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ORIGINAL PAPER

Physiological behaviour, oxidative damage and antioxidative protection of olive trees grown under different irrigation regimes

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Abstract Irrigation effects were investigated on an 8-year-old olive (*Olea europaea* L., cv. Cobrançosa) commercial orchard located in northeast Portugal. Trees were subjected to a rainfed control (T0) and three treatments (T1, T2, T3) that received a seasonal water amount equivalent to 30%, 60% and 100% of the estimated local evaporative demand by a drip irrigation system. Irrigation increases the photosynthetic activity of olive trees, in association with increases in water status, and reduces the midday and afternoon depression in gas exchange. The closely association between photosynthetic rate (A) and stomatal conductance (g_s) revealed that the decline in net photosynthesis over the course of the day was largely a consequence of stomatal limitation. However, the ratio of intercellular to atmospheric CO_2 concentration increased markedly from morning to midday in non-irrigated plants, in spite of lower g_s, suggesting that non-stomatal limitations of photosynthesis also occur when environmental conditions become more stressful. The occurrence of perturbations at chloroplastic level in rainfed plants was demonstrated by a lower maximum photochemical efficiency of photosystem II during the afternoon. Chlorophyll fluorescence measurements also revealed the occurrence of a dynamic photoinhibition in irrigated trees, mainly in T2 and T3, which seemed to be effective in protecting the photosynthetic apparatus from photodamage. Irrigation enhances antioxidant protection and decreases the oxidative damage at leaf level. Leaves grown under rainfed conditions revealed symptoms of oxidative stress, like the reduction (14%) in chlorophyll concentration and the increased levels (57%) of lipid peroxidation. We also found that the scavenging function of superoxide dismutase was impaired in rainfed plants. In contrast, the low thiobarbituric acid reactive substances concentration in T3 indicates that irrigation enhances the repairing mechanisms and decreases the oxidative damage by lipid peroxidation. Accordingly, leaves in T3 treatment had high levels of –SH compounds and the highest

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antioxidant potential. Meanwhile, the finding that guaiacol peroxidase activity increased in rainfed plants, associated with the appearance of oxidative damage, suggests that this enzyme has no major antioxidative function in olive.

Keywords Antioxidant enzymes · Chlorophyll fluorescence · Gas exchange · Lipid peroxidation · Photoinhibition · Radical scavenging activity

Introduction

During the summer, Olive (*Olea europaea* L.) is often subjected to periods of severe drought and leaves exhibit large reductions in relative water content (RWC) and water potential (Nogués and Baker 2000). Frequently, large decreases in photosynthetic activity are associated with such changes in water status. Upon mild and moderate water deficit conditions, photosynthesis decreases in olive plants mainly due to stomatal closure, whereas non-stomatal factors would limit carbon assimilation under severe drought (Angelopoulos et al. 1996). Even under irrigation, stomatal conductance can substantially restrict CO_2 entry into the leaves as a result of the interaction of low vapour pressure deficits in the atmosphere with high temperature and irradiance (Osório et al. 2006). It has been demonstrated that the combination of these factors predispose plants to photoinhibition or down-regulation process. In particular, a CO_2 deprivation at the chloroplast level by stomatal closure during the warmest period of the day could enhance the sensitivity of the photosynthetic apparatus to high irradiance (Faria et al. 1998; Flexas et al. 1998).

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 reactive oxygen species (ROS), mainly in the photosynthetic (Asada 1999) and mitochondrial electron transport chains (Møller 2001). Plants are endowed with a complex antioxidant system to cope with ROS (Smirnoff 1993). However, when the accumulation of ROS under water stress conditions exceeds the removing capacity of the antioxidant system, the effects of oxidative damage arise, including oxidation of cellular lipids and proteins, destruction of photosynthetic pigments and inactivation of photosynthetic enzymes (Smirnoff 1993).

Previous studies reported that water supply minimizes the negative effects of water stress on olive performance (Fernández and Moreno 1999), and reduces the oxidative damage in young olive plants growing in pots (Bacelar et al. 2006). However, the response of field-grown olive trees to irrigation is not well documented at this level. The aim of this work was to evaluate the benefits of irrigation in mature olive trees growing under Mediterranean conditions. We hypothesize that irrigation improve carbon assimilation and protect the photosynthetic apparatus from the high risk of photodamage occasioned by the superimposed stresses to which the plants were subjected under field conditions. To test this hypothesis we compared plant water status, gas exchange and chlorophyll fluorescence in olive trees growing under different irrigation regimes. We also hypothesize that irrigation enhances antioxidant protection and decreases the oxidative damage at leaf level. To this aim we have carried out measurements of the leaf concentration of photosynthetic pigments (chlorophylls, carotenoids), soluble sugars (SS), starch, thiobarbituric acid reactive substances (TBARS), total phenols, UV-B absorbing compounds, total soluble proteins (SP) and total thiols. We also evaluated the radical scavenging activity (RSA) of leaf extracts and antioxidant enzymes activities.

