

Physiological and structural changes in response to altered precipitation regimes in a Mediterranean macchia ecosystem

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Abstract Significant decrease in precipitation up to 15–20% has been observed in the Mediterranean area in the last two decades as a consequence of climate change. To simulate an analogous scenario, the precipitation regime was altered in replicated experimental plots in a Mediterranean macchia dominated by *Arbutus unedo* L. species. Two different levels of soil water content (SWC) were obtained during the summer: a mean value of 7% was obtained in water-depleted (*D*) plots by a partial (−20%) rain exclusion treatment using rain gutters; while a mean value of 14% in SWC was obtained in watered (*W*) plots supplying water by a sprinkler net. The physiological and structural changes were investigated over the course of two consecutive years by measurement of water potential, gas exchange leaf carbon isotopes, leaf pigments and growth. Apart from short-term responses, mainly related to the

elastic response of stomatal conductance to soil water, a more long-lasting and significant acclimation to water availability was observed as a result of the increase in hydraulic resistance in the soil–plant continuum, which persisted even after the return to full water availability during the fall and winter. This response involved the permanent down-regulation of stomatal conductance and photosynthesis, accumulation of photo-protective pigments, as well as a reduction in shoot growth, leaf area index and an increase in shoot-bearing flowers in *D* plots. This acclimation response prevented the onset of any runaway damage thereby reducing the forest vulnerability to drought. Furthermore, the imposed drought induced a slight increase or no change in intrinsic water-use efficiency (WUE_{int}), as a result of the parallel increase in stomatal and non-stomatal limitations; conversely integrated WUE (i.e., estimated from leaf carbon isotopes) was not affected by drought.

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Introduction

Observational climatic data and models are already indicating that rainfall patterns are changing in the Mediterranean region as a consequence of climate change, with a marked reduction in precipitation up to 15–20% mainly occurring during the summer (IPCC 2001, 2007; Sarris et al. 2007). This is particularly relevant because forest ecosystem types already have to cope with frequent hot and dry summers. As far as we know, water availability is the most important factor controlling plant functions

(Rambal et al. 2003) and limiting plant productivity under dry climates (Boyer 1982; Churkina and Running 1998; Nemani et al. 2003). It has been stated that drier summers, may increase the physiological stress and as a consequence enhance vulnerability and forest die-back. Thus, a clear need exists to explore how forests may behave under the new climatic scenario and what might be the impact on physiology, growth and productivity.

Although the tree response to drought has been extensively studied in Mediterranean environment (Grieu et al. 1988; Lebourgeois et al. 1998; Irvine et al. 1998; Fernández et al. 2000; Chaves et al. 2003, etc.), very few investigations have been carried out in a natural forest by experimental manipulation of rainfall pattern (Borghetti et al. 1998; Llorens et al. 2004a; Ogaya and Peñuelas 2007; De Dato et al. 2008).

For example, from the previous experiments, we know that over the short-term (1) partial stomatal closure is one of the key mechanisms developed by plants to save water by reducing transpiration and to keep the leaf water potential within a safe range to prevent cavitation risk (Tyree and Sperry 1989; Saliendra et al. 1995); and (2) this efficient stomatal control may result in a negative impact on plant carbon balance through the increase of stomatal limitation to photosynthesis (Wong et al. 1979; Jarvis and Davies 1998; Rambal et al. 2003). Our knowledge of the mechanisms that underlie this response and how the relationship between stomatal conductance and photosynthesis is affected by drought is still limited. For example, structural changes by the reduction of leaf area index (LAI) or the increase of hydraulic resistance (Cochard 1992; Irvine et al. 1998) may alleviate the direct effects of water stress and change the relationship between stomatal and CO₂ assimilation rate (Geiger and Servaites 1991; Cinnirella et al. 2002).

It has also been suggested that functional acclimation mechanisms, other than structural changes, could be involved over the medium and long term. In fact, non-stomatal limitations [i.e., mesophyll conductance and enzyme catalytic rates (Rubisco, etc.)] may be involved even under mild to moderate water stress (Flexas and Medrano 2002; Grassi and Magnani 2005). Furthermore, drought, associated with high temperatures and an excess of light during summer may result in a chronic photo-inhibition or down-regulation of photosynthesis, causing a marked reduction of CO₂ assimilation (Osmond 1994; Demmig-Adams and Adams 1996). Nevertheless, this negative effect may be counterbalanced by the seasonal variations in pigment content in leaves [i.e., xanthophylls, carotenoids, chlorophyll (Chl *a* + *b*)]. It has been shown that summer accumulation of xanthophyll pigments may protect the photosynthetic apparatus from photo-inhibition during drought (Krause and Weis 1991).

Thus, how the relationship between CO₂ assimilation and stomatal conductance is affected may have a strong impact on water-use efficiency (WUE, the amount of carbon gained per water transpired) (Dewar 1997; Fernández et al. 2000), which is an important trait under Mediterranean environmental conditions.

The general objective of this research was to explore how *Arbutus unedo* L. coppice forests may respond to a reduction in precipitation pattern by physiological and structural changes in order to reduce the risks of xylem embolisms and foliage dieback. *A. unedo* is a common evergreen species in Mediterranean macchia that has a vast distribution in Italy ($\approx 500,000$ ha). The physiological changes have been assessed over the course of 2 years by measurements of water potential, gas exchanges, leaf carbon isotopes and leaf pigments. Structural changes were assessed by measurements of LAI, apical growth and flowering. In particular, we hypothesised that (1) the reduced water availability, as simulated in this study by a partial restriction of precipitation (-20%), may induce an acclimation response in leaf gas exchange rates and an increase in photo-protective pigments, in order to avoid the onset of run-away damage and forest vulnerability, (2) the variations in hydraulic conductance may have a role in the functional acclimation mainly by affecting stomatal conductance, and (3) the down-regulation of maximum CO₂ assimilation and stomatal conductance may reduce the growth i.e., shoot and leaf growth similarly affect the changes in WUE.

