Antioxidant defences in olive trees during drought stress: changes in activity of some antioxidant enzymes

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Abstract. The effects of drought on the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (POD), indoleacetate oxidase (IAAox) and polyphenol oxidase (PPO) were studied in 2-year old *Olea europaea* L. (cv. 'Coratina') plants grown under high temperatures and irradiance levels and gradually subjected to a controlled water deficit. After 20 d without irrigation, mean predawn leaf water potential fell from -0.37 to -5.37 MPa, and decreases in net photosynthesis and transpiration occurred. The activities of SOD, APX, CAT and POD increased in relation to the severity of drought stress in both leaves and roots. In particular, a marked increase in APX activity was found in leaves of plants at severe drought stress. CAT activity increased during severe water deficit conditions in leaves and fine roots. The patterns of POD and IAA oxidase activity ran in parallel and showed increases in relation to the degree of drought. In contrast, PPO activity decreased during the progression of stress in all the tissues studied. The results show that the ability of olive trees to up-regulate the enzymatic antioxidant system might be an important attribute linked to drought tolerance. This could limit cellular damage caused by active oxygen species during water deficit.

Keywords: ascorbate peroxidase, catalase, guaiacol peroxidase, indoleacetate oxidase, polyphenol oxidase, superoxide dismutase.

Introduction

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 levels (Ingram and Bartels 1996).

Very low water contents, resulting from severe dehydration, are often associated with increased levels of activated oxygen species (AOS), such as superoxide anion $(O_2^{\bullet-})$, hydrogen peroxide (H_2O_2), hydroxyl radical $(HO^{\bullet})$ and singlet oxygen $(^1O_2)$, which, in turn, damage cellular structures and macromolecules (Smirnoff 1993), 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). The enzymatic system includes superoxide dismutases (SOD; EC 1.15.1.1), which catalyse 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, oxidise *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).

Superoxide dismutases and APXs 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 the chloroplast (Asada 1999). Catalases — 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. Guaiacol peroxidases 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, acting also as IAA oxidase (IAAox) (Shinshi and Noguchi 1975), while 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).

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). 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 1993).

The olive tree (*Olea europea* L.) is one of the most typical and economically important tree culture species belonging to the Mediterranean basin, where water shortage occurs with regularity, and often lasts throughout the spring–summer period. This evergreen, sclerophyllous tree shows a high degree of drought tolerance (Lo Gullo and Salleo 1988), a conservative consumption of soil water (Moreno *et al.* 1996) and a higher ratio of transpiration rate to leaf surface area compared with other fruit tree species in both ideal and in water shortage conditions (Tombesi *et al.* 1986; Nogués and Baker 2000). Olive trees are able to resist drought stress by lowering the water content and water potentials of their tissues, establishing a high water potential gradient between leaves and roots. During water deficit conditions, olive trees stop shoot growth and reduce transpirative and photosynthesis-related processes (Xiloyannis *et al.* 1988; Angelopoulos *et al.* 1996). This allows the continued production of assimilates as well as their accumulation in the various plant parts, in particular in the root system, creating a higher root / leaf ratio compared with well-watered plants (Fernández *et al.* 1992).

The response of olive plants to drought stress is a well-documented process, but most studies have focused on its physiological aspects (Xiloyannis *et al.* 1988; Angelopoulos *et al.* 1996; Fernández *et al.* 1997; Moriana *et al.* 2002). The aim of this work was to study the variations of antioxidant enzyme activities (SOD, CAT, APX, POD, IAAox, PPO) and to examine the physiological parameters (photosynthetic rate, transpiration rate, stomatal conductance and substomatal CO_2 level) in olive trees grown in environmental conditions characterised by high temperature and irradiance levels and with an imposed, progressive water shortage.

Materials and methods

Study site and experimental design

Trials were conducted on own-rooted 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 23 July 2001.

