Partial root zone drying: regulation of photosynthetic limitations and antioxidant enzymatic activities in young olive (*Olea europaea*) saplings

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Summary The effect of partial root drying (PRD) irrigation on split-root olive (*Olea europaea* L. cv Picholine marocaine) saplings was investigated. An irrigated control and two PRD regimes were applied (control: irrigation applied on both sides of the root system to keep the soil water content close to field capacity; PRD50: irrigation applied at 50% of the control amount on one side of the root system and irrigation withheld from the other side, with irrigation regimes switched between the sides of the root system every 2 weeks; and PRD100: irrigation applied at 100% of the control amount on one side and irrigation withheld on the other side, with irrigation regimes switched between the sides of the root system every 2 weeks. Only saplings in the PRD50 regime were subjected to water-deficit irrigation. The PRD treatments significantly affected water relations and vegetative growth throughout the growing season. Predawn leaf water potential and relative water content differed significantly between the PRD50 and PRD100 saplings, leading to reduced stomatal conductance, carbon assimilation, shoot length and leaf number in PRD50 saplings. However, the PRD50 water-deficit treatment did not affect the capacity of the saplings to assimilate CO2. Activities of superoxide dismutase, soluble and insoluble peroxidase (POX) and polyphenol oxidase were up-regulated by the PRD50 and PRD100 treatments compared with control values. The higher activities of both soluble and insoluble POX observed in PRD50 saplings may reflect the greater inhibitory effect of this treatment on vegetative growth. Up-regulation of the detoxifying systems in the PRD100 and PRD50 saplings may have provided protection mechanisms against irreversible damage to the photosynthetic machinery, thereby allowing the photosynthetic apparatus to function and preventing the development of severe water-stress. We also measured CO2 assimilation rate/attached leaves to very low [CO2] (~ 50 μmol mol⁻¹) to force stomatal opening. These results confirmed that, under conditions of moderate water stress, the sum of the diffusional resistances (i.e., stomatal and mesophyll resistances) sets the limit to photosynthetic rates. Assessing photosynthetic capacity without removing the diffusional limitations may lead to an overestimation of the biochemical limitations to photosynthesis in sclerophyllous plants.

Keywords: antioxidant defence, diffusional limitations, drought, growth, photosynthesis, PRD, water relations, water stress.

Introduction

The inhibition of plant growth and development during water stress is well-documented (Hsiao 1973). Water availability is the most important environmental resource limiting plant growth in Mediterranean regions. In a strongly seasonal climate, the length of the dry season may be crucial for crop production. Moreover, current global change models predict lower frequency of rainfall days and longer dry periods associated with increased temperature in Mediterranean regions (IPCC 2007). This will cause higher potential evapotranspiration, which is expected to lower the soil water content (SWC) and increase its variations in time (IPCC 2007). Increased crop production in the Mediterranean region will therefore be increasingly dependent on improvements in water management. Irrigation must be carried out efficiently, and agronomic practices must be implemented to save water and to improve plant growth while reducing evaporation from soil and transpiration from leaves (Centritto et al. 2000, Costa et al. 2007).

Water supplied in suboptimal amounts often results in the activation of a variety of physiological responses that may...
result in impairment of carbon assimilation (Lawlor and Cornic 2002). A detailed knowledge of the mechanisms that regulate photosynthetic capacity under different water regimes is of great interest because it may provide insights into the regulation of plant water-use efficiency (WUE) and, ultimately, help improve crop yield and quality. Studies on olive (Olea europaea L.) trees subjected to water deficits indicate that photosynthesis is mainly limited by stomatal conductance ($g_s$) under moderate water-stress conditions, whereas it is mainly limited by biochemical factors under severe water-stress conditions (Angelopoulos et al. 1996). However, because the analysis of stomatal versus non-stomatal limitations on photosynthetic efficiency under water-stress conditions cannot clearly discriminate between the diffusive and biochemical limitations to photosynthesis (Centritto et al. 2003), it can result in an overestimation of the drought-induced decrease in biochemical photosynthetic capacity, and in particular RuBP regeneration (Centritto et al. 2003, Flexas et al. 2008). Based on a study of adult olive trees subjected to soil drying conditions in the field, Centritto et al. (2005) concluded that the estimation of photosynthetic limitations under non-saturating CO$_2$ conditions should be made after measuring the total diffusional limitations (i.e., $g_s$ and mesophyll conductance to CO$_2$, $g_m$). The importance of estimating both $g_s$ and $g_m$, and consequently total conductance ($g_t$), for correctly determining the photosynthetic limitations of olive leaves, was first identified by Bongi and Loreto (1989), and this has also been shown in salt-stressed young olive plants (Centritto et al. 2003, Loreto et al. 2003) and in water-stressed adult olive trees growing in the field (Diaz-Espejo et al. 2007). In young salt-stressed olive plants, Loreto et al. (2003) found that the low chloroplast CO$_2$ concentration set by both $g_s$ and $g_m$ was the main limitation on photosynthesis. Diaz-Espejo et al. (2007) showed that the seasonal pattern of photosynthetic capacity of water-stressed olive trees was significantly affected by the physical limitations to CO$_2$ diffusion.