Methods

Site description

The study was performed during 2004 and 2005 at the Allumiere experimental site, in a 50 ha natural evergreen forest located approximately 100 km north of Rome (Italy) (42°11' N, 11°56' E; 180 m a.s.l.). The area's climate is a Mediterranean type, with the long term (1951–2005) mean annual precipitation and temperature of 919 mm and 13.6°C, respectively (Viterbo climatic station). Precipitation is mainly distributed during autumn-winter, with a dry period occurring between June and August (~ 120 mm).

The soil is classified as Andosol (Soil Taxonomy; USDA Soil Survey Staff 1999) and is essentially acidic (pH = 4) due to the presence of aluminium minerals. The depth to the bedrock at this location is approximately 31 cm and about 90% of tree roots are concentrated in this layer. Climatic data (temperature and precipitation) were obtained from the meteorological station within the measurement site.

The forest is a 25-year-old Mediterranean macchia, which originated after a fire that completely destroyed the

vegetation. Since then, it has been growing undisturbed and unmanaged. The plant community is dominated by the coppice *A. unedo*, which covers 65% of the surface. The stand contains other less-abundant species: *Erica arborea* L. (13%), *Fraxinus ornus* L. (8%), *Quercus pubescens* L. (5%), *Q. cerris* L. (4%), *Q. ilex* L. (2%), etc.

A field campaign carried out before the beginning of the experiment showed the following characteristics that were similar within experimental plots: total basal area $19.6 \text{ m}^{-2} \text{ ha}^{-1}$, density of the re-sprouted stems $4,070 \text{ ha}^{-1}$, mean canopy height 5 m, average stem diameter 5 cm and LAI $5.5 \text{ m}^2 \text{ m}^{-2}$.

Experimental design

A 6.2 ha plateau area was chosen because of its uniform slope, consistent soil, lack of water table and a uniform distribution of vegetation. Within this area, two plots ($8 \times 12 \text{ m}$) replicated three times were randomly identified and the rainfall regime was altered in order to obtain two different levels of volumetric soil water content (SWC, volume of water per volume of soil multiplied by 100) during the summer (June–August). In one treatment, the SWC was decreased by a partial rain exclusion treatment (–20%) using a system of pipes suspended at about 1.8 m above the forest floor re-routing the intercepted water far away from the plot area (water-depleted plots, *D*). The experimental manipulation started on 10 April 2004. In the other treatment, the SWC was increased by irrigation through a sprinkler net designed to supply water uniformly to the soil in order to simulate rain events (watered plots, *W*). Water from precipitation events was collected in a slough and used for the irrigation. As about half of the water was lost outside the plots during irrigation from the eight sprinklers positioned at the edge of each plot, only half of the water was accounted from those.

Irrigation treatment was conducted from June to August in order to keep the SWC above the 10% threshold that indicates well-watered conditions, except for 2004 when irrigation system was in place only in July. This threshold was established in a pretreatment experiment carried out in 2003. Predawn water potentials were compared with corresponding values of SWC at each plot, so as to calibrate a field retention curve and estimate predawn water potentials from continuous measurements by time-domain reflectometry (TDR). This information was used to define irrigation targets.

One scaffold tower was located in the central position of the *D* and *W* replicates, allowing access to at least six trees with a canopy of 5–6 m height. On each tree, we performed measurements on the same branches of 6–8 experimental trees per replicate throughout both years.

Water relations

Soil water content was measured continuously within each replicate in the *D* and *W* treatments by CS616-L probes connected to measurement electronics (Campbell Scientific, INC, Logan, UT, USA). The probes consisted of two 30-cm long stainless steel rods, fully inserted into the soil at six different locations per replicate. The readings were then translated to a measure of SWC with TDR data in accordance with the procedure described by Topp and Davis (1985).

Eight intensive field campaigns, of 2–3 days were carried out between June 2004 and September 2005 to determine plant water status. Predawn (Ψ_{pd}) and midday leaf water potential (Ψ_{md}) were measured on the same 6–8 experimental trees per replicate on 5–6 fully expanded leaves with a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR, USA).

Tree hydraulic conductance was measured indirectly as:

$$K_{\text{tree}} = E / (\Psi_{\text{pd}} - \Psi_{\text{md}}) \quad (1)$$

where E is leaf transpiration rate and Ψ_{pd} was used as a proxy of soil water potential (Ripullone et al. 2007).

Gas exchange and leaf pigment content

Gas exchange measurements were carried out on the same experimental trees for each replicate plot used for assessment of Ψ_{pd} and Ψ_{md} . Measurements were restricted to trees growing in the central portion of each plot to avoid “edge effects”. Measurements were performed on ten leaves, similar in size and quality, located at positions of comparable relative photosynthetic photon flux density (PPFD) in the upper sunlit part of the canopy, with each leaf considered an individual independent sample of the tree, using a portable infrared gas analyzer (CIRAS 1, PP Systems, Hitchin, UK). Net carbon assimilation (A), stomatal conductance (g_s), leaf transpiration rate (E) and intercellular CO_2 concentration (C_i) were recorded after steady-state conditions had been attained after 3–5 min at ambient CO_2 ($370 \mu\text{mol mol}^{-1}$). Measurements were made on clear and sunny days between 11:30 a.m. and 15:30 p.m., when changes in environmental conditions were relatively small and gradual. All gas exchange measurements were taken under natural PPFD ranging between 1,200 and 2,000 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ and leaves were placed in the leaf chamber in their natural orientation. Light-saturated photosynthesis at $370 \mu\text{mol mol}^{-1}$ was considered a maximum assimilation rate (A_{max}). Temperature and humidity in the gas exchange chamber were kept similar to environmental conditions. Intrinsic water-use efficiency (WUE_{int}) was calculated from gas exchange measurements, as the ratio of A_{max} and g_s (Saurer and Siegwolf 2007).