Olive plants grew uniformly outdoors in 0.016 m^3 containers filled with a mixture of loam, peat and sand (in a 1 : 1 : 1 ratio). Pots were covered with plastic film and aluminium foil in order to avoid evaporation from the soil surface and to minimize temperature increase inside the containers. 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 water-holding capacity of the pot by integrating the amount of water lost through transpiration during the day. Plants were fertilised at 25 d intervals throughout the period of vegetative growth with 3.5 g of slow release nitrogen complex fertiliser (Nitrophoska Gold 15 N : 9 P : 16 K + 2 Ca + 7 Mg; Compo Agricoltura, Cesano Maderno, MI, Italy).

The plants were divided in two groups: 36 drought-stressed plants (SP) and 14 control plants (CP). In CP, the amount of water added daily during the whole experimental period was equal to the amount transpired. During the first 10 d of the experimental period, SP were subjected to a gradual controlled water depletion, with a daily reduction of 10% less than the total transpired water being applied. In the subsequent 10 d, irrigation ceased. The degree of drought stress in plants was measured by means of the leaf water potentials (LWP) measured predawn. Values of LWP between –0.5 and –2.4 MPa, corresponding to 4 and 8 d from the beginning of the drought stress period, respectively, were defined as 'mild' drought stress. LWP values between –2.5 and –4.9 MPa (12 and 16 d from the beginning of the drought stress period, respectively) were described as 'moderate' drought stress, and those between –5.0 and –6.0 MPa (20 d from the beginning of the drought stress period) as 'severe' drought stress.

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 (RH), vapour pressure deficit (VPD) and photosynthetic photon flux density (PPFD) for each day of the experimental period were taken. PPFD was recorded at 1-min intervals and daily integrated values were logged.

Three CP and three SP were randomly chosen to measure physiological parameters at 0, 4, 8, 12, 16 and 20 d from the beginning of the drought stress period 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 0400–0500 h) using a Sholander pressure chamber (PMS Instrument Co. Corvallis, OR), according to the method by Turner (1981).

The measurements of transpiration and photosynthetic rate, stomatal conductance and substomatal CO_2 concentration were conducted using the portable photosynthesis system LCA-4 (ADC, Herts, UK) operated at 200 µmol m^{-2} s^{-1} flow rate, under natural temperature, relative humidity and full sunlight conditions (at 1300–1400 h) at 0, 4, 8, 12, 16 and 20 d from the beginning of the drought stress period.

Enzyme activities

Three SP plants with similar LWPs and three CP were selected in each date for tissue sampling. Leaves were collected early in the

morning (at 0600–0700 h) at 0, 4, 8, 12, 16 and 20 d from the beginning of the drought stress period. Each sample contained eight fully expanded leaves taken from each plant along the median segment of new-growth shoots. The same three SPs and CPs as previously selected were then destructively harvested in order to collect roots. Roots were sampled at 0600–0700 h at 0, 8, 16 and 20 d from the beginning of the drought stress period, and divided in two groups: 'fine roots' (FR, 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 used for extraction of the different enzymes. All procedures for enzyme extraction and determination of enzyme activities were conducted at 0°C in an ice bath unless otherwise stated. The values of enzyme activities were expressed as units per mg dry weight. A comparison between the values of enzyme activities in frozen and fresh tissue samples was conducted during the experiment.

SOD, APX, CAT pre-extraction

An aliquot (1.0 g) of frozen powder was added to 10 mL of cold absolute ethanol 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.

SOD extraction and activity

The extracted pellet was 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 according to methods by 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. The reaction was started by exposing the reaction mixture to four white fluorescent lamps (Leuci, Lecco, Italy, 15 W preheat, daylight 6500°K) in a box ($80 \times 50 \times 50$ cm) with aluminium foil-coated walls. 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 enzyme extract causing 50% inhibition in colour development was taken as one unit of SOD activity.

APX extraction and activity

The pellet was 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 (Sigma-Aldrich, Milano, Italy) 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*. (1997). APX activity was estimated excluding the contribution of POD activity in the extract to the oxidation of ascorbate. Hydroxylamine, a selective inhibitor of APX, was used to determine the POD activity. One unit of APX activity was defined as the amount of enzyme that oxidises 1 μmol of ascorbate per min at room temperature. An absorption coefficient of 2.47 mM^{-1} cm^{-1} was used for calculations.