Materials and methods

Site description, plant material and treatments

The experiment took place during three consecutive years, from 2002 to 2004, on an 8-year-old olive (*Olea europaea* L., cv. Cobrançosa) commercial orchard located at Vilarelhos (41°21′ N and 6°45′ W; at an elevation of 320 m above sea level), a typical olive-growing area in northeast Portugal. The sandy-loam soil of the experimental site (organic matter 0.74%, P_2O_5 102 mg kg^{-1}, K_2O 89 mg kg^{-1} and pH 5.8) was characterized by

volumetric water content of 23.6% at field capacity (soil matric potential of –0.03 MPa) and 9.7% at wilting point (soil matric potential of –1.5 MPa). Tree arrangement was 6 m × 6 m and the experimental design was a complete randomized block (one tree per block), replicated four times. During the first years after planting, all plots were irrigated equally to guarantee the uniform tree development. Differentiation of irrigation levels started in May 2002. Four irrigation treatments were applied: a rainfed control (T0) and three treatments (T1, T2, T3) that received a seasonal water amount equivalent to 30%, 60% and 100% of the estimated local evaporative demand. The trees were drip-irrigated daily by two drippers per tree, each with a flow rate of 4 l h^{-1}. Trees were irrigated in the following periods: May 17 to September 30 in 2002, June 26 to September 30 in 2003, and June 4 to October 6 in 2004. Olive trees have been trained, pruned and managed every year according to standard commercial procedures suited to high-density plantings.

The climate in the zone is typically Mediterranean (Fig. 1), with 520 mm of average rainfall, concentrated mainly from autumn to spring (INMG 1991). The warmer months are July/August and the coldest are December/January, with average daily temperatures of 23.6/22.9 °C and 6.3/6.1 °C, respectively.

The effects of irrigation on the physiology of olive trees were analysed in 2002, 2003 and 2004. Since the results were quite similar in the three studied years, only the data obtained in 2003 (the driest and the hottest year from May to September) are presented. To facilitate the study, the more time-consuming measurements were made only for the extreme irrigation treatments T0 and T3.

Leaf water status and stem water potential

Six mature leaves per treatment were detached in a similar position to determine leaf water status. After cutting, the petiole was immediately immersed in demineralized water inside a glass tube, which was immediately sealed. The tubes were then taken to the laboratory where the increased weight of the tubes was used to determine leaf fresh mass (FM). After 48 h in the dark, the leaves were weighed to obtain FM at full turgor (TM). Dry mass (DM) was measured after oven-drying at 70 °C to a constant weight. Leaf water status was assessed by the RWC, calculated as RWC = (FM – DM)/(TM – DM) × 100, and the water content at saturation (WCS = (TM – DM)/DM).

Stem water potential measurements were used to evaluate tree water status. Predawn stem water potentials (Ψ_{PD}) were measured, on 6 sun-exposed shoots using a pressure chamber (PMS, Corvallis, OR), according to Scholander et al. (1965). Care was taken to minimize water loss during the transfer of the shoot to the chamber by enclosing it in a plastic bag immediately after excision (Turner and Long 1980).

Gas exchange and chlorophyll fluorescence measurements

On August 6, 7, 27 and September 10, 2003, leaf gas exchange rates were measured at natural incident photosynthetic photon flux density (PPFD) in the field using a portable IRGA (ADC-LCA-3, Analytical Development, Hoddesdon, U.K.), operating in the open mode. Diurnal patterns of gas exchange rates were very similar throughout the season, so only the data obtained on September 10 (severe drought period, minimum photosynthesis) are presented. Measurements were performed on 8 well exposed current year leaves (fully expanded, of the same stage of development), during the morning (09:30–10:30 h), midday (13:30–14:30 h) and afternoon (17:30–18:30 h). PPFD was always higher than 1,000–1,200 μmol m^{-2} s^{-1} (1,450, 1,900 and 1,520 μmol m^{-2} s^{-1} at morning, midday and afternoon, respectively), which is known to be over the saturation point in olive (Natali et al. 1991). The values of vapour pressure deficit in the same periods (VPD) were 2.08, 3.79 and 4.09 kPa, respectively. Net CO_2 assimilation rate (A), stomatal conductance (g_s) and the ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a) were estimated from gas exchange measurements using the equations developed by von Caemmerer and Farquhar (1981). Intrinsic water use efficiency was calculated as the ratio of A/g_s.

