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Changes in foliar carbon isotope composition and seasonal stomatal conductance reveal adaptive traits in Mediterranean coppices affected by drought

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Abstract: We estimated water-use efficiency and potential photosynthetic assimilation of Holm oak (*Quercus ilex* L.) on slopes of NW and SW aspects in a replicated field test examining the effects of intensifying drought in two Mediterranean coppice forests. We used standard techniques for quantifying gas exchange and carbon isotopes in leaves and analyzed total chlorophyll, carotenoids and nitrogen in leaves collected from Mediterranean forests managed under the coppice system. We postulated that responses to drought of coppiced trees would lead to differential responses in physiological traits and that these traits could be used by foresters to adapt to predicted warming and drying in the Mediterranean area. We observed physiological responses of the coppiced trees that suggested acclimation in photosynthetic potential and water-use efficiency: (1) a significant reduction in stomatal conductance ($p<0.01$) was recorded as the drought increased at the SW site; (2) foliar $\delta^{13}C$ increased as drought increased at the SW site ($p<0.01$); (3) variations in levels of carotenoids and foliar nitrogen, and differences in foliar morphology were recorded, and were tentatively attributed to variation in photosynthetic assimilation between sites. These findings increase knowledge of the capacity for acclimation of managed forests in the Mediterranean region of Europe.

Keywords: Mediterranean forest ecosystems, forest acclimation, stable isotopes, leaf gas exchanges, water-use efficiency, foliar traits, adaptive silviculture.

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Introduction

Most Mediterranean forests are broadleaved forests managed for centuries under the coppice system. Historically, coppice forests have been subjected to intensive management on short rotations for firewood and charcoal. Most of these forests have been abandoned over the last 60 years because of the depopulation of rural areas, the change in socio-economical conditions, and the replacement of firewood by fossil fuels. These ecosystems survived intensive management because of their ability to adapt to different light regimes and drought conditions by maximizing their photosynthetic potential and water-use efficiency (WUE). The main role currently attributed to the unmanaged coppice forests is the restoration of more functional cover in terms of biodiversity, societal and ecosystem services, landscape quality and climate change mitigation.

Recent global warming caused a significant upward elevational shift of growth potential of forest ecosystems. This trend is predicted to continue in future but many forest species have limited capacity to migrate to higher elevations due to their sensitivity to late frosts (Lindner et al. 2010; Maxime and Hendrik 2011). Trees in particular are susceptible to climate change due to their longevity that extends from decades to centuries. Mediterranean evergreen species such as *Quercus ilex* are characterized by high

leaf plasticity that may explain their wide distribution under varying ecological environments. This attribute might be advantageous in response to altered environmental conditions such as global climate change (Gratani et al. 2006). Climate projections indicate drying trends due to decreasing precipitation and increasing evaporation on most continents, and these are supported by current evidence in the Mediterranean region (Christensen et al. 2007).

The coppice system is still widespread in European Mediterranean countries and covers about 8.5 million hectares (Morandini 1998). A typical "coppice with standards" forest consists mainly of shoots in the growth form of "simple coppice" with fewer scattered mature standard trees. Standard trees are the long-lived trees originating mainly from seed and untouched by coppicing or thinning for a few cycles. Shoots are the younger trees regenerating from root stocks resprouting after each coppicing. Although of different ages, standard trees and shoots live together in the same stand.

Coppices are adapted to a wide range of environments and soil water conditions because of their deep rooting and stomatal control of gas exchange. Coppices tolerate negative water potentials by osmotic adjustment and other morphological and physiological adaptations (Damesin et al. 1998; Tognetti et al. 2007). Coppices are also characterized by trees of varying ages that can capture different water sources, thereby avoiding water limitation (Valentini et al. 1992). The repeated cuts (coppicing) create an 'artificial disturbance regime' similar to the periodical natural opening of large canopy gaps. Since the abandonment of coppicing on a portion of the managed forest area over the last decades, new forest structures have been created through the conversion of former coppice into high-forest physiognomies from which one shoot grows from each stump. Resulting structures are similar to single-story young stands and scattered standard trees.