CAT extraction and activity

The pellet was 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 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, IAAox, PPO extraction and activity

An aliquot (1.0 g) of frozen powder was added to 10.0 mL of cold 200 mM NaP$_i$, pH 7.0, 5 mM Na$_2$EDTA, 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 according to Chance and Maehly (1955). The activity of the mixture was determined spectrophotometrically at 470 nm after 10 min at 20°C. IAAox activity, due to POD, was spectrophotometrically measured at 247 and 254 nm according to Ricard and Job (1974). PPO activity was assayed according to Cañal *et al*. (1988) by reading absorbance at 420 nm.

Total activities for POD, IAAox and PPO were expressed as increases in absorbance per minute.

Statistical analysis

The values of physiological parameters were represented as means of nine measurements ($\pm$ SE) from three selected plants (three measurements per plant), while the values of enzyme activities were expressed as means of three measurements ($\pm$ SE) from three plants having a similar level of drought stress (one measurement per plant and three replications of each measurement). Statistical analysis was performed using ANOVA. Significant differences between values of enzyme activity in CP and SP were determined at $P \leq 0.05$, according to Duncan's multiple range test.

Results

Environmental conditions and physiological parameters

The highest value of maximum air temperature was 33.8°C on 12 July and the mean of all the daily maximum values was 31.2°C. Minimum relative humidity pattern showed the highest value (40.8%) on 5 July, with a mean of 30.1%. Vapour pressure deficit range was between 2.2 (on 5 July) and 4.1 kPa (on July 22), with a mean value of 3.2 kPa. PPFD levels fluctuated within a range from 22.21 to 28.34 mol m^{-2} d^{-1} (on 7 July and 4 July, respectively) (Fig. 1). The mean value of predawn LWP in CP was –0.36 MPa. LWP values in SP declined during the whole period of water deficit, reaching a minimum of –5.73 MPa after 20 d of stress (Fig. 2).

Stressed plants showed a gradual and continuous decrease of net photosynthesis rate at the mild stress level, followed by a rapid decline at the moderate stress level to almost zero value reached at the severe stress level (Fig. 3*A*). Transpiration rate and stomatal conductance patterns in SP showed a similar trend, as both displayed a marked decrease at the mild stress level and subsequently a gradual drop during moderate and severe drought stress (Fig. 3*B–C*). Substomatal CO_2 concentration gradually increased at mild stress level, subsequently reached a plateau during moderate stress and
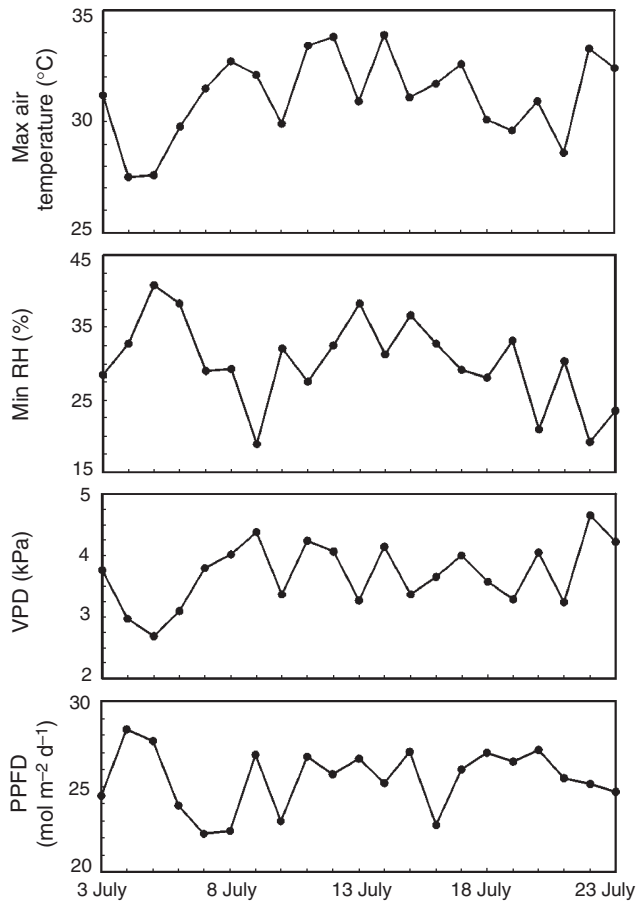
Fig. 1. Maximum air temperature, minimum relative humidity (RH), vapour pressure deficit (VPD) and daily integrated photosynthetic photon flux density (PPFD) at the field site during the experimental period.
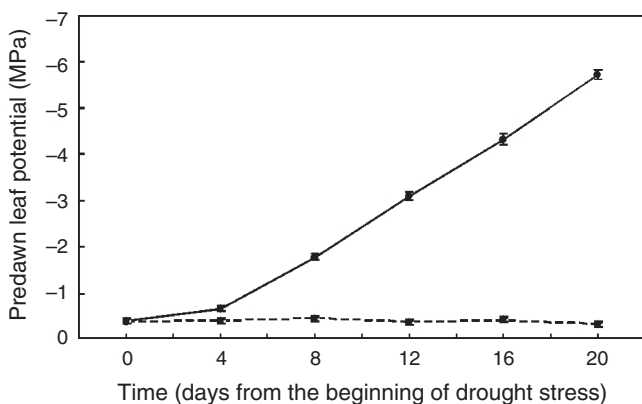

