

Leaf physiological responses to extreme droughts in Mediterranean *Quercus ilex* forest

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ABSTRACT

Global climate change is expected to result in more frequent and intense droughts in the Mediterranean region. To understand forest response to severe drought, we used a mobile rainfall shelter to examine the impact of spring and autumn rainfall exclusion on stomatal (S_L) and non-stomatal (NS_L) limitations of photosynthesis in a *Quercus ilex* ecosystem. Spring rainfall exclusion, carried out during increasing atmospheric demand and leaf development, had a larger impact on photosynthesis than autumn exclusion, conducted at a time of mature foliage and decreasing vapour pressure deficit. The relative importance of NS_L increased with drought intensity. S_L and NS_L were equal once total limitation (T_L) reached 60%, but NS_L greatly exceeded S_L during severe drought, with 76% NS_L partitioned equally between mesophyll conductance (M_{CL}) and biochemical (B_L) limitations when T_L reached 100%. Rainfall exclusion altered the relationship between leaf water potential and photosynthesis. In response to severe mid-summer drought stress, A_n and V_{cmax} were 75% and 72% lower in the spring exclusion plot than in the control plot at the same pre-dawn leaf water potential. Our results revealed changes in the relationship between photosynthetic parameters and water stress that are not currently included in drought parameterizations for modelling applications.

Key-words: climate change; maximum carboxylation rate; mesophyll conductance; photosynthesis; rainfall exclusion; stomatal conductance.

INTRODUCTION

Increasing atmospheric greenhouse gas concentrations are expected to raise the global mean near-surface air temperature by 1.8 to 4.0 °C during the 21st century, accompanied by a 7% increase in mean precipitation (IPCC 2007). Under a variety of emissions scenarios, simulations with both coupled atmosphere-ocean regional climate models and coarser resolution global circulation models indicate

substantial drying and warming of the Mediterranean region (Gao, Pal & Giorgi 2006; Giorgi & Lionello 2008; Somot *et al.* 2008). These changes would result in increased drought frequency, intensity and duration, especially during the warm season (Sheffield & Wood 2008). The response of vegetation to extreme climatic events remains poorly understood, because their natural occurrence is rare and field experimentation to induce such conditions is difficult to operate on a large scale without introducing bias (Fay *et al.* 2000). The acclimatory capacity of vegetation can be surpassed during extreme events, as response thresholds are exceeded, triggering non-linearities, reduced recovery, hystereses and cascading effects lasting several years (Gutschick & BassiriRad 2003; Breda *et al.* 2006; Galle, Haldimann & Feller 2007; Galmés, Medrano & Flexas 2007; Granier *et al.* 2007; Haldimann, Galle & Feller 2008). Current ecosystem models are limited in their capacity to account for these effects, especially at large spatial scales, because they were mostly developed and calibrated with data collected under standard climate conditions.

Globally, photosynthesis is an important process controlling the climate system, because it is responsible for the removal of 120 Gt year⁻¹ of carbon from the atmosphere. In Mediterranean regions, drought is the main factor limiting canopy photosynthesis at both interannual and seasonal time scales. At lower levels of organization, canopy photosynthesis is driven by leaf photosynthesis and the effect of typical seasonal droughts is mediated by three physiological processes. Firstly, stomatal closure is the fastest rate process and is widely recognized as the primary limitation on carbon assimilation during drought (Chaves 1991; Chaves, Maroco & Pereira 2003). Secondly, mesophyll conductance has been shown to decrease rapidly under water stress, thereby limiting CO₂ diffusion to the chloroplast stroma (Roupsard, Gross & Dreyer 1996; Grassi & Magnani 2005; Galmés *et al.* 2007; Flexas *et al.* 2008). Thirdly, under long-lasting drought, photosynthesis may be limited by biochemical impairments such as decreasing photosynthetic enzyme activity and regeneration (Bota, Medrano & Flexas 2004; Flexas *et al.* 2004). In previous field studies, carried out in deciduous and evergreen species of a variety of climate zones, stomatal limitation has ranged from 30 to 50%, with mesophyll limitation of about 15 to 30% and biochemical

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	Control	Autumn drought	Spring drought
Number of trees ha ⁻¹	8125	7641	8744
Diameter at breast height ± SD (cm)	6.1 ± 3.1	6.4 ± 3.4	6.0 ± 3.2
Basal area (m ² ha ⁻¹)	29.8	31.7	31.5
Mean tree height ± SD (m)	4.0 ± 0.9	4.1 ± 1.0	4.2 ± 1.0
Leaf area index ± SD (m ² m ⁻²)	2.5 ± 0.2	2.9 ± 0.5	2.6 ± 0.3

SD, standard deviation.

limitation of about 10 to 20% (Wilson, Baldocchi & Hanson 2000; Nogues & Alegre 2002; Grassi & Magnani 2005; Limousin *et al.* 2010).

Declining leaf-level net photosynthesis (A_n) is known to result from throughfall exclusion and partial rainfall exclusion (Wilson *et al.* 2000; Wilson, Baldocchi & Hanson 2001; Llorens, Penuelas & Estiarte 2003; Limousin *et al.* 2010). Decreases in A_n can be accompanied by higher photosynthetic water use efficiency, expressed as the ratio of A_n to water loss by transpiration (E ; Ogaya & Penuelas 2003). However, no functional changes have been observed in the relationship between photosynthesis and leaf water potential. Limousin *et al.* (2010) measured stomatal (S_L) and non-stomatal photosynthetic limitation (NS_L) in a 27% throughfall exclusion experiment during two successive years, and demonstrated that the relationships between leaf water potential (ψ_p), stomatal conductance (g_s), mesophyll conductance (g_m) and leaf photosynthetic parameters remained identical after 6 years of increased drought. In the same exclusion experiment, Misson *et al.* (2009) found that the relationship between spring rainfall and annual gross primary productivity (GPP) was the same in the dry treatment as for interannual variations of GPP derived from eddy-covariance measurements in the control plot (see also Allard *et al.* 2008). Such throughfall exclusion experiments are valuable, because they allow the study of long-term acclimation to increasing drought severity in a field setting. However, these experiments were restricted to moderate drought conditions that fall within the normal, present-day interannual range. It is unknown whether the results obtained in these studies can be applied to the more extreme conditions expected under future climate scenarios. Furthermore, because water inputs are partially excluded on a continuous basis in throughfall exclusion field studies, interseasonal comparisons of experimental drought impacts have not been carried out before this study.

