Available online at www.sciencedirect.com
SCIENCE DIRECT®
Plant Science 166 (2004) 293–302
PLANT SCIENCE
www.elsevier.com/locate/plantsci

Effects of different irradiance levels on some antioxidant enzymes and on malondialdehyde content during rewatering in olive tree

Adriano Sofo [a,*], Bartolomeo Dichio [a], Cristos Xiloyannis [a], Andrea Masia [b]

[a] *Università degli Studi della Basilicata, Dipartimento di Produzione Vegetale, Campus Macchia Romana, 85100 Potenza, Italy*
[b] *Università degli Studi di Bologna, Dipartimento di Colture Arboree, viale Fanin 46, 40127 Bologna, Italy*

Received 1 July 2003; received in revised form 27 September 2003; accepted 29 September 2003

Abstract

The effects of water recovery on the activities of superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), guaiacol peroxidase (POD; EC 1.11.1.7), polyphenol oxidase (PPO; EC 1.30.3.1) and lipoxygenase (LOX; EC 1.13.11.12), and on malondialdehyde (MDA) levels were investigated in 2-year-old *Olea europaea* L. (cv. "Coratina") plants grown in environmental conditions characterized by high temperatures and irradiance levels and gradually subjected to a controlled water deficit. After reaching the maximum level of water stress, plants were subjected to a rewatering treatment for 30 days, under both environmental irradiance and semi-shade conditions. The activities of SOD, CAT, APX, POD and LOX, and MDA levels decreased during the rewatering period in both leaves and roots and these decrements were faster in plants rewatered in semi-shade conditions (SHP) than in plants under environmental light (NSHP). In contrast, PPO activity increased during rewatering in both leaf and root tissues. Thus, the lower expression of the enzymatic antioxidant system in SHP with respect to NSHP may be due to a reduced need of activated oxygen species removal. On the contrary, in NSHP, higher enzyme activities are required for a better protection against a more pronounced oxidative stress.

Keywords: Antioxidant enzymes; Lipoxygenase; Malondialdehyde; *Olea europaea*; Rewatering; Water stress

1. Introduction

The response of plants to water stress is a species- and cultivar-dependent characteristic. In Mediterranean ecosystems, in which the summer months are characterized by elevated temperatures, high irradiance levels and lack of precipitation, plants are subjected to a continuous and severe water deficit [1]. Under these adverse environmental conditions, photoinhibition, photooxidation and photorespiration occur [2,3].

Plants are particularly susceptible to photoinhibition when exposed to bright light [4–6] and environmental stresses as drought [7], chilling [8,9] and heat [10]. The synergic action of high irradiance level and water stress reduces the capacity of the photosynthetic systems to utilize incident radiation, leading to a higher degree of photodamage [2,11]. Under environmental conditions, where the photon energy is in excess of CO_2 assimilation, photosystem II (PSII) is the primary target for photoinhibition, while PSI is more stable than PSII, receiving a damage usually less significant and strictly related to the rate of electron flow from PSII and the presence of oxygen [12–14].

The limitation of CO_2 assimilation in water-stressed plants causes the over-reduction of photosynthetic electron chain. This excess of reducing power determines a redirection of photon energy into processes that favour the production of activated oxygen species (AOS), mainly in the photosynthetic [15] and mitochondrial electron transport chains [16]. Being toxic for the cells, AOS are efficiently eliminated by non-enzymatic (α-tocopherol, β-carotene, phenolic compounds, ascorbate, glutathione) and enzymatic antioxidants [17,18]. The enzymatic antioxidant system include superoxide dismutase (SOD; EC 1.15.1.1), which catalyzes the reaction from $O_2^{\bullet -}$ to H_2O_2, and catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and guaiacol peroxidase (POD; EC 1.11.1.7), which are able to detoxify the H_2O_2 produced [2].

All the enzymes mentioned above exist in plant tissues in multiple forms. The three known types of SOD are classified by their metal cofactor and are mainly located

* Corresponding author. Tel.: +39-339-1563355; fax: +39-883-489681.
E-mail address: adriano.sofo@libero.it (A. Sofo).

0168-9452/$ – see front matter © 2003 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2003.09.018

in chloroplasts, cytosol and mitochondria [12,19]. APX isozymes are generally located in chloroplasts, but microsomal, peroxisomal and membrane-bound forms, as well as soluble cytosolic and apoplastic isozymes, also exist [18]. CAT isoforms are particularly abundant in the glyoxysomes and in the peroxysomes [20]. POD have vacuolar and apoplastic forms which can use a wide range of substrates [21], including indoleacetic acid (IAA), and are so involved in auxin catabolism [22]. Polyphenol oxidase (PPO; EC 1.30.3.1), a copper enzyme located in plastids, acts on phenols in the presence of oxygen, catalysing the oxidation of *o*-diphenols into *o*-chinones [23]. When the accumulation of AOS under water stress conditions exceeds the removing capacity of the antioxidant system, the effects of oxidative damage arise, including peroxidation of membrane lipids, destruction of photosynthetic pigments and inactivation of photosynthetic enzymes [17].

Lipoxygenases (LOX; EC 1.13.11.12), located in cytosol [24], microsomes [25], plasma membrane [26] and oil bodies [27], catalyse the dioxygenation of polyunsaturated fatty acids containing a *cis*,*cis*-1,4-pentadiene moiety, producing hydroperoxy fatty acids, which are highly reactive compounds, toxic to the cell. Different LOX isozyme forms are involved in many physiological processes such as flowering [28], seed germination [29], formation of flavours and aroma in plant products [30], plant growth and development [31], pigment bleaching, senescence, pest resistance, wound stress and biosynthesis of regulatory molecules such as traumatin and jasmonic acid, which have growth-inhibitory properties analogous to those of abscisic acid [24].

Malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids hydroperoxides, has been utilized very often as a suitable biomarker for lipid peroxidation [32], which is an effect of oxidative damage. Nonetheless, lipids are not the only targets for MDA action; in fact MDA damages DNA, forming adducts to deoxyguanosine and deoxyadenosine [33].

Olive (*Olea europaea* L.) is a sclerophyll tree species with a high degree of drought tolerance [34]. In olive tree, severe drought stress causes closure of stomata [35], inhibition of photosynthesis [7] and transpiration [36], reduction of gas exchange [37] and changes in root and canopy dynamics [38,39], but little is known about the effects of water deficit on antioxidant enzyme activities and oxidative damage in this species. Olive tree is able to restore leaf water potentials (LWP) and chlorophyll fluorescence after rewatering, but the rapid recovery of tissue water status is often coupled to a non-recovery of leaf functionality that lasts several days and is correlated with the level of stress previously reached [7]. The persisting deficit in leaf gas exchange found in this species could not be imputed to the non-recovery of cell turgor but, as observed in other species, to other factors probably involving the biochemical [40,41] and hormonal balance [42,43]. A complete understanding of the biochemical and metabolic factors involved in olive tree's defence strategies against drought stress is of paramount importance to crop improvement but is, at present, lacking. The aim of this study was to investigate the influence of different levels of irradiance on antioxidant enzymes and LOX activities and on MDA level in olive tree during rewatering. We hypothesize that semi-shade conditions could minimize the oxidative effect of the damage following a period of water stress.