Fig. 2. Trend of leaf water potential in drought stressed (——) and irrigated (- - - -) plants, measured at 0400–0500 h, at 0, 4, 8, 12, 16 and 20 d from the beginning of the drought stress period. Each data point represents the mean of three measurements ($\pm$ SE) from three selected plants.

then slowly decreased under severe drought stress (Fig. 3*D*). Midday net photosynthetic rate, transpiration rate, stomatal conductance and substomatal CO_2 concentration of CPs remained rather stable during all the 20-d experimental period (Fig. 3).

Activities of antioxidant enzymes

SOD activity (Tables 1–3) increased significantly in relation to the degree of drought stress in all the tissues tested but subsequently decreased in leaves during the last severe drought stress phase. At the highest level of drought stress, SOD activity in leaves, FR and TR was 1.9, 3.9 and 2.5 times the CP values, respectively. The values of APX activity in leaf tissues began to rise at mild drought stress and then showed a marked increase at severe drought stress. In particular, during severe drought stress, leaves of SP presented nearly 5-fold greater APX activity than CP leaves. APX activity in roots of SP showed less marked increments and very low absolute values if compared with those in the leaves. CAT activity increased in leaves and FR during mild and moderate drought stress. Subsequently, CAT activity maintained a relatively constant value in leaves but decreased during severe drought stress in FR. In MR, CAT activity did not change significantly.

The patterns of POD activity and POD-dependent IAAox activity were roughly parallel in all the tissues examined, showing a gradual increase during the mild and moderate drought stress period. Subsequently, at severe drought stress, POD activity decreased slightly in leaves and MR, while the decline of IAAox activity was more pronounced in leaves and FR. At the highest level of drought stress, POD activity in leaves, FR and MR was 1.4, 2.3 and 1.9 times the CP values, respectively, whereas IAAox activity was 1.4, 2.2 and 2.0 times the CP values, respectively. The effect of drought stress was particularly evident when POD and IAAox activities were measured in root tissues. Generally, SOD, APX, CAT and POD were up-regulated during water deficit.

PPO activities were inversely related to drought stress levels in all the tissues studied. FR and MR showed a marked decline of PPO activity during moderate and severe drought stress, while in leaves the activity decrease was less sharp. PPO was the only enzyme clearly down-regulated by water deficit conditions.

In CP, the activities of the enzymes above mentioned did not change significantly during the 20-d experimental period with the exception of PPO in MR, which rapidly doubled activity after 8 d of drought stress and subsequently remained relatively stable. Frozen tissue samples did not differ significantly in their enzyme activities when compared with fresh samples (data not shown).

