

Do photosynthetic limitations of evergreen *Quercus ilex* leaves change with long-term increased drought severity?

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ABSTRACT

Seasonal drought can severely impact leaf photosynthetic capacity. This is particularly important for Mediterranean forests, where precipitation is expected to decrease as a consequence of climate change. Impacts of increased drought on the photosynthetic capacity of the evergreen *Quercus ilex* were studied for two years in a mature forest submitted to long-term throughfall exclusion. Gas exchange and chlorophyll fluorescence were measured on two successive leaf cohorts in a control and a dry plot. Exclusion significantly reduced leaf water potential in the dry treatment. In both treatments, light-saturated net assimilation rate (A_{\max}), stomatal conductance (g_s), maximum carboxylation rate (V_{\max}), maximum rate of electron transport (J_{\max}), mesophyll conductance to CO_2 (g_m) and nitrogen investment in photosynthesis decreased markedly with soil water limitation during summer. The relationships between leaf photosynthetic parameters and leaf water potential remained identical in the two treatments. Leaf and canopy acclimation to progressive, long-term drought occurred through changes in leaf area index, leaf mass per area and leaf chemical composition, but not through modifications of physiological parameters.

Key-words: acclimation; drought; Mediterranean evergreen; mesophyll conductance; photosynthesis limitation; photosynthetic capacity; seasonality; throughfall exclusion.

INTRODUCTION

Water availability has a dominant or co-dominant effect on the productivity of most biomes on earth (Churkina & Running 1998; Nemani *et al.* 2003), and extreme drought severely impacts the carbon balance of a wide range of terrestrial ecosystems (Ciais *et al.* 2005). Drought impacts are particularly important for Mediterranean evergreen woodlands and forests, where water availability is a major factor controlling vegetation structure and function (Rambal 2001). Recent climate projections for the Mediterranean basin include a decline in precipitation by more than 20% during spring and summer, combined with higher evaporative demand due to warmer temperatures (Giorgi

2006; Rowel & Jones 2006). Mediterranean ecosystems are therefore likely to experience more frequent and intense drought with ongoing climate change.

Carbon assimilation can be impaired by drought through a variety of mechanisms occurring from leaf to ecosystem scales. Photosynthetic response to drought has been extensively studied, and several limitations have been identified and quantified (Chaves *et al.* 2002; Flexas & Medrano 2002; Lawlor & Cornic 2002). Stomatal closure occurs rapidly and is widely recognized as the primary effect of drought on carbon assimilation (Chaves 1991; Chaves *et al.* 2002). CO_2 diffusion to the chloroplast stroma is also limited by mesophyll conductance, which has been shown to decrease rapidly under water stress, further reducing the availability of CO_2 (Roupsard, Gross & Dreyer 1996; Grassi & Magnani 2005; Flexas *et al.* 2007; Galmés, Medrano & Flexas 2007). Under severe or long-lasting drought, photosynthesis is also limited by several metabolic impairments, such as decreased ribulose 1,5-bisphosphate-carboxylase-oxygenase (Rubisco) activity and ribulose-1,5-bisphosphate (RuBP) regeneration capacity (Bota, Medrano & Flexas 2004; Flexas *et al.* 2004). Decreases in Rubisco activity can occur in response to deactivation or decreased Rubisco content, the latter resulting from either a smaller allocation of nitrogen to Rubisco (Wilson, Baldocchi & Hanson 2000a; Grassi *et al.* 2005) or a lower nitrogen content in the leaves (Xu & Baldocchi 2003; Misson *et al.* 2006). At longer time scales, these mechanisms are coordinated with adjustments of leaf morphology and chemical content (Castro-díez *et al.* 1997) or even of the whole-ecosystem leaf area index (Grier & Running 1977; Gholz 1982). It is still unknown how these mechanisms are temporally and spatially associated at different levels of organization.

Photosynthesis is commonly simulated by the biochemical model of Farquhar, von Caemmerer & Berry (1980). This model can be used to simulate carbon assimilation from leaf to continental scales through the estimation of maximum Rubisco carboxylation (V_{\max}) and maximum photosynthetic electron transport (J_{\max}) rates (Wullschlegel 1993). Seasonal variations of V_{\max} and J_{\max} have been shown to strongly influence plant carbon uptake (Wilson, Baldocchi & Hanson 2001), and several studies have aimed to describe these variations among species (Wilson *et al.* 2000a; Nogués & Alegre 2002; Xu & Baldocchi 2003; Grassi *et al.* 2005; Misson *et al.* 2006). Collectively, these studies

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demonstrate a lack of consensus regarding the influence of drought stress on seasonal variations of V_{cmax} and J_{max} under field conditions. This is probably because these variations are influenced by multiple biotic factors, such as species and phenology, as well as abiotic factors, including radiation, temperature and atmospheric VPD . Moreover, these parameters, called apparent V_{cmax} and apparent J_{max} in this paper, have been estimated using substomatal CO_2 response curves of assimilation, the so-called $A-C_i$ curve, neglecting the possibility of finite mesophyll conductance. In several cases, mesophyll conductance has been shown to strongly influence the apparent seasonality of leaf photosynthetic parameters (Grassi & Magnani 2005; Montpied, Granier & Dreyer 2009). However, measuring seasonal time courses of ecophysiological parameters during annual drought development may be inappropriate to reveal long-term adjustments that are expected to occur as a consequence of declining precipitation, as predicted by climate models (Limousin *et al.* 2009). Experimental rainfall manipulations can be used to combine the effects of seasonal drought with those of long-term precipitation decreases, but very few studies have measured the seasonality of leaf photosynthetic capacity using such experiments (Wilson *et al.* 2000a).

In this study, leaf photosynthetic capacity was monitored during two consecutive growing seasons, on different-aged cohorts of leaves from mature evergreen *Quercus ilex* growing in a Mediterranean woodland ecosystem exposed to 5 years of reduced precipitation, by throughfall exclusion. This experiment reduced the average precipitation input to the soil by 27% on an annual basis (Limousin *et al.* 2008), resulting in lower transpiration and leaf area index (Limousin *et al.* 2009). Our specific objectives were (1) to describe the seasonal pattern of leaf photosynthetic capacity, (2) to investigate the mechanisms underlying this seasonality and (3) to determine how long-term increased drought modifies leaf photosynthetic capacity and its sensitivity to water limitation. We hypothesized that five consecutive years of increased drought severity would modify leaf photosynthetic capacity in the dry treatment of the throughfall exclusion experiment through changes in leaf morphology, chemical composition, mesophyll conductance and water use efficiency.

MATERIALS AND METHODS

Experimental site

The experimental site is located 35 km northwest of Montpellier (southern France) in the Puéchabon State Forest on a flat plateau (3°35'45"E, 43°44'29"N, 270 m a.s.l.). The area has a Mediterranean-type climate with a mean annual temperature of 13.4 °C, the coldest month being January (5.5 °C) and the hottest month, July (22.9 °C). The mean annual precipitation is 907 mm with a range of 556–1549 mm recorded over the 1984–2008 period. Rainfall mainly occurs during autumn and winter, with about 80% between September and April (Allard *et al.* 2008). The site

supports a *Q. ilex*-dominated evergreen forest, which has been managed as a coppice for centuries and clear cut for the last time in 1942. The top canopy height is about 5.5 m and the density of resprouts was 6393 stems·ha⁻¹ in 2007. The soil is extremely rocky from hard Jurassic limestone origin; the average volumetric fractional content of stones is about 0.75 for the top 0–50 cm, and 0.90 below. The stone-free fine fraction within the 0–50 cm layer is a homogeneous silty clay loam (Rambal *et al.* 2003).