2. Materials and methods

2.1. Study site and experimental design

Trials were conducted on self-planted 2-year-old *Olea europaea* L. plants, cv. 'Coratina', measuring 130–150 cm in height. The study site was located at the 'Pantanello' Agricultural Experiment Station in Metaponto (southern Italy, Basilicata region—40°24′N, 16°48′E). The experimental period started on 3 July and ended on 22 August, 2001.

Olive plants grew uniformly outdoors in 0.016 m^3 containers filled with a mixture of loam, peat and sand (in proportion of 1:1:1). Pots were covered with plastic film and aluminium foil in order to avoid evaporation from the soil surface and to minimize temperature increases inside the containers. All plants were weighed each evening in order to calculate the amount of water transpired daily by each plant by means of weight difference. Soil water content was maintained at a constant value of around 85% of water-holding capacity of the pot by integrating the amount of water lost through transpiration during the day. Plants were fertilized at 25-day intervals throughout the period of vegetative growth with 3–4 g of slow-release nitrogen complex fertilizer (Nitrophoska Gold 15N − 9P − 16K + 2Ca + 7Mg; Compo Agricoltura, Cesano Maderno, MI, Italy).

The plants were initially divided in two groups: 15 water-stressed plants and 30 control plants (CP). In CP, the amount of water daily added during the whole experimental period was equal to the amount transpired. During the first 10 days of the experimental period, water-stressed plants were subjected to a gradual controlled water depletion, applying a daily reduction of 10% less than the total transpired water. In the subsequent 10 days, irrigation ceased.

After reaching the maximum level of water stress (−5.73 Mpa on July 23), measured by LWPs, water-stressed plants were divided into two groups of 15 plants each: shaded plants (SHP) and non-shaded plants (NSHP). SHP were subjected to a rewatering treatment in semi-shade conditions of about 60% of environmental photosynthetic photon flux density (PPFD) by means of a plastic shading paper film, whereas NSHP were re-irrigated in environmental PPFD conditions. The rewatering period lasted for 30 days (24 July–22 August) and the amount of water added daily to SHP and NSHP during this period was equal to the transpired amount.

We defined the 10th day from the beginning of the rewatering period as 'first level of rewatering', the 20th day as

'second level of rewatering' and the 30th day as 'third level of rewatering'.

2.2. Environmental and physiological parameters

Environmental parameters were monitored by a weather station placed within 20 m of the experimental plot. Measurements of maximum air temperature, minimum relative humidity, vapour pressure deficit and PPFD for each day of the experimental period were taken. PPFD was recorded at 1-min intervals and daily integrated values were logged.

Three plants per treatment (SHP, NSHP and CP) were randomly chosen to measure LWPs and physiological parameters at each level of rewatering using three fully expanded leaves selected from each plant along the median segment of new-growth shoots and marked at the beginning of the experiment. LWPs were measured predawn (at 04:00–05:00 h) using a Sholander pressure chamber (PMS Instrument Co. Corvallis, OR, USA), according to Turner [44].

The measurements of transpiration and photosynthetic rate, stomatal conductance and substomatal CO_2 concentration were carried out at each level of rewatering using the leaf chamber analyser LCA-4 (ADC, UK) operated at 200 $\mu mol\, m^{-2}\, s^{-1}$ flow rate, under natural climate and full sunlight conditions (at 13:00–14:00 h) and expressed on a leaf area basis.

2.3. Enzyme activities and malondialdehyde content measurements

In SHP, NSHP and CP, leaves were collected early in the morning (at 06:00–07:00 h) at each level of rewatering. Three plants having similar LWPs were selected for tissue sampling. Each sample contained 10 fully expanded leaves taken from each tree along the median segment of new-growth shoots. Roots were sampled at 06:00–07:00 h, after plant destruction, at each level of rewatering, and divided into two groups: 'thin roots' (TR, with a diameter <1 mm) and 'medium roots' (MR, with a diameter between 1 and 5 mm). Leaf and root samples were washed with distilled water, dried with filter paper, immediately frozen in liquid nitrogen and then stored at −80 °C.

Frozen tissues were finely ground in liquid nitrogen using a mortar and pestle previously chilled with liquid nitrogen, and the frozen powder was immediately used for the extraction of the different enzymes. All procedures for enzyme extraction and determination of enzyme activities were carried out at 0 °C in an ice bath unless otherwise stated.

Measurements of enzyme activities and MDA levels on extracts from each sample were repeated three times. Enzyme activities were expressed as units per milligram of dry weight. Such a basis appeared to be appropriate since the overall dry weight remained largely unaffected during drought treatment, whereas fresh weight and total soluble protein concentration were more variable (data not shown).

2.3.1. SOD

A 1.0 g aliquot of frozen powder was added to 10 ml of cold ethanol absolute for 30 min, then centrifuged at 0 °C and 10,000 × *g* for 10 min and the supernatant discarded. The ethanol extraction was repeated twice. The pellet was then resuspended in 5.0 ml of cold 100 mM sodium–potassium phosphate buffer ($NaKP_i$), pH 7.0, 0.1% (w/v) polyvinylpolypyrrolidone (PVPP), prepared and stored at 4 °C the day before and, after 30 min, centrifuged at 4 °C and 10,000 × *g* for 30 min. The supernatant was recovered and used for the enzyme activity assay.

Total SOD activity was measured after Madamanchi et al. [45]. For each sample assayed, six tubes were set up containing 10, 20, 40, 60, 80 and 500 μl of the enzyme extract. The reaction mixture contained 2 μM riboflavine, 10 μM L-methionine, 50 μM nitro blue tetrazolium (NBT), 20 μM KCN, 6.6 μM Na_2EDTA, 10–500 μl of the enzyme extract, and 65 μM $NaKP_i$, pH 7.8, to give a total volume of 3.0 ml. SOD activity was assayed by measuring the capacity of the enzyme extract to inhibit the photochemical reduction of NBT to blue formazan. Glass tubes were thermostated at 25 °C for 10 min in absence of direct light. The reaction was started by exposing the mixture to four white fluorescent lamps (Leuci, 15 WTS preheat, daylight 6500 °K) in a box (80 cm × 50 cm × 50 cm) with aluminium-foil-coated walls. Blanks were obtained with non-illuminated duplicates. The blue colour developed in the reaction was spectrophotometrically measured at 560 nm and the corresponding non-exposed samples were used as blank. The volume of sample causing 50% inhibition in colour development was taken as one unit of SOD activity.

2.3.2. APX

A 1.0 g aliquot of frozen powder was added to 10 ml of cold ethanol absolute for 30 min, then centrifuged at 0 °C and 10,000 × *g* for 10 min and the supernatant discarded. This extraction was repeated twice. The pellet was then resuspended in 10.0 ml of 50 mM potassium phosphate buffer, pH 7.8, 100 μM EDTA, 500 μM ascorbate, 0.1% (w/v) PVPP, prepared and stored at 4 °C the day before and, after 30 min, centrifuged at 4 °C and 15,000 × *g* for 30 min. The supernatant was recovered, desalted on a Sephadex™ G-25M column pre-equilibrated with grinding medium, which included 100 μM ascorbate, and used for the enzyme activity assay.