To study the impact of severe drought on leaf photosynthesis in a Mediterranean forest, we constructed a retractable rainfall shelter to experimentally induce exceptional spring and autumn drought conditions in a holm oak (*Quercus ilex*) forest of southern France. The general objective of this study was to quantify leaf-level photosynthetic parameters, to assess the relative importance of S_L and NS_L in response to spring and autumn rainfall exclusion. We addressed the following questions: (1) are levels and ranks of photosynthesis limitations that have been reported in natural settings under moderate drought stress similar to those observed in response to extreme drought?; (2) does

Table 1. Dendrometric characteristics of the three experimental plots in winter 2007

extreme drought modify the fundamental relationships between ψ_p and photosynthesis limitations?; (3) does drought severity affect the rate of recuperation of leaf-level photosynthetic parameters?

MATERIALS AND METHODS

Experimental site

The experimental site of Puéchabon State Forest is located on a flat plateau (3°35'45"E, 43°44'29"N, 270 m. a.s.l.), 35 km north-west of Montpellier, France. The area has a Mediterranean-type climate, with a mean annual temperature of 13.4 °C. The coldest month is January (5.5 °C) and the hottest month is July (22.9 °C). Mean annual precipitation is 907 mm, with a range of 556–1549 mm recorded during 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 *Quercus 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 (mean is 4.1 m) and the stem density was 8170 ha⁻¹ in 2007. The soil is extremely stony, with bedrock parent material of Jurassic limestone origin (Rambal *et al.* 2003).

Rainfall exclusion experiment

In 2007, a mobile rainfall shelter was built, to exclude 100% of rainfall at different periods of the year, without changes in other meteorological variables. The experimental design consisted of three 195 m² plots, including spring rainfall exclusion, fall rainfall exclusion and a control (Table 1). Rainfall exclusion was achieved using a retractable, 15 × 13 m rainfall shelter, programmed to slide automatically along 60 m long rails to prevent rainfall in the desired plot and return to its parking position at the end of each event (Supporting Information Fig. S1). The two exclusion plots were situated at opposing ends of the rails, whereas the middle section served as a parking position for the shelter in the absence of rain. The control plot was situated 20 m to the east of the exclusion plots.

The framework of the rainfall shelter was built with galvanized steel. Transparent greenhouse PVC sheeting was used as a roofing material to prevent rainfall, but allow light penetration during rainfall. The shelter rested on wheels over the rails, which stood just above the canopy on 40 adjustable galvanized steel scaffolds, fixed to the ground

and reinforced by wire cables. The rainfall shelter automatically moved from the parking position to the exclusion plot during rainfall. A tipping bucket rain gauge with 0.25 mm resolution was installed above the canopy and was connected to a data logger. A generator was initiated in response to the first tip of the rain gauge, to power the movement of the mobile shelter. To increase exclusion efficiency and avoid repeated movement of the shelter, it was programmed to remain over the exclusion plots for 4 h after the last tip of the tipping bucket. Plexiglass panels were installed vertically on the side of the rainfall shelter to avoid lateral penetration of windblown rain. Rainwater intercepted by the shelter was collected and redirected by gutters 15 m away from the plots.

Two rainfall exclusion treatments were performed. The first treatment, henceforth referred to as the autumn exclusion, simulated an extreme autumn-winter drought with no rainfall for 6 months, from July to December 2008. In this treatment, soil water shortage increased whereas atmospheric demand was decreasing. The second treatment, referred to below as the spring exclusion, simulated an extreme winter-spring drought with no rainfall for 6 months, from February to July 2009. In this treatment, soil water shortage increased simultaneously with atmospheric demand. In each plot, soil water content was measured every 5 min in the 0–6 cm soil layer using three ML2X ThetaProbe Soil Moisture Sensors (Delta-T Devices Ltd., Cambridge, UK) and in the 0–30 cm soil layer using six CS616-L Water Content Reflectometers (Campbell Scientific, Logan, UT, USA).

Tree sampling

Six trees were sampled in each plot. Because the soil has a high stone content, it was not possible to dig trenches around the plots to prevent lateral roots of the sampled trees from accessing water from outside of the exclusion plots. Previous excavation of whole woody root systems has shown that lateral roots of *Quercus ilex* extend outward up to four meters (Rambal and Joffre, personal communication). Sampled trees with trunks located at least four meters from the boundary of the plot were, thus, chosen, to limit water acquisition from soil adjacent to the exclusion plots. Upper branches of the sampled trees were accessed using permanent horizontal platforms mounted on scaffolds 1.5 m below the top of the trees.

Phenology

Leaf phenological phases were monitored every 1 to 2 weeks on each sampled tree, during the new leaf development period in 2007, 2008 and 2009. For each tree, three branches and two growth flushes per branch were randomly sampled and monitored repeatedly. Seven phenophases were defined similarly to Dumerle & Mazet (1983), in which phase 1 is the closed bud or winter stage, phase 2 corresponds to bud swelling, phase 3 refers to budburst, phase 4 is the period of shoot lengthening, phase 5 is the time of new

leaf opening, phase 6 refers to the period of fully open but immature foliage and phase 7 is the stage of fully developed and mature leaves.

Leaf sampling for gas exchange

One leaf from the upper canopy of each sampled tree was measured on two dates before the start of the treatments in 2007, and then every 2 to 6 weeks throughout 2008 and 2009. Measurements were made on 1-year-old leaves until the end of June, and then on current-year leaves when they had reached maturity, by the beginning of July. In 2009, new leaves did not reach maturity in three trees of the spring exclusion plot, as a consequence of increased drought in this treatment. After 1 July 2009, measurements in this treatment were performed on three 1-year-old leaves and three current-year leaves.

To account for the impact of extreme drought on phenology, a separate experiment was carried out to investigate the effect of leaf age, leaf water potential and their interaction on photosynthesis. Gas exchange was measured simultaneously on current-year and 1-year-old leaves of six trees, at leaf water potentials varying from –0.5 to –4.0 MPa (Limousin *et al.* 2010). A non-linear regression analysis was carried out, to examine the relationship between water potential and physiological parameters on these two leaf cohorts, with the difference between regression coefficients tested using a Student's *t*-test.

A–C_i curves

Leaf gas-exchange measurements were performed with a portable photosynthesis system (LI-6400, Li-Cor Biosciences, Lincoln, NE, USA), equipped with a leaf chamber fluorometer (LI-6400-40). Leaves were first acclimated in the chamber for 15 min at ambient temperature, ambient CO₂ concentration (375 μmol CO₂ mol⁻¹ air) and a saturating photosynthetic photon flux density (PPFD) of 1500 μmol m⁻² s⁻¹. Light-saturated net assimilation at ambient CO₂ concentration (*A*_n) and stomatal conductance (*g*_s) were obtained at the end of the 15 min acclimation period, before measuring the photosynthetic response to varying substomatal CO₂ concentration (*A*–*C*_i curve). Each curve consisted of nine steps, starting from the ambient CO₂ concentration and 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.