A throughfall exclusion experiment was set up at the site in March 2003. Two 140 m² plots were subjected to different precipitation regimes: one was a control treatment facing natural drought conditions, whereas the other was a dry treatment subjected to throughfall exclusion. In the dry plot, throughfall exclusion was achieved by using PVC gutters hung under the canopy and by reducing the net input of precipitation to the soil by 27% compared with the control treatment (Limousin *et al.* 2008). In the control plot, identical gutters had been set up upside down, so that the albedo and the understorey microclimate were as close as possible in both treatments. The leaf area index of *Q. ilex* inside the experimental plots was assessed from leaf litterfall and found to be 1.9 m² m⁻² in the control treatment and 1.6 m² m⁻² in the dry treatment. The average leaf lifespan was not significantly affected by the throughfall exclusion, and the average fraction of leaves that are more than 1 year old was around 0.47 in both treatments (Limousin *et al.* 2009).

Predawn leaf water potential

Predawn leaf water potential (Ψ_{pd}) was monitored at 2–4 week intervals between April and November 2007 and 2008 with a pressure chamber (PMS 1000, PMS Instruments, Corvallis, OR, USA). Measurements were started one hour before sunrise and completed by dawn. Four trees were sampled per treatment, including two leaves per tree. When the observed difference between the two leaves was greater than 0.2 MPa, a third leaf was measured.

A-C_i curves and apparent photosynthetic parameters

Leaf gas-exchange measurements were performed on the upper canopy of three trees per treatment during the 2007 and 2008 growing seasons, on dates of predawn leaf water potential assessment. Measurements were taken on 1-year-old leaves until the end of June, and then on both 1-year-old and current-year leaves, as soon as current-year leaves were fully developed. Leaf gas exchange was measured with two portable photosynthesis systems (Li-6400, Li-Cor, Lincoln, NE, USA) equipped with a light source (6200-02B LED, Li-Cor). Leaves were first acclimated in the chamber for more than 20 min at ambient temperature, ambient CO_2 concentration (375 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ air) and a saturating photosynthetic photon flux density (PPFD) of 1500 $\mu\text{mol m}^{-2} \text{ s}^{-1}$. During gas-exchange measurements, leaf

temperatures were held constant between 18 and 35 °C, and the vapour pressure deficit (*VPD*) was 1.0–3.5 kPa, depending on leaf temperature and transpiration rate. Light-saturated net assimilation rate (A_{\max}) and stomatal conductance (g_s) were measured at the end of the 20 min acclimation period, before obtaining photosynthetic response to varying substomatal CO₂ concentration (*A-C_i* curves). Each curve consisted of nine steps, starting with the ambient concentration of 375 μmol CO₂ mol⁻¹ and then decreasing to 200, 100 and 50 μmol CO₂ mol⁻¹, before increasing to 300, 500, 700, 1000 and 1400 μmol CO₂ mol⁻¹. Leaves were allowed to equilibrate for at least 3 min at each step before logging data. After the completion of the *A-C_i* curve, the light was switched off, and leaf respiration rate was measured after 3 min at ambient CO₂ concentration. A and C_i values at each step were corrected for CO₂ diffusion leaks, following the manufacturer's recommendation and using a diffusion correction term (k) of 0.46 μmol s⁻¹ (Li-Cor Inc. 2004). In total, more than 200 *A-C_i* curves were performed over the two growing seasons in both treatments of the throughfall exclusion experiment. Apparent V_{\max} , the apparent maximum rate of carboxylation of Rubisco, and apparent J_{\max} , the apparent maximum rate of electron transport, were estimated from these *A-C_i* curves, according to the Farquhar *et al.* (1980) model of leaf photosynthesis. The Michaelis–Menten constants for CO₂ and O₂ (respectively K_c and K_o) and the CO₂ compensation point in the absence of mitochondrial respiration (Γ^*), as well as their temperature dependencies, were taken from Bernacchi *et al.* (2001).

Chlorophyll fluorescence measurements

During the 2008 growing season, 104 additional gas-exchange and chlorophyll fluorescence measurements were carried out with the same sampling methodology and at the same frequency as other *A-C_i* curves, using a Li-Cor 6400 equipped with a LI-6400-40 Leaf Chamber Fluorometer (Li-Cor). The procedure for gas exchange was identical to that described previously. Additionally, the photochemical efficiency of photosystem II (Φ_{PSII}) was determined at every step of the *A-C_i* curves by measuring steady-state fluorescence (F_s) and maximum fluorescence (F_m') during a light-saturating pulse, as $\Phi_{\text{PSII}} = (F_m' - F_s)/F_m'$. The rate of photosynthetic electron transport (J_{ETR}) was then calculated, following Krall & Edwards (1992), as $J_{\text{ETR}} = 0.5 \cdot \Phi_{\text{PSII}} \cdot \alpha \cdot \text{PPFD}$, where 0.5 is a factor accounting for the distribution of light between the two photosystems and α is the leaf absorbance, which was taken as 0.93 for *Q. ilex* leaves under the spectral composition of the Li-Cor light source (Niinemets *et al.* 2005). The relationship between Φ_{PSII} and J_{ETR} was not calibrated in this study but has previously been shown to be valid for *Q. ilex* leaves (see Niinemets *et al.* 2006 for discussion and sensitivity analysis), and any error in J_{ETR} estimates would have been systematic among treatments, leaf cohorts and seasons. Changes in leaf absorbance with water stress were assumed to be negligible because the chlorophyll content, which was assessed with a SPAD chlorophyll metre (SPAD-502, Konica Minolta

Sensing, Osaka, Japan), was found to remain constant over the drought stress period (data not shown).

Mesophyll conductance and *A-C_c*-based photosynthetic parameters

The internal mesophyll diffusion conductance from the substomatal cavities to the chloroplasts (g_m) was estimated by following the variable electron transport rate method of Harley *et al.* (1992b):

$$g_m = \frac{A}{C_i - \frac{\Gamma^*[J_{\text{ETR}} + 8(A + R_d)]}{J_{\text{ETR}} - 4(A + R_d)}} \quad (1)$$

The CO₂ compensation point in the absence of mitochondrial respiration (Γ^*), used in the calculation of g_m , was taken from Bernacchi *et al.* (2002), and the rate of non-photorespiratory respiration continuing in the light (R_d) was taken as half of the rate of respiration measured in the dark (Piel *et al.* 2002; Niinemets *et al.* 2005). The g_m values were calculated for every step of the *A-C_i* curves and then used to convert them to *A-C_c* curves, C_c being the CO₂ concentration in the chloroplast stroma:

$$C_c = C_i - A/g_m \quad (2)$$

This method takes into account the variations of g_m encountered along the C_i range, and was chosen because it allows better estimates of photosynthetic parameters from *A-C_c* curves than assuming a constant g_m , especially under water stress conditions (Flexas *et al.* 2007). *A-C_c* curves were used to estimate V_{\max} and J_{\max} from fitting the equations of the Farquhar *et al.* (1980) model. K_c , K_o and Γ^* values for *A-C_c* curve-fitting were taken from Bernacchi *et al.* (2002). Calculations of g_m , and therefore of V_{\max} and J_{\max} , may be biased if non-uniform stomatal closure affects the estimation of C_i (Laisk 1983; Beyschlag, Pfanz & Rysel 1992). However, non-uniform stomatal closure is generally negligible for g_s values above 0.04 mol m⁻² s⁻¹ (Flexas *et al.* 2002; Grassi & Magnani 2005). In the present study, g_s lower than 0.04 mol m⁻² s⁻¹ occurred only under severe water stress and on a few measurement dates. However, in these cases, g_m values were also low, and low values of g_m are less sensitive to errors in C_i (see Galmés *et al.* 2007 for a sensitivity analysis), so g_m , V_{\max} and J_{\max} estimations were kept.