APX activity was assayed by recording spectrophotometrically the decrease in ascorbate content at 290 nm, according to Ushimaru et al. [46] with some modifications. A 1.0 ml aliquot of enzyme extract was treated with 100 μl of 10 mM H_2O_2 and 100 μl of 80 mM hydroxylamine for 5 min at room temperature. Hydroxylamine is a selective inhibitor of APX, so the 'genuine' APX activity was estimated excluding the contribution of POD activity in the extract to the oxidation of ascorbate. The same procedure, but with 200 μl of distilled water, was used to determine

total (APX + POD) activity in a second 1.0 aliquot of the same extract. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 500 μM ascorbate, 100 μM EDTA, 100 μM H_2O_2 and 100 μl of enzyme extract, in a final volume of 3.0 ml. The reaction started with the addition of H_2O_2. One unit of APX activity was defined as the amount of enzyme that oxidizes 1 μmol of ascorbate per minute at room temperature. An absorption coefficient of 2.47 mM^{-1} cm^{-1} was used for calculations.

2.3.3. CAT

A 1.0 g aliquot of frozen powder was added to 10 ml of cold ethanol absolute for 30 min and then centrifuged at 0 °C and 10,000 × *g* for 10 min, and the supernatant discarded. This extraction was repeated three times. The pellet was then resuspended in 10.0 ml of 10 mM cold $NaKP_i$, pH 7.0, 0.1% (w/v) PVPP, prepared and stored at 4 °C the day before and, after 30 min, centrifuged at 4 °C and 15,000 × *g* for 15 min. The supernatant was recovered and used for the enzyme activity assay.

CAT activity was assayed after Aebi [47]. The enzyme extract was thermostated at 20 °C for 5 min. The activity was measured in a reaction mixture (3.0 ml final volume) composed of 30 mM H_2O_2 in 50 mM $NaKP_i$, pH 7.0, and 2.0 ml of enzyme extract. Samples without H_2O_2 were used as a blank. 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 [20].

2.3.4. POD, IAAox, PPO

A 1.0 g aliquot of frozen powder was added to 10.0 ml of cold 200 mM NaP_i, pH 7.0, 5 mM Na_2EDTA, 0.1% (w/v) PVPP, 3 mM dithiothreitol, 15 mM β-mercaptoethanol, 10 mM sodium metabisulfite, prepared and stored at 4 °C the day before and, after 30 min, centrifuged at 15,000 × *g* for 30 min. The supernatant was recovered and used for the enzyme activity assay.

POD activity was measured after Chance and Maehly [48]. The reaction mixture (3.0 ml final volume) consisted of 50 μl of 10 mM guaiacol, 2.9 ml of 10 mM NaP_i, pH 7.0, 10 μl of 40 mM H_2O_2. A 40 μl aliquot of the crude enzyme extract was then added to start the reaction. The activity of the mixture was determined spectrophotometrically at 470 nm after 10 min at 20 °C.

Indoleacetate oxidase (IAAox) activity, due to POD, was spectrophotometrically measured at 247 and 254 nm, after an incubation at 30 °C for 30 min, using a reaction mixture containing 1 mM $MnCl_2$, 100 μM *p*-coumaric acid, 50 mM NaP_i, pH 7.0, and 50 μM IAA [49,50]. The reaction mixture was prepared by mixing 5 ml of 10 mM $MnCl_2$, 5 ml of 1 mM *p*-coumaric acid, 5 ml of 500 mM NaP_i, 2.5 ml of 1 mM IAA. The mixture pH was led to 4.5 with H_3PO_4, and distilled water was added to reach a final volume of 50 ml. A 30 μl aliquot of 10 mM sodium dithionite was added to 3.0 ml of the mixture in order to inhibit PPO activity, and the reaction was initiated by adding 30 μl of enzyme extract.

PPO activity was assayed according to Cañal et al. [51] with some modifications. The reaction mixture (3.0 ml final volume) consisted of 0.1 ml of 25 mM pyrogallol, 2.8 ml of 100 mM NaP_i, pH 7.0, and 100 μl of the enzyme extract. The mixture was maintained at 30 °C for 30 min and the activity was read at 420 nm.

Total activity for these enzymes was expressed as increase in absorbance per minute.

2.3.5. LOX

LOX activity was assayed following the method of Williams et al. [30] with some modifications. A 1.0 g aliquot of frozen powder was suspended in 10 ml of acetone at −20 °C for 30 min, centrifuged at 10,000 × *g* for 15 min and then the supernatant was discarded. This extraction was repeated three times. The pellet was resuspended in 10 ml of 50 mM HEPES, pH 7.2, containing 3 mM dithiothreitol 10 mM MgCl and 1 mM EDTA, for 1 h, and then centrifuged at 10,000 × *g* for 10 min. The resultant supernatant was desalted on a Sephadex™ G-25M column and used for the enzyme activity assay. Linoleate hydroperoxidation activity was photometrically determined at 25 °C by monitoring the increase of A_{234}, due to the conversion of linoleate into the corresponding hydroperoxide. LOX activity was measured in a reaction mixture consisting of 120 μl of 10 mM linoleate, 1.25 ml of 100 mM NaP_i, pH 6.5, 0.1% (w/v) Tween 80 and 25 μl of enzyme extract. One unit of enzyme was defined as the quantity that generates 1 μmol of conjugate diene per minute at 25 °C.

2.3.6. MDA

MDA content was measured following the method of Du and Bramlage [52] and expressed as nmol per g of dry weight. A 0.5 g aliquot of frozen powder was added to 5.0 ml of 0.1% (w/v) trichloroacetic acid and centrifuged at 10,000 × *g* for 5 min. A 1 ml aliquot of supernatant was added to 4.0 ml of 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid. The mixture was heated at 100 °C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000 × *g* for 10 min, A_{532}, A_{600} and A_{440} of the supernatant were recorded. The value for non-specific absorption at 600 nm was subtracted and a standard curve of sucrose (from 2.5 to 10 μmol ml^{-1}) used to rectify the results from the interference of soluble sugars in samples, reading A_{532} and A_{440}. MDA content was calculated using its absorption coefficient of 157 $mmol^{-1}$ cm^{-1} and expressed as nmol MDA g^{-1} (DW).

2.4. Statistical analysis

The values of physiological parameters were represented as means of nine measurements (±SE) from three selected plants (three measurements per plant). The values of enzyme activities and MDA level were expressed as means of

three measurements (±SE) from three plants having a similar level of water stress (one measurement per plant and three replications of each measurement).