The main cause of the biochemical limitation under water-stress conditions is the accumulation of active oxygen species (AOS) and free radicals (Chaves and Oliveira 2004) that damage cell membranes and lead to the accumulation of lipid peroxides (Smirnoff 1993, Edreira 2005). The toxicity of AOS is normally counteracted by efficient scavenging by both non-enzymatic and enzymatic antioxidants present in the plant cells. Enzymatic antioxidants in leaves include superoxide dismutase (SOD; EC 1.15.1.1), which catalyzes the dismutation of superoxide radicals to H$_2$O$_2$ and O$_2$ together with catalase (CAT; EC 1.11.1.6), and ascorbate peroxidase (APX; EC 1.11.1.11), which detoxify the H$_2$O$_2$ produced by SOD. Guaiacol-type peroxidases (POX; EC 1.11.1.7) are also effective antioxidants and play an important role in the regulation of cell wall expansion (Mac Adam et al. 1992, Fry 1995). Finally, polyphenol oxidase (PPO; EC 1.3.3.1) isoenzymes, which oxidize o-diphenolic substrates to o-quinones (Kuwabara and Katoh 1999), are involved in the metabolism of phenols that are also important antioxidants (Rice-Evans et al. 1997). As shown by Sofo et al. (2005), the ability of olive trees to up-regulate the enzymatic antioxidant system may be an important factor underlying the drought tolerance of this species. Sofo et al. (2005), who studied olive saplings grown at high temperature and irradiances and gradually subjected to water deficit, found that the activities of SOD, APX, CAT and guaiacol peroxidase increased with increasing drought severity, whereas PPO activity decreased. A recent study on olive saplings subjected to soil drying showed that the activities of SOD, soluble POX and insoluble POX were up-regulated by water deficits, and that PPO activity also increased in water-stressed leaves (Aganchich et al. 2007).

Young saplings of olive, which is an important agricultural and timber crop in Mediterranean regions, were chosen to study the effects of partial root drying (PRD) irrigation on growth and physiology. The PRD is a water-saving irrigation system that has been developed to increase WUE in agriculture (i.e., the maximum net revenue per unit water used rather than per land unit) by exposing plants simultaneously to both wetted and drying soil (Dry et al. 1996, Davies et al. 2002, Kang and Zhang 2004, Fereres and Soriano 2007). The theory behind this approach is to utilize the plant’s long-distance signaling system, which plays an important role in the regulation of stomatal behavior, to control plant growth and to prevent the development of the physiological effects associated with severe water deficits (Davies and Zhang 1991, Stoll et al. 2000, Davies et al. 2002, Kang and Zhang 2004, Tahli et al. 2007). In earlier studies on the effects of PRD irrigation treatments on field-grown, adult olive trees, we confirmed that carbon assimilation is reduced in PRD saplings, but they have significantly higher WUE than control plants (Centritto et al. 2005, Wahbi et al. 2005); however, the physiological mechanisms underlying these responses were not investigated. In an attempt to identify these physiological mechanisms, we focused on photosynthetic limitations and antioxidant defence activities in olive saplings subjected to PRD treatments.