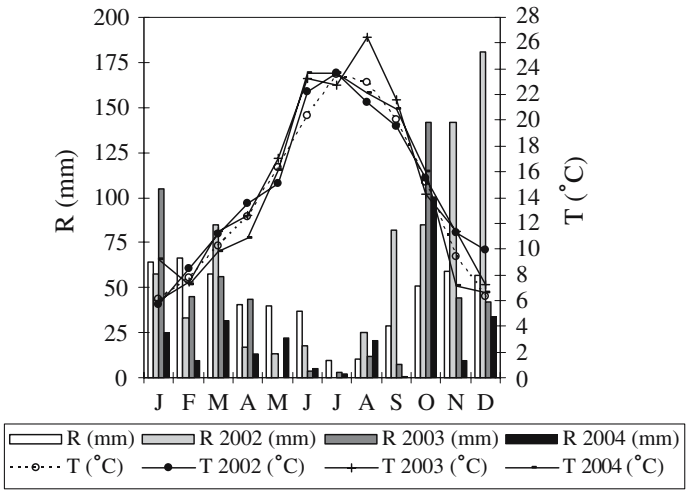

Fig. 1 Rainfall (R = mean for the period 1951–1980; R 2002 = rainfall for the year of 2002; R 2003 = rainfall for the year of 2003 and R 2004 = rainfall for the year of 2004) and monthly air temperatures (T = mean for the period 1951–1980; 2002 = mean for the year of 2002; T 2003 = mean for the year of 2003 and T 2004 = mean for the year of 2004) at the study site

In vivo chlorophyll fluorescence was measured with a portable chlorophyll fluorometer (Plant Stress Meter, BioMonitor SCI AB, Umeå, Sweden) at morning, midday and afternoon on the same leaves used for gas exchange measurements. Prior to the measurements, a small part of the leaves was kept in the dark for 30 min using cuvettes for dark adaptation. A 5-s light pulse at 400 μmol m^{-2} s^{-1} was used. Maximum quantum yield of PSII was estimated by the F_v/F_m ratio (Krause and Weis 1991).

Photosynthetic pigments, SS and starch

All metabolic compound analyses were made with leaf discs taken at morning (10:00 h) in the same date of gas exchange measurements from six fully expanded leaves of comparable physiological age, thereby eliminating developmental effects. Leaf sections of a known area were ground in 80% acetone for chlorophyll and carotenoid determination. Total chlorophyll (Chl_{a+b}) and Chl_a/Chl_b ratio was determined according to Sesták et al. (1971) and total carotenoids (Car) according to Lichtenthaler (1987).

Total SS were extracted by heating leaf discs in 80% ethanol, according to Irigoyen et al. (1992). SS were analysed by the reaction of 200 μl of the alcoholic extract with 3 ml of fresh anthrone and placed in a boiling water bath for 10 min. After cooling, the absorbance at 625 nm was determined. After the extraction of the soluble fractions, the solid fraction was used for starch analysis. Starch was extracted with 30% perchloric acid, according to Osaki et al. (1991). The starch concentration was determined by the anthrone method as described above. Glucose was used as a standard for both SS and starch.

Lipid peroxidation

The lipid peroxidation products in olive leaves were estimated following the method of Heath and Packer (1968). More details can be found in Bacelar et al. (2006). The total TBARS concentration was calculated using an extinction coefficient of 155×10^{-3} M^{-1} cm^{-1} (Costa et al. 2002).

Total phenol, UV-B absorbing compounds, SP and total thiol

Total phenol concentration (TP) in leaf extracts was determined on the same extract used for pigment analysis, according to the Folin-Ciocalteu's procedure (Singleton and Rossi 1965).

The ultraviolet-B absorbing compounds ($UV\text{-}B_{AC}$) were extracted by heating leaf discs in 10 ml of methanol:HCl:water (79:1:20 (v/v)) according to Mirecki and Teramura (1984). After centrifugation ($3{,}000 \times g$ for 10 min), the supernatant was used for absorbance measurement at 300 nm, peak of maximum absorption.

The amount of SP was quantified using the method of Bradford (1976). Leaf discs were

homogenized in a grinding medium that contained 50 mM phosphate buffer (pH 7.8), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 100 μM phenylmethylsulfonyl fluoride (PMSF) and 2% polyvinylpyrrolidone (PVP) (w/v). Bovine serum albumin was used as a standard.