3. Results

3.1. Environmental conditions and physiological parameters

The highest value of maximum temperature was 37.5 °C on 7/29 and the mean of all the daily maximum values was 32.6 °C; RH pattern showed the highest value (44.4%) on 8/22, with a mean of 29.2%; VPD range was between 2.2 (on 7/5) and 5.0 kPa (on 8/11), with a mean value of 3.6 kPa; PPFD level showed a slight decrement during the experimental period, especially in the last 3 days of the trial (Fig. 1).

The mean predawn LWP in CP was −0.34 MPa. LWP in SHP increased during the whole rewatering period, starting from −5.89 MPa and subsequently reaching a maximum of −0.36 MPa at the third level of rewatering. The trend of LWP in NSHP was similar to that of SHP, showing an increase of LWP values from −5.84 to −0.39 MPa during the whole rewatering period. At the first level of rewatering, both SHP and NSHP recovered LWPs almost completely (Fig. 2A).

SHP showed an increase of net photosynthesis rate from 0.33, at the beginning of the experiment, to 15.04 μmol

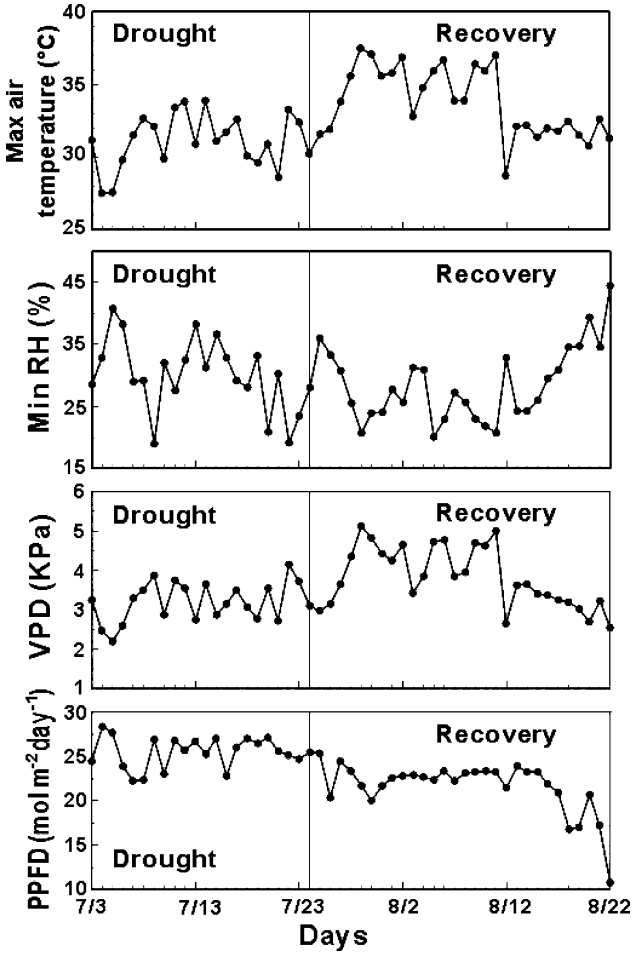

Fig. 1. Maximum air temperatures, minimum relative humidity (RH), vapour pressure deficit (VPD) and photosynthetic photon flux density (PPFD) at the field site during the water stress period (values before vertical line) and during the rewatering period (values after vertical line).

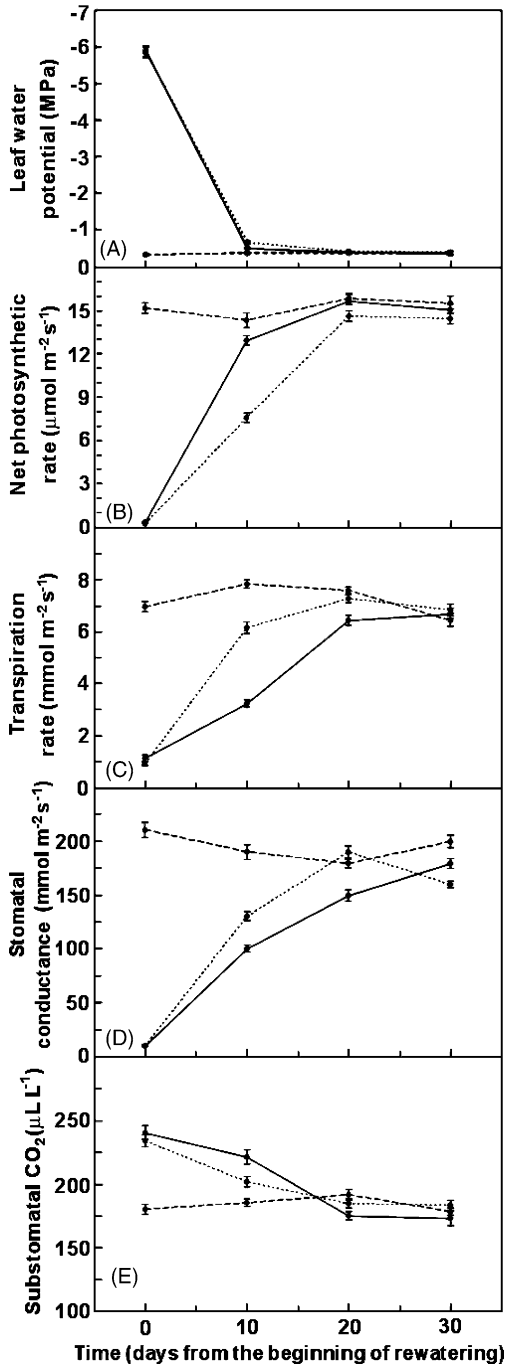

Fig. 2. Trends of leaf water potential (A) net photosynthesis (B), transpiration (C), stomatal conductance (D) and substomatal CO_2 concentration (E) in shaded (continuous line), non-shaded (dotted line) and control plants (dashed line) at 0, 10, 20 and 30 days from the beginning of the rewatering period. Each data point represents the mean of nine measurements (±S.E.).

$m^{-2}\,s^{-1}$, at the third level of rewatering. These values are comparable to those of NSHP, with the difference that SHP had a faster recovery of net photosynthesis than NSHP: at the first level of rewatering, SHP showed net photosynthesis rates similar to those in CP, whereas NSHP values were lower than those of CP. After 20 days of rewatering, NSHP recovered almost completely the photosynthetic functionality (Fig. 2B). Transpiration rate patterns of SHP and NSHP were different (Fig. 2C). NSHP recovered transpiration faster than SHP: at the first level of rewatering, transpiration rate in NSHP was 79% of that in CP, whereas transpiration rate in SHP was 41% of the value in CP; at the second level of rewatering, transpiration values in SHP were still lower than those of NSHP. SHP completely recovered the transpirative functionality after 30 days from the beginning of trials. Trends of stomatal conductance in SHP and NSHP ran in parallel, increasing at the first and second level of rewatering, and differed only at the third level of rewatering, when NSHP showed a slight decrease (Fig. 2D). In particular, stomatal conductance values in SHP increased gradually from 10 mmol $m^{-2}\,s^{-1}$, at the beginning of the experiment, to 180 mmol $m^{-2}\,s^{-1}$, at the third level of rewatering, while the corresponding values in NSHP were 10 and 160 mmol $m^{-2}\,s^{-1}$, respectively. In both SHP and NSHP, substomatal CO_2 concentration decreased during the whole rewatering period, showing a faster recovery in NSHP than that in SHP: at the first level of rewatering, NSHP presented a CO_2 substomatal concentration of 201.5 $\mu l\,l^{-1}$, whereas SHP a value of 221.3 $\mu l\,l^{-1}$ (109 and 119% of CP values, respectively) (Fig. 2E). In CP, net photosynthetic rate, transpiration rate, stomatal conductance and substomatal CO_2 concentration remained relatively stable during the 30-day rewatering period.