Materials and methods

Plant material and experimental protocol

The experiment was conducted in the greenhouse of the Faculty of Sciences Semlalia, Marrakech (Morocco) 31°83’ N and 88°04’ W, altitude: 411.6 m a.s.l. Three-year-old olive (O. europaea L. cv Picholine marocaine) saplings, with a mean height of 40 cm, were planted in January 2003 in 7-dm$^3$ pots. Saplings were kept at pot water capacity until the end of May, when each root system was divided vertically into two parts and each part was potted in a 100 cm-long PVC pipe (7.0 dm$^3$), containing a 1:1:1 (v/v) mixture of sand:peat:silt-clay, to produce a split-root system. Before initiating the irrigation treatments, the
saplings were allowed to adapt to the new split-root systems by keeping the two root compartments constantly well-watered for 1 year to ensure uniformity of plant development. Then, at the beginning of January 2004, three irrigation treatments were imposed – an irrigated control and two PRD regimes. (1) Controls were irrigated with 100% of evapotranspiration in both root pots of each sapling. The SWC was monitored in the pots every other day with a Tetraprobe (Delta-T Devices, Cambridge, UK). Irrigation was applied to keep the SWC close to field capacity (0.45 v/v) in both pots. (2) In the PRD_{50} treatment (hereafter also referred to as the water-deficit treatment), saplings were subjected to water deficit by irrigating with only 50% of the water supplied to controls on one side of the root system only, while irrigation was withheld from the other side of the root system. Every 2 weeks the PRD_{50} treatment was switched to the unirrigated side of the root system. (3) In the PRD_{100} treatment, saplings were irrigated with 100% of the water supplied to controls on one side of the root system only, while irrigation was withheld from the other side of the root system. Every 2 weeks the PRD_{100} treatment was switched to the unirrigated side of the root system. To avoid drainage losses, the terminal part of each PVC pipe was sealed. Only saplings in the PRD_{50} regime were subjected to water-deficit irrigation.

**Growth measurements**

Stem length was measured every 2 weeks from mid-April (Day 95) to mid-September (Day 249) on one current-year stem per sapling (eight saplings per treatment). Leaf number was measured on the same stems. At the end of the experiment, leaf length (L) and width (W) were measured and leaf area (LA) was determined as LA = k LW, where k is a constant whose value (k = 0.67) was determined as described by Van Arkel (1978) based on a sample of 100 leaves (Montgomery 1911, Aganchich et al. 2007).

**Measurements of water status, gas exchange and fluorescence**

Predawn stem water potential (Ψ) was measured with a Scholander pressure chamber (SKPD 1400, Skye Instruments, Powys, UK). A branch with four newly expanded leaves per sapling (six saplings per treatment) was detached, enclosed in a plastic bag. Before determining the balancing pressure the branch was recut under water.

At midday every 2 weeks throughout the growing season, stomatal conductance (g_s) was measured with a portable porometer (Delta-T AP4, Delta-T Devices, Cambridge, UK). The device was calibrated before use with the supplied calibration plate. The terminal part of the main leaf lobe was placed in the cup on the head unit which was positioned normal to the sun. Measurements were made during cloudless periods.

During summer, between Days 242 and 247, gas exchange was measured in situ between 1100 and 1500 h on the central section of a newly expanded leaf in five saplings per irrigation treatment with a portable infrared analyzer (IRGA) (LiCor 6400, Li-Cor, Lincoln, NE) equipped with a 6-cm² cuvette with a transparent window to allow illumination by natural light. The cuvette window was modified to accommodate a fluorescence probe (MiniPAM, Walz, Effeltrich, Germany). The tip of the optic fibre of the MiniPAM was inserted in one corner of the cuvette window at an angle of 45° to the surface. The optic fibre could be placed about 1 cm from the leaf without shading it (cf. Loreto et al. 2003). A leaf was clamped in the cuvette and was exposed to ambient irradiance until the photosynthetic rate reached a steady state. Then, gas exchange parameters and chlorophyll fluorescence yielded were measured simultaneously. The fluorescence yield (i.e., the quantum yield of PSII in the light, ΔF/F_m) was measured following a saturating pulse (10,000 μmol m⁻² s⁻¹) of white light (Genty et al. 1989). The electron transport rate was measured by fluorescence as described by Genty et al. (1989). Mesophyll conductance (g_m) was calculated by the variable Δ method, as described by Harley et al. (1992). Measurements of dark respiration (R_d) were made after maintaining the leaves in darkness for 10 min. The CO₂ compensation point to photosynthesis (F*) used in the gas exchange algorithm was calculated with the Rubisco specific factor estimated for woody evergreen by Galmar et al. (2005). Because ΔF/F_m is a remarkably conservative parameter (Harley et al. 1992), we assumed that the value used in the gas exchange algorithm did not affect the estimation of g_m. Although the diffusion leaks through channel-foam gaskets were taken into account and corrected for according to the manufacturer’s suggestions (Li-Cor Inc. 2004), it was impossible to rule out the occurrence of other measurement errors (Flexas et al. 2008). However, Centritto et al. (unpublished results) have recently calculated g_m by using both the variable Δ method and carbon isotope discrimination in recently synthesized sugars in water-stressed rice genotypes, and found that the two methods yielded congruent estimations of g_m, confirming the reliability of the technique based on simultaneous measurements of gas exchange and fluorescence parameters. Total conductance (g_t) was calculated as g_t = g_s g_m/(g_s + g_m).