The carbon isotope ratio in organic material is a useful tool for identifying medium- and long-term effects of environmental factors on gas exchange in plants. Changes in stomatal conductance (gs) and/or photosynthetic capacity (A) are reflected in changes in the pi/pa ratio (intercellular and atmospheric partial pressures of CO_2), affecting the carbon isotope composition of the plant ($\delta^{13}C$) (Farquhar et al. 1982 and references therein). Changes in foliar $\delta^{13}C$ along environmental gradients have been reported for a wide range of forest ecosystems (Körner et al. 1991; Hultine and Marshall 2000; Sah and Brume 2003; De Lillis et al. 2004; Shi et al. 2006, Klein et al. 2013). Correlations have also been reported along elevational gradients between $\delta^{13}C$ and abiotic factors such as soil moisture (Beerling et al. 1996; Sun et al. 1996), air temperature (Panek and Waring 1995), atmospheric CO_2 concentration (Gale 1972; Ehleringer and Cerling 1995) and barometric pressure (Marshall and Zhang 1994). The variation in $\delta^{13}C$ along environmental gradients has been correlated to morphometric and physiological features that vary with drought and elevation, such as leaf thickness (Vitousek et al. 1990; Cordell et al. 1998; Kogami et al. 2001), foliar nitrogen content (Evans 1989; Friend et al. 1989; Morecroft and Woodward 1996; Cordell et al. 1999; Kogami et al. 2001; Hikosaka et al. 2002; Shi et

al. 2006) and stomatal density (Körner et al. 1988; Hultine and Marshall 2000; Paridari et al. 2013).

We conducted a replicated field trial in two Mediterranean coppice forests of southern Italy to: (1) characterize the interacting effects of aspect and intensifying drought on foliar $\delta^{13}C$; (2) quantify temporal variation in gs. Our hypothesis was that intensifying drought leads to physiological responses of coppiced trees at both sites. We tested six parameters to represent tree physiology (carbon isotope composition, stomatal conductance) and tree photosynthetic potential (total chlorophyll, carotenoids, nitrogen and leaf morphology). Observations were made over three years (i.e., 2004, 2005 and 2006) with focus on June-September, when plant physiological activities ranged between summer minima and summer maxima.

Materials and Methods

Study site, climate and experimental design

The study was conducted in a mature 9,500 ha Holm oak (*Quercus ilex* L.), *Arbutus unedo* L. and *Phillyrea latifolia* L. coppice forest at Pula in the southern Sulcis region of Sardinia, Italy (39° 03' N, 8° 50' E) between September 2004 and September 2006. *Q. ilex* is a deep-rooted tree widely distributed in the sub-humid areas of the Mediterranean basin at 0–1400 m elevation. *A. unedo* is an evergreen laurophyllous shrub or tree, occurring mainly on siliceous soils. Both *Q. ilex* and *A. unedo* are species of the Mediterranean sclerophyllous vegetation characterized by deep root systems for summer drought survival (Cubera and Moreno 2007; David et al. 2007). Our two experimental sites were located on opposing sides of the same valley. Mean annual air temperature was 15.2 °C, and mean temperature of the warmest months was 21.0 °C. Mean annual precipitation was 976 mm, mostly occurring in winter and spring. Due to the evidence that during the study period the meteorological conditions were drier than their long-term average (Table 1), we assumed an intensifying of drought conditions at both sites.

Soil profiles were characterized as *Eutric Cambisol* or Mediterranean brown earths of xerophytic and mesophytic forests, whereas volcanic porphyritic rocks constituted the main geological substrate (Palaeozoic) (Amorini et al. 1996).

At one site, a set of experimental plots faced northwest (NW) and at the other site plots faced southwest (SW). Rainfall varied across the valley with higher total precipitation at the NW site (1,179 mm) than at the SW site (773 mm). Relative humidity over the drought season (May to August) was higher at the NW site (72.3%) than at the SW site (42.5%) (Fabbio et al. 1996; ARPA Sardinia). Dry summer periods are on average very long, from May to September, and the lowest mean monthly rainfall is recorded in July. The SW site was affected by one additional month of dry summer than the NW site.

Both coppice forests were historically intensively coppiced for firewood and charcoal production and then progressively abandoned over the last 60-70 years. Hence *Q. ilex* occurred both as a standard tree (hereafter "uncoppiced tree") and as a shoot (here-

after "coppiced trees"). *A. unedo* occurred only as a coppiced tree. In 2005, trees at both sites had reached average ages of 30-40 yrs for coppiced trees and 70 or more for uncoppiced trees. During the pre-sampling 2004 inventory, trees had mean diameter at breast height (DBH) of 7.1 cm and density of about 7,560 stems ha^{-1} at the NW site, compared to mean DBH of 5.5 cm and density of 8,286 stems·ha^{-1} at the SW site. Total basal area (BA) of trees varied between sites from 44.1 m^2·ha^{-1} at the NW site to about 30 m^2·ha^{-1} at the SW site (Table 2).