Temperature corrections for photosynthetic parameters

Because of the large range of leaf temperatures encountered during the field campaign, we did a preliminary survey to describe the impact of leaf temperature on apparent V_{\max} and J_{\max} . During the 2006 growing season, *A-C_i* curves were performed on 10-year-old *Q. ilex* saplings irrigated weekly with 50 mm of water to prevent any water limitation (see Lavoie *et al.* (2009) for details). Forty-four curves were obtained on mature current-year leaves of three trees, with

leaf temperatures ranging from 18 to 40 °C depending on hour and date. The equation and parameters given in Harley *et al.* (1992a) for V_{cmax} and J_{max} temperature responses showed the best agreement to our data, with $r^2 = 0.83$ and $P < 0.001$ for V_{cmax} , and $r^2 = 0.56$ and $P < 0.001$ for J_{max} . These temperature response functions were, thus, used to normalize these parameters to the reference temperature, 25 °C. The equation presented in Bernacchi *et al.* (2002) was used to normalize the estimates of g_m to 25 °C.

Leaf chemical and structural analyses

The leaves used for gas-exchange measurements were collected for analysis of area, mass per area (*LMA*) and nitrogen content. Leaf area was determined with an optical area metre (Delta-T devices, Cambridge, UK). The leaves were then oven-dried at 60 °C for at least 48 h and weighed for their dry mass. Leaf nitrogen content per dry mass (N_M) was measured using an elemental analyser (Flash EA1112 Series, ThermoFinnigan, Milan, Italy).

LMA and N_M values were used together with V_{cmax} and J_{max} estimates to calculate the apparent nitrogen fraction in Rubisco, P_R , and in the rate-limiting proteins of photosynthetic electron transport, P_B (Niinemets & Tenhunen 1997):

$$P_R = \frac{V_{\text{cmax}}}{6.25 \times V_{\text{cr}} \times N_M \times LMA} \quad (3)$$

$$P_B = \frac{J_{\text{max}}}{8.06 \times J_{\text{mc}} \times N_M \times LMA} \quad (4)$$

where V_{cr} is the maximum rate of ribulose-1,5-bisphosphate carboxylation per unit Rubisco protein [$V_{\text{cr}} = 20.5 \mu\text{mol CO}_2$ (g Rubisco) $^{-1} \text{ s}^{-1}$ at 25 °C], J_{mc} is the capacity for photosynthetic electron transport per unit cytochrome f [$J_{\text{mc}} = 156 \text{ mol e}^-$ (mol cyt f) $^{-1} \text{ s}^{-1}$ at 25 °C], and scaling coefficients of 6.25 and 8.06 are based on the stoichiometry of nitrogen content of proteins and rate-limiting proteins (Niinemets & Tenhunen 1997).

Quantitative limitation analysis

The relative limitations on assimilation imposed by stomatal conductance (S_L), mesophyll conductance (MC_L) and biochemical processes (B_L) under water stress conditions were separated following the approach proposed by Grassi & Magnani (2005), which explicitly includes the mesophyll conductance limitation in previously described limitation analysis methods (Jones 1985; Wilson, Baldocchi & Hanson 2000b). This approach requires the measurement of A_{max} , g_s , g_m and V_{cmax} and expresses limitations as a proportion of a maximum assimilation rate that is defined as a standard. It considers A_{max} as limited by substrate availability and is expressed following the model of Farquhar *et al.* (1980). From the derivative of this model, any change in A_{max} can be expressed in terms of the concurrent changes in V_{cmax} and C_c , changes in C_c being themselves a function of changes in

g_s , g_m and A_{max} . In this approach, relative changes in g_s , g_m and V_{cmax} at a given date, compared with the maximum value over the season, are weighted by a corresponding relative limitation or sensitivity, with a value between 0 and 1, to calculate S_L , MC_L and B_L . As the analysis was performed here to assess the seasonal variations of S_L , MC_L and B_L with progressively increasing water limitation over the growing season, a different maximum assimilation rate was taken for each treatment and leaf cohort (1-year-old or current-year leaves).

Statistical analyses

Effects of treatment and leaf age on *LMA* and N_M were tested by two-way analysis of variance (ANOVA). However, the same methodology could not be applied to the physiological variables (A_{max} , g_s , g_m , V_{cmax} and J_{max}), which showed strong variations with $\Psi_{\text{l,pd}}$. To determine whether treatment or leaf age affected these variables, we fit linear models, assuming (1) only a $\Psi_{\text{l,pd}}$ effect, (2) $\Psi_{\text{l,pd}}$ and treatment effects, (3) $\Psi_{\text{l,pd}}$ and leaf age effects and (iv) $\Psi_{\text{l,pd}}$, treatment and leaf age effects. The inference of each model was assessed by the value of the Akaike Information Criterion (AIC), and the model with the lowest AIC was considered as the best to explain the data set. Statistical analyses were performed with R software (R-Development-Core-Team 2007).

RESULTS

Climate and predawn leaf water potential

Marked summer drought occurred at the site during the two study years, with drier conditions in 2007 than in 2008. Total precipitation in 2007 was 681 mm, with 295 mm occurring between March and June, which is the most active growth period for vegetation (Allard *et al.* 2008). In 2008, total precipitation was 1231 mm, with 386 mm occurring between March and June. Summer temperatures and *VPD* were similar for both years. Maximum temperature rarely exceeded 30 °C and never exceeded 35 °C, while *VPD* was always below 3.0 kPa (data not shown). Predawn leaf water potential ($\Psi_{\text{l,pd}}$) remained high (around -0.5 MPa) and was similar in both treatments during the wet periods (Fig. 1). An important decline in $\Psi_{\text{l,pd}}$ was observed in both years during summer drought, before a full recovery in response to early autumn rainfall. Minimum values of $\Psi_{\text{l,pd}}$ were -3.55 and -4.41 MPa in the control and dry treatment, respectively, during September 2007. Higher values of -3.17 and -3.89 MPa were observed in September 2008. $\Psi_{\text{l,pd}}$ was systematically lower in the dry plot during the drought periods, and the differences in $\Psi_{\text{l,pd}}$ between treatments were statistically significant ($P < 0.02$) in both years.