Total thiol content (–SH) of SP extract was assayed according to Ellman (1959), using 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). Absorbance was determined at 412 nm and the –SH concentration was calculated using an extinction coefficient of 13,600 M^{-1} cm^{-1} (Ellman 1959).

Radical scavenging activity

The effect of the olive leaf extracts on 1,1-diphenyl–2-picrilhydrazyl (DPPH) radical was studied employing an modified method described earlier by Kitts et al. (2000). The RSA of leaf extracts was measured as a decrease in the absorbance of DPPH at 517 nm and was calculated using the equation: RSA = $(1 - A_{sample}\ 517\ nm/A_{control}\ 517\ nm) \times 100$.

Antioxidant enzymes

Extracts for determination of antioxidant enzymes superoxide dismutase (SOD, EC 1.15.1.1) and guaiacol peroxidase (GPX, EC 1.11.1.7) activities were prepared from leaf discs homogenized with a mortar and pestle in 1.5 ml of ice-cold 50 mM potassium-phosphate buffer (pH 7.4), containing 0.1 mM EDTA and 0.2% PVP (w/v). SOD activity was measured according to the method described by Flohé and Otting (1984) using xanthine–xanthine oxidase system. GPX activity was calculated following the oxidation of guaiacol at 470 nm using an extinction coefficient of 26.6×10^{-3} M^{-1} cm^{-1} (Laloue et al. 1997).

All reagents and chemicals used were of the highest grade of purity commercially available.

Statistics

All data were subjected to an analysis of variance with prior data transformation when required. Proportional data expressed as ratio data were log transformed. Significant different means were separated using the Fisher's LSD test ($P < 0.05$).

Results

Leaf water status and stem water potential

The RWC of T0 was significantly lower than all the other irrigation treatments (Table 1). Moreover, no significant variation among the treatments was observed in the WCS. Like the RWC, the Ψ_{PD} was significantly lower in T0 plants.

Gas exchange and chlorophyll fluorescence measurements

The typical diurnal course of gas exchange rates for the different irrigation treatments is shown in Fig. 2. The *A* values measured in the morning period were significantly different among treatments. The highest *A* was observed in T2 and T3 (19.0 and 18.4 μmol m^{-2} s^{-1}, respectively) and the lowest in T0 (7.8 μmol m^{-2} s^{-1}). T1 presented an intermediate photosynthetic rate (15.3 μmol m^{-2} s^{-1}). Then, a progressive decline in *A* was observed until midday in all irrigation regimes. Moreover, the importance of water availability in olive photosynthesis is more evident at midday. In fact, at midday *A* is 594% higher in T3 than in T0 whereas in the morning it was 136% higher. We verify again that the midday *A* values of T2 are similar to those of T3. On the other hand, *A* of T1 is now clearly lower than *A* of T2 and T3, but still higher than *A* of T0. Furthermore, no recovery of *A* was observed in the afternoon in all treatments. At this period of the day, the photosynthetic rate of T1 is at the same level of T0.

The g_s exhibited maximal values between 71 (in T0) and 235 (in T3 and T2) mmol m^{-2} s^{-1} in the morning, followed by a decline thereafter, in a

Table 1 Effects of irrigation on RWC, water content at saturation (WCS) and stem water potential at predawn (Ψ_{PD}) ($n = 6$)

	RWC (%)	WCS (g H_2O g^{-1})	Ψ_{PD} (MPa)
Irrigation treatment			
T0	65.3 b	0.97 a	–2.27 b
T1	87.4 a	0.98 a	
T2	87.9 a	1.02 a	
T3	91.2 a	1.06 a	–1.05 a

Means within a column flanked by a different letter are significantly different at $P < 0.05$ (LSD test)