For pigment analysis, disks were punched from the same sunlit leaves used for gas exchange measurements in June, July and September 2005. After harvesting, disks were immediately stored at -190°C in a special container filled with liquid nitrogen, and subsequently preserved in a freezer at -80°C . Chlorophyll and carotenoid pigments were extracted and analysed with high-performance liquid chromatography (HPLC; model LC-10AS with a detector SPD-10AV, Shimadzu, Kyoto, Japan) according to Baraldi et al. (2008). Xanthophyll de-epoxidation state (DEPS), which consists in the conversion of violaxanthin (V) to zeaxanthin (Z) via antheraxanthin (A), was calculated as the ratio $(Z + 0.5A)/(V + A + Z)$, following Müller et al. (2006). Xanthophylls were also expressed as VAZ normalised to chlorophyll.

Growth and LAI

Tree height, apical shoot growth and flowering were monitored throughout the experiment on the same trees used for gas exchange and biochemical analysis. Canopy LAI was also monitored in each replicate plot by the hemispherical photography technique. The images were taken at grid points 4 m apart using a digital camera equipped with a Nikon FC-E8 fisheye adapter, immediately followed by a film camera shot (Sigma 8 mm F4 fisheye lens). Images were digitized and analysed using a Gap Light Analyzer and converted into LAI (Frazer et al. 2001). Measurements were then normalised for any pretreatment differences.

Carbon isotope analysis

Samples of partially expanded leaves were collected five times in the 2004 and 2005 summer field campaigns (June, July 2004 and June, July, September 2005) for carbon isotope analysis. Six samples were randomly collected from six trees of each plot in the upper part of the crown at the same position of the leaf used for gas exchange and leaf water potential measurements.

For each sample two or three leaves, similar in size and quality, were collected, dried and ground to a fine powder, and then $\delta^{13}\text{C}$ was measured by a continuous-flow isotope ratio mass spectrometer (DeltaPlus, Finnigan, Germany):

$$\delta^{13}\text{C} = [R_{\text{sample}}/R_{\text{standard}} - 1] \times 1,000 \quad (2)$$

where R is the isotope ratio, and $^{13}\text{C}/^{12}\text{C}$ referred to the sample and to the standard, respectively. The isotope values are expressed in delta notation, relative to V-PDB for carbon. The standard deviation for replicate analysis was $\pm 0.3\text{‰}$.

Statistical analysis

To test the effect of treatments (W , D) on physiological variables, we performed repeated-measures analysis of variance (ANOVA) using the mean of the three replicates per each date of measurement and treatment as the independent factor. Mean comparisons were carried out using the Bonferroni test. Statistical significance was defined as $P \leq 0.05$ and $P \leq 0.01$. Furthermore, the correlation analysis was made using the Pearson's coefficient of correlation. All statistics were computed with the SPSS statistical package (SPSS, Chicago, IL).

Results

Soil and plant water status

Although, the study period was slightly wetter (i.e., 1,095 mm in 2004 and 935 mm in 2005) (Fig. 1a) than the long-term average (919 mm), the summer (June–August) (i.e., 90 mm in 2004 and 80 mm in 2005) was drier than the long-term average (~ 120 mm). In all, 72 and 370 mm of water were supplied to each plot in summer 2004 and 2005, respectively (Fig. 1b), which corresponded to an increase in percentage of about 10% in 2004 (calculated from the beginning of the manipulation) and about 40% in 2005.

As an effect of treatment, SWC showed variations during the study period (Fig. 1c); the lowest value (about 5%) was reached in summer 2005, whilst the maximum values (about 30%) were reached in autumn–winter in both years, following the rainfall distribution. Treatment effect on SWC was significant only in summer 2005 ($F_{1,59} = 64.5$, $P = 0.0001$), while in 2004 treatment effect was not significant probably due to either generally wetter conditions in 2004 comparing to 2005 or the irrigation system that was in place only in July. The mean value of SWC was ca. 10% in D and ca. 12% in W for summer 2004; while SWC was ca. 5% in D and ca. 15% in W for summer 2005. A 10% SWC was a threshold below which conditions were water stressed.

As expected, vapour pressure deficit (VPD) was minimum in winter–spring and maximum in summer (Fig. 2a), although July 2004 (4.8 kPa) was drier compared with July 2005 (3.5 kPa).

In general, predawn water potentials (Ψ_{pd}) followed the fluctuations of SWC over the 2 years of the experiment (Fig. 2b). No statistical differences in Ψ_{pd} were observed in 2004 and spring 2005 between the D and W plots, with the exception of July 2004 when the difference was significant ($P < 0.05$). In July 2005, Ψ_{pd} of stressed trees was lower than the watered trees demonstrating the