To estimate the biochemical limitations to photosynthesis (Farquhar et al. 1980), we measured CO₂ assimilation rate (A) as a function of internal leaf CO₂ concentration (C_i) at CO₂ concentrations between 40 and 2100 μmol mol⁻¹. The A/C_i measurements were made by moving the saplings to a laboratory, to ensure a constant leaf temperature of 30 °C and a relative humidity in the leaf cuvette of about 50%, and all measurements were made between 1000 and 1700 h. To measure light-saturated photosynthetic rates, leaves were illuminated with a red–blue light source attached to the gas-exchange system that generated a photosynthetic photon flux (PPF) of 1400 μmol m⁻² s⁻¹. A first set of standard A/C_i curves was obtained based on short-term measurements (~ 10 min for each data point), starting at a [CO₂] of...
375 μmol mol⁻¹ (ambient [CO₂], Cₐ), and progressively reducing the [CO₂] to 40 μmol mol⁻¹. Subsequently, the [CO₂] was progressively increased up to 2100 μmol mol⁻¹. By the time a [CO₂] of 2100 μmol mol⁻¹ was reached, gₛ decreased to values as low as 0.015 mmol m⁻²s⁻¹. Finally, to remove the effect of stomatal limitation on A (and to estimate photosynthetic capacity at high gₛ), another set of A/Cᵢ curves was generated on three to four saplings in each of the control, PRD₅₀ and PRD₁₀₀ treatments, as described by Centritto et al. (2003). After measuring A at Cₐ, the [CO₂] was reduced to 50 μmol mol⁻¹ and leaves were exposed at this [CO₂] for up to 90 min to force stomatal opening. When gₛ approached the maximum value (Figure 1), [CO₂] was progressively increased to 375 μmol mol⁻¹. After the measurement at 375 μmol mol⁻¹, the [CO₂] was immediately increased to 2100 μmol mol⁻¹, and then progressively decreased back to 375 μmol mol⁻¹.

The photosynthetic parameters Aₘₐₓ (net CO₂ assimilation rate under conditions of saturating PPF and CO₂), apparent Vₗₘₐₓ (i.e., apparent RuBP-saturated rate of Rubisco: estimate of the carboxylation efficiency of Rubisco determined from the slope of the A/Cᵢ curve at [CO₂] of 40–200 μmol mol⁻¹, assuming that the resistance to CO₂ diffusion inside the leaf mesophyll is taken as zero, i.e., gₚ = ∞) and Jₘₐₓ (maximum rate of electron transport) were estimated by fitting the mechanistic model of CO₂ assimilation proposed by Farquhar et al. (1980) to individual A/Cᵢ response data by the method developed by de Pury and Farquhar (1997). The fitting model was run using the in vivo Rubisco kinetics parameters measured by Bernacchi et al. (2001) and Galmés et al. (2005). Fitting the model involved adjusting the parameter values until the sum of residuals between observed and modeled assimilation values over a range of Cᵢ were minimal.

**Enzymatic assays**

The enzymatic assays were carried out every 4 weeks on six saplings per treatment. Each sample consisted of two fully expanded leaves taken from newly developed shoots. The leaf samples were immediately frozen in liquid nitrogen and then stored at –80°C until used. The frozen leaves were weighed (1.5 g of fresh mass), and ground in an ice-cold mortar and pestle with 1.5 ml of 0.1 M Na₂HPO₄/NaH₂PO₄ (pH 6.5) buffer containing 5% (w/v) PVP. The homogenates were centrifuged for 10 min at 10,000g. The

![Figure 1](https://academic.oup.com/treephys/article-abstract/29/5/685/1683053)
supernatants were assayed for SOD, soluble POX and PPO activities.

To extract cell-wall-associated peroxidase (POXins), the pellet was washed twice in the same volume of 0.1 M Na₂HPO₄/NaH₂PO₄ to remove cytoplasmic peroxidase activity, before being resuspended in an equal volume of the final extraction buffer containing 0.1 M Na₂HPO₄/NaH₂PO₄ and 1 M sodium chloride.

We assayed POX activity in 100 μl of the supernatant by the Guaiacol test as described by Chance and Maehly (1955). The supernatant was added to 1 ml of 20 mM sodium phosphate buffer (pH 6), containing 276 μl of guaiacol per 50 ml of buffer. The reaction was started by adding 200 μl of 0.03% hydrogen peroxide in distilled water (w/w). After 3-min incubation, absorbance of the reaction mixture was measured spectrophotometrically at 470 nm.