Discussion

Plants subjected to drought stress, combined with high irradiance levels and high temperature, undergo an excess of
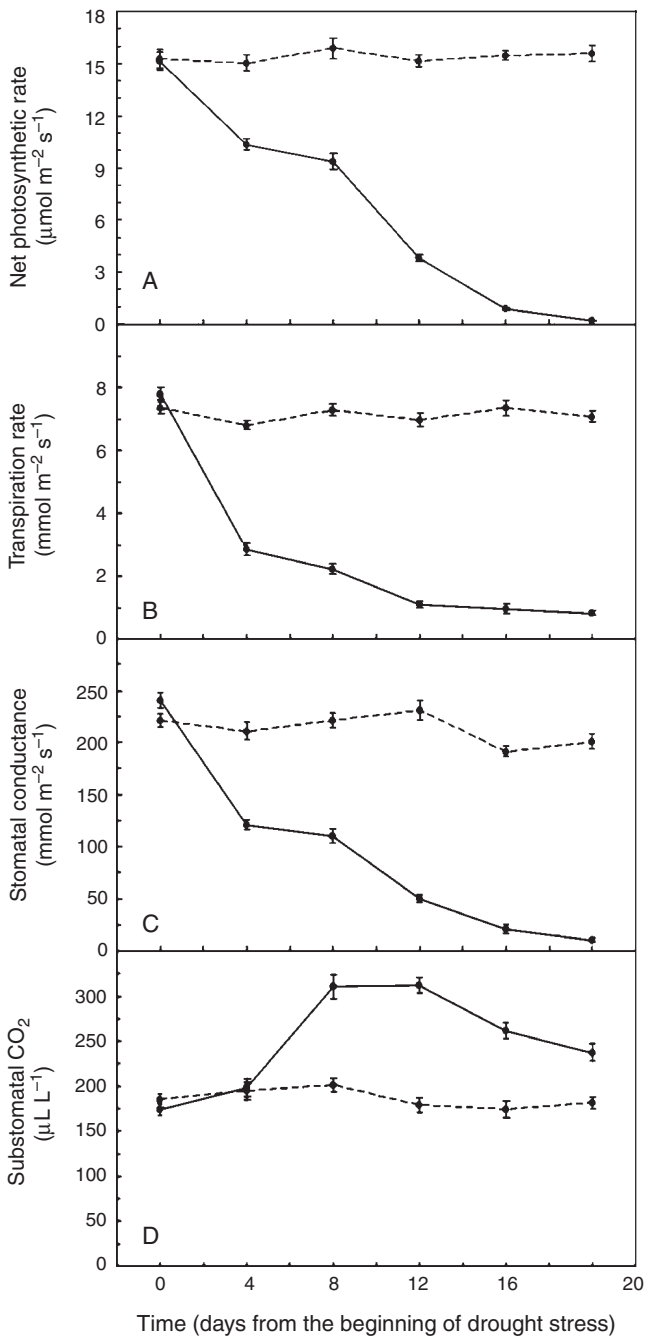
Fig. 3. Trends of net photosynthesis (*A*), transpiration (*B*), stomatal conductance (*C*) and substomatal CO$_2$ concentration (*D*) in drought stressed (——) and irrigated (- - - -) plants, measured at 1300–1400 h, at 0, 4, 8, 12, 16 and 20 d from the beginning of the drought stress period. Each data point represents the mean of three measurements (± SE) from three selected plants.

reducing power, due to limitation of CO$_2$ assimilation, which in turn causes increased levels of AOS and accumulation of free radicals (Vranová *et al.* 2002). During these stressful conditions, increased activities of AOS-scavenging enzymes might be expected. In fact, the activities of all the enzymes in

olive plants changed significantly from mild to severe drought stress and moreover were related to the plant tissue considered (Tables 1–3). Drought treatment produced a marked decrease in LWP (Fig. 2) paralleled by a substantial decrease in net photosynthetic rate (Fig. 3*A*). The rapid decrease in transpiration rate (Fig. 3*B*) was paralleled by the reduction in stomatal conductance (Fig. 3*C*). These results confirm previous findings in olive trees (Nogués and Baker 2000; Moriana *et al.* 2002).