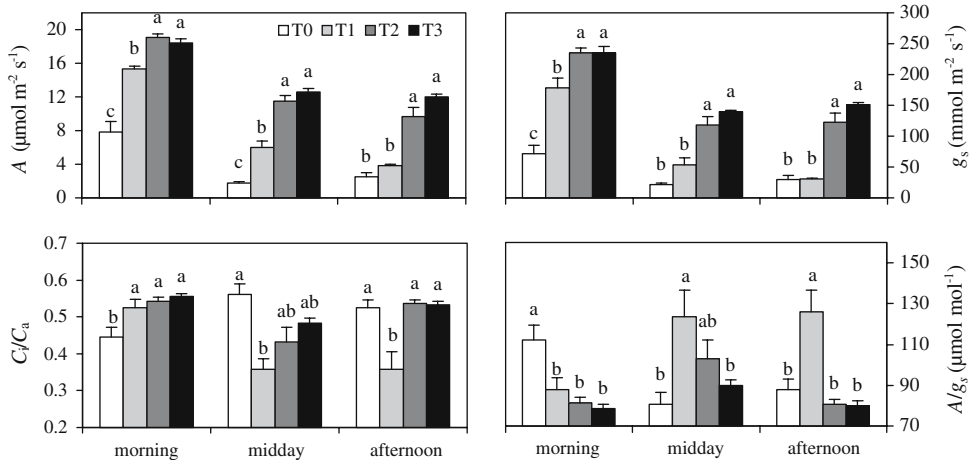

Fig. 2 Diurnal evolution of leaf net CO_2 assimilation rate (A), stomatal conductance (g_s), ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a) and intrinsic water use efficiency (A/g_s) in the four irrigation treatments. Columns are means (n = 8) and vertical bars represent the standard error. Measurements of each diurnal period followed by a different letter are significantly different at $P < 0.05$ (LSD test)

closely association with the decrease of A (Fig. 2). The minimum values of g_s were obtained at midday, ranging from 22 (in T0) to 140 (in T3) mmol m^{-2} s^{-1}. Thus, stomatal closure might be one of the factors responsible for the reduction in A. Nevertheless, the relative influence of irrigation on A and g_s differed among treatments and changed during the diurnal period. Intrinsic water use efficiency was higher in T0 in the morning, whereas in the other periods was higher in T1 plants. Interestingly, A/g_s decreased along the day in T0 and increased in the other treatments, mainly at midday, in a negative association with the behaviour of C_i/C_a. In fact, C_i/C_a decreased from morning to midday in T1, T2 and T3 (Fig. 2). From midday to the afternoon the C_i/C_a increased considerably in T2 and T3, but did not changed in T1. T0 revealed a different trend of C_i/C_a. It increased markedly from morning to midday suggesting that non-stomatal limitations are also responsible for the decrease in A in T0 plants.

Daily recordings of chlorophyll fluorescence are shown in Fig. 3. The morning values of F_v/F_m were high (varying between 0.71 and 0.74) and not significantly different across the irrigation treatments. Then, a decline in F_v/F_m was observed at midday, mainly in irrigated plants. Interestingly, a recovery of F_v/F_m was observed in the afternoon in irrigated plants, namely in T2 and T3.

Lipid peroxidation

The concentrations of TBARS in the leaves of olive trees are shown in Fig. 4. The TBARS concentration was higher in T0 leaves (12.4 nmol cm^{-2}), showing signs of oxidative damage. Irrigated plants revealed low levels of TBARS. Meanwhile, no significant differences were registered among T1, T2 and T3. For the succeeding assays, only the T0 and T3 treatments were used to explore the effect of irrigation in olive trees.

Photosynthetic pigments, SS and starch

The $Chl_{(a+b)}$ amount per unit leaf area and the Chl_a/Chl_b ratio were higher in T3 than in T0 (Table 2). However, no differences between treatments were observed in the Car concentration and in Chl/Car ratio. Concerning the SS concentration, no differences between irrigation treatments were observed, but the T3 leaves contained high concentration of starch than T0 leaves (Table 2), in a straight association with their high A rates (Fig. 2).

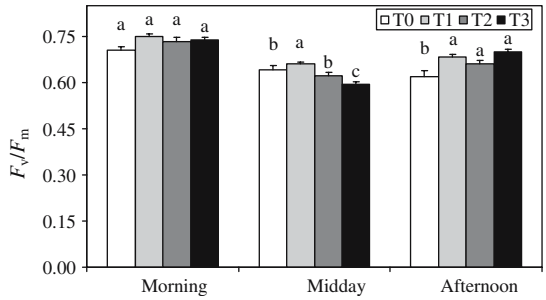

Fig. 3 Diurnal evolution of maximum quantum yield of PSII (F_v/F_m) in the four irrigation treatments. Columns are means (n = 8) and vertical bars represent the standard error. Measurements of each diurnal period followed by a different letter are significantly different at $P < 0.05$ (LSD test)