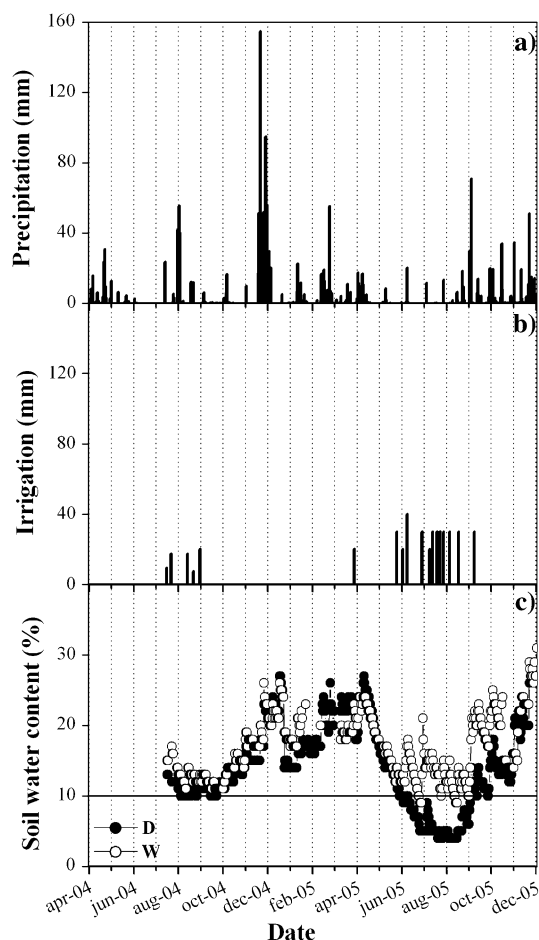


Fig. 1 Seasonal course of: **a** daily precipitation, **b** irrigation and **c** soil water content (SWC) in watered (*W* open circles) and water-depleted (*D* closed circles) plots at the study site during the overall studied period (from 10 April 2004 to November 2005). SWC below the 10% threshold represents soil under water stress conditions. Symbols of SWC are daily or bi-daily values (%). Treatment effect on SWC was significant in summer 2005 ($P < 0.05$)

effectiveness of the applied treatments, even if the mean values for *D* plots were never less than -1.2 MPa. Minimum Ψ_{pd} in the *D* treatment was similar in both years showing similar drought conditions. With the onset of rainfall at the end of summer 2004, Ψ_{pd} values in *D* plots were similar to those measured in the *W* plots. Thus *A. unedo* had no difficulty in recovering its water status after the summer drought. In contrast, midday water potential (Ψ_{md}) did not significantly differ amongst treatments along the experiment. Furthermore, no clear and significant relationship emerged between Ψ_{md} and Ψ_{pd} (data not shown).

In contrast, hydraulic conductance (K_{tree}) was significantly affected and reduced by drought treatment (*D* 1.95 ± 0.13 mol $m^{-2} s^{-1} MPa^{-1}$; *W* 2.51 ± 0.17 mol $m^{-2} s^{-1} MPa^{-1}$; $P < 0.05$) over the experiment (Fig. 3). These differences between treatments were maintained

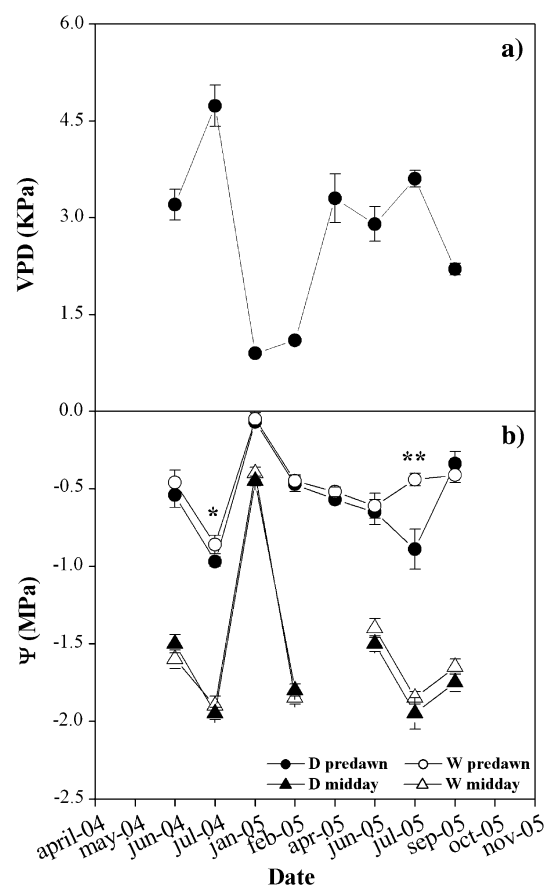


Fig. 2 Seasonal course of **a** vapour pressure deficits (VPD), **b** predawn water potential in *W* (open circles) and *D* (closed circles) plots and mid-day water potential in *W* (open triangles), *D* (filled triangles) plots. Vertical bars indicate mean \pm SE ($n = 30$ in VPD and $n = 15$ in water potential). Significant differences between treatments are marked by asterisks (* $P < 0.05$) and (** $P < 0.01$)

even over the winter under full water availability. It is worth noting that on the first date (June 2004), K_{tree} in the *D* treatment was actually greater than in *W* treatment.

Gas exchange measurements, isotope and leaf pigment analysis

As a consequence of the water restriction imposed in July 2004, stomatal conductance (g_s) and light-saturated CO_2 assimilation (A_{max}) of *D* diverged from *W* plots (Fig. 4a, b). This divergence was significantly maintained for g_s ($F_{1,40} = 4.7$, $P = 0.05$) and A_{max} ($F_{1,40} = 4.2$, $P = 0.05$) over the whole experimental period irrespective of improved soil water conditions during the winter and spring.