Activity of SOD was determined by measuring the percent inhibition of nitroblue tetrazolium (NBT) reduction by SOD (Beauchamp and Fridovich 1971). The activity was expressed in units where 50% inhibition is equivalent to one unit of SOD activity. Xanthine oxidase was used to generate O₂⁻ during the conversion of xanthine to uric acid. One millilitre of 0.3 mM xanthine, 500 μl of 0.06 mM EDTA, 500 μl of 0.15 mM NBT and 100 μl of tissue extract were mixed in a cuvette and the spectrophotometer was zeroed at 560 nm before adding xanthine oxidase and recording absorbance at 5-s intervals for 1 min with a Beckman DU-64 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) fitted with a Sof-Pac Kinetics module.

Activity of PPO was assayed as described by Taneja and Sachar (1974). The reaction mixture (4 ml final volume) comprised 200 μl of enzyme extract, 2 ml of catechol 1% and 1.8 ml of 0.05 M sodium phosphate buffer (pH 6.6). After mixing and a 3-min incubation period, the absorbance of the reaction mixture was measured spectrophotometrically at 430 nm.

Statistical analyses

Pots were randomized by a completely randomized block design, with eight replicates and three treatments (control, PRD₁₀₀ and PRD₅₀). Data were subjected to one-way analysis of variance, followed by the least significant difference (LSD) test at P = 0.05 to compare the means. All analyses were made using SPSS 10.0 for Windows software (SPSS, Chicago, IL).

Results and discussion

Effects of irrigation treatments on water status of olive saplings

The irrigation treatments significantly influenced predawn stem Ψ (Figure 2A). The control saplings showed a constant and high Ψ (more than −0.80 MPa) throughout the study, whereas predawn stem Ψ in PRD saplings was significantly lower than that in control saplings starting 140 days after beginning the treatments. Although predawn Ψ was consistently higher in PRD₁₀₀ saplings than in saplings subjected to the PRD₅₀ water-deficit treatment, the differences were not statistically significant during most of the growing season. The minimal values of predawn Ψ reached in PRD₁₀₀ and PRD₅₀ saplings were −1.13 and −1.23 MPa, respectively, indicating that the saplings were not severely water stressed (Fernández et al. 1997). Significant differences in either predawn or midday Ψ between well-watered saplings and PRD₅₀ saplings have been found in numerous studies, including in olive (Aganchich et al. 2007) and in both field-grown (de Souza et al. 2003, 2005, Marsal et al. 2008) and potted (Stoll et al. 2000) grapevines. However, conflicting results have been reported for field-grown adult olive trees growing in an arid climate. On the one hand, Wahbi et al. (2005) found that, in field-grown olive plants, midday leaf Ψ was similar in the PRD₁₀₀ and control treatment plants and significantly higher than that in the PRD₅₀ treatment, whereas leaf Ψ decreased substantially during the summer reaching values as low as −2.7 and −3.4 MPa in the PRD₁₀₀ and PRD₅₀ treatments, respectively, indicating that the saplings were severely water stressed. On the other hand, Fernández et al. (2006) found only small and random differences in predawn stem Ψ between well-watered plants and plants that received about 30% of the water supplied to the well-watered plants.
Liu et al. (2006) reported that PRD decreased root $\Psi$ significantly more in greenhouse-grown potato plants than in well-watered plants, whereas in the field there was no difference in leaf $\Psi$ between treatments. Plant age, degree of water stress and root volume, as well as specific split-root effects on $\Psi$, which may be exacerbated in potted plants, may account for these conflicting results.

Stomatal conductance, which we assessed throughout the growing season with a portable porometer (Figure 2B) and on specific dates with an infrared gas analyzer (Table 1), was significantly reduced by the PRD treatments ($P < 0.001$), which accords with the results of many studies (e.g., Dry et al. 2000, Stoll et al. 2000, de Souza et al. 2003, 2005, Aganchich et al. 2007, Tahi et al. 2007, Marsal et al. 2008) showing that PRD treatments result in a mild stress that significantly affects $g_s$. We found significant differences in $g_s$ between the PRD$_{50}$ and PRD$_{100}$ treatments, reflecting the treatment differences seen in predawn stem $\Psi$, and confirming previous observations on field-grown adult olive trees (Centritto et al. 2005, Wahbi et al. 2005, Fernández et al. 2006). The PRD treatments significantly reduced $g_s$ already at the beginning of the treatment, whereas the reduction in leaf $\Psi$ occurred only later during the treatment (Figure 2), supporting the general observation that stomatal closure in response to drought occurs before any significant change in leaf water status is detectable (Gollan et al. 1985, Davies et al. 2002). We found that $g_s$ was more closely related to SWC than to $\Psi$ (cf. Jones 1992, Centritto et al. 1999). Giorio et al. (1999) reached a similar conclusion working with field-grown olive trees subjected to water deficit. Natali et al. (1991) reported that rewatering of severely water-stressed olive resulted in a prompt recovery in $\Psi$ that was not accompanied by an increase in $g_s$.