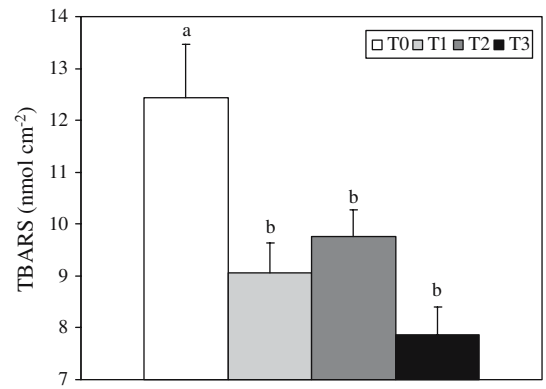

Fig. 4 Effect of irrigation on TBARS concentration of leaf extracts. Columns are means (n = 6) and vertical bars represent the standard error. Columns followed by a different letter are significantly different at $P < 0.05$ (LSD test)

Total phenol, UV-B absorbing compounds, SP and total thiol

Irrigation had no statistically significant effects on TP and UV-B_{AC} (Table 3). Meanwhile, T0 plants revealed higher SP concentration and lower levels of –SH.

Radical scavenging activity

The results obtained in the present study clearly demonstrate that olive leaf extracts contain antioxidant compounds, which can effectively scavenge various ROS/free radicals under in vitro conditions (Table 3). Nevertheless, the antioxidant activity of T3 leaf extract was 31% higher than in T0 extract.

Antioxidant enzyme activities

Water availability significantly changed the specific activities of SOD and GPX (Table 3). The drought-stressed leaves had 27% lower SOD activity than irrigated plants, whereas GPX was 52% higher.

Discussion

The extremely low values of WCS observed (0.97 g H_2O g^{-1} in T0) were even lower than those reported by Abd-El-Rahman et al. (1966) and Bacelar et al. (2004), suggesting that the cultivar used in this study had the capacity to withstand arid environments. Meanwhile, we found an increase in RWC from 65 (in T0) to 91% (in T3), which is of particular significance in photosynthesis. The diurnal trends of g_s and A in the field-grown olive trees, either irrigated or non-irrigated, followed a typical pattern described for woody Mediterranean vegetation (Fernández and Moreno 1999; Ogaya and Peñuelas 2003; Tognetti et al. 2004), with maximum values during the morning hours (Fig. 2). Over the course of the day, even under irrigation, g_s substantially restrict CO_2 entry into the leaves, as a result of the interaction of high vapour pressure deficits in the atmosphere with high temperature and solar irradiance. Thus, a decrease in photosynthesis and a cascade of different processes contributing to the protection of chloroplasts may occur. However, irrigation significantly decreased the midday and afternoon depression of A and g_s. In fact, T3 plants exhibited by midday decreases of 32% and 41% (in A and g_s, respectively) in relation to morning values, as compared to decreases of 77% and 69% observed in T0 plants. Similar influence of water availability on A was obtained by Jorba et al. (1985) who found that a reduction in RWC from 96% to 65% induced an 85% reduction in photosynthesis of potted olive trees.

Table 2 Photosynthetic pigments, SS and starch of leaf extracts of T0 and T3 ($n = 6$)

	$Chl_{(a+b)}$ (mg dm^{-2})	Chl_a/Chl_b	Car (mg dm^{-2})	Chl/Car	SS (mg dm^{-2})	Starch (mg dm^{-2})
Irrigation treatment						
T0	6.66 b	2.62 b	1.53 a	4.40 a	147.5	31.4 b
T3	7.75 a	2.94 a	1.71 a	4.53 a	141.2	61.1 a

Means within a column flanked by a different letter are significantly different at $P < 0.05$ (LSD test)

Table 3 Total phenols (TP), ultraviolet-B absorbing compounds ($UV\text{-}B_{AC}$), total SP, total thiol concentration (–SH), RSA and antioxidant enzymes superoxide dismutase (SOD) and guaiacol peroxidase (GPX) activities of leaf extracts of T0 and T3 ($n = 6$)

	TP (mg dm^{-2})	$UV\text{-}B_{AC}$ (A300 cm^{-2})	SP (mg dm^{-2})	–SH (nmol mg $protein^{-1}$)	RSA (%)	SOD (units mg $protein^{-1}$)	GPX (units mg $protein^{-1}$)
Irrigation treatment							
T0	96.2 a	3.08 a	340.3 a	38.0 b	9.86 b	51.5 b	0.150 a
T3	104.5 a	3.16 a	200.9 b	76.5 a	12.87 a	70.2 a	0.072 b

Means within a column flanked by a different letter are significantly different at $P < 0.05$ (LSD test)

The closely association between A and g_s suggests that the decline in net photosynthesis over the course of the day is largely a consequence of stomatal limitation. Since stomatal limitation of photosynthesis is exerted through the control of intercellular CO_2 concentration, a decrease in C_i/C_a along the day was observed in irrigated plants (Fig. 2). However, we observed that C_i/C_a increased markedly from morning to midday in T0, in spite of lower g_s, suggesting that non-stomatal limitations to photosynthesis also occur when environmental conditions become more stressful. Similar results were obtained by Giorio et al. (1999) in a study with field-grown olive trees under water deficit conditions.