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 $J_{\text{ETR}} = 0.5 \Phi_{\text{PSII}} \cdot \alpha \cdot \text{PPFD}$, where 0.5 is a factor accounting for the distribution of light between the two photosystems and α is the leaf absorptance, which was taken as 0.93 for *Q. ilex* leaves under the spectral

composition of the Li-Cor light source (Niinemets *et al.* 2005; Niinemets *et al.* 2006). After the completion of the $A-C_i$ curve, the light source was switched off and the chamber air was reset to the ambient CO_2 concentration. Leaf respiration rate was measured after at least 3 min had passed and chamber CO_2 concentration had stabilized. Photosynthesis rate was corrected for CO_2 diffusion leaks at each step using a diffusion correction term (k) of $0.46 \mu\text{mol s}^{-1}$ (Li-Cor Inc., 2004). Pre-dawn leaf water potential (ψ_p) was monitored on the same trees with a pressure chamber (PMS 1000, PMS Instruments, Corvallis, OR, USA).

Mesophyll conductance and photosynthetic parameters

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

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

The CO_2 compensation point in the absence of mitochondrial respiration (Γ^*) was taken from Bernacchi *et al.* (2002). 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). Mesophyll conductance values were calculated for every step of the $A-C_i$ curves and used to produce $A-C_c$ curves, where C_c is the CO_2 concentration in the chloroplast stroma:

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

$A-C_c$ curves were used to estimate the maximum carboxylation rate (V_{cmax}) and the maximum rate of electron transport (J_{max}), by fitting the equations of the Farquhar model (Farquhar, von Caemmerer & Berry 1980). However, because most ecophysiological models and the quantitative limitation analysis described in the following consider A_n as limited solely by substrate availability, only the results for V_{cmax} are presented here. The Michaelis–Menten constants for CO_2 (K_c) and O_2 (K_o) and the CO_2 compensation point in the absence of mitochondrial respiration (Γ^*) were taken from Bernacchi *et al.* (2002) for $A-C_c$ curve-fitting. The Harley *et al.* (1992b) parameters for the V_{cmax} temperature response showed the best agreement to previous data measured on *Quercus ilex*, with $r^2 = 0.83$ and $P < 0.001$ (Limousin *et al.* 2010). This temperature response function was used to normalize V_{cmax} to the reference temperature (25°C), whereas the Bernacchi *et al.* (2002) formulation was used to normalize the g_m estimates to 25°C .

Calculations of g_m , and therefore of V_{cmax} , 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 $0.04 \text{ mol m}^{-2} \text{ s}^{-1}$ (Flexas *et al.* 2002; Grassi & Magnani 2005). In the present study, g_s lower than $0.04 \text{ mol m}^{-2} \text{ s}^{-1}$ occurred only during severe water stress. In these cases, g_m values were also low, and sensitivity to errors in C_i is minimal when g_m values are low (see Galmés *et al.* 2007 for a sensitivity analysis). Hence, all g_m and V_{cmax} estimates were included.

Quantitative limitation analysis

The relative limitations to assimilation imposed by stomatal conductance (S_L), mesophyll conductance (MC_L) and biochemical processes (B_L) were separated, following the approach proposed by Grassi & Magnani (2005) and applied to *Quercus ilex* by Limousin *et al.* (2010). This approach requires the measurement of A_n , g_s , g_m and V_{cmax} , and expresses limitations as a proportion of a maximum assimilation rate. It considers A_n 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_n can be expressed in terms of the concurrent changes in V_{cmax} and C_c . Changes in C_c are themselves a function of changes in g_s , g_m and A_n . Using this approach, relative changes in g_s , g_m and V_{cmax} on a given date, compared with the maximum value over the season, are weighted by a corresponding relative limitation or sensitivity, with a value between zero and one, to calculate S_L , MC_L and B_L . Non-stomatal limitation (NS_L) was calculated as $MC_L + B_L$. Total limitation (T_L) was calculated as $S_L + MC_L + B_L$.

Experimental design and statistics

We employed a Before–After Control–Impact (BACI) experimental design (Stewart-Oaten, Murdoch & Parker 1986; Stewart-Oaten & Bence 2001) with data from pairs of plots, including one control and one impact (autumn or spring exclusion plots), collected both before (2007) and after (2008 and 2009) the start of the treatments (Smith, Orvos & Cairns 1993; Carpenter *et al.* 1996; Cottingham & Carpenter 1998; Smith 2002). Because we found no difference in leaf level gas exchange between the plots in 2007, before the application of the treatments and for various levels of seasonal water stress (not shown), differences between plots after the application of the treatments were tested and interpreted as treatment effects. The Wilcoxon signed-rank test was used whenever the difference between values in the control plot was tested against values in one of the treatment plots (autumn exclusion or spring exclusion). This non-parametric test is used as an alternative to the paired Student's t -test when the number of replications is less than 20, or when the population cannot be assumed to be normally distributed (Wilcoxon 1945).

RESULTS

Rainfall exclusion

Rainfall exclusion efficiency was 97% and 87% in the autumn and spring exclusion treatments, respectively.

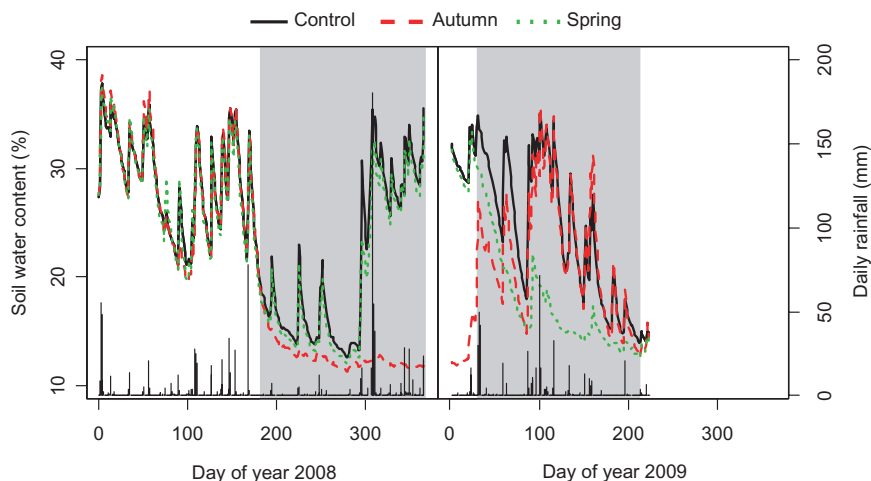


Figure 1. Seasonal variation of topsoil water content at 0–6 cm (lines, y-left axis) in the different treatments and daily precipitation (mm) (vertical bars, y-right axis). The shaded areas show the timing of the autumn exclusion (2008) and of the spring exclusion (2009).