Light-saturated photosynthetic rate, stomatal conductance and intrinsic water use efficiency

Maximum photosynthetic rates during well-watered periods were similar among treatments, leaf cohorts and

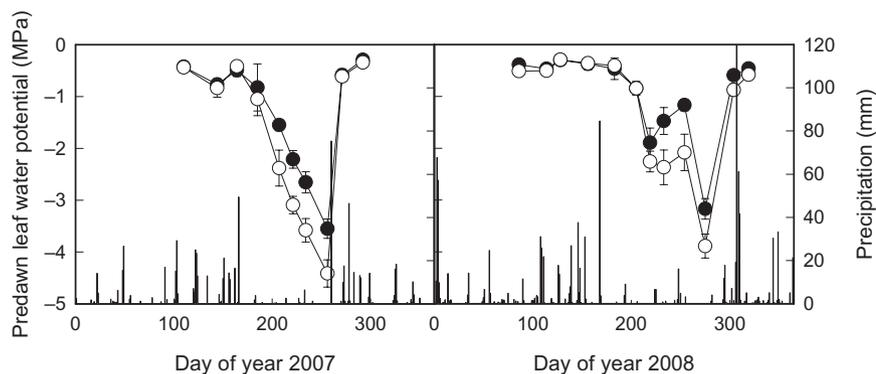


Figure 1. Seasonal variation of daily precipitation (mm, vertical bar) and predawn leaf water potential (MPa) in the control treatment (closed symbols) and the dry treatment (open symbols) of the throughfall exclusion experiment. Error bars are standard deviations.

study years, with values of around $13 \mu\text{mol m}^{-2} \text{s}^{-1}$, except for lower values in 1-year-old leaves of the control treatment during spring 2008 (Fig. 2, Table 1). There was a strong and rapid decline in A_{max} during summer as leaves experienced water stress, with a more severe impact in the dry treatment, as indicated by the higher percentage reduction of photosynthesis (98%; Table 1). In autumn 2007, a full recovery of photosynthesis with restored soil water condition was observed in current-year leaves, but only a partial recovery for 1-year-old leaves. Autumn recovery of current-year leaves seemed faster in the control than in the dry treatment for both years 2007 and 2008 (Fig. 2). The relationship between A_{max} and $\Psi_{\text{l,pd}}$ (Fig. 3a) presented a lower AIC when incorporating a leaf age effect, but not a treatment effect, in the model (Table 2). A rapid decline of g_s with decreasing $\Psi_{\text{l,pd}}$ was observed in the two treatments (Fig. 3b). Maximum values of g_s were typically $0.250 \text{ mol m}^{-2} \text{ s}^{-1}$ in current-year leaves of both treatments and slightly lower in 1-year-old leaves. The ratio of A_{max} to g_s defines the intrinsic water use efficiency (WUE_i). WUE_i increased with increasing water stress until $\Psi_{\text{l,pd}}$ reached approximately -3 MPa , and was reduced below this threshold because photosynthesis was strongly impaired (Fig. 3c). However, WUE_i for moderate drought stress was similar in both treatments.

Mesophyll conductance

Mesophyll conductance to CO_2 was strongly reduced by water stress, and a significant correlation ($P < 0.001$) was observed between g_m and $\Psi_{\text{l,pd}}$ (Fig. 4a). Reduction of g_m with drought was more pronounced in the dry than in the control treatment (Table 1). As for the other photosynthetic parameters, the highest values of g_m were recorded in 1-year-old leaves of the dry treatment, followed by current-year leaves of the control treatment. The relationship between g_m and $\Psi_{\text{l,pd}}$ was not explained better when accounting for a treatment effect, a leaf age effect or interaction between age and treatment (Table 2). Mesophyll conductance to CO_2 was higher than g_s , except for one date in current-year leaves of the dry treatment (Fig. 4b), and a significant correlation was found between the two conductances ($P < 0.001$).

Variations of V_{cmax} and J_{max}

A significant correlation with $\Psi_{\text{l,pd}}$ ($P < 0.001$) was observed for V_{cmax} and J_{max} , as well as for apparent V_{cmax} and apparent J_{max} (Fig. 5). However, the decrease with $\Psi_{\text{l,pd}}$ was stronger for apparent V_{cmax} and apparent J_{max} than for V_{cmax} and J_{max} . Percent ranges of variation between maximum and

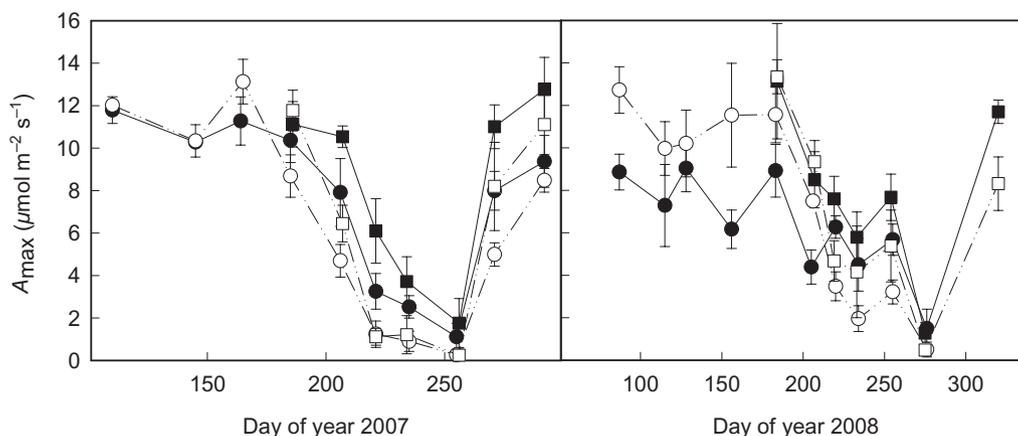


Figure 2. Seasonal variation of light-saturated net assimilation rate (A_{max}) for current year leaves of the control (■) and the dry treatment (□), and one-year-old leaves of the control (●) and dry treatment (○). Error bars are standard errors.

Table 1. Mean maximum and minimum values of A_{\max} , V_{cmax} , J_{\max} , g_m , P_R and P_B , recorded during the 2008 study year (average of three trees \pm SE)

Treatment	Leaf age (year)	A_{\max} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)			V_{cmax} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)			J_{\max} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)		
		Max	Min	Var	Max	Min	Var	Max	Min	Var
Control	0	13.1 \pm 2.7	1.3 \pm 0.5	90	76.2 \pm 18.9	54.0 \pm 14.2	29	131.7 \pm 8.2	54.7 \pm 19.8	58
	1	11.8 \pm 0.6	1.1 \pm 0.6	91	105.2 \pm 5.3	33.8 \pm 4.6	68	165.0 \pm 7.1	39.1 \pm 4.8	76
Dry	0	13.3 \pm 0.8	0.2 \pm 0.3	98	78.5 \pm 3.0	44.9 \pm 3.6	43	106.3 \pm 7.4	68.9 \pm 3.0	35
	1	13.1 \pm 1.1	0.3 \pm 0.2	98	100.6 \pm 1.6	39.2 \pm 10.8	61	150.4 \pm 9.6	51.0 \pm 12.7	66
		g_m ($\text{mol m}^{-2} \text{s}^{-1}$)			P_R (g g^{-1})			P_B (g g^{-1})		
		Max	Min	Var	Max	Min	Var	Max	Min	Var
Control	0	0.202 \pm 0.06	0.056 \pm 0.02	72	0.238 \pm 0.03	0.115 \pm 0.04	52	0.058 \pm 0.01	0.016 \pm 0.01	72
	1	0.192 \pm 0.01	0.035 \pm 0.02	82	0.293 \pm 0.01	0.128 \pm 0.04	56	0.047 \pm 0.00	0.015 \pm 0.00	68
Dry	0	0.175 \pm 0.01	0.024 \pm 0.02	86	0.254 \pm 0.04	0.149 \pm 0.07	41	0.063 \pm 0.00	0.022 \pm 0.01	76
	1	0.241 \pm 0.04	0.012 \pm 0.01	95	0.288 \pm 0.01	0.117 \pm 0.03	59	0.058 \pm 0.01	0.010 \pm 0.00	83

The percent range of variation (Var; %), calculated as $100 \times (\text{Max} - \text{Min})/\text{Max}$, is also given.