The A/g_s of olive plants differed among the irrigation treatments (Fig. 2). According to Passioura's theory of plant water-use behaviour (1982), T2 and T3, with high g_s, high C_i/C_a and low A/g_s, appear to employ a prodigal or non-conservative strategy, whereas T0 (at morning) and T1 (at midday and afternoon), with high A/g_s, appear to employ a conservative strategy in the use of water. The prodigal water-use behaviour enables a plant to grow quickly and the conservative water-use behaviour is beneficial in conditions where a long dry period prevails, enabling the plant to use the available water efficiently.

The analysis of chlorophyll fluorescence rates reveals that non-stomatal limitations are also responsible for the low of A in T0 plants (Fig. 3). The occurrence of perturbations at chloroplastic level in T0 plants is demonstrated by a low F_v/F_m during the afternoon and by a high minimal fluorescence along the day (data not shown). Thus, T0 plants had lower maximum photochemical efficiency of PSII and lower absorption efficiency of photons by chlorophyll a in the light-harvesting complex (Giorgieva and Yordanov 1993).

No alterations occurred in morning values of F_v/F_m, indicating that the maximal PSII primary photochemistry was not permanently affected by the stressful conditions previously experienced by the trees. At midday, the decrease of F_v/F_m, mainly in T2 and T3, revealed the occurrence of a dynamic photoinhibition, which seemed to be effective in protecting the photosynthetic apparatus from the high risk of photodamage occasioned by the superimposed stresses to which the plants were subjected under field conditions (Souza et al. 2004). Thus, the excessive excitation energy is deflected away from PSII and dissipated harmlessly, primarily as heat (Osmond 1994). Such down-regulation in T2 and T3 leaves may be the response to excessive radiation. Down-regulation of photochemical efficiency around midday

has also been shown in *Quercus suber* L. trees under field conditions (Faria et al. 1996).

Interestingly, T0 plants were able to maintain a similar amount of leaf SS as in T3 plants (Table 2), in spite of the low *A* (Fig. 2), at the expenses of leaf starch, which drastically decreased. This may be linked to their ability to perform tissue osmotic adjustment, as observed in potted olive trees (Bacelar et al. 2006). In fact, upon exposure to drought and extreme temperatures, plants accumulate osmotic compounds including SS, amino acids like proline, aspartic and glutamic acid, methylated quaternary ammonium compounds (e.g. glycine betaine and olanine betaine), hydrophilic proteins (e.g. late embryogenesis abundant, LEA) and cycitols (e.g. pinitol, manitol) (Chaves et al. 2003). One of the main sources of these osmolytes is the starch reserves (Amundson et al. 1993), which supply hydrolysed sugars.

As photosynthesis decreased under water stress, an excess of reducing power is frequently generated and, thus, over-reduction of photosynthetic electron chain may result in the formation of ROS that can cause oxidative damage. In fact, leaves grown under rainfed conditions revealed signs of oxidative stress. One sign was the large reduction (14%) in leaf $Chl_{(a+b)}$ (Table 2). According to Smirnoff (1993), the decrease of chlorophyll content (chlorophyll bleaching) is a typical symptom of oxidative stress and may be the result of chlorophyll degradation or be due to chlorophyll synthesis deficiency together with changes of thylakoid membrane structure. Bussis et al. (1998) indicated that expanded leaves exposed to water deficit started to degrade their photosynthetic apparatus, possibly to mobilize resources for the production of new acclimated leaves. Furthermore, the higher Chl_a/Chl_b ratio in T3 than in T0 plants (Table 2) reflects the relative increase of the chlorophyll a containing reaction centre complexes at the expense of the light harvesting chlorophyll a/b proteins (Evans 1993).

Another sign of oxidative stress in T0 leaves was the increased levels (57%) of lipid peroxidation (Fig. 4). Several studies have been shown lipid peroxidation in water-deficit conditions, including studies with *Olea europaea* L. plants (Sofo et al. 2004; Bacelar et al. 2006). Lipid peroxidation is a natural metabolic process under normal aerobic conditions and is one of the most investigated ROS actions on membrane structure and function (Blokhina et al. 2003). It is widely reported that ROS bring about peroxidation of membrane lipids leading to membrane damage (Shalata and Tal 1998). Considering the TBARS concentration as a biochemical marker for the ROS mediated injury, results suggest that irrigation reduces drastically the oxidative damage on cell membranes by lipid peroxidation, validating our working hypothesis.