**Effects of irrigation treatments on growth of olive saplings**

In many studies, including several based on the split-root system method, it has been found that roots can sense soil drying and produce chemical signals (e.g., abscisic acid and pH) that are transported in the transpiration stream to the shoots where they affect reductions in stomatal conductance and vegetative growth (Davies and Zhang 1991, Kang and Zhang 2004, Tahi et al. 2007). Similarly, in our study, vegetative growth was strongly affected by the irrigation treatments ($P < 0.001$). Temporal variations in both shoot lengths and leaf numbers of PRD saplings are shown in Figure 3. Not only did the PRD$_{50}$ water-deficit treatment significantly decrease shoot length and leaf number, but significant differences were also found between PRD$_{100}$ and control saplings. The reduction in leaf number caused by both the lower amount of water applied and the irrigation method resulted in a significantly decreased leaf area (Table 1). At the end of the experiment, the PRD$_{50}$ water-deficit treatment caused reductions in shoot length, leaf number and leaf area of about 56.10%.
However, A-lings (Table 1). There is growing evidence that confirmed that both the PRD 50 water deficit and PRD 100 Measurements of gas exchange under ambient conditions properties of olive leaves Effects of irrigation treatments on photosynthetic which, in turn, may also affect olive growth in response system increases the resistance of olive trees to drought, over, our observations are in agreement with the results obtained from several studies on partial root zone drying (Dry et al. 2000, Stoll et al. 2000, Aganchich et al. 2005) observed similar results in a study on the effects of PRD irrigations on field-grown adult olive trees. Moreover, our observations are in agreement with the results obtained from several studies on partial root zone drying experiments (Dry et al. 2000, Stoll et al. 2000, Aganchich et al. 2007, Du et al. 2008). Recently, Sofo et al. (2005) pointed out that up-regulation of the antioxidant defence system increases the resistance of olive trees to drought, which, in turn, may also affect olive growth in response to soil drying.

Effects of irrigation treatments on photosynthetic properties of olive leaves

Measurements of gas exchange under ambient conditions confirmed that both the PRD50 water deficit and PRD100 treatments decreased gs, by 33% and 16%, respectively. However, A was significantly inhibited only in PRD50 saplings (Table 1). There is growing evidence that gs is generally more sensitive to PRD irrigation than A (Liu et al. 2006). This may be because diffusional limitations to photosynthesis are set not only by stomatal resistances, but also by resistances to CO2 diffusion toward the carboxylation sites inside the mesophyll. Although gm was not significantly affected by the irrigation method, it increased by about 18% in PRD100 saplings (Table 1). This is to our knowledge one of the few cases (Shi et al. 2006, 2008) in which gm and gs become uncoupled. Generally, diffusive resistances increase concurrently in response to drought (Flexas et al. 2004) or salinity (Centritto et al. 2003, Loreto et al. 2003). The contrasting effects of the PRD100 treatment on gs and gm led to a similar total conductance g of PRD irrigations on field-grown adult olive trees. More- Effects of irrigation treatments on photosynthetic and consequently to similar total diffusional limitations to photosynthesis in PRD100 and control saplings. Given that diffusive limitations most often limit photosynthesis in the leaves of water-stressed plants (Flexas et al. 2004), the increase in gm compensating for the reduction in gs may explain why the PRD100 treatment had no effect on A.

In contrast to the effect of the PRD100 treatment, the total diffusional limitations to photosynthesis significantly increased in saplings subjected to the PRD50 water-deficit treatment, and this together with the absence of an effect on apparent Vcmax may explain the inhibition in A in the PRD50 treatment. The irrigation methods did not significantly affect dark respiration or its contribution to net photosynthesis (Table 1). Overall, these results confirm previous findings that, when the diffusional limitations of photosynthesis are correctly estimated, A of olive leaves is significantly reduced only in PRD50 saplings (Centritto et al. 2005). A similar negative effect of deficit irrigation on A was observed by Fernández et al. (2006) in adult olive trees grown in the field and receiving about 30% of the water supplied to well-watered plants. Inhibition of A in response to deficit irrigation treatments has been reported in many other papers (e.g., de Souza et al. 2003, Liu et al. 2006), but, because more severe water stress treatments were applied than in our study, direct comparisons with these studies are not possible. However, our observation confirmed that the sum of diffusional resistances often effectively contributes to limit photosynthesis in well-watered and mildly water-stressed olive plants (Loreto et al. 2003).