During the 6-month autumn exclusion treatment from July to December 2008, rainfall was 575 mm, of which 16 mm was not excluded. During the 6-month spring exclusion treatment from February to July 2009, rainfall was 491 mm, of which 56 mm was not excluded. The excluded rainfall represents 50% of the 1127 mm that fell during 2008 (autumn exclusion), and 55% of the 749 mm that fell during 2009 (spring exclusion). Rain did not enter the plot laterally, except when rainfall intensity and wind speed were very high. When this occurred in November 2008, lateral entry was observed up to 2 m inside the plot.

Soil water content in the 0–6 cm soil layer was similar in the three plots during winter and spring 2008, before the application of the treatment (Fig. 1). The soils of the three plots dried substantially in late June, just before the rainfall exclusion period. Soil moisture responded to small summer 2008 rainfall events in the control and spring exclusion plots, but continued to decrease in the autumn exclusion plot. Rainfall in September and October 2008 were lower than normal and minimum soil water content was similar in the three plots at the end of October 2008. The soils of the control and spring exclusion plots were rewetted to saturation by 290 mm of rainfall in early November. Topsoil water content remained low in the autumn exclusion plot until end of January 2009 (Fig. 1).

At the beginning of the spring exclusion in February 2009, soil moisture was similar in the control and spring exclusion plots, and only slightly lower in the autumn exclusion plot, following a 120 mm rainfall event (Fig. 1). Soil water content responded to rainfall in the control and autumn exclusion plots in 2009, but continued to decrease in the spring exclusion plot. In 2009, two power failures occurred, thus delaying the movement of the roof: 18.5 mm of rain was not excluded on 2 and 3 April 2009 and 8.5 mm of rain was not excluded on 8 June 2009. Following a hot and dry period in August, minimum topsoil water content of the control and autumn exclusion plots decreased to values only slightly above those of the spring exclusion plot.

Leaf water potential

Pre-dawn leaf water potential was similar in the three plots during 2007, before the application of the treatments (not shown). In 2008, ψ_p decreased to near -3.0 MPa in all plots by end of September, but recovered to -0.6 MPa in the control and spring exclusion plots following 290 mm of rain in early November, during a period of decreasing atmospheric demand (Fig. 2). The ψ_p recovery was less pronounced in the autumn exclusion plot (-1.3 MPa) and the standard error about the mean was high, largely because one tree failed to recover at all. The maximum observed difference in ψ_p between plots (0.8 MPa) occurred at this time. In 2009, during a period of increasing atmospheric demand, ψ_p was lower in the spring exclusion plot by mid-May. The difference became larger with time until

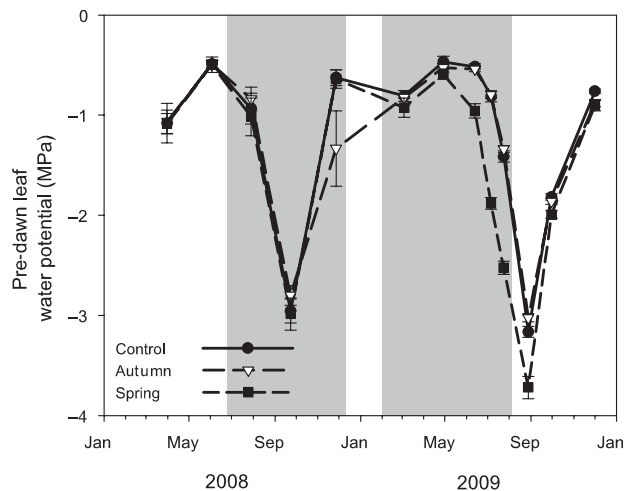


Figure 2. Pre-dawn leaf water potential in the different treatments. Points are the average of six trees per plot. Standard errors are shown. The shaded areas show the timing of the autumn exclusion (2008) and of the spring exclusion (2009).

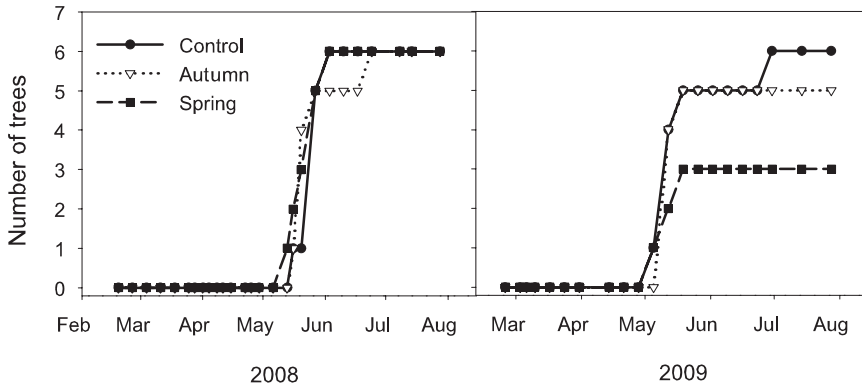


Figure 3. Seasonal pattern of leaf development based on the number of trees per treatment (max = 6) with fully mature leaves.

August, when ψ_p was -1.4 MPa in the control plot and -2.4 MPa in the spring exclusion plot.

Phenology

Trees reaching a phenophase higher than four are considered to have fully-developed and mature foliage (Dumerle & Mazet 1983). In 2007, all sampled trees reached this phase by the beginning of May (not shown). The same was true by the beginning of July in 2008 (Fig. 3). In 2009, all trees in the control plot reached phenophase 4, but three trees in the spring exclusion plot did not, including one tree that only achieved bud-burst (phase 3) and two trees that only showed signs of bud swelling (phase 2).