Our results show that activities of SOD, CAT, APX and POD increased in parallel to the severity of drought stress in all the tissues examined (Tables 1–3). Drought stress causes perturbations in plant metabolism and decreases the capacity of the photosynthetic electron transport system (Asada 1999). In olive leaf, drought stress induces the inactivation of the primary photochemistry associated with PSII (Angelopoulos *et al.* 1996) and the down-regulation of PSII electron transport (Nogués and Baker 2000). This could explain the decrease in net photosynthetic rates found in SP (Fig. 3*A*).

Under water deficit conditions, the production of AOS is increased because the energy intercepted by photosynthetic pigments exceeds their capacity for thermal energy dissipation (Foyer *et al.* 1994; Tambussi *et al.* 2002). During drought, O$_2$$^{\bullet-}$ and H$_2$O$_2$, in combination or separately, induce different genes, confirming that AOS are also efficacious signal molecules during stress responses (Van Breusegem *et al.* 2001; Vranová *et al.* 2002). Moreover, O$_2$$^{\bullet-}$ and H$_2$O$_2$ are able to regulate their own level by changing the redox state of specific transcription factors (Bowler *et al.* 1992). Therefore, the increase in AOS concentration likely causes the up-regulation of some antioxidant enzymes during the drought period.

Among all the enzymes examined, APX and SOD showed an increase of activity directly related to the degree of drought stress in all the tested tissues (Tables 1–3). Thus, our results show that drought-related oxidative stress up-regulated the activity of SOD and APX. This can be an important protection mechanism of olive tree against the excessive increase of AOS during drought stress. SOD isoforms play a key role in AOS scavenging by regulating the levels of O$_2$$^{\bullet-}$ produced in chloroplasts, mitochondria and cytosol (Bowler *et al.* 1992). Moreover, SOD activity reduces the possibility of HO$^{\bullet}$ formation, a very strong oxidant with a very high affinity for biological molecules, through Haber–Weiss and / or the metal-catalysed Fenton reaction (Smirnoff 1993). APX is one of the most widely distributed antioxidant enzymes in the plant cell and it reduces H$_2$O$_2$, a strong inhibitor of photosynthesis, to water, using ascorbate as the electron donor (Mehlhorn *et al.* 1996). Ascorbate can then be regenerated by the ascorbate–glutathione cycle (Foyer and Mullineaux 1998; Smirnoff 2000). Leaves of SP at severe drought stress showed a considerable increase of APX activity (Table 1), while APX in roots of SP presented low

Table 1. Antioxidant enzyme activities of leaves from drought-stressed (SP) and control (CP) plants

Samples were collected, at 0600–0700 h, at 0, 4, 8, 12, 16 and 20 d from the beginning of the drought stress period. Each value represents the mean of three measurements ($\pm$ SE) from three plants having a similar level of drought stress. *Significant differences at the 5% level between control and drought-stressed plants ($P \leq 0.05$, according to Duncan's multiple range test)