To measure in vivo the multi-enzyme kinetic properties of photosynthesis and, thus, to estimate the biochemical limitations to photosynthesis, we used A/Ci responses. Centritto et al. (2003) showed that increased diffusional resistances in olive, caused by salt stress, were fully overcome by exposing attached leaves to very low [CO2] (∼50 μmol mol−1). Similarly, in our study, the photosynthetic capacity of well-watered saplings (Figure 4A) and of saplings subjected to PRD (Figure 4B and C) was significantly increased by exposing attached leaves to very low [CO2]. By comparing the standard and pre-conditioned A/Ci curves, it is evident that apparent Rubisco activity, i.e., Vcmax estimated from the slope of the A/Ci curve assuming gm = ∞, was similar in the two sets of A/Ci curves (see also the initial slope of the A/Ci curves, as an estimate of the carboxylation efficiency). Although the A/Ci responses do not account for mesophyll conductance,
apparent $V_{cmax}$ was clearly unaffected by stomatal resistances that may negatively influence diffusive resistance in water-stressed leaves (Flexas et al. 2004). In contrast, the estimates of $A_{max}$ (net CO₂ assimilation rate under conditions of saturating PPF and [CO₂]) and $J_{max}$ (maximum rate of electron transport) were higher in the $A/C_i$ curves of saplings pre-conditioned at low [CO₂], and in which diffusive limitations were reduced to a minimum, than in the standard $A/C_i$ curves (Table 2). This is further evidence that, at least in sclerophyllous plants, assessing photosynthetic capacity without removing the diffusive limitations may lead to misleading conclusions about biochemical limitations to photosynthesis (Centritto et al. 2003, 2005). The influence of diffusive limitations on the apparent biochemical limitations to photosynthesis was independent of leaf water status, because there were no differences in photosynthetic capacity between well-watered saplings and PRD saplings (Table 2).

Effects of irrigation treatments on the antioxidant status of olive leaves

Water stress is partially a result of increased exposure to secondary oxidative stress that damages cellular membranes and macromolecules (Smirnoff 1993, Edreva 2005). The capacity to avoid or repair oxidative damage during dehydration is pivotal for the maintenance of membrane integrity, which, in turn, allows plants to remain functional for longer under soil drying conditions. Recent studies by Sofo et al. (2005) and Aganchich et al. (2007) have demonstrated up-regulation of the antioxidant defence system in young olive plants subjected to different degrees of water stress. Similarly, in our study, there was a marked stimulation in the activity of SOD, PPO and both soluble and insoluble POX in response to the irrigation treatments (Figure 5). However, there were also consistent differences in antioxidant enzymatic activities between the PRD50 and PRD100 saplings. With the exception of SOD activity (Figure 5D), the activities of soluble POX (Figure 5A), insoluble POX (Figure 5B) and PPO (Figure 5C) were

![Figure 4. Standard $A/C_i$ (□) and pre-conditioned $A/C_i$ (●) measured after exposing O. europaea saplings to a [CO₂] of $\sim$ 50 μmol mol$^{-1}$ for about 45 min to force stomatal opening (see Figure 1) in the control (A), PRD50 (B) and PRD100 (C) irrigations. Measurements were made at saturating PPF ($\sim$ 1200 μmol m$^{-2}$ s$^{-1}$) on three to four plants per treatments.](https://academic.oup.com/treephys/article-abstract/29/5/685/1683053)