Leaf gas exchange

Net photosynthesis and g_s were similar in all plots during the spring and summer of 2007, for values of pre-dawn leaf water potential ranging from -1.0 to -3.5 MPa (not shown). In 2008, A_n and g_s were similar among plots from April to mid-August, but were lower in the autumn exclusion plot by November, just after the first heavy post-summer rainfall event (Wilcoxon signed-rank test, $P < 0.05$; Fig. 4a, b). At that time, A_n was $4.3 \mu\text{mol m}^{-2} \text{s}^{-1}$ lower in the autumn exclusion plot, which represents a difference of 40%. Recovery was complete in all three plots in March 2009, following several winter rainfall events (Fig. 4a, b). A_n and g_s started to diverge between the control and the spring

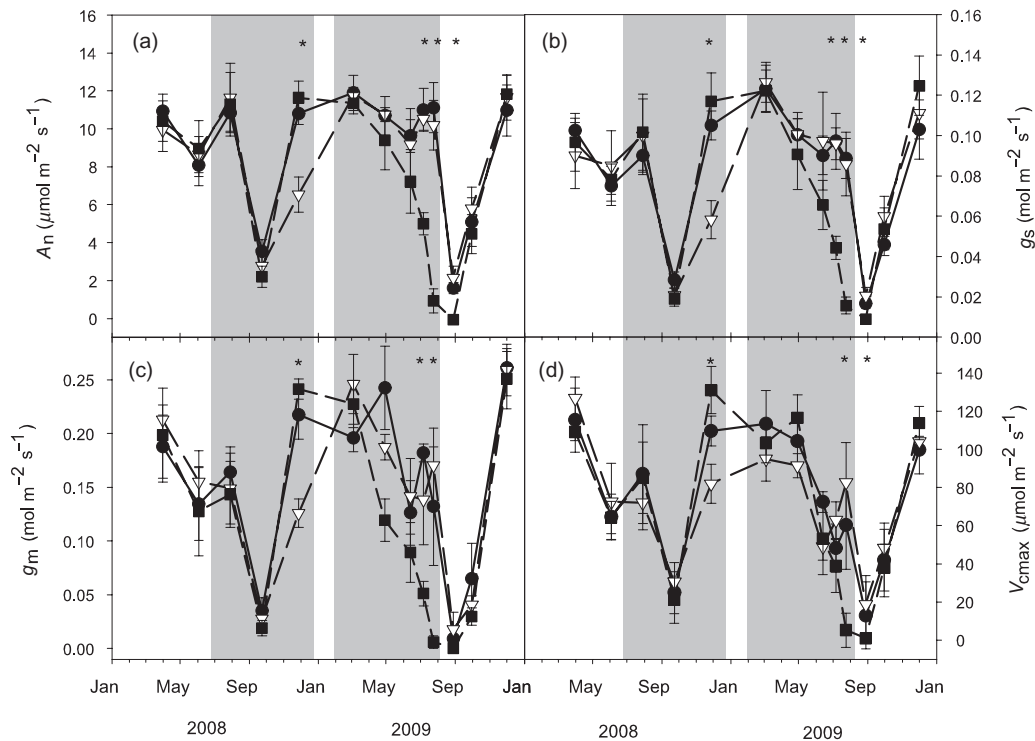


Figure 4. Photosynthetic parameters in the different treatments: (a) net assimilation (b) stomatal conductance (c) mesophyll conductance (d) maximum rate of carboxylation. Points are the average of 6 trees per plot. Standard errors are shown. The shaded areas show the timing of the autumn exclusion (2008) and of the spring exclusion (2009).

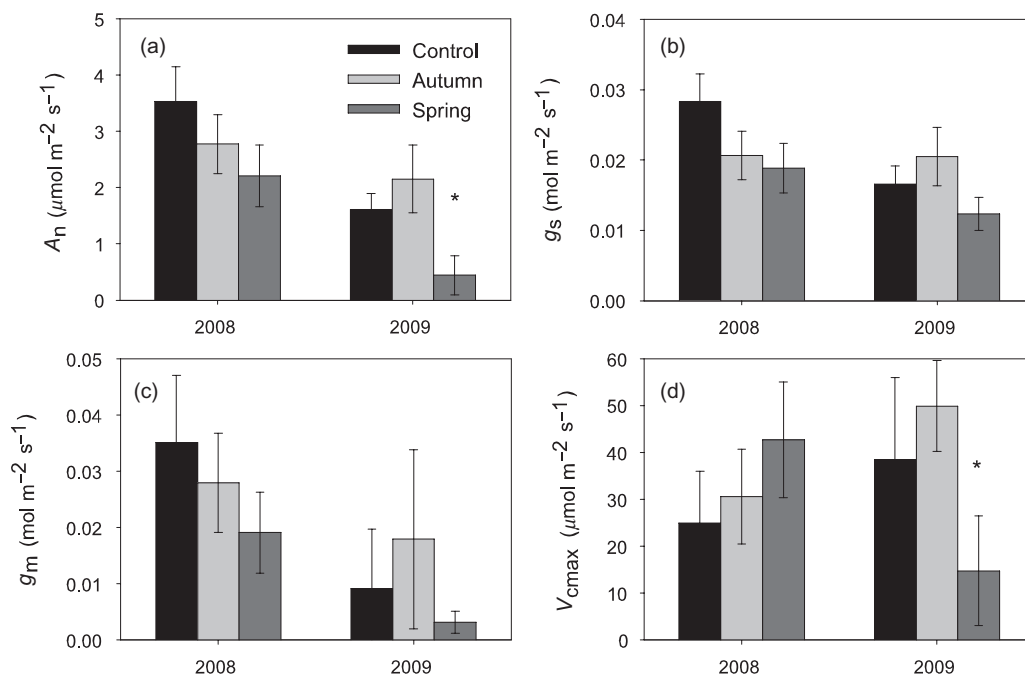


Figure 5. Mid-summer physiological parameters in 2008 and 2009 corresponding to the same pre-dawn water potential of -3 MPa ($-3.2 < \psi_p < -2.8$) in the different treatments: (a) net assimilation (b) stomatal conductance (c) mesophyll conductance (d) maximum rate of carboxylation. Bars represent the average of 6 to 12 trees per plot. Standard errors are shown. Stars (*) indicate a significant difference from the control (Wilcoxon signed-rank test, $P < 0.05$).

exclusion plot at the end of April 2009, but values were not statistically different until the beginning of July. The difference in A_n between the spring exclusion and control plots reached $10.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ (-92%) at the end of July 2009. Values remained significantly lower in the spring exclusion plot through the end of August, but were equal among plots at the end of September 2009, after 30 mm of rainfall facilitated partial recovery. Total recovery for A_n and g_s was observed at the beginning of December 2009, after 112 mm of rain fell at the end of October.

Mesophyll conductance and V_{cmax} showed the same tendencies as A_n and g_s over the course of the study, but fewer statistically-significant differences were observed between plots, because g_m showed higher among-sample variability, and differences in V_{cmax} were proportionally smaller (Fig. 4c, d). In 2008, trees of the control plot exhibited g_m values nearly double those of the autumn exclusion plots after the first heavy autumn rain event in November. Similarly, V_{cmax} was about 25% higher in the control plot at this time. In 2009, g_m was significantly higher in the control plot than in the spring exclusion plot throughout July, but not at the end of August, when values were very low in all plots (Fig. 4c). Differences in V_{cmax} between the control and the spring exclusion plots were statistically significant at the end of July and August 2009 (Fig. 4d). Mesophyll conductance and V_{cmax} were similar in the three plots at the end of September and total recovery was observed in December 2009.