3.2. Activities of antioxidant enzymes

The variations in the activities of all the evaluated enzymes were related to the progression of the rewatering period and to the plant tissues analysed (Fig. 3).

In SHP, SOD activity started from high values at the start of the experiment and subsequently decreased during the whole rewatering period in all the tissues, reaching the levels of CP at the third level of rewatering (Fig. 3A). In NSHP, SOD activity showed a similar trend, though both leaves and roots showed values higher than those of SHP in all the levels of rewatering.

The values of APX activity decreased in all the tissues of SHP during rewatering, whereas the decrease in NSHP started from the second level of rewatering (Fig. 3B). NSHP maintained high values of APX activity during the first level of rewatering in comparison with SHP (196, 173 and 141% in leaves, TR and MR, respectively).

CAT activity changed in both SHP and NSHP during the rewatering period (Fig. 3C). In particular, at the first level of rewatering, CAT activity increased slightly in both leaves and roots of SHP and NSHP; subsequently, CAT activity began to decline as from the second level of rewatering in all the tissues studied. The decrease of CAT activity in SHP was slower than that in NSHP and, at the third level of rewatering, CAT activities in SHP were higher in comparison to NSHP (156, 114 and 119% of the values recorded in leaves, TR and MR).

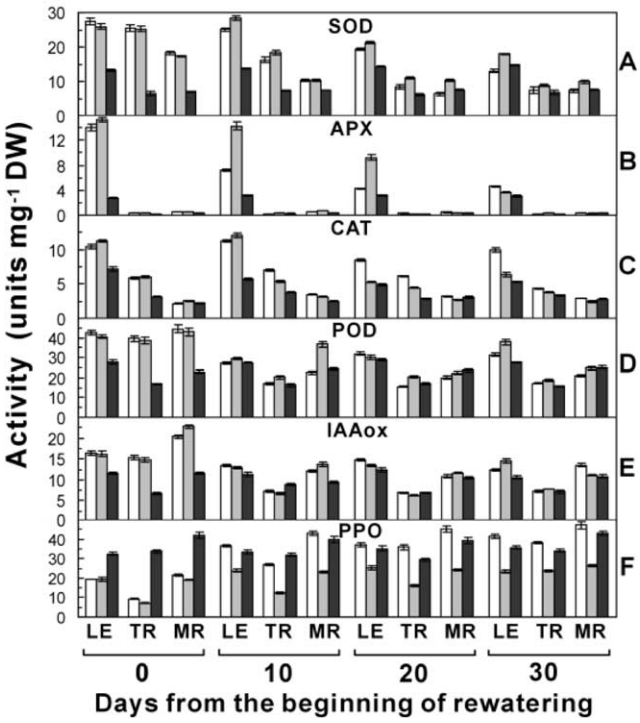

Fig. 3. Activities of superoxide dismutase (A), ascorbate peroxidase (B), catalase (C), guaiacol peroxidase (D), indolacetate oxidase (E) and polyphenol oxidase (F) in leaves (LE), thin roots (TR) and medium roots (MR) from shaded (white), non-shaded (grey) and control plants (black). Samples were collected at 06:00–07:00 h at 0, 10, 20 and 30 days from the beginning of the rewatering period. Each value represents the mean of three measurements (±S.E.).

The patterns of POD activity and POD-dependent IAAox activity ran in parallel (Fig. 3D and E). In all the tissues of SHP and NSHP, both the activities showed a strong decrease with effect from the first level of rewatering (99, 106 and 93% of CP values for POD activity, and 119, 86 and 129% of CP values for IAAox activity in leaves, TR and MR of SHP, respectively). The values of POD and IAAox activities in leaves and TR of NSHP at the third level of rewatering differed from those in SHP, showing a further increase.

PPO activities appeared to be directly related to rewatering in all the tissues studied (Fig. 4E). Both SHP and NSHP showed a marked increase of PPO activity during the whole rewatering period, but a total recovery of enzyme activity occurred at the third level of rewatering in SHP (116, 112 and 110% of CP values in leaves, TR and MR of SHP, respectively), whereas NSHP maintained values lower than those of CP (65, 70 and 61% of CP values in leaves, TR and MR).

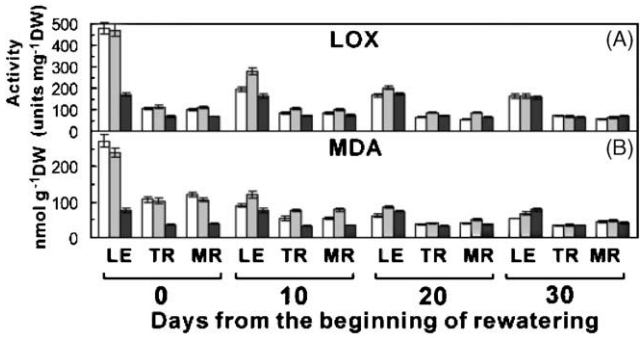

Fig. 4. Lipoxygenase activity and malondialdehyde content in leaves (LE), thin roots (TR) and medium roots (MR) from shaded (white), non-shaded (grey) and control plants (black). Samples were collected at 06:00–07:00 h at 0, 10, 20 and 30 days from the beginning of the rewatering period. Each value represents the mean of three measurements (±S.E.).

In CP, all the activities of the enzymes described above did not show marked changes during the 30-day rewatering period (Fig. 3).

3.3. LOX activity and malondialdehyde content

Progressive rewatering caused marked decrease in LOX activity in both SHP and NSHP (Fig. 4A). The decrease in LOX activity was faster in SHP than in NSHP: at the first level of rewatering, the values of LOX activity in SHP were comparable to those in CP (120, 114 and 119% in leaves, TR and MR, respectively), while the values present in NSHP at the same rewatering level were higher than those of CP (171, 145 and 137% in leaves, TR and MR, respectively).

In both SHP and NSHP, MDA levels declined in relation to the level of rewatering in all the tissues examined (Fig. 4B). MDA levels in leaves, TR and MR of SHP at the first level of rewatering were 75, 74 and 68%, respectively, of those present in NSHP; less marked differences between SHP and NSHP were detected in tissues collected after the second level of rewatering. Leaves of SHP, at the second and third level of rewatering, showed MDA levels lower than those of CP (83 and 68%, respectively).

CP did not show any marked changes in LOX activity and MDA concentration during all the rewatering period.