Table 1: Mean monthly precipitation (P) and mean monthly air temperature (T) recorded during the study period (i.e., 2004, 2005 and 2006) at both sites compared with their long-term averages. Precipitation and mean air temperature were either recorded at two climate stations located approximately few km distance from the experimental sites or collected directly from regional meteorological services (i.e., ARPA Sardinia website: http://www.sar.sardegna.it/pubblicazioni/riepiloghimensili/mensili.asp).

Month	P (mm)	T (°C)	P (mm)	T (°C)
	NW site		SW site	
January	143.9	7.1	70.2	6.5
February	83.3	4.2	53.3	7.4
March	85.9	10.2	46.7	9.6
April	187.4	11.6	100.2	13.1
May	54.7	17.9	26.7	16.3
June	7.6	24.2	7.7	21.5
July	15.3	27.3	1.7	25.0
August	16.4	28.2	7.4	23.8
September	44.9	19.4	63.4	21.8
October	51.7	16.4	56.7	19.3
November	126.2	12.3	93.4	14.2
December	210.2	7.6	113.4	11.3
2004-2006 average	**1027.6**	**15.5**	**640.8**	**15.8**
long-term average	**1179.0**	**15.0**	**773.0**	**15.4**

Table 2. Forest mensurational parameters for the experimental sites. DBH = mean diameter at breast height; BA= basal area.

Site	Density (stems·ha^{-1})	DBH (cm)	BA (m^2·ha^{-1})
NW	7560	7.1	44.1
SW	8286	5.5	30.0

Sampling procedures

A 1,000 m^2 plot was established at each experimental site and 15 trees were sampled randomly on the basis of three growth forms: *Q. ilex* uncoppiced trees (mean tree height: 12.2 m), *Q. ilex* coppiced trees (mean tree height: 8.6 m) and *A. unedo* coppiced trees (mean tree height: 5.3 m). Altogether, 45 trees were sampled at each site (15 trees of each of 3 growth forms).

Fully expanded sun-exposed leaves from the middle-upper part of the crown were selected to measure seasonal *gs*, foliar δ^{13}C, total chlorophyll, carotenoids, foliar nitrogen and foliar morphology.

Total chlorophyll and carotenoids analyses

In order to quantify total chlorophyll (a + b) and carotenoid concentrations on a leaf-area basis, fifteen sunlit leaves per growth form were sampled at each site (*n* = 15 leaves × 15 trees × 3 growth forms). Immediately after collection, samples were sealed in cryovials and stored in a dark environment under liquid nitrogen and transported to the lab. Total chlorophyll and carotenoid extraction was performed using 95% ethanol. Absorbance measurements were performed with a spectrophotometer (Lambda 3B, Perkin Elmer, Norwalk, USA) at wavelengths of 664.2 nm and 648.6 nm. Total chlorophyll and carotenoid contents were calculated following Lichtenthaler (1987).

Foliar carbon isotope composition analyses (δ^{13}C)

We ground the foliar samples using a Retsch mill (Mixer Mills, MM 200, Haan, Germany). The resulting powder was placed in tin capsules and then weighed (approximately 1.5 mg) using a microbalance (Mettler AT 21 Comparator, Mettler Toledo AG, Greifensee, Switzerland). Foliar carbon isotope ratios were determined by combustion of the samples in an Elemental Analyzer (Carlo Erba, model 1108EA, Milan, Italy) coupled to an isotopic ratio mass spectrometer (Finnigan MAT, model S delta, Bremen, Germany). Analysis precision was greater than ± 0.11‰. The results were expressed as delta (δ^{13}C) in ‰ relative to the Pee Dee Belemnite (PDB) standard where: [δ^{13}C= (R$_{sample}$/R$_{standard}$ −1) × 1000], and R$_{sample}$ and R$_{standard}$ represent the ^{13}C/^{12}C molar ratios of the sample and the standard, respectively.

Stomatal conductance measurements

We measured stomatal conductance at both sites during spring and summer (between June and September). Measurements were made at three times during the day (08:00, 12:00 and 16:00 hrs) on sunlit leaves formed in the previous growing season along the upper-middle portion of the crown using a steady-state Porometer LI-COR, LI-1600 (Li-Cor Inc, Lincoln, NE, USA). Stomatal conductance measurements were carried out under constant PAR values between 1,000 and 1,200 μE·m^{-2}·s^{-1} and under leaf temperatures in the range of 28–30°C.