A_{\max} , light-saturated net assimilation rate; V_{cmax} , maximum carboxylation rate; J_{\max} , maximum photosynthetic electron transport; g_m , mesophyll conductance to CO_2 ; P_R nitrogen fraction in Rubisco; P_B , nitrogen fraction proteins limiting photosynthetic electron transport.

minimum values were lower for V_{cmax} than for J_{\max} , except for current-year leaves of the dry treatment (Table 1). As a result, the ratio of J_{\max} to V_{cmax} generally decreased with drought. Percent ranges of variation for V_{cmax} and J_{\max} were lower in current-year leaves than in 1-year-old leaves, probably because young leaves were already suffering from water stress when they were measured for the first time at the beginning of summer. The maximum drought impairment of V_{cmax} and J_{\max} was not stronger in the dry treatment (Table 1). The relationships between V_{cmax} and $\Psi_{\text{l, pd}}$ (Fig. 5a) and between J_{\max} and $\Psi_{\text{l, pd}}$ (Fig. 5c) had a lower AIC when not accounting for a treatment or a leaf age effect (Table 2). As for A_{\max} , the relationship between apparent V_{cmax} and $\Psi_{\text{l, pd}}$ (Fig. 5b) presented a lower AIC when accounting for a leaf age effect, but not a treatment effect, whereas the relationship between apparent J_{\max} and $\Psi_{\text{l, pd}}$ (Fig. 5d) was better explained by the model with no treatment or leaf age effect (Table 2). As a consequence of finite g_m , V_{cmax} obtained from fitting the Farquhar *et al.* (1980) model to the $A-C_c$ curves was significantly higher ($P < 0.001$) than apparent V_{cmax} fitted on $A-C_i$ curves (Fig. 6). Contrarily, J_{\max} was not significantly different from apparent J_{\max} (data not shown). A linear correlation was found between V_{cmax} and apparent V_{cmax} ($V_{\text{cmax}} = 1.08 \cdot \text{apparent } V_{\text{cmax}} + 18.1$; $r^2 = 0.77$; $P < 0.001$; $n = 30$) with a slope not significantly different from 1.

Leaf nitrogen content and investment in photosynthesis

Leaf mass per area (LMA) was significantly higher ($P = 0.02$) in the dry treatment than in the control treatment, with average values of $230 \pm 13 \text{ g m}^{-2}$ and $221 \pm 15 \text{ g m}^{-2}$, respectively. Because only mature leaves were considered in this study, LMA was not significantly different between leaf

cohorts and remained constant throughout the study period without being affected by drought and seasonality. Leaf nitrogen content was significantly higher in the dry treatment than in the control treatment, whether it was expressed on a mass basis, N_M ($P = 0.04$), or on a per unit leaf area, N_A ($P = 0.003$), but no effect of leaf age was observed. Average N_A was $2.58 \pm 0.30 \text{ g m}^{-2}$ in the control treatment and $2.77 \pm 0.31 \text{ g m}^{-2}$ in the dry treatment (mean \pm SD). N_A was unaffected by drought and no correlation could be established between N_A and $\Psi_{\text{l, pd}}$. However, a significant linear correlation was found between N_A and V_{cmax} ($r^2 = 0.20$; $P = 0.012$) when pooling data from the two treatments. This correlation between N_A and V_{cmax} was stronger than the one between N_A and apparent V_{cmax} ($r^2 = 0.10$; $P = 0.080$). Apparent leaf nitrogen investment in P_R and in P_B was of a similar order of magnitude in both treatments, and higher in 1-year-old leaves for P_R and in current-year leaves for P_B (Table 1). P_R and P_B strongly decreased in response to drought and exhibited percentage reductions similar to those observed for V_{cmax} and J_{\max} (Table 1). Nitrogen use efficiency (NUE) is not presented but clearly decreased with drought, as A_{\max} decreased despite constant N_A .

Quantitative limitation analysis

The proportional reduction of A_{\max} resulting from stomatal closure, reduced mesophyll conductance and biochemical impairment increased with drought intensity (Fig. 7). The maximum assimilation rate, chosen as a reference, occurred on the first measurement date in early July for current-year leaves of both treatments, at the end of March for 1-year-old leaves in the control treatment, and at the beginning of June for 1-year-old leaves in the dry treatment. Stomatal limitation reduced photosynthesis more than non-stomatal limitation (NS_L) in response to decreasing $\Psi_{\text{l, pd}}$. Of the NS_L

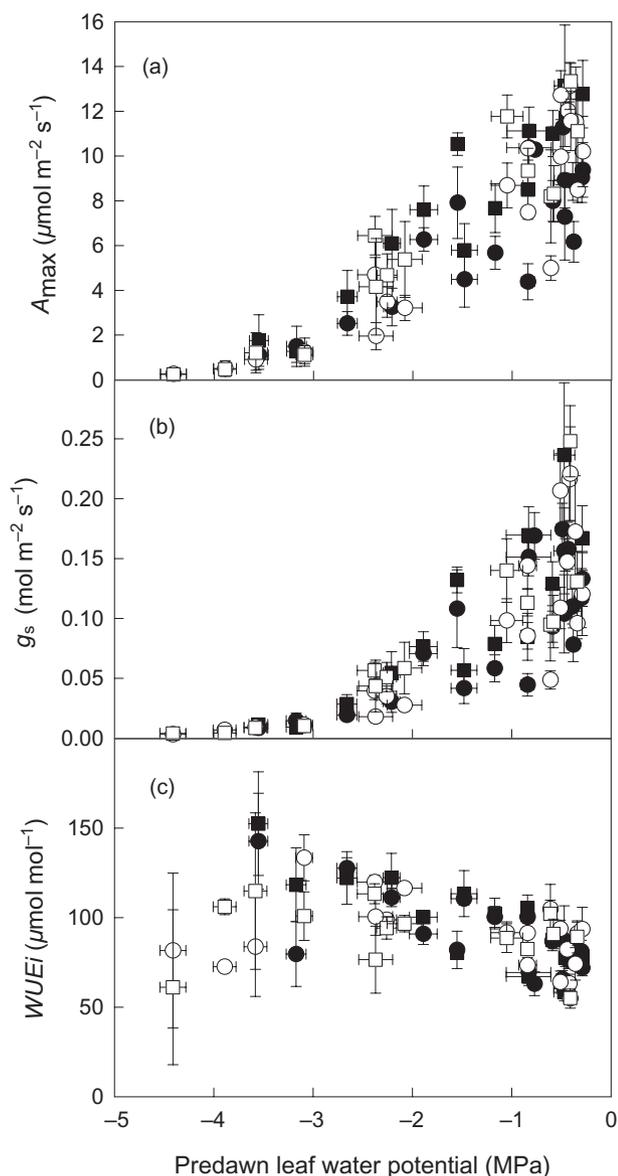


Figure 3. Relationship between predawn leaf water potential and (a) light-saturated net assimilation rate (A_{\max}) (b) stomatal conductance at ambient CO_2 concentration (g_s) and (c) intrinsic water-use efficiency ($WUE_i = A_{\max}/g_s$) in the control treatment (current-year leaves: ■; 1-year-old leaves: ●) and the dry treatment (current-year leaves: □; 1-year-old leaves: ○). Error bars are standard errors. Data from 2007 and 2008 were pooled.

effects, MC_L had a similar or greater impact on A_{\max} than biochemical limitation (B_L). The relative contributions of S_L , MC_L and B_L to the maximum limitation recorded in 2008 are shown in Table 3. Even at severe water stress, NS_L remained lower than S_L , especially in current-year leaves of the control treatment. Maximum MC_L was higher in the dry treatment than in the control treatment, but no trend was found for maximum B_L . Leaf age affected the relative contribution of the different limitations, with higher NS_L and lower S_L for older leaves (Table 3).