During the study period, g_s reached the maximum value (0.16 mol $m^{-2} s^{-1}$) in spring 2005 when environmental conditions were favourable, while the lowest minimum rate was monitored in July 2004 (0.04 mol $m^{-2} s^{-1}$) under air

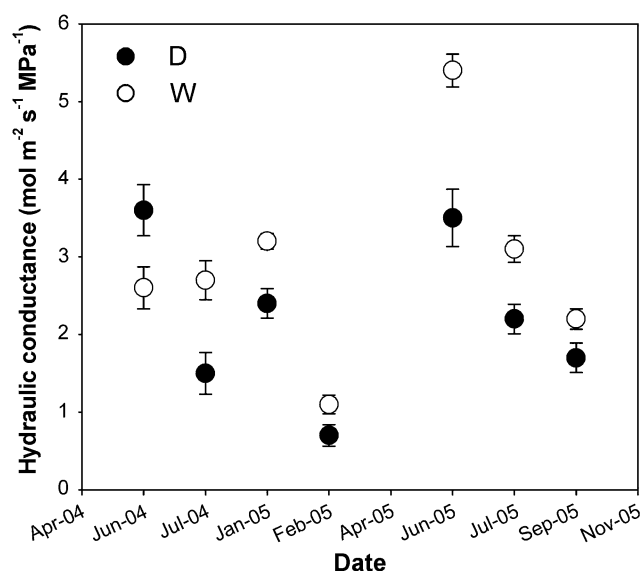


Fig. 3 Seasonal changes of hydraulic conductance (K_{tree}) in W and D treatments; mean treatment \pm SE ($n \geq 18$). Differences are all statistically significant at $P < 0.05$

and soil humidity stress. The difference in g_s measured in D plots between July 2004 and 2005 (0.04 and 0.08 $\text{mol m}^{-2} \text{s}^{-1}$, respectively), despite the same Ψ_{pd} , was likely due to the difference in VPD monitored between the two dates (see Fig. 1b).

Under the combination of natural and artificial drought, A_{max} values from D plots did not fall below 60% of those observed in the watered treatment; this was the consequence of the realistic nature of the drought treatment imposed (i.e., only 20% reduction in precipitation). Despite full water availability, A_{max} during the winter was similar to values measured in drought plants during the summer, as a result of low temperatures.

A_{max} and g_s were linearly and significantly correlated ($R = 0.94$, $P < 0.0001$) over the summer and in response to water availability (Fig. 5). As a result, computed intercellular CO_2 (C_i) concentration (Fig. 4c) and intrinsic water-use efficiency (WUE_{int}) did not significantly differ between treatments (Fig. 6), in contrast to what would have been expected as a result of stomatal limitation alone. A slight increase in WUE_{int} was observed in D plots during July 2004 and 2005 and February 2005. However, WUE_{int} showed large fluctuations over the season, which appeared to be mainly related to changes in VPD (see inset in Fig. 6).

The stable carbon isotope composition ($\delta^{13}\text{C}$) were not affected ($F_{1,24} = 0.42$, $P = 0.52$) by changes in soil water availability through the experiment (Fig. 7), except for June 2005, when only slight differences ($P < 0.05$) were shown between D and W plots. This result was consistent with our gas exchange findings, in which either

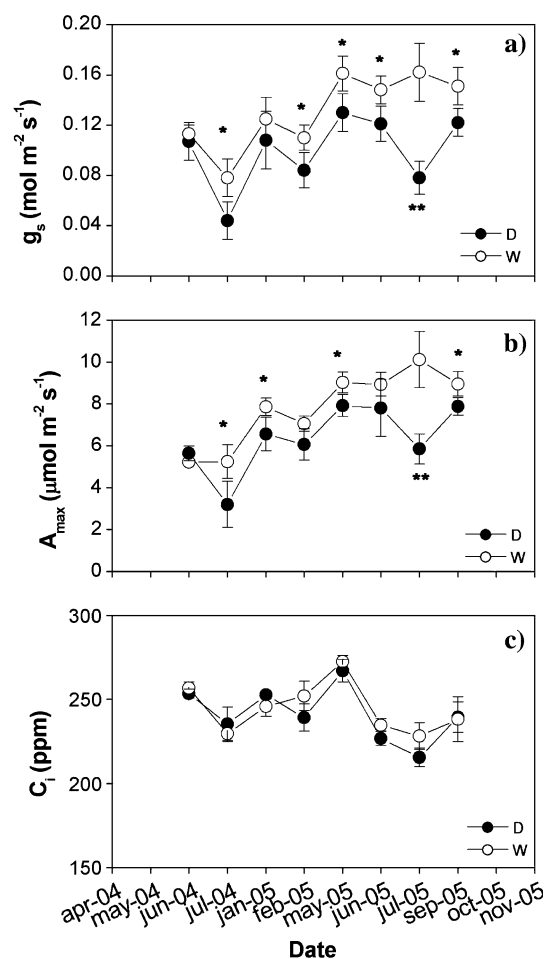


Fig. 4 Seasonal dynamics of **a** midday stomatal conductance (g_s), **b** light-saturated carbon assimilation (A_{max}) and **c** intercellular CO_2 concentration (C_i) under clear sky conditions in D (filled circles) and W (open circles) treatments. Mean \pm SE ($n = 30$). Significant differences are marked by asterisks (* $P < 0.05$) and (** $P < 0.01$)

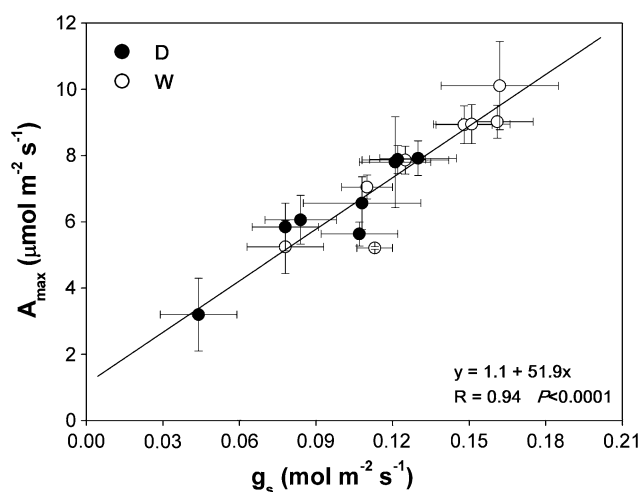


Fig. 5 Correlation between midday stomatal conductance (g_s) and light-saturated carbon assimilation rate in D (filled circles) and W (open circles) treatments. Mean \pm SE ($n = 30$)