Mid-summer values of A_n , g_s , g_m and V_{cmax} were compared among plots, for the same range of pre-dawn water potential (-2.8 to -3.2 MPa), and in both study years, to test the effect

of rainfall exclusion on the relationship between ψ_p and these four physiological parameters during drought stress. Six to nine values were selected per plot, with mean ψ_p ranging from -2.95 (± 0.09 SD) to -3.16 MPa (± 0.04 SD). Comparison between the control plot and each treatment allowed us to test whether the exclusion changed the relationship between pre-dawn water potential and the physiological parameters for stressed conditions. Average values for all parameters were similar among plots in 2008, but lower values were observed in the spring exclusion plot than in the control plot during summer 2009, including significantly lower A_n and V_{cmax} (Wilcoxon signed-rank test, $P < 0.05$; Fig. 5). Area-based leaf N was significantly lower in the spring rainfall exclusion plot ($2.9 \pm 0.3 \text{ g m}^{-2}$) than in the control plot ($3.3 \pm 0.2 \text{ g m}^{-2}$) during mid-summer 2009 ($P = 0.01$), but not during wetter periods, when pre-dawn leaf water potential was above -0.8 MPa ($3.2 \pm 0.5 \text{ g m}^{-2}$ versus $3.1 \pm 0.4 \text{ g m}^{-2}$, respectively; $P = 0.69$). Leaf nitrogen did not differ between the autumn exclusion and control plots, and no differences in leaf N were observed on a mass basis.

To determine whether the exclusion resulted in acclimatory changes affecting the relationship between pre-dawn leaf water potential and physiological parameters in the absence of drought stress, the same analysis was performed for mid-winter measurements, when pre-dawn water potential was above -0.8 MPa. Fifteen to 21 values were selected per plot, with average ψ_p ranging from -0.53 (± 0.02 SD) to -0.56 MPa (± 0.02 SD). No among-plot differences were observed in the four physiological parameters in either study year (not shown).

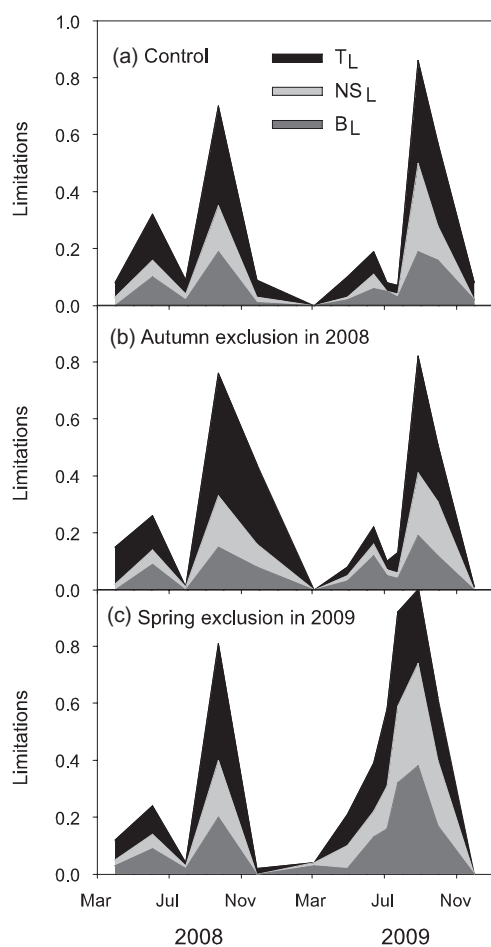


Figure 6. Photosynthetic limitations (fraction) in the different treatments: (a) Control (b) Autumn exclusion in 2008 (c) Spring exclusion in 2009. B_L , biochemical limitation, NS_L , non-stomatal limitation (biochemical + mesophyll limitations); T_L , total limitation (biochemical + mesophyll + stomatal limitations).

The seasonal course of stomatal (S_L), mesophyll (MC_L) and biochemical (B_L) limitations is shown in Fig. 6. Total limitation (T_L) was similar in all plots in mid-summer 2008, at about 75%, with S_L near 40%, and both MC_L and B_L around 18%. Thus, photosynthesis was limited by an almost equal proportion of stomatal (S_L) and non-stomatal ($NS_L = MC_L + B_L$) limitation at the peak of summer 2008. Differences between plots were marked at the beginning of

November 2008, after the first post-summer rain. T_L was around 5% in the control and spring exclusion plots, but was 44% in the autumn exclusion plot, including 28% S_L and 16% NS_L , evenly split between MC_L and B_L (Fig. 6). T_L was zero in all plots by March 2009, but increased thereafter. By late summer 2009, maximum T_L reached 84% in the control and autumn exclusion plots, and 100% in the spring exclusion plot (Fig. 6). Non-stomatal limitation was more important in 2009 than in 2008, especially in the spring exclusion plot, where NS_L reached 76% but S_L reached only 24%. NS_L consisted of an equal amount (38%) of MC_L and B_L in this plot. By December 2009, T_L was zero in all plots. The relationships between ψ_p and photosynthetic limitations are shown in Fig. 7. The slopes of the linear regressions between ψ_p and photosynthetic limitations were not statistically different between the control and the autumn exclusion plots (Table 2). In contrast, slopes were significantly steeper for NS_L and especially B_L in the spring exclusion plot compared with the control, but no among-plot differences were observed in the relationships between ψ_p and either S_L or T_L (Fig. 7, Table 2).

In response to increased drought severity, three trees of the spring exclusion plot failed to develop new leaves to full maturity in spring 2009 (Fig. 3). Measurements in this treatment were, thus, performed on three 1-year-old leaves and three current-year leaves, after 1 July 2009. Current year leaves were measured in the two other plots. Differences between treatments in 2009 could be caused by three factors: lower ψ_p in the spring exclusion plot, among-plot differences in leaf age, and interaction between these two factors. Leaf water potential strongly influenced all leaf physiological parameters (A_n , g_s , g_m and V_{cmax}), but the effect of leaf age was primarily restricted to V_{cmax} and g_s (Fig. 8). Furthermore, no effect of leaf age was observed on any physiological parameter at low ψ_p . This clearly demonstrates interaction between ψ_p and leaf age, with water stress tending to homogenize the physiological parameters among current-year and 1-year-old leaves below ψ_p of about -1.5 MPa. We fitted a non-linear regression between pre-dawn ψ_p and each physiological parameter, and tested for between-cohort differences in the regression coefficients, a and b (Table 3). The b parameter did not differ between leaf cohorts, but the intercept a was significantly higher for younger leaves for g_s and especially V_{cmax} (Table 3).