DISCUSSION

Variation of leaf photosynthetic capacity with seasonal drought stress

Leaf gas-exchange rates recorded in this study were consistent with previously published data for *Q. ilex* (Sala & Tenhunen 1996; Damesin, Rambal & Joffre 1998; Peñuelas *et al.* 1998; Peña-Rojas, Aranda & Fleck 2004; Lavoire *et al.* 2009). Net assimilation rate generally decreases linearly with reductions in predawn leaf water potential as drought progresses (Damesin *et al.* 1998; Ogaya & Peñuelas 2003), while g_s exhibits a more hyperbolic decline (Damesin *et al.* 1998; Rambal *et al.* 2003) (Fig. 3). The relative contribution of S_L and MC_L to diffusive limitation varies among species during drought, with S_L much higher than MC_L in some Mediterranean species, but not in the most sclerophyllous plants, which exhibit higher MC_L (Galmés *et al.* 2007). Our quantitative limitation analysis demonstrated that S_L was the most limiting process on photosynthesis under drought stress conditions in *Q. ilex*, followed by MC_L and then B_L (Fig. 7, Table 3). These observations confirm the results of earlier studies that have found S_L to be more limiting than NS_L in temperate deciduous trees (Wilson *et al.* 2000b), including oaks (Grassi & Magnani 2005), but contradict a recent modelling exercise inferring that NS_L reaches 80% at our site (Keenan, Sabate & García 2010).

It is commonly hypothesized that the decrease of apparent V_{cmax} and apparent J_{max} with drought could be an artefact of decreasing g_m in stressed leaves, causing C_c to be much lower than C_i (Flexas *et al.* 2004). We found that g_m decreased markedly with drought (Fig. 4, Table 1) but remained higher than g_s regardless of the stress intensity. This contradicts the observation of Galmés *et al.* (2007) that g_m is lower than g_s in Mediterranean plants, but confirms the Niinemets *et al.* (2006) finding that g_m is slightly higher than g_s in leaves of *Q. ilex*. Our maximum values of g_m in *Q. ilex* are higher than those reported by Niinemets *et al.* (2005, 2006), but are within the range observed by Rouspard *et al.* (1996). Ignoring the effect of mesophyll conductance on CO_2 concentration in the chloroplasts resulted in an underestimation of V_{cmax} and, to a lesser extent, J_{max} . Nevertheless, the decrease in g_m with increasing drought was not fully responsible for the decrease in apparent V_{cmax} because (1) V_{cmax} also decreased with Ψ_{lpd} , although to a lesser extent than apparent V_{cmax} , and (2) the relationship between V_{cmax} and apparent V_{cmax} had a slope close to 1 (Fig. 6). A proportional decline in V_{cmax} and g_m with drought stress was previously reported in a field study by Grassi & Magnani (2005). It can be hypothesized that, under field conditions with a slowly imposed drought, g_m and photosynthetic capacity maintain a co-limitation of photosynthesis so that the CO_2 drawdown from internal air space to the chloroplasts remains relatively unaffected by water stress.

Seasonality of leaf photosynthesis can result from leaf ontogeny, light availability, temperature changes and drought. Wilson *et al.* (2001) showed that the seasonal variations of V_{cmax} and J_{max} strongly influence photosynthetic

Physiological variable	AIC			
	$\Psi_{1, \text{pd}}$	$\Psi_{1, \text{pd}} \times \text{Tr}$	$\Psi_{1, \text{pd}} \times \text{Lc}$	$\Psi_{1, \text{pd}} \times (\text{Tr} + \text{Lc})$
A_{max}	281.9	285.2	275.7	278.9
g_s	-245.6	-242.3	-243.8	-238.2
g_m	-133.1	-131.1	-129.2	-130.8
V_{cmax}	255.5	258.3	255.6	260.5
J_{max}	283.1	285.0	284.0	287.1
Apparent V_{cmax}	520.5	522.8	512.0	516.4
Apparent J_{max}	602.2	603.2	602.9	606.0

Table 2. Results of the Akaike Information Criterion (AIC) comparison of the linear models for seven measured physiological variables (A_{max} , g_s , g_m , V_{cmax} , J_{max} , apparent V_{cmax} and apparent J_{max})

Four different models were tested, the first one assuming only a predawn leaf water potential effect ($\Psi_{1, \text{pd}}$), the second one assuming an interaction between $\Psi_{1, \text{pd}}$ and a treatment effect ($\Psi_{1, \text{pd}} \times \text{Tr}$), the third one assuming an interaction between $\Psi_{1, \text{pd}}$ and a leaf age effect ($\Psi_{1, \text{pd}} \times \text{La}$), and the fourth one assuming an interaction between $\Psi_{1, \text{pd}}$ and both a treatment and a leaf age effect [$\Psi_{1, \text{pd}} \times (\text{Tr} + \text{La})$]. The models producing the best fit to the measured data have the lowest AIC and are indicated in bold.

A_{max} , light-saturated net assimilation rate; g_s , stomatal conductance; g_m , mesophyll conductance to CO_2 ; V_{cmax} , maximum carboxylation rate; J_{max} , maximum photosynthetic electron transport.

carbon uptake, so that the description of seasonality can be as important as quantifying maximum values at peak seasons. In this study, all measurements were performed on mature leaves, at constant light intensity and normalized to 25 °C, so that the observed seasonality was only attributable to drought. The range of seasonal variation displayed by apparent V_{cmax} and apparent J_{max} (Fig. 5) was similar to previous observations in several species of a temperate deciduous forest (Wilson *et al.* 2000a), two Mediterranean evergreen shrubs (Nogués & Alegre 2002) and the Californian deciduous oak *Quercus douglasii* (Xu & Baldocchi 2003). In response to less severe water stress, Grassi *et al.* (2005) and Misson *et al.* (2006) observed a lower range of variation, but the linear decrease in apparent V_{cmax} with soil water potential (Grassi *et al.* 2005) is consistent with the results of the present study (Fig. 5).