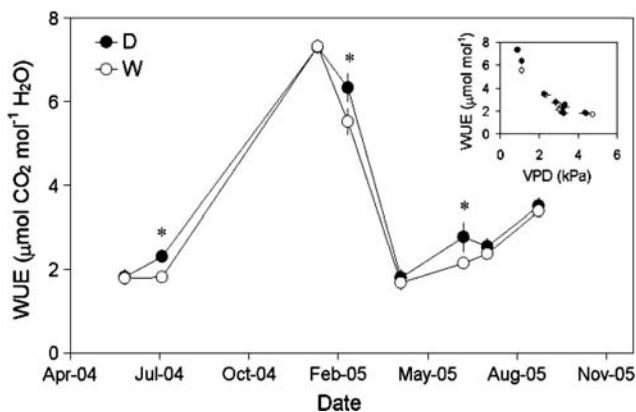


Fig. 6 Seasonal dynamics of intrinsic water-use efficiency (WUE, A_{\max}/g_s) as determined from gas exchange measurements, in *D* (filled circles) and *W* (open circles) treatments; mean \pm SE ($n = 30$). Significant differences are marked by an asterisk ($P < 0.05$). Inset panel relationship between WUE and vapour pressure deficit (VPD)

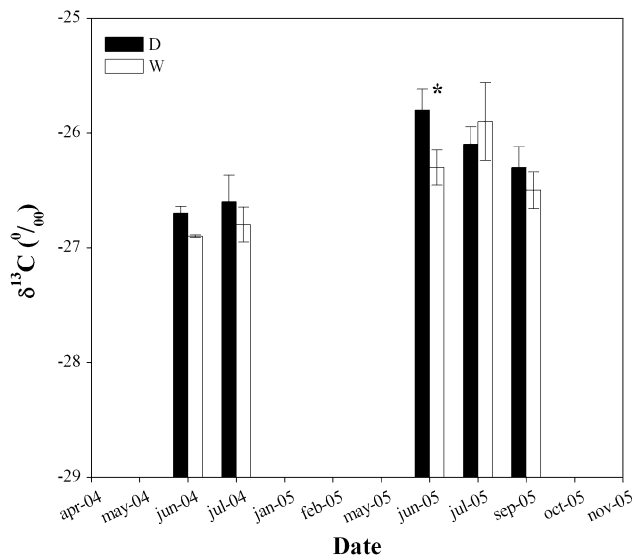


Fig. 7 Variations in carbon isotope composition ($\delta^{13}\text{C}$) measured on samples coming from 2 to 3 composited leaves in summer 2004 and 2005. Vertical bars indicate mean treatment \pm SE ($n = 18$). Significant differences are marked by asterisks ($P < 0.05$)

intercellular CO_2 concentration (C_i) or the linearly related WUE_{int} did not differ between treatments.

Xanthophyll de-epoxidation state (DEPS) under sunlit conditions diverged between treatments in July 2005 (Fig. 8a); the difference persisted in September, despite the full recovery in predawn water potentials in the *D* plots. However, a summer accumulation of xanthophyll was observed, although it was not significant ($P < 0.063$). Biochemical analyses also demonstrated a pattern in the absolute amount of pigments in the two treatments over the season (Fig. 8b). The ratio of total xanthophyll to

chlorophyll content sharply declined over the season in the watered treatment, possibly as a result of leaf maturation, but a relative accumulation of these protective compounds was observed in the *D* treatment. Furthermore, leaf chlorophyll content ($a + b$) diverged significantly ($P < 0.05$) between treatments only in September 2005 (3.0 and 3.7 nmol g^{-1} DM in *D* and *W* plots, respectively).

Growth, flowering and LAI

Differences in water relations and CO_2 assimilation also translated to a depression of total height increment in the study period (Table 1). Moreover, imposed drought was also found to affect the apical growth of vegetative shoots more than for reproductive shoots. On an average, the apical growth of drought trees was about 50% that of watered trees (Table 1). Partial rainfall exclusion also resulted in a substantial increase in the fraction of shoots bearing flowers, a typical response to stress. In fact, the incidence of shoots bearing flowers was about 50% higher in drought trees than in watered trees (Table 1). Stand LAI was also significantly lower in the *D* plots than in the *W* plots (Table 1).

Discussion

The experimental periods i.e., summer 2004 and 2005 were drier compared to long-term mean annual precipitation by -25 and -33% , respectively. Thus, a further reduction of 20% imposed in water-depleted (*D*) plots during the summer had strong effects on physiological and structural activities over the short and medium term, although this result may have been slightly affected by the reduced nutrient content re-routed away from the system with the water (Gundersen et al. 1995). However, this significant effect was obtained despite predawn water potential (Ψ_{pd}) values not falling below -1.2 MPa. Comparable results have been found in similar experiments, in which a more severe water stress was induced by a higher rainfall exclusion (Borghetti et al. 1998; Hanson et al. 2001; Llorens et al. 2004b; Ogaya and Peñuelas 2007).