Degree of drought	Day	SOD SP	SOD CP	APX SP	APX CP	CAT SP	CAT CP	POD SP	POD CP	IAAox SP	IAAox CP	PPO SP	PPO CP
None	0	15.21±0.82	14.23±0.52	3.88±0.20*	3.13±0.15	4.52±0.18	4.45±0.15	29.21±1.24	28.72±1.17	14.30±0.49	13.23±0.13	33.49±0.87	34.66±1.70
Mild	4	18.24±0.50	16.35±0.38	3.24±0.05*	2.97±0.12	5.06±0.13	4.50±0.12	30.73±1.35	27.43±0.95	16.15±0.75*	11.27±0.45	31.87±1.06	33.42±1.29
Mild	8	30.73±0.89*	15.87±0.65	7.36±0.07*	2.43±0.12	6.81±0.05*	4.87±0.12	39.06±1.97*	28.84±0.91	19.00±0.47*	12.70±0.56	26.55±0.85*	35.21±0.96
Moderate	12	34.34±1.27*	14.39±0.19	11.06±0.46*	2.76±0.10	11.94±0.74*	5.33±0.10	45.80±1.77*	27.56±1.28	22.56±0.60*	13.76±0.46	25.47±1.04*	35.73±0.85
Moderate	16	31.69±1.90*	15.49±0.41	12.11±0.26*	2.62±0.15	11.44±0.15*	6.53±0.15	48.17±2.01*	28.52±1.05	16.28±0.34*	10.61±0.28	24.13±1.34*	34.13±1.20
Severe	20	25.17±1.06*	12.90±0.97	13.77±0.55*	2.80±0.14	11.78±0.18*	7.36±0.14	40.66±0.73*	28.67±0.85	16.50±0.41*	11.49±0.24	20.36±0.91*	33.26±0.66

Enzyme activity (units mg^{-1} dry weight)

Table 2. Antioxidant enzyme activities of fine roots from drought stressed (SP) and irrigated (CP) plants

Samples were collected, at 0600–0700 h, at 0, 8, 16 and 20 d from the beginning of the drought stress period. Each value represents the mean of three measurements ($\pm$ SE) from three plants having a similar level of drought stress. *Significant differences at the 5% level between values of control and drought stressed plants ($P \leq 0.05$, according to Duncan's multiple range test)

Degree of drought	Day	SOD SP	SOD CP	APX SP	APX CP	CAT SP	CAT CP	POD SP	POD CP	IAAox SP	IAAox CP	PPO SP	PPO CP
None	0	7.23±0.15	6.96±0.26	0.23±0.01	0.19±0.01	2.86±0.09	2.98±0.05	18.77±0.31	17.68±0.74	8.56±0.14	7.54±0.12	30.31±0.42	32.12±0.12
Mild	8	17.32±0.38*	7.56±0.10	0.24±0.01	0.20±0.01	4.57±0.20*	3.26±0.09	26.51±0.34*	18.77±0.65	12.19±0.63*	7.28±0.11	28.47±0.47	31.79±0.48
Moderate	16	19.37±0.66*	6.31±0.18	0.32±0.02*	0.21±0.01	8.16±0.13*	3.74±0.13	39.37±1.37*	19.61±0.51	17.11±0.60*	8.73±0.38	10.90±0.34*	31.72±0.34
Severe	20	25.00±1.12*	6.42±0.17	0.34±0.01*	0.22±0.01	5.98±0.13*	3.14±0.12	39.52±0.90*	16.86±0.70	14.76±0.45*	6.77±0.11	7.16±0.12*	33.63±0.79

Enzyme activity (units mg^{-1} dry weight)

Table 3. Antioxidant enzyme activities of medium roots from drought stressed (SP) and irrigated (CP) plants

Samples were collected, at 0600–0700 h, at 0, 8, 16 and 20 d from the beginning of the drought stress period. Each value represents the mean of three measurements ($\pm$ SE) from three plants having a similar level of drought stress. *Significant differences at the 5% level between values of control and drought stressed plants ($P \leq 0.05$, according to Duncan's multiple range test)

Degree of drought	Day	SOD SP	SOD CP	APX SP	APX CP	CAT SP	CAT CP	POD SP	POD CP	IAAox SP	IAAox CP	PPO SP	PPO CP
None	0	7.99±0.20	8.12±0.38	0.36±0.01	0.37±0.02	2.08±0.05	2.35±0.04	23.88±0.34	25.62±0.85	11.22±0.13	10.67±0.35	38.19±0.99	19.76±1.22
Mild	8	11.20±0.28*	7.61±0.35	0.42±0.01*	0.33±0.01	3.10±0.07*	2.36±0.07	39.05±1.56*	23.52±1.35	15.74±0.51*	9.36±0.48	35.63±0.74	40.46±1.28
Moderate	16	15.94±0.59*	8.27±0.20	0.56±0.02*	0.40±0.02	2.74±0.10*	2.57±0.22	47.77±2.11*	25.09±0.67	24.00±1.04*	10.04±0.13	21.43±0.43*	39.61±1.60
Severe	20	17.19±0.49*	6.85±0.09	0.51±0.02*	0.35±0.02	2.58±0.07*	2.25±0.13	44.56±1.27*	22.97±0.85	19.05±0.33*	11.81±0.41	41.88±1.84	23.17±0.56

