the high APX activity likely determined low level of AsA (Tables 16.3 and 16.4), which is the main substrate of APX and is then regenerated by MDHAR, whereas the fast turnover of AsA and MDHA, due to high activities of APX and DHAR caused low levels of MDHA and, indirectly, of DHA (Tables 3 and 4). We hypothesize that the similar behaviour of the clones 6–5 and 7–7 could be due to the sharing of one parent (*Prunus cerasifera* L. 'P2175'), common to both the genotypes.

The increase of DHAR and GR activities during in SP suggests a strict relationship of these enzymes with drought stress conditions in all the hybrids studied (Fig. 16.4). DHAR is a monomeric thiol enzyme that reduces DHA to AsA at expense of GSH as an electron donor, with the consequent production of GSSG (Foyer and Mullineaux, 1998; Urano *et al.*, 2000). The isoforms of GR are flavoenzymes with a redox cystine residue in their active sites which maintain the intracellular glutathione pool in the reduced status catalysing the NADPH-dependent reduction of GSSH to GSH (Stevens *et al.*, 1997). DHAR and GR activities, were higher in the hybrids 3605 and 8–9 than those found in 6–5 and 7–7 (Fig. 16.4). The high levels of GHS in these clones, in particular at high degrees of drought stress (Fig. 16.5) are directly related to the observed increase of GR at severe drought stress (Fig. 16.4). This highlights the regulative action of GR in the homeostasis of glutathione pool in *Prunus* plants subjected to water deficit conditions. The results of 3605 and 8–9, together with the relative low activities of APX and MDHAR with respect to those found in 6–5 and 7–7, show that antioxidant protection in these clones is mainly due to the enzymes and molecules involved in glutathione metabolism. Therefore, the production of DHA deriving from MDHA disproportionation and it consequent reduction by DAHR plays a key role in these hybrids. Both the patterns

and levels of H_2O_2 were similar in all the *Prunus* hybrids (Fig. 16.5). This confirm that the drought-related reduction of CO_2 assimilation (Fig. 16.3A) causes an excess of H_2O_2 deriving from the photoreduction of O_2 to $O_2^{\cdot -}$ in PSI and the following disproportionation of $O_2^{\cdot -}$ catalyzed by SOD (Smirnoff, 2005). The results show that the progressive loss of water from leaf tissues (Fig. 16.3) caused an increase of H_2O_2-related oxidative stress (Fig. 16.5) which in turn enhanced ascorbate accumulation and AsA-dependent detoxification processes (Tabes 16.3 and 16.4). The increase of glutathione pool in SP (Fig. 16.5) confirms the important role of GSH in plant protection against oxidative stress and could be necessary to regulate the levels of AsA and DHA.

CONCLUSION

Experimental results provided an overview of the biochemical antioxidant mechanisms developed by olive and *Prunus* trees subjected to water limited conditions. Olive trees have the capacity of maintaining higher photosynthetic rates during water deficit if compared to *Prunus* plants with the same values of Ψ_w (Figs. 16.1 and 16.3). This reflects the different physiological response of olive and *Prunus* to drought. In contrast, both in olive and *Prunus* we have found evidence for an up-regulation of AOS-scavenging enzymes as plants enter water deficit conditions (Table 16.1 and Fig. 16.4). On the basis of other studies, this regulation of antioxidant enzymes in olive and *Prunus* leaves is also related to the level of exposition to light (about 30% lower in shaded leaves) and it is reversible after a rewatering phase (Sofo *et al.*, 2004, 2005b). The observed increases of lipoxygenase activity and malondialdehyde levels during the progression of water deficit (Table 16.2) highlight that the cell membrane's damage is a direct consequence of water deficit. The results obtained in this investigation underline the important role of some antioxidant enzymes and compounds in protecting cellular apparatus during water deficit conditions. They may be important for a more complete understanding of the behaviour of the vegetative growth of olive and *Prunus* plants grown in semi-arid regions and for the selection for drought resistance of cultivars and rootstocks.

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