4. Discussion

Rewatering treatment following the water stress phase produced a rapid increase in LWP both in SHP and NSHP paralleled by slower increases in photosynthetic gas exchange (Fig. 2B–E). The delay in recovery of photosynthetic rate during rewatering, more marked in NSHP, is probably due to a light-dependent inactivation of the primary photochemistry associated with PSII occurred during the previous water stress [7] or to the downregulation of electron transport, as recently underlined by Nogués and Baker [1].

The rapid increase in transpiration rate (Fig. 2C) was paralleled by the surge in stomatal conductance (Fig. 2D). The delay in transpiration recovery observed in SHP with respect to NSHP could be due to the slower recovery of full stomatal conductance for SHP likely depending from a greater inability to refill the vessels cavitated during the water stress period [53]. It has to be outlined that plants growing in shade conditions generally present less lignified tissues and therefore are more prone to vessel collapse. Moreover, plants growing in shade tend to etiolate and present less lignified tissues in comparison with NSHP.

The evolution of stomatal conductance showed that both SHP and NSHP recovered slowly after the water stress period. This result is in contrast with that obtained by Fernández et al. [35], which observed a rapid recovery of stomatal conductance during rewatering—though on 26-year-old water-stressed olive plants (cv. Manzanillo) with rainfall as their only source of water supply. This discrepancy may be related to many different factors such as the different tree ages, the surely different root system architecture (pot versus field conditions), and to a lower level of tissue lignification in young pot-grown trees when compared with old field-grown ones.

Our results show that the activities of SOD, CAT, APX, POD and LOX decreased during the rewatering phase in all the tissues tested, while PPO is the only enzyme clearly induced by rewatering treatment (Fig. 3). The different values of enzyme activities among tissues of SHP and NSHP point out the key role played by light intensity in the response of plants during rewatering. In general, SHP showed lower activity values for SOD, APX and LOX, and lower MDA levels than those present in NSHP, especially at the first level of rewatering.

Since SOD and APX are the main antioxidant enzymes of chloroplasts [19,21], the more marked reduction of SOD and APX activities in SHP with respect to that in NSHP at the same rewatering level (Fig. 3A and B) suggests that lower PPFD levels induce a different response of olive tree to oxidative stress because in semi-shade conditions the need of antioxidant defences is reduced. Our data highlight that the response of NSHP during the rewatering phase involves the ability to maintain high values of SOD and APX activities. This allows the cell apparatus to exhibit a better response to oxidative stress due to AOS action. Since the values of transpiration rate and stomatal conductance found in SHP are lower than those in NSHP in the first two levels of rewatering, the higher photosynthetic rate in SHP could be due to a lower degree of photoinhibition and not to stomatal regulation.

Our results show that CAT activity was affected by different PPFD levels (Fig. 3C): in fact, in both SHP and NSHP, rewatering caused marked decreases in CAT activity, but the reduction in NSHP was faster, probably because CAT is very susceptible to photoinactivation and degradation under high levels of irradiance [54]. Since CAT is mainly confined to microbodies, and particularly in peroxisomes [20], a decrease in its activity could be considered as an indirect evidence of a reduced photorespiration, also confirmed by

the pattern of substomatal CO_2 concentration in SHP and NSHP.

Similarly to the enzymes described above, decreases in POD and IAAox activities under rewatering occurred in both SHP and NSHP (Fig. 3D and E). The results show that POD and IAAox activities were scarcely influenced by the PPFD levels during rewatering, except for POD activity in TR and MR, in which NSHP values were higher than those of SHP at all the levels of rewatering. Different POD isoforms have a higher affinity for H_2O_2 if compared with CAT but require some phenolic compounds (e.g. guaiacol) as substrates [21]. They are involved in lignin biosynthesis and in the modulation of cell wall properties during plant growth [55,56]. Moreover, POD catalyses the oxidation of IAA, a plant hormone that also acts as a growth-regulatory molecule, causing the increase of POD activity and hence of lignin deposition in cell wall [57]. Therefore, the increased total POD activity during water stress contributes to the restriction in cell expansion, which in turn causes growth limitation of shoots, observed by Dichio et al. [39], and changes in root dynamics [38]. In this case, the decrease of POD activity during the rewatering phase following a drought period could allow plants the renewal of growth, in particular in SHP, whose POD activity values in roots are lower than those of NSHP.

In contrast to the other enzymes studied, an increment in PPO activity was detected in plants during the rewatering phase (Fig. 3F). This increase was more marked in SHP than in NSHP likely related to a lower lignification level of tissues present in shadow-grown plants. Since PPO has a main proteolytic activity [23], its increase during rewatering, particularly evident in SHP, allows the olive plants to remove the proteins damaged by AOS during water stress and repair the cell wall by means of cross-linking of matrix polymers [58]. Moreover, PPO activity can regulate the redox state of phenolic compounds. Phenolics are very abundant in olive tree leaves [59,60]: they are physiologically active secondary compounds with a non-enzymatic antioxidant action and are involved in many different physiological aspects such as auxin protection or its catabolism [61,62], in the modulation of the cell wall plasticity [58] and in gas exchange dynamics [63].

The tissues studied showed different dynamics of enzyme activities: leaves presented more marked changes, due to the synergic effect of irradiance level and loss of cellular water; TR appeared to be more sensitive to water recovery and its consequent effects, while MR showed less reactivity. These different patterns confirm the different function of TR, involved in water absorption and thus more reactive to water content changes, and MR, with a higher degree of lignification, in which the carbohydrates produced in leaves are stored.

Our results about LOX activity and MDA levels pointed out a limited oxidative damage, when measured in terms of lipid peroxidation, in SHP with respect to NSHP (Fig. 4). The enhanced production of AOS during water stress may activate specific LOX isozymes [28], and this effect is more pronounced in olive plants subjected to environmental PPFD level. Although olive trees are very resistant to water stress [35,37,39], the observed loss of photosynthetic functionality could be due to AOS-induced lipid peroxidation. This hypothesis is confirmed by the greater accumulation of MDA and the higher LOX activity in water-stressed plants [64]. The observed decrement of MDA concentration in SHP and NSHP (Fig. 4B) indicates that during water recovery, following a water-deficit period, repairing mechanisms start to keep pace with oxidative damage. The changes in lipid peroxidation observed herein are in agreement with the results of other studies [32,65] and support the hypothesis that rewatering after a severe water deficit can reduce membrane-lipid peroxidation.

The results obtained in this study confirm that light intensity plays a key role in the functionality of olive plants subjected to water stress conditions, as underlined by Schansker and van Rensen [5] and Hideg and Murata [6]. The repairing of damage due to oxidative stress, generated by drought stress and high irradiance levels, was associated with a different antioxidant response in plants grown in semi-shade conditions or under environmental irradiance. We can affirm that SHP faced a lower oxidative stress with respect to NSHP and that low levels of PPFD allowed an efficacious antioxidant protection. This was confirmed by the lower MDA levels and LOX activity in SHP. We can therefore assert that the observed reduction of photosynthetic functionality is accentuated when the synergic action of water deficit and high light intensity occurs.