Significant reductions of stomatal conductance (g_s) and light-saturated CO_2 assimilation (A_{\max}) were observed over the 2 years of the experiment. This result confirmed, as with other Mediterranean species, that the efficient mechanism of stomatal closure allows trees to cope with seasonal water deficits (Aussenac and Valette 1982; Acherar et al. 1991). Evidence of strong stomatal control was the rather low threshold (-0.6 MPa) of Ψ_{pd} for the onset of stomatal closure, in accordance with the previous results on the same species (Castell et al. 1994; Peñuelas et al. 1998). The high sensitivity of g_s indicates that *A. unedo* operates above the

Fig. 8 **a** Xanthophylls de-epoxidation state at midday in *D* and *W* treatments before, during and after the onset of summer drought in 2005 and **b** changes in total xanthophyll content per unit of total chlorophyll (VAZ/Chl) in *D* and *W* treatments. Vertical bars represent mean \pm SE ($n = 30$). Significant differences are marked by an asterisk ($P < 0.05$)

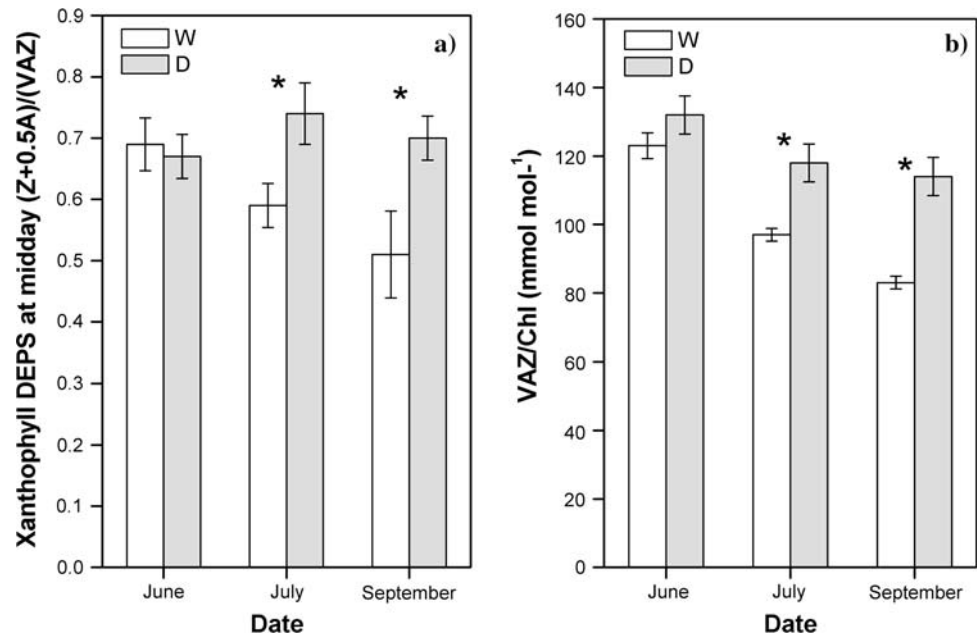


Table 1 Differences in growth of total height between the beginning and end of the experiment, summer growth of apical shoots, incidence of shoots bearing flowers and leaf area index (LAI) between the beginning and end of the experiment in *D* and *W* treatments

| | <i>D</i> | <i>W</i> |
|--------------------------|-------------------|-------------------|
| Total height (m) | 4.63 \pm 0.36 b | 5.27 \pm 0.12 a |
| Shoot summer growth (cm) | 9.8 \pm 0.7 b | 18.5 \pm 0.7 a |
| Incidence of flowers (%) | 50.5 \pm 5 a | 32.4 \pm 4 b |
| Leaf area index | 3.35 \pm 0.15 b | 4.65 \pm 0.18 a |

Vertical bars represent mean \pm SE ($n = 30$, except for LAI $n = 50$)
Mean values not sharing common letters are significantly different ($P < 0.05$)

threshold for xylem embolism under water stress, and that g_s is tightly regulated to prevent reductions in midday leaf water potential (Ψ_{md}) that could induce run-away embolism (Tyree and Sperry 1989). In fact, the lack of a clear relationship emerged between Ψ_{md} and Ψ_{pd} , indicating that *A. unedo* may effectively control the leaf water status over a range of soil water potentials. The mechanism of functional homeostasis is typical for total or partial-isohydric species where minimum Ψ_{md} is kept almost constant irrespective of any changes in soil or air humidity (Tardieu and Simonneau 1998).

The maintenance of differences in g_s and A_{max} amongst treatments, despite the return to full water availability during the winter 2005 indicates that physiological activity is controlled over the medium term as well. This functional acclimation appears to be linked to the parallel down-regulation of tree hydraulic conductance (K_{tree}), as shown in Fig. 3. Experimental evidence shows that acclimation of

K_{tree} in response to drought may be due to the control of transpiration by the adjustment of stomatal aperture or carbon allocation (Cinnirella et al. 2002; Mencuccini 2003; Uemura et al. 2004). Hydraulic adjustments may occur by a decrease in total leaf area or by a change in allometry (i.e., total leaf to sapwood area) (Mencuccini and Grace 1995), as recently confirmed by Martínez-Vilalta et al. (unpublished) on different European populations of *Pinus sylvestris*. Evidence of strict relationships between plant leaf area and whole plant hydraulic conductance has been found in several plant life forms (i.e., coniferous, broad-leaf, shrubs, etc.) (Bunce 1996; Hubbard et al. 2001). Although the mechanisms controlling this feedback were not investigated here, the reduced investment in foliage production in response to drought, which resulted in a substantial decline in LAI in the *D* plots, is consistent with literature findings (Cinnirella et al. 2002). Further, the significant reduction of total height and summer growth of shoots suggests that changes in allometry may have had an effect on hydraulic acclimation. Thus, the down-regulation of K_{tree} may have pushed the root system to explore deeper soil for available water and may have increased the level of stomatal regulation determined by short-term episodes of drought (Ewers et al. 2000). Other experiments support the hypothesis of alteration in ionic composition of the xylem sap in the regulation of K_{tree} in response to hydraulic perturbations that induce physicochemical changes of pit membranes (Gasco et al. 2006). Several studies have experimentally shown that any factor of disturbance that causes a reduction in K_{tree} , induces a parallel decrease in g_s (Sperry and Pockmann 1993; Saliendra et al. 1995; Ripullone et al. 2007). This feedforward response is

necessary to ensure the balance between evaporative demand and hydraulic properties (Sperry et al. 2002). Conversely, the down-regulation of K_{tree} in an adult stand may consequently leave more water available in the soil for younger individuals to exploit during the summers.