Foliar nitrogen analyses

The same foliar powder obtained for carbon isotope analyses was used for nitrogen elemental analyses. Powder was placed in tin capsules and then weighed (approximately 2.5 mg) using a microbalance (Mettler AT 21 Comparator, Mettler Toledo AG, Greifensee, Switzerland). Foliar nitrogen concentration was determined by combustion of the samples in an Elemental Analyzer (Carlo Erba, model 1108EA, Milan, Italy). Data were processed using Carlo Erba software and reported as the dry weight percentage for each sample.

Foliar morphological analyses

The measured leaf morphology variables were: leaf area, leaf length and specific leaf area (SLA) (the ratio of surface area to dry weight). Leaf area was measured using a Leaf Area Meter (LI-3100, Li-Cor Inc, Lincoln, NE, USA). In order to determine

the foliar dry weight, samples were dried at 70 °C to constant dry weight.

Statistics

All measured and calculated variables were compared between sites, species and growth forms, and analysed by analysis of variance (ANOVA) using PRISM software version 4.01 (Graph-Pad Software, Inc. San Diego, CA, USA). Measurements of individual trees were used as observations, whereas site, species, growth forms and their interactions were defined as factors. Because stomatal conductance was measured in four months (i.e., June, July, August and September) by species, growth form and site, a four-way ANOVA was performed with time as the fourth factor. Means were compared using Tukey's HSD test, reported as letters indicating significant differences in Fig. 1.

Results

Total chlorophyll, carotenoids and nitrogen

Total chlorophyll and carotenoid contents differed significantly by growth form ($p < 0.01$) and species ($p < 0.05$), and carotenoids differed significantly between sites ($p < 0.01$). We found significant interactions Si × Gf in carotenoids ($p < 0.05$) and Si × Sp for both total chlorophyll and carotenoids ($p < 0.05$) (Table 3). Significant total chlorophyll differences ($p < 0.01$) were found between uncoppiced (87.5 µg cm⁻²) and coppiced trees (82.8 and 84.1 µg cm⁻², for *Q. ilex* and *A. unedo*, respectively). Mean foliar nitrogen values were similar for uncoppiced (1.1%) and coppiced trees (1.0%).

Table 3: P values (probability > F, an effect is significant if P <0.05, in bold) from ANOVA for the effects of site, species, growth forms, time and their interactions on main response parameter.

Variable	Chlorophyll (mg·cm⁻²)	Carotenoids (mg·cm⁻²)	Foliar N (%)	SLA (cm²·g⁻¹)	FL (cm²)	Foliar d¹³C (‰)	gs (mmol·m⁻²·s⁻¹)
Site	n.s.	<0.01	n.s.	<0.01	<0.05	<0.05	<0.001
Species	<0.05	<0.05	n.s.	<0.001	<0.001	<0.01	n.s.
Si x Sp	<0.05	<0.05	n.s.	<0.01	<0.05	<0.05	n.s.
Growth forms	<0.01	<0.05	n.s.	<0.01	<0.01	n.s.	n.s.
Gf x Si	n.s.	<0.05	n.s.	<0.01	<0.05	n.s.	n.s.
Time							<0.05

Specific Leaf Area and Leaf Length

Specific leaf area (SLA) and leaf length (FL) differed between sites ($p < 0.01$ and $p < 0.05$, respectively). FL and SLA were significantly lower in uncoppiced than in coppiced trees ($p < 0.01$). Both FL and SLA showed significant Si × Gf and Si × Sp interactions, indicating high variability between sites (Table 3).

Foliar carbon isotope composition

Foliar carbon isotope ratios were higher at the SW site ($p < 0.05$) (Table 3). δ¹³C values differed by species with higher mean values recorded for coppiced trees of *A. unedo* (-25.1‰) compared to *Q. ilex* (-26.3‰) ($p < 0.01$).