Our data highlight that the lower expression of the enzymatic antioxidant system in SHP with respect to NSHP could be due to a reduced need for AOS removal. On the contrary, in NSHP, a high stress level induces an impairment on the equilibrium between AOS production and cellular defence machinery, with a greater AOS formation, and consequently a higher degree of protection against oxidative damage is required. Given that photoinhibition is a function of both photodamage and repair [14], research into variations in the activities of other antioxidant molecules and measurements of electrolyte leakage and photoinhibition may give a more exhaustive picture of the response of olive trees against water stress.

Acknowledgements

We thank Dr. Giuseppe Montanaro for his help in LCA-4 use and Prof. Elvira Di Nardo for her contribution in statistical analysis. We are grateful to Antonio Ditaranto and Angelo Mossuto for technical assistance.

References

[1] S. Nogués, N.R. Baker, Effects of drought on photosynthesis in Mediterranean plants grown under enhanced UV-B radiation, J. Exp. Bot. 51 (348) (2000) 1309–1317.

[2] C.H. Foyer, M. Lelandais, K.J. Kunert, Photooxidative stress in plants, Physiol. Plant 92 (1994) 696–717.
[3] B. Osmond, M. Badger, K. Maxwell, O. Björkman, R. Leegood, Too many photons: photorespiration, photoinhibition and photooxidation, Trends Plant Sci. 2 (4) (1997) 119–121.
[4] D.H. Greer, W.A. Laing, Photoinhibition of photosynthesis in intact kiwifruit (*Actinidia deliciosa*) leaves. Changes in susceptibility to photoinhibition and recovery during the growth season, Planta 186 (1992) 418–425.
[5] G. Schansker, J.J.S. van Rensen, Mechanism of photoinhibition in pea thylakoids: effects of irradiance level and pH, Bioch. Biophys. Acta 1276 (1996) 239–245.
[6] E. Hideg, N. Murata, The irreversible photoinhibition of the photosystem II complex in leaves of *Vicia faba* under strong light, Plant Sci. 130 (1997) 151–158.
[7] K. Angelopoulos, B. Dichio, C. Xiloyannis, Inhibition of photosynthesis in olive trees (*Olea europaea* L.) during water stress and rewatering, J. Exp. Bot. 47 (301) (1996) 1093–1100.
[8] K.A. Solhaug, J. Haugen, Seasonal variation of photoinhibition of photosynthesis in bark from *Populus tremula* L., Photosynthetica 35 (3) (1998) 411–417.
[9] F.C. Lidon, G. Ribeiro, H. Santana, H. Marques, K. Correia, S. Gouveia, Photoinhibition in chilling stressed leguminosae: comparison of *Vicia faba* and *Pisum sativum*, Photosynthetica 39 (1) (2001) 17–22.
[10] R.D. Law, S.J. Crafts-Brandner, Inhibition and acclimation of photosynthesis to heat stress is closely correlated with activation of ribulose-1,5-bisphosphate carboxylase/oxygenase, Plant Physiol. 120 (1999) 173–181.
[11] A. Wingler, P.J. Lea, W.P. Quick, R.C. Leegood, Photorespiration: metabolic pathways and their role in stress protection, Philos. Trans. R. Soc. London B 355 (2000) 1517–1529.
[12] C. Bowler, M. Van Montagu, D. Inzé, Superoxide dismutase and stress tolerance, Annu. Rev. Plant Physiol. Plant Mol. Biol. 43 (1992) 83–116.
[13] J.M. Anderson, Y. Park, W.S. Chow, Unifying model for the photoinactivation of photosystem II in vivo under steady-state photosynthesis, Photosynth. Res. 56 (1998) 1–13.
[14] A. Melis, Photosystem-II damage and repair cycle in chloroplasts: what modulates the rate of photodamage in vivo? Trends Plant Sci. 4 (1999) 130–135.
[15] K. Asada, The water–water cycle in chloroplasts scavenging of active oxygens and dissipation of excess photons, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 601–639.
[16] I.M. Møller, Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species, Annu. Rev. Plant Physiol. Plant Mol. Biol. 52 (2001) 561–591.
[17] N. Smirnoff, The role of active oxygen in the response to water deficit and desiccation, New Phytol. 125 (1993) 27–58.
[18] G. Noctor, C.H. Foyer, Ascorbate and glutathione: keeping active oxygen under control, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 249–279.
[19] R.G. Alscher, N. Erturk, L.S. Heath, Role of superoxide dismutases (SODs) in controlling oxidative stress in plants, J. Exp. Bot. 53 (372) (2002) 1331–1341.
[20] E.A. Havir, N.A. McHale, Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves, Plant Physiol. 84 (1987) 450–455.
[21] H. Mehlhorn, M. Lelandais, H.G. Korth, C.H. Foyer, Ascorbate is the natural substrate for plant peroxidases, FEBS Lett. 378 (1996) 203–206.
[22] H. Shinshi, M. Noguchi, Relationship between peroxidase, IAA oxidase and polyphenol oxidase, Phytochemistry 14 (1975) 1255–1258.
[23] T. Kuwabara, Y. Katoh, Involvement of the binuclear copper site in the proteolytic activity of polyphenol oxidase, Plant Cell Physiol. 40 (10) (1999) 1029–1035.
[24] J.N. Siedow, Plant lipoxygenase: structure and function, Annu. Rev. Plant Physiol. Plant Mol. Biol. 42 (1991) 145–188.
[25] I. Feussner, H. Kindl, Particulate and soluble lipoxygenase isozymes. Comparison of molecular and enzymatic properties, Planta 194 (1994) 22–28.
[26] F. Macri, E. Braidot, E. Petrusa, A. Vianello, Lipoxygenase activity associated to isolated soybean plasma membranes, Biochim. Biophys. Acta 1215 (1994) 109–114.
[27] M.P. Rodríguez-Rosales, L. Kerkeb, N. Ferrol, J.P. Donaire, Lipoxygenase activity and lipid composition of cotyledons and oil bodies of two sunflower hybrids, Plant Physiol. Biochem. 36 (4) (1998) 285–291.
[28] Z. Ye, R. Rodriguez, A. Tran, H. Hoang, D. de los Santos, S. Brown, L. Vellanoweth, The developmental transition to flowering repress ascorbate peroxidase activity and induces enzymatic lipid peroxidation in leaf tissue in *Arabidopsis thaliana*, Plant Sci. 158 (2000) 115–127.
[29] Y. Suzuki, U. Matsukura, Lipoxygenase activity in maturing and germinating rice seeds with and without lipoxygenase-3 in mature seeds, Plant Sci. 125 (1997) 119–126.
[30] M. Williams, J.J. Salas, J. Sanchez, J.L. Harwood, Lipoxygenase pathway in olive callus cultures (*Olea europaea*), Phytochemistry 53 (2000) 13–19.
[31] D.F. Hildebrand, G.B. Versluys, G.B. Collins, Changes in lipoxygenase isozyme levels during soybean embryo development, Plant Sci. 75 (1991) 1–8.
[32] C. Bailly, A. Benamar, F. Corbineau, D. Dôme, Changes in malondialdehyde content and in superoxide dismutase, catalase and glutathione reductase activities in sunflower seed as related to deterioration during accelerated aging, Physiol. Plant 97 (1996) 104–110.
[33] L.J. Marnett, Lipid peroxidation—DNA damage by malondialdehyde, Mutat. Res. 424 (1999) 83–95.
[34] A.M. Lo Gullo, S. Salleo, Different strategies of drought resistance in three Mediterranean sclerophyllous trees growing in the same environmental conditions, New Phytol. 108 (1988) 267–276.
[35] J.E. Fernández, F. Moreno, I.F. Girón, O.M. Blázquez, Stomatal control of water use in olive tree leaves, Plant Soil 190 (1997) 179–192.
[36] A. Moriana, F.J. Villalobos, E. Fereres, Stomatal and photosynthetic responses of olive (*Olea europaea* L.) leaves to water deficits, Plant Cell Environ. 25 (2002) 395–405.
[37] C. Xiloyannis, B. Pezzarossa, J. Jorba, P. Angelici, Effects on soil water content on gas exchange in olive trees, Adv. Hort. Sci. 2 (1988) 58–63.
[38] J.E. Fernández, F. Moreno, J. Martin-Aranda, E. Fereres, Olive-tree root dynamics under different soil water regimes, Agricoltura Mediterranea 122 (1992) 225–235.
[39] B. Dichio, M. Romano, V. Nuzzo, C. Xiloyannis, Soil water availability and relationship between canopy and roots in young olive trees (cv Coratina), Acta Hortic. 586 (2002) 255–258.
[40] E.A. Bray, Molecular responses to water deficit, Plant Physiol. 103 (1993) 1035–1040.
[41] K. Shinozaki, K. Yamaguchi-Shinozaki, Gene expression and signal transduction in water-stress response, Plant Physiol. 115 (1997) 327–334.
[42] P.K. Busk, M. Pagès, Regulation of abscisic acid-induced transcription, Plant Mol. Biol. 37 (1998) 425–435.
[43] J. Leung, J. Giraudat, Abscisic acid signal transduction, Annu. Rev. Physiol. Plant Mol. Biol. 49 (1998) 199–222.
[44] N.C. Turner, Techniques and experimental approaches for the measurement of plant water status, Plant Soil 58 (1981) 339–366.
[45] N.R. Madamanchi, J.L. Donahue, C.L. Cramer, R.G. Alscher, K. Pedersen, Differential response of Cu, Zn superoxide dismutases in two pea cultivars during a short-term exposure to sulfur dioxide, Plant Mol. Biol. 26 (1994) 95–103.
[46] T. Ushimaru, Y. Maki, S. Sano, K. Koshiba, K. Asada, H. Tsuji, Induction of enzymes involved in the ascorbate-dependent antioxidative system, namely, ascorbate peroxidase, monodehydroascorbate reductase and dehydroascorbate reductase, after exposure to air of rice