The underlying processes responsible for the decrease in V_{cmax} and J_{max} are still a matter of debate (Flexas &

Medrano 2002; Lawlor & Cornic 2002; Flexas *et al.* 2004). Strong correlations between the seasonality of V_{cmax} and N_A have been reported (Xu & Baldocchi 2003; Misson *et al.* 2006). Environmental stresses are thought to have produced the decline in V_{cmax} by affecting the structural and biochemical properties of leaves. However, V_{cmax} showed a strong seasonality despite almost constant N_A in our study. It is therefore unlikely that leaf nitrogen translocation was responsible for the decrease in V_{cmax} . Instead, the apparent leaf nitrogen investment in P_R and in proteins limiting P_B showed important variations over the summer drought (Table 1), as observed by Wilson *et al.* (2000a). V_{cmax} may be driven by N_A during leaf ontogeny or senescence, but this relationship is generally not significant during drought development (Wilson *et al.* 2000a; Grassi *et al.* 2005), as was the case in our study. In addition to the decline in nitrogen allocation to the photosynthetic apparatus, as indicated by the variations of P_R and P_B (Table 1), several mechanisms

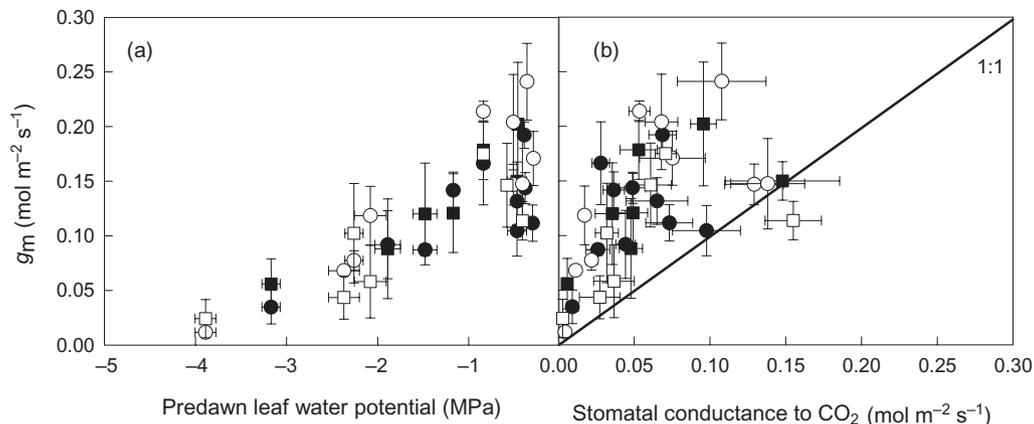


Figure 4. Relationship between (a) mesophyll conductance to CO_2 (g_m) and predawn leaf water potential, and (b) g_m and stomatal conductance to CO_2 (calculated as $g_s/1.6$) for the year 2008. Data presented are from the control treatment (current-year leaves: ■; 1-year-old leaves: ●) and the dry treatment (current-year leaves: □; 1-year-old leaves: ○). Error bars are standard errors.

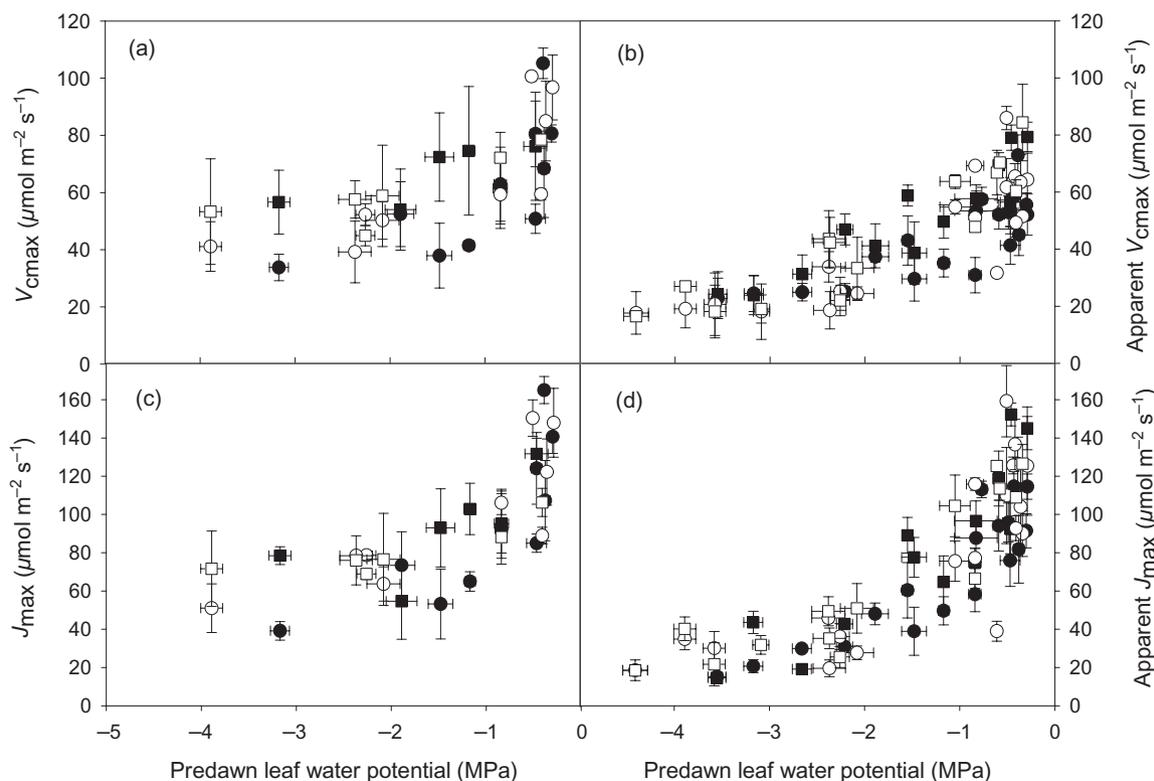


Figure 5. Relationship between predawn leaf water potential and (a) maximum carboxylation rate (V_{cmax}) (b) apparent V_{cmax} (c) maximum electron transport rate (J_{max}) and (d) apparent J_{max} in the control treatment (current-year leaves: ■; 1-year-old leaves: ●) and the dry treatment (current-year leaves: □; 1-year-old leaves: ○). Error bars are standard errors. Both parameters were normalized to 25 °C and data from 2007 and 2008 were pooled.

can be proposed to explain the decrease in V_{cmax} and J_{max} under drought stress. These include the inactivation of Calvin cycle enzymes by limitation of ATP and RuBP synthesis (Lawlor & Cornic 2002); the inhibition of Rubisco

activity (Parry *et al.* 2002); the down-regulation of photochemistry, due to dissipation of excitation energy through processes other than photosynthesis (Chaves *et al.* 2002); and the drought-induced inhibition of growth causing a negative feedback on photosynthetic capacity (Paul & Foyer 2001).

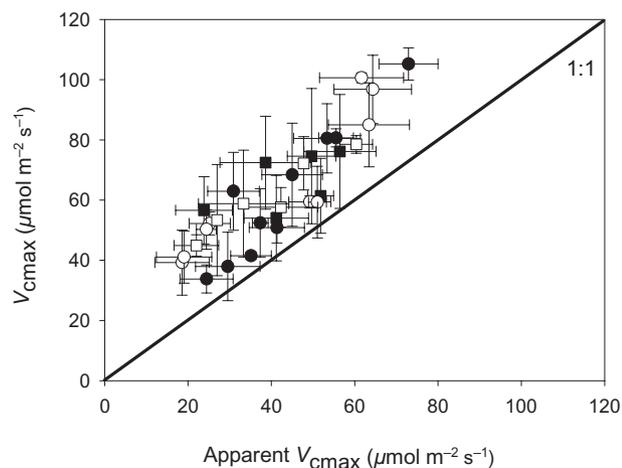


Figure 6. Relationship between maximum carboxylation rate (V_{cmax}) estimated from $A-C_c$ curves, and apparent V_{cmax} , estimated from $A-C_i$ curves. Data presented are from the control treatment (current-year leaves: ■; 1-year-old leaves: ●) and the dry treatment (current-year leaves: □; 1-year-old leaves: ○) for the year 2008. Error bars are standard errors.