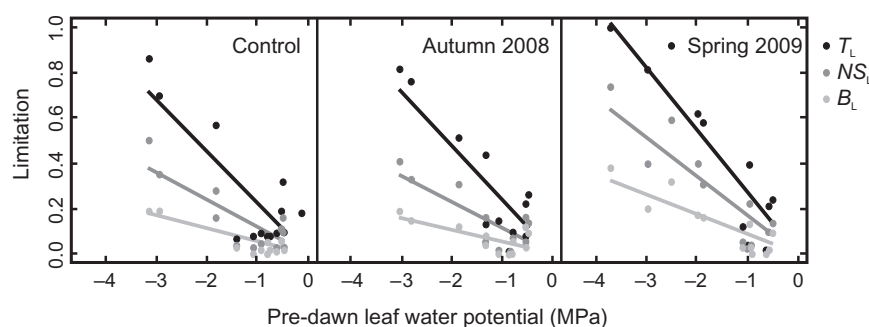


Figure 7. Photosynthetic limitations (fraction) as a function of pre-dawn leaf water potential in the different treatments: (a) control; (b) autumn exclusion in 2008; (c) spring exclusion in 2009. Linear regressions are presented in Table 2. B_L , biochemical limitation, NS_L , non-stomatal limitation (biochemical + mesophyll limitations); T_L , total limitation (biochemical + mesophyll + stomatal limitations).

Table 2. Slopes and standard errors (SEs) for the linear regressions ($y = \text{slope} \cdot x$) between fractional limitations and pre-dawn leaf water potential (MPa) (see Fig. 7 for regression lines)

	Control		Autumn		Spring		Student's <i>t</i> -test Control versus Autumn	Student's <i>t</i> -test Control versus Spring
	Slope	SE	Slope	SE	Slope	SE		
S_L	-0.11	0.013	-0.12	0.015	-0.10	0.012	0.79	-0.18
MC_L	-0.06	0.009	-0.06	0.008	-0.08	0.008	-0.09	1.69
B_L	-0.06	0.008	-0.05	0.008	-0.09	0.009	-0.32	2.53*
NS_L	-0.12	0.015	-0.11	0.015	-0.17	0.017	-0.22	2.27*
T_L	-0.23	0.027	-0.24	0.026	-0.27	0.025	0.32	1.30

Student's *t*-tests were performed to compare slopes between the control and exclusion treatments (d.f. = 22, *denotes a significant difference at $P < 0.05$).

B_L , biochemical limitation; S_L , stomatal limitation; MC_L , mesophyll limitation; NS_L , non-stomatal limitation ($MC_L + B_L$); and T_L , total limitation ($S_L + MC_L + B_L$).

DISCUSSION

The extreme drought conditions produced by the rainfall exclusion experiments revealed important leaf physiological changes that have not been observed in previous field studies involving less severe water stress. Firstly, the photosynthetic impact of spring rainfall exclusion was much stronger than the effect of autumn rainfall exclusion. Secondly, rainfall exclusion altered the relationship between leaf water potential and photosynthesis, with lower A_{\max} and V_{\max} observed at a given ψ_p in the exclusion plots. Lastly, it was demonstrated that non-stomatal limitation of photosynthesis can greatly exceed stomatal limitation in response to extreme drought.

Physiological parameters (A_n , g_s , g_m and V_{\max}) were similar in the three plots before the onset of the rainfall

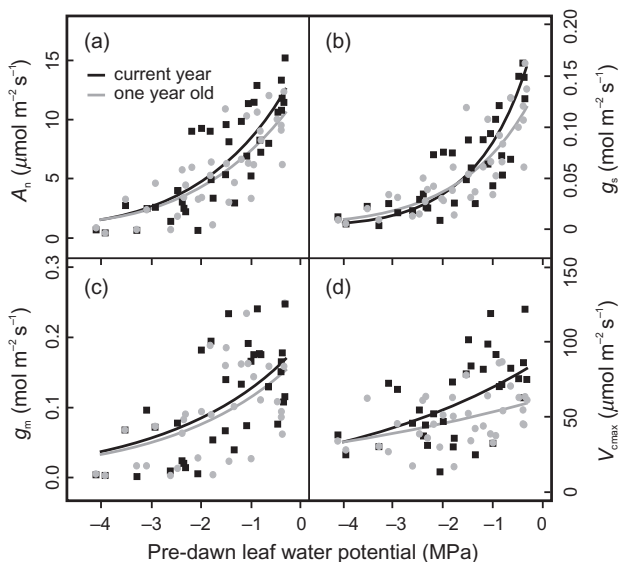


Figure 8. Photosynthetic parameters as a function of pre-dawn leaf water potential for 2 leaf cohorts, current year-old (black) and 1-year-old (gray): (a) net assimilation; (b) stomatal conductance; (c) mesophyll conductance; (d) maximum carboxylation rate. Non-linear regressions are presented in Table 3.

exclusion experiments. Among-plot differences that occurred during exclusion were therefore interpreted as treatment effects. The autumn rainfall exclusion had a relatively weak impact on A_n , that only appeared after 4 months of exclusion. The main reasons for this delay were as follows: (1) September and October 2008 were abnormally dry, so that little rainfall was excluded during the first half of the experiment; (2) strong winds caused lateral rainfall entry up to two metres into the autumn exclusion plot during a 290 mm rainfall event in early November 2008; and (3) water content decreased simultaneously with atmospheric demand, decoupling soil and atmospheric drought. Nevertheless, A_n , g_s , g_m and V_{\max} were about 40% lower in the autumn exclusion plot than in the control plot in November 2008. Differences in photosynthesis between the autumn exclusion and control plots likely lasted until the end of January 2009, when the first significant post-treatment rainfall event occurred.

The spring exclusion had a much stronger effect, despite lower rainfall in the control plot (491 mm versus 575 mm) and less effective rainfall exclusion in the treatment plot (436 mm versus 559 mm, or 87% versus 97%). The main difference between the two exclusion periods was that soil water content decreased simultaneously with increasing atmospheric demand during spring exclusion. Under these conditions, ψ_p rapidly decreased to lower values than were observed during the autumn exclusion (Fig. 2). Based on leaf water potential data for the 1998–2009 period, ψ_p usually falls below -1 MPa around the beginning of July at this site (Rambal *et al.* 2003; Limousin *et al.* 2009). This threshold was reached 1 month earlier in response to spring rainfall exclusion.