The permanent down-regulation of g_s induced a parallel decrease in A_{max} , suggesting that stomata strongly limit carbon assimilation. However, a similar C_i found in this experiment in *D* and watered (*W*) plots, indicates also that photosynthesis may have been down-regulated in the medium term, and that non-stomatal limitations may have played an important role (Lawlor and Cornic 2002). By a study of quantitative limitation analysis carried out in July 2004 on the same experimental site, it was shown that non-stomatal limitations largely affected A_{max} (unpublished data), accounting for 41% of total limitations [9% biochemical (carboxylation and electron transport rate), 26% resistance to CO_2 from intercellular spaces to carboxylation sites and 6% leaf temperature]. Several studies showed evidence that non-stomatal limitations are far from negligible, and often are the most important under moderate water stress (e.g., Loreto et al. 1992; Warren et al. 2004; Grassi and Magnani 2005). The limited recovery of mesophyll conductance after the return to full water availability may have contributed to incomplete recovery of photosynthesis (Galmés et al. 2007). On the other hand, the activation of photoprotective mechanisms in *A. unedo*, as shown by the analysis of leaf pigments (i.e., xanthophyll and chlorophyll), should exclude permanent damage to photosystem II. The summer accumulation of xanthophyll pigments may have played an important role in the dissipation of the excess of excitation energy to avoid damage to photochemical efficiency (Jefferies 1994; Demmig-Adams et al. 1995). In fact, while the ratio of total xanthophyll to chlorophyll content sharply declined over the season in *W* plots, possibly as a result of leaf maturation, a relative accumulation of these protective compounds was observed in *D* plots. Furthermore, the limited chlorophyll bleaching observed in *D* plots only at the end of the summer was probably the result of the accumulation of the effects of excess light over the summer. This should be viewed more as a photo-protective mechanism than as damage. In fact, photo-inhibition is consistent with a decrease in chlorophyll fluorescence (Zunzunegui et al. 1999).

The increase of non-stomatal limitation observed, even at earlier stages of leaf dehydration and maintained through the seasons irrespective of full recovery of well-watered conditions, may explain the linear relationship between g_s and A_{max} leading to slight or no statistical differences in WUE_{int} . In agreement with these results, we did not find significant effects on integrated WUE (deduced from $\delta^{13}\text{C}$ values), except for June 2005 when the difference was significant. Several works have been published showing the

robustness of this relationship (Farquhar et al. 1989; Ripullone et al. 2004). Similarly, Llorens et al. (2004a) did not find significant effect of drought treatment on WUE in some species of Mediterranean macchia, as a consequence of proportional changes in A_{max} and g_s . In contrast, many other studies showed a robust increase in WUE even under mild or moderate water deficit (Ogaya and Peñuelas 2003; Lloret et al. 2004). This increase resulted from the non-linear relationship between CO_2 assimilation and g_s , with water loss restricted more intensely than the inhibition of photosynthesis. The increase in WUE is often interpreted as an adaptation to drought-prone environments that facilitates the maintenance of a positive carbon balance under dry conditions (Cowan and Farquhar 1977; Raven 2002). In contrast, decline of WUE was shown in *Q. ilex* and *P. latifolia* (Serrano and Peñuelas 2005) as a result of increased temperature during drought periods and the subsequent increase in photorespiration (Reichstein et al. 2002). These results confirm that there is no unique relationship between A_{max} and g_s , and that the response to drought is not only dependent upon species, but also on environmental parameters whose influence operates at the cell to the whole plant level.

Water-use efficiency also showed large fluctuations over the season, but this appeared to be mainly related to changes in VPD. VPD is considered an important parameter driving gas exchange in Mediterranean environments (Martinez-Vilalta et al. 2003), which in addition to soil water deficit, influences WUE (Law et al. 2001).

Final remarks and conclusions

It has been stated, that the marked reduction in precipitation occurring during the summer, as a consequence of climate change, should enhance the risk of vulnerability and forest die-back. Our results suggested an onset of physiological acclimation in response to drought induced by our partial rain exclusion treatment, as the observed reduction in stomatal conductance and CO_2 assimilation persisted even after the return to full water availability during the fall and winter. Further, the permanent down-regulation of stomatal conductance and photosynthesis resulted in a significant reduction of shoot growth and leaf area in the medium term. The parallel decrease in hydraulic conductance maintained even during winter/spring may mainly have been involved in this acclimation response. At the same time, the acclimation response prevented the onset of any run-away damage and reduced the tree vulnerability, as confirmed also by the accumulation of photoprotective pigments.

On the other hand, the imposed drought induced a parallel increase in stomatal and non-stomatal limitations.

This behaviour may result in a negative effect on WUE, which would not significantly increase in case of enhanced drought.

Finally, although long-term experiments are needed (up to 5–6 years) to confirm our findings, this study revealed the importance of rainfall manipulation experiments to predict the real behaviour of adult trees in a natural environment under a likely scenario of climate change. Further, leaf nutrients are another important factor that should also be considered in long-term rainfall manipulation experiments, because of re-routing of minerals nutrients away from the system with the water thereby influencing the physiology.

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