(*Oriza sativa*) seedlings germinated under water, Plant Cell Physiol. 38 (5) (1997) 541–549.
[47] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121–126.
[48] B. Chance, A.C. Maehly, Assay of catalases and peroxidases, Methods Enzymol. 2 (1955) 764–775.
[49] P.H. Rubery, Studies on indole acetic acid oxidation by liquid medium from crown gall tissue culture cells: the role of malic acid and related compounds, Biochim. Biophys. Acta 261 (1972) 21–34.
[50] J. Ricard, D. Job, Reaction mechanisms of indole-3-acetate degradation by peroxidases (a stopped-flow and low-temperature spectroscopic study), Eur. J. Biochem. 44 (1974) 359–374.
[51] M.J. Cañal, R.S. Tamés, B. Fernández, Peroxidase and polyphenol oxidase activities in *Cyperus esculentus* leaves following glyphosate applications, Physiol. Plant 74 (1988) 125–130.
[52] D. Du, W.J. Bramlage, Modified thiobarbituric acid assay for measuring lipid oxidation in sugar-rich plant tissue extracts, J. Agric. Food Chem. 40 (1992) 1566–1570.
[53] F. Moreno, J.E. Fernández, B.E. Clothier, S.R. Green, Transpiration and root water uptake by olive trees, Plant Soil 184 (1996) 85–96.
[54] W. Shang, J. Feierabend, Dependence of catalase photoinactivation in rye leaves on light intensity and quality and characterization of a chloroplast-mediated inactivation in red light, Photosynth. Res. 59 (1999) 210–213.
[55] T.M. Lee, Y.H. Lin, Changes in soluble and cell-wall-bound peroxidase activities with growth in anoxia-treated rice (*Oryza sativa* L.) coleoptiles and roots, Plant Sci. 106 (1995) 1–7.
[56] M.A. Bacon, D.S. Thompson, W.J. Davies, Can cell wall peroxidase activity explain the leaf growth response of *Lolium temulentum* L. during drought? J. Exp. Bot. 48 (317) (1997) 2075–2085.
[57] F. Sitbon, S. Hennion, C.H.A. Little, B. Sundberg, Enhanced ethylene production and peroxidase activity in IAA-overproducing transgenic tobacco plants is associated with increased lignin content and altered lignin composition, Plant Sci. 141 (1999) 165–173.
[58] S.C. Fry, Cross-linking of matrix polymers in the growing cell walls of angiosperms, Annu. Rev. Plant Physiol. 37 (1986) 165–186.
[59] B. Le Totour, D. Guedon, Antioxidative activities of *Olea europaea* leaves and related phenolic compounds, Phytochemistry 31 (4) (1992) 1173–1178.
[60] D. Ryan, M. Antolovich, P. Prenzler, K. Robards, S. Lavee, Biotransformation of phenolic compounds in *Olea europaea* L., Sci. Hortic. 92 (2002) 147–176.
[61] O. Faivre-Rampant, C. Kevers, T. Gaspar, IAA-oxidase activity and auxin protectors in nonrooting, *rac*, mutant shoots of tobacco in vitro, Plant Sci. 153 (2000) 73–80.
[62] M. Hrubcová, M. Cvikrová, J. Eder, J. Zoñ, I. Macháèková, Effect of inhibition of phenylpropanoid biosynthesis on peroxidase and IAA-oxidase activities and auxin content in alfalfa suspension cultures, Plant Physiol. Biochem. 38 (2000) 949–956.
[63] S. Purohit, M.M. Laloraya, S. Bhartu, Effect of phenolic compounds on abscisic acid-induced stomatal movement: structure–activity relationship, Physiol. Plant 81 (1991) 79–82.
[64] A.L.S. Lima, F.M. DaMatta, H.A. Pinheiro, M.R. Totola, M.E. Loureiro, Photochemical responses and oxidative stress in two clones of *Coffea canephora* under water deficit conditions, Environ. Exp. Bot. 47 (2002) 239–247.
[65] C.G.S. Queiroz, A. Alonso, M. Mares-Guia, A.C. Magalhães, Chilling-induced changes in membrane fluidity and antioxidant enzyme activities in *Coffea arabica* L. roots, Biol. Plant 41 (3) (1998) 403–413.