Effects of long-term increasing drought and leaf aging on leaf photosynthesis

Despite the high sensitivity of leaf photosynthetic capacity to drought stress observed in this study, no clear trend of acclimation appeared in the dry treatment of the throughfall exclusion experiment after 5 years of recurrently increased drought severity. Lower leaf water potentials ($\Psi_{l,pd}$) occurred every year in the dry treatment (Fig. 1), but this only resulted in lower values of A_{max} during limited time periods (Fig. 2). Furthermore, no treatment effect was observed in the relationships between photosynthetic parameters and $\Psi_{l,pd}$ (Fig. 5). Very few studies have aimed to measure the seasonality of photosynthesis in mature trees submitted to different water regimes in the same environment. Wilson *et al.* (2000a, 2000b) studied the seasonality of photosynthesis in a temperate deciduous forest submitted to three different water regimes in a throughfall displacement experiment, but decided that the differences in soil water were too small to evaluate any effect. Nogués

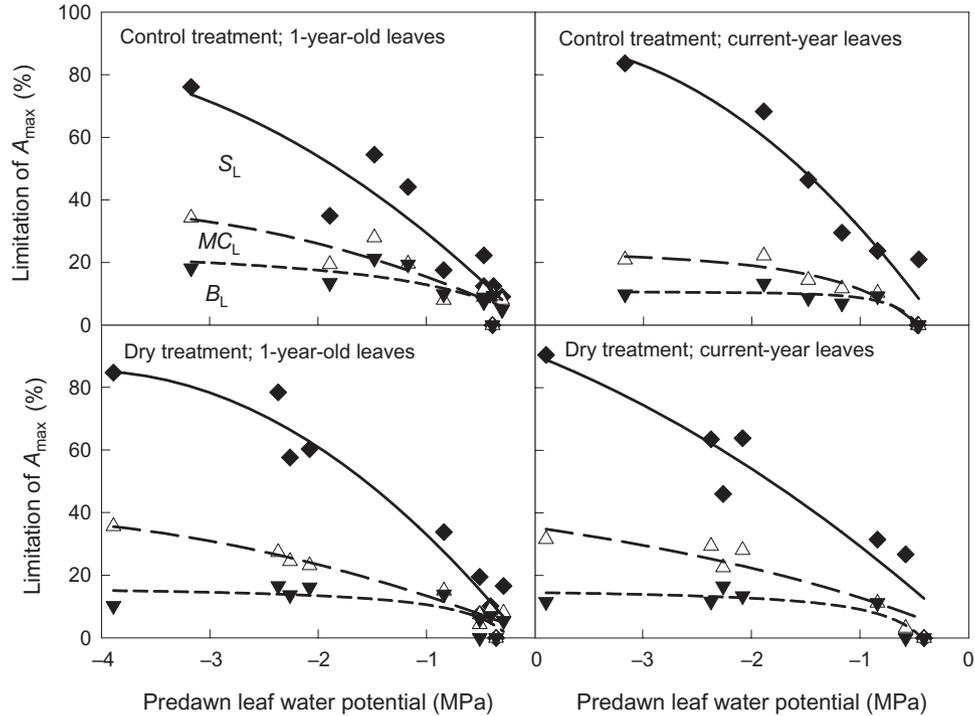


Figure 7. Relationship between the limitations of A_{\max} and predawn leaf water potential for the control and the dry treatments and the two leaf ages. Each point has been calculated from three measurements on a given date. The symbols are as follows: total limitation (closed diamonds), non-stomatal limitation (open triangles), biochemical limitation (closed triangles). Stomatal limitation (S_L = total limitation – non-stomatal limitation), mesophyll conductance limitation (MC_L = non-stomatal limitation – biochemical limitation) and biochemical limitation (B_L) are indicated in the left-upper panel.

& Alegre (2002) observed no differences in the seasonality of leaf photosynthetic capacity in Mediterranean shrubs submitted to natural drought conditions or irrigation. Finally, Ogaya & Peñuelas (2003) measured the seasonal variations of photosynthesis in *Q. ilex* trees submitted to a throughfall exclusion in Spain and found a slight acclimation of photosynthesis. Nevertheless, the relationship between A_{\max} and $\Psi_{l, pd}$ was similar between dry and control treatments. These observations, together with our results, suggest that increasing drought severity does not modify the functional relationships between leaf photosynthesis and water stress.

A treatment effect was observed on leaf morphology and chemical composition, with slightly higher values of LMA

and leaf nitrogen content in the dry treatment. However, such changes had no effect on leaf photosynthetic capacity. Increasing LMA with increasing water stress has already been described for *Q. ilex* leaves along rainfall gradients (Castro-díez *et al.* 1997; Damesin, Rambal & Joffre 1997). Castro-díez *et al.* (1997) found that variations of LMA in *Q. ilex* species were driven by leaf thickness rather than density. As LMA is a product of leaf thickness and density, greater thickness increases photosynthetic rates per unit area without impairing internal diffusion of CO_2 , whereas greater density decreases photosynthetic potentials per unit mass and reduces internal CO_2 conductance (Mediavilla, Escudero & Heilmeyer 2001; Niinemets 2001). Thus, if the increase of LMA in the dry treatment is a result of

Treatment	Leaf age (year)	Limitation			
		Total (T_L)	Stomatal (S_L)	Mesophyll conductance (MC_L)	Biochemical (B_L)
Control	0	84	63	11	10
	1	76	42	16	18
Dry	0	90	58	20	12
	1	85	49	25	11

Table 3. Maximum stomatal and non-stomatal limitations of A_{\max} recorded in 2008, expressed as percentage reductions from maximum values for the control and dry treatments, and the two cohorts of leaves

T_L , total limitation; S_L , stomatal conductance limitation; MC_L , mesophyll conductance limitation; B_L , biochemical limitation.

increased leaf thickness, this would have no drawbacks on g_m and photosynthetic parameters. The leaf area index in the dry treatment was reduced by 18% compared with the control, as a consequence of the throughfall exclusion (Limousin *et al.* 2009). The increased N_M in the dry treatment may be related to the smaller leaf area index resulting in larger nitrogen availability for individual leaves.

In Mediterranean evergreen oaks, leaf age generally produces an increase in LMA and a decrease in V_{cmax} , J_{max} and g_m for relatively time-independent leaf nitrogen content (Niinemets, Tenhunen & Beyschlag 2004; Niinemets *et al.* 2005, 2006). Our results do not confirm these earlier findings regarding maximum values of V_{cmax} , J_{max} and g_m (Table 1) because drought occurred before maturity of current-year leaves in 2008, so that maximum possible values were likely not reached in current-year leaves. However, A_{max} and apparent V_{cmax} were lower in older leaves in the two treatments, except at very low leaf water potentials (Fig. 2, Table 2). Autumn recovery of photosynthetic capacity was also stronger in current-year leaves than in 1-year-old leaves (data not shown).

In conclusion, we observed that five consecutive years of recurrently increased drought severity did not modify photosynthetic sensitivity to water limitation. Leaf acclimation to long-term increased drought occurred through changes in leaf morphology and chemical composition, while functional relationships between photosynthetic capacity and water potential were conserved. Such a result is particularly important for modelling studies because current drought-dependent parameterization, as proposed by Wilson *et al.* (2000a, 2001), may remain valid in the future under projected climate scenarios.

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