Meanwhile, in our study, T0 plants showed a lower –SH concentration (Table 3), probably due to the oxidation of non-proteic –SH groups. Oxidative stress depletion on non-proteic thiols in T0 enhances the susceptibility to membrane damage by TBARS and may trigger ROS irreversible negative effects on cellular function (Ali et al. 2005). In opposition, T3 plants revealed a high level of –SH compounds (e.g. glutathione) that may function as reducers of oxidative damage (Ali et al. 2005).

According to the DPPH test, the highest antioxidant potential was observed in T3 leaf extract (Table 3), reflecting their low susceptibility to oxidation. Similar results were focused by Marron et al. (2002) in a *Populus* × *euramericana* clone. Antioxidants play an important role delaying or preventing the oxidation of cellular oxidable substrates (Singh and Rajini 2004). They exert their effects by scavenging ROS, activating a battery of detoxifying proteins, or preventing the generation of ROS (Halliwell et al. 1992). Natural antioxidants constitute a broad range of compounds including phenolic compounds, nitrogen compounds and carotenoids (Velioglu et al. 1998). Olive leaves are rich in phenols, such as oleuropein, verbascoside, ligstroside, tyrosol and hydroxytyrosol, which have exhibited antioxidant and antimicrobial properties (Caturla et al. 2005). However, Yu et al. (2002) claim that there is no correlation between the TP and the radical scavenging capacity. Our results support this claim. In fact, the TP concentration was similar in T0 and T3 leaves (Table 3).

Interestingly, T0 plants revealed higher SP concentration (69%) than T3 plants (Table 3). Changes in SP concentration are important to

understand the impact of stress on cell proteolysis and protein synthesis (Santos and Caldeira 1999). During drought periods, plants undergo many physiological changes and induce a large number of genes for adaptation (Ingram and Bartels 1996). Under water-deficit conditions, a typical change in gene expression is the induction of genes involved in the synthesis of various osmolytes and low-molecular-weight proteins, e.g., dehydrins and late embryogenic-abundant proteins (Ingram and Bartels 1996). The accumulation of leaf proteins under water deficit may also represent a reserve of nitrogen to be used during the recovery after summer drought (Millard 1988).

Under mild and/or moderate drought stress some adapted species exhibit increases in activities of antioxidant enzymes, such as SOD and peroxidase (Lima et al. 2002; Liang et al. 2003). However, severe drought stress may cause damage to cells by inducing active oxygen production or by disrupting the scavenging systems that quench active oxygen and eliminate the detrimental effects (Van Breusegem et al. 1998). In our study, the decline in SOD activity in T0 plants (Table 3) indicated that the scavenging function of SOD was impaired, which would favour the accumulation of O_2^-. Similar results were achieved in water-stressed Norway spruce (Kronfuβ et al. 1998) and in maritime pine and pendunculate oak (Schwanz et al. 1996). Meanwhile, the finding that GPX activity increased in rainfed plants (Table 3), associated with the appearance of oxidative damage, suggests that this enzyme has no major antioxidative function in olive as in chieves (Egert and Tevini 2002), which is consistent with Asada's classification of peroxidases (Asada 1992) into ''antioxidatively'' and ''physiologically'' (e.g. during the synthesis of lignin) function ones. In fact, the increased peroxidase activity was reported in water-stressed marigold (*Tagetes erecta* L.), accompanying with increased lignin concentration (Kurup et al. 1994).

In conclusion, results fit well with the hypotheses presented. All irrigation levels improved carbon assimilation of field-grown olive trees and protected the photosynthetic apparatus by down-regulation of photochemical efficiency around midday. Furthermore, irrigation enhanced antioxidant protection and decreased the oxidative damage at leaf level. Additionally, results demonstrated that olive trees irrigated with 30% of the estimated local evaporative demand had high A/g_s at midday and afternoon, saving consistent amounts of water and revealing an RWC similar to T2 and T3 trees. Moreover, this level of irrigation seemed to be sufficient to reduce oxidative damage at leaf level. This is an interesting result since a sagacious irrigation approach is essential for viable olive industry due to the limited water resources available in the Mediterranean region. Further studies are needed to compare the effects of different irrigation levels in terms of productivity and oil quality.

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