![Table 2. Photosynthetic parameters derived from the individual $A/C_i$ curve responses (shown in Figure 4) of O. europaea saplings subjected to control and PRD irrigations. The values were obtained by fitting the Farquhar et al. (1980) model of leaf photosynthesis to standard $A/C_i$ curves and after exposing plants at [CO₂] of $\sim$ 50 μmol mol$^{-1}$ for about 45 min to force stomatal opening (pre-conditioned $A/C_i$). Abbreviations: $A_{max}$ (μmol m$^{-2}$ s$^{-1}$), maximum photosynthetic rate at saturating PPF and [CO₂]; $J_{max}$ (μmol m$^{-2}$ s$^{-1}$), potential rate of electron transport per unit leaf area, and apparent $V_{cmax}$ (μmol m$^{-2}$ s$^{-1}$), photosynthetic Rubisco capacity per unit leaf area. Values are means of three to four plants per treatment $\pm$ 1 SEM; within a column, means followed by different letters differ significantly at $P < 0.05$.](https://academic.oup.com/treephys/article-abstract/29/5/685/1683053)
significantly and consistently higher in PRD<sub>50</sub> saplings than in PRD<sub>100</sub> saplings. Because SOD is an essential component of a plant’s defence mechanism against AOS produced by the photosynthetic electron transport chain in chloroplasts (Asada 1999), increased SOD activity in response to both PRD<sub>50</sub> and PRD<sub>100</sub> likely indicates that the chloroplast antioxidant apparatus is immediately activated in response to a stress signal, and that this activation may occur long before the stress is detectable by physiological measurements. In plants subjected to the PRD<sub>100</sub> treatment, no stress may ever occur at the photosynthetic level, even though the leaves up-regulate their antioxidant defence system: the latter response may be a useful defensive trait against additional and coevolving stresses that also generate dangerous AOS (e.g., high temperatures and irradiances).

In general, stimulation of the antioxidant defence in response to the PRD treatments mirrored the decreases in predawn stem Ψ (Figure 2A) and g<sub>s</sub> throughout the growing season (Figure 2B). Similarly, Sofo et al. (2005) showed that the activities of antioxidant enzymes increase with increasing severity of water stress, with the exception of PPO activity, a result that conflicts with our findings. However, Shivashankar (1988) observed that PPO activity in rain-fed palms increased with the development of water stress, whereas there was little change in PPO activity in irrigated palms.

Previous studies have implicated high soluble and insoluble POX activities in the regulation of plant growth rate through biochemical modifications of cell wall properties causing reduced rates of cell wall expansion (Mac Adam et al. 1992, Fry 1995). Many studies have provided evidence that peroxidases play a role in lignification (Whetten et al. 1998, Quiroga et al. 2000). Bacon et al. (1997) found that drought reduced leaf elongation of <i>Lolium tumelentum</i> and suggested that this was associated with a 200–300% increase in cell-wall-associated POX activity in the leaf elongation zones. Sofo et al. (2005) argued that, under water-stress conditions, high POX activity limits olive growth by increasing lignification rates and consequently inactivating auxin, and that this process is correlated to drought adaptation. The large increases in the activities of soluble and insoluble POX may also account for the reduction in growth (Figure 3) of our olive saplings subjected to the irrigation treatments. Moreover, the significant differences in activities of both soluble and insoluble POX between PRD<sub>50</sub> and PRD<sub>100</sub> saplings may explain the higher inhibitory effect of PRD<sub>50</sub> on vegetative growth.

Conclusions

We analyzed the responses of young olive saplings to water saving irrigation treatments. Predawn stem Ψ was significantly reduced by the irrigation treatments, and PRD<sub>100</sub> saplings had consistently higher predawn Ψ than PRD<sub>50</sub>
saplings. These differences in plant water relations caused reductions in stomatal conductance, instantaneous assimilation rates and, in turn, vegetative growth. However, photosynthetic capacity was unaffected by the irrigation treatments even in the PRD_{50} saplings that received only about 50% of the water supplied to the control and PRD_{100} saplings. We estimated the photosynthetic capacity of water-stressed saplings in vivo by separating the diffusional limitations from the non-diffusional limitations and found that the moderate water stress caused by the PRD_{50} deficit irrigation treatment did not affect the biochemical capacity of the saplings to assimilate CO_{2}. Therefore, in the PRD_{50} saplings, the sum of the diffusional resistances (i.e., stomatal and mesophyll resistances) sets the limit to photosynthetic rates. We also demonstrated that, at least in sclerophyllous plants, assessing photosynthetic capacity without removing the diffusional limitations may lead to an overestimation of the biochemical limitations to photosynthesis.

Up-regulation of the antioxidant defence system was found in response to the PRD_{100} treatment and especially to the PRD_{50} treatment and may provide protection mechanisms that enable saplings to maintain the functionality of their photosynthetic apparatus, thereby preventing the development of severe water stress. In general, plants subjected to water stress undergo increased exposure to AOS which, in turn, damage functional proteins, membrane and cellular structures. Overall, we found that, in response to soil drying, olive saplings activate a complex detoxifying system that likely prevents irreversible damage to the photosynthetic machinery caused by an excessive increase in AOS. Because water saving irrigation treatments lead to the activation of this important protection mechanism, it may be advantageous to implement deficit irrigation techniques to the olive-growing conditions of Morocco and, in general, to the drought-prone areas of the Mediterranean basin.

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