Enzyme activity (units mg^{-1} dry weight)

levels of activity if compared with leaves (Tables 2, 3). This suggests that the APX activity could be attributed mainly to the chloroplast-located enzyme (chlAPX) of leaf tissues. The huge 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).

CAT activity, after an increase at mild and moderate water deficit in leaves and FR, remained rather stable in leaves and declined slightly during severe water deficit in roots, possibly because of CAT inactivation and degradation (Feierabend *et al*. 1992). 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, under 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, a non-enzymatic antioxidant compound (Wingler *et al*. 2000). Therefore, the high substomatal CO_2 concentration observed in SP (Fig. 3*C*) could be the result of an increase in photorespiratory processes together with a slowed Calvin cycle activity (Angelopoulos *et al*. 1996).

PODs can oxidise and thus scavenge H_2O_2 using preferably some phenolic compounds (e.g. guaiacol) as primary reducing agents (Mehlhorn *et al*. 1996). 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). Moreover, IAA seems to cause an increase of POD activity, and hence, also of lignin deposition in cell walls (Sitbon *et al*. 1999). POD isoenzymes 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). The observed increases in POD activity (Tables 1–3) could reflect the changed mechanical properties of the cell wall, which, in turn, can be correlated with drought adaptation. The high degree of POD activity may cause a limitation of growth in olive plants under drought stress for two reasons: increased level of lignification and oxidation, and the consequent inactivation of IAA. This could explain the inhibitory effect of water deficit on the growth of olive tree canopies (Dichio *et al*. 2002) and its influence on root dynamics (Fernández *et al*. 1992).

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). Phenolics are physiologically active secondary compounds with a non-enzymatic antioxidant action. They are abundant in the leaves of olive trees (Ryan *et al*. 2002) and in olive oil (Owen *et al*. 2000), being found in cytoplasm, vacuoles and cell walls. Phenolic compounds are also involved in auxin protection or catabolism (Hrubcová *et al*. 2000) and in the modulation of the cell wall plasticity (Fry 1986). Furthermore, abscisic acid-induced stomatal closure is reversed by some phenolics (Purohit *et al*. 1991), suggesting an important role of these compounds in gas exchange dynamics. For all these reasons, the regulative action of PPO plays an important role in the physiological aspects of plants subjected to water deficit conditions. Drought stress can improve the antioxidant action of phenols by inhibiting PPO activity (Tables 1–3) and consequently by maintaining the phenol compounds pool in the reduced state. Additionally, the proteolytic activity of PPO (Kuwabara and Katoh 1999) suggests that the enzyme could be involved in removing the proteins damaged by AOS.

Our results highlight the olive tree's capacity to withstand arid environments and to maintain high photosynthetic rates at moderate drought stress when the transpiration rate declines significantly. The different values of enzyme activities found in leaves, fine roots and medium roots confirm their different functions: leaf tissues showed more pronounced changes, owing to the synergical effect of high irradiance levels and loss of cellular water; FR were more sensitive to drought stress and its consequent effects, while MR maintained a prolonged functionality and presented less reactivity, even at severe drought stress. In this investigation into antioxidant defence enzyme activities of olive tree, we have found evidence for an up-regulation of AOS-scavenging enzymes as plants enter water deficit conditions. The results obtained underline the important role of some antioxidant enzymes in protecting cellular apparatus during water deficit conditions. Research into variations in the activities of other antioxidant enzymes and the action of other molecules with a non-enzymatic action may give a more complete picture of the response of olive tree to drought and better explain its high resistance to this specific abiotic stress.

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