In response to severe mid-summer drought stress, A_n and V_{\max} were 75% and 72% lower in the spring exclusion plot than in the control plot for the same pre-dawn leaf water potential (Fig. 5). Furthermore, NS_L increased with decreasing ψ_p more steeply in the spring exclusion plot than in the control (Fig. 7, Table 2). Past field studies have either shown no change in the fundamental relationship between water stress and photosynthesis (Wilson *et al.* 2000; Llorens *et al.* 2003; Grassi *et al.* 2005; Limousin *et al.* 2010), or have

	Current year leaves		1-year-old leaves		Student's <i>t</i> -test
	Regression coef.	SE	Regression Coef.	SE	
A_n					
<i>a</i>	15.04	1.36	12.55	1.19	1.38
<i>b</i>	0.58	0.09	0.54	0.09	0.37
g_s					
<i>a</i>	0.21	0.02	0.15	0.02	2.49*
<i>b</i>	0.93	0.12	0.70	0.11	1.39
g_m					
<i>a</i>	0.19	0.03	0.17	0.03	0.46
<i>b</i>	0.41	0.12	0.41	0.14	-0.01
V_{cmax}					
<i>a</i>	88.79	9.98	62.17	6.02	2.28*
<i>b</i>	0.25	0.08	0.16	0.06	0.93

Table 3. Coefficients of regression [$y = a \cdot \exp(b \cdot x)$] and standard error (SE) between pre-dawn leaf water potential (MPa) and photosynthetic parameters for two leaf cohorts: current-year and 1-year-old leaves (see Fig. 8 for regression lines)

Student's *t*-tests were carried out, to compare slopes between cohorts (d.f. = 68, *denotes a significant difference at $P < 0.05$).

A_n = net assimilation, g_s = stomatal conductance, g_m = mesophyll conductance, and V_{cmax} = maximum carboxylation rate.

revealed higher A_n at a given soil moisture in the dry plots of rainfall exclusion experiments (Ogaya & Penuelas 2003; Llorens *et al.* 2004). Collectively, these results suggest that the impact of drought acclimation on the relationship between water stress and photosynthetic productivity may depend on drought severity. Given that both the frequency and duration of drought events are expected to increase in Mediterranean regions as a consequence of global climate change, further research is needed to identify extreme drought thresholds beyond which acclimatory changes may negatively impact relationships between water stress indicators and photosynthetic productivity.

The strong decrease in V_{cmax} observed during spring rainfall exclusion may be the result of leaf N translocation. Consistent with this hypothesis, area-based leaf N content ($g\ m^{-2}$) was slightly lower in the spring rainfall exclusion plot than in the control plot during mid-summer 2009 ($P = 0.01$). Alternatively, declining V_{cmax} might have resulted from lower apparent leaf N investment in ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco; Niinemets & Tenhunen 1997), from inhibition of Rubisco activity (Parry *et al.* 2002) or as a negative feedback of drought-induced growth inhibition (Paul & Foyer 2001).

Increased leaf retention rate and lifespan have been observed in response to drought in holm oak forests (Busotti *et al.* 2003). At Puéchabon, three trees in the spring rainfall exclusion plot retained older leaves and failed to develop new leaves to maturity in 2009. On these trees, gas exchange was measured on the 1-year-old leaf cohort throughout the season, whereas current-year leaves were measured on all six trees of both the control and autumn exclusion plot after July 1. It was therefore important to consider whether the differences in physiological parameters between the plots beyond this date were the result of differences in the mean age of the sampled leaves. Niinemets *et al.* (2005) showed that older leaves of *Quercus ilex* have lower g_m , V_{cmax} and J_{max} (see also Niinemets,

Tenhunen & Beyschlag 2004 and Niinemets *et al.* 2006). However, the Niinemets *et al.* (2005) study did not include testing of the interaction of age and drought stress on physiological parameters. At Puéchabon, differences between age groups were minor under stressed conditions (Fig. 8 & Table 3). Considering that leaf age effects were negligible at low ψ_p , our results indicate that extreme water stress acted on physiological parameters through lower leaf water potential rather than higher leaf age.

Non-stomatal limitation reached 30–76% in the spring exclusion treatment, when total limitation was in the 60–100% range. The NS_L was evenly split between MC_L and B_L , which were both stronger than previously reported in field settings (Wilson *et al.* 2000; Grassi *et al.* 2005; Limousin *et al.* 2010). Wilson *et al.* (2000) found maximum values of $NS_L = 20\%$ and $S_L = 50\%$ during seasonal drought in a temperate deciduous forest. Maximum values attributed to seasonal drought by Grassi & Magnani (2005) on ash in Italy were 14, 12 and 32% for MC_L , B_L and S_L , respectively. Based on partial throughfall exclusion in a *Quercus ilex* forest, Limousin *et al.* (2010) found maximum values of 20, 18 and 52% for MC_L , B_L and S_L . Our data demonstrate that NS_L can exceed S_L , when drought intensity is increased beyond levels that are normally observed under current climate conditions. Similar results were obtained by Galmés *et al.* (2007) in a potted experiment on ten Mediterranean plants under severe water stress. However, in contrast with our results, they showed systematically stronger MC_L (36%) than B_L (25%). Collectively, our data demonstrate that stomatal closure operates as a fast rate process, explaining as much as 30 to 50% of the decrease in assimilation, whereas MC_L and B_L adjust more slowly, but can reach 60 to 75% under severe drought intensity in field settings.

The speed and extent of photosynthetic recovery can be affected by the intensity and duration of antecedent water stress (Miyashita *et al.* 2005). Recovery of A_n and g_s can be slow, lasting 20 d in *Quercus pubescens* and 30 d for *Fagus*

sylvatica, based on the results of potted plant experiments (Galle *et al.* 2007). Delayed photosynthetic recovery has been attributed to persistent stomatal closure, damage to mesophyll tissue and increased resistance to xylem water transport following severe water stress (Kaiser *et al.* 1981). MC_L can be the strongest limitation to photosynthetic recovery in Mediterranean plants, with recovery extents ranging from <10 to 70% (Galmés *et al.* 2007). Despite the severity of the water stress imposed by the spring rainfall exclusion at Puéchabon, we observed complete photosynthetic recovery in *Q. ilex*, but not until 2 months after the treatment (Fig. 4).

Most previous field studies have shown no functional changes in the relationships between photosynthesis parameters and water stress under moderate to strong drought intensity (Wilson *et al.* 2000; Llorens *et al.* 2003; Grassi *et al.* 2005; Limousin *et al.* 2010). However, we have demonstrated that more severe droughts can result in strong non-stomatal limitation of photosynthesis, as well as lower A_n and V_{cmax} at a given leaf water potential. These observations are important for modelling vegetation responses to climate change in Mediterranean ecosystems, because existing drought parameterizations may be invalid if droughts become longer, more frequent and more severe.

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SUPPORTING INFORMATION

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Figure S1. Rainfall shelter and experimental design.

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