Stomatal conductance

Stomatal conductance was higher in June and September than in July and August (Figures 1a and 1b) at both sites. July and August were characterized by lower ($p < 0.05$) *gs* values for all three growth forms at both sites. Stomatal conductance values were markedly lower at the SW site during summer when they were consistently <100 mmol·m⁻²·s⁻¹. Conversely, summer was characterized by higher *gs* values at the NW site, always >100 mmol·m⁻²·s⁻¹ with a maximum value of 164.9 mmol·m⁻²·s⁻¹ recorded in July on coppiced *Q. ilex* trees.
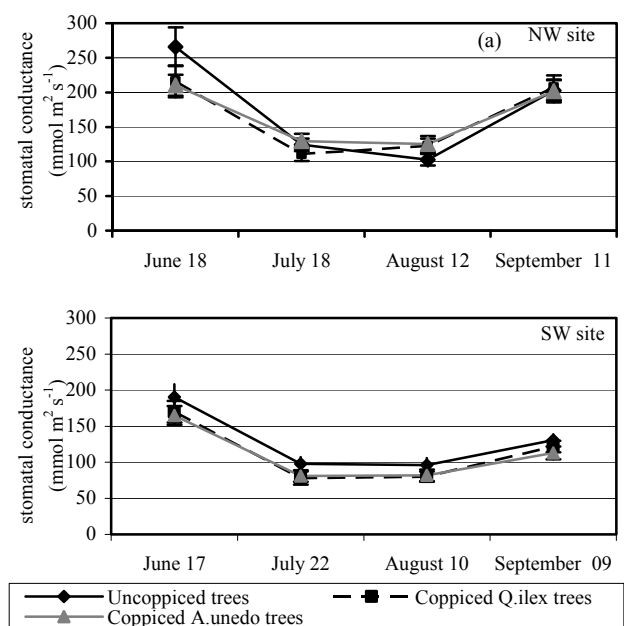
Fig. 1: Spring and summer pattern of stomatal conductance (June to September) for the experimental sites. The reported values refer to the average daily figures, the same trees having been measured at 8.00 a.m., again at noon and again at 4.00 p.m. Bars indicate the standard error of the mean. Different letters denote significant differences between growth forms (sample size = 45, $p < 0.05$, Tukey's HSD test).

Discussion

Physiological differences between sites

The hypothesis that intensifying drought caused physiological changes to Mediterranean coppice species and growth forms was supported by the data. Changes in the six selected parameters between sites and growth forms indicated two lines of adjustment (Fig. 2): (1) a trait indicated acclimation by difference in values between sites and growth forms, e.g., large differences by site in carotenoids, foliar $\delta^{13}C$, gs and SLA, and small differences by growth form in total chlorophyll, foliar nitrogen (coppiced trees), and foliar $\delta^{13}C$ (both uncoppiced and coppiced trees); (2) a trait indicated marked acclimation by differences in value between sites and between growth forms, e.g., gs, foliar $\delta^{13}C$, SLA and leaf length.
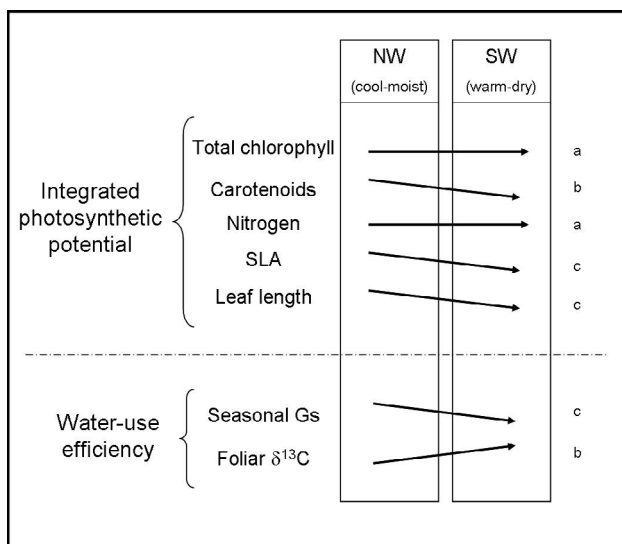

Fig. 2: Physiological parameters describing the photosynthetic potential and water-use efficiency of growth forms for the experimental sites. The direction of the arrows indicates the increase or decrease of the parameters measured between NW and SW aspect. a) Similar trait: refers to small changes between sites; b) Acclimation trait: refers to the larger site differences and small growth forms differences; c) Marked acclimation trait: refers to the differences between sites and among growth forms.

Growth-form traits showed acclimation by differences in total chlorophyll, carotenoids and foliar morphology. This was probably because (1) they had different potential photosynthetic responses at both sites, and (2) the coppiced trees were subject to greater shading than the uncoppiced trees.

Garzia-Plazaola et al. (1997) and Faria et al. (1998) reported that carotenoid concentration provide an index for dissipation of excessive thermal energy in response to drought, e.g., when carbon assimilation is limited by reduced stomatal conductance the observed variability in carotenoid would indicate a physiological adjustment, suggesting that photo-protection processes were the primary physiological responses to differences between sites and

drought intensity or duration. Faria et al. (1998) and Oliveira & Penuelas (2000) reported significantly lower total chlorophyll in Mediterranean forest stands populated mainly by *A. unedo*, *Q. ilex* and *Q. suber* than we recorded here. In Mediterranean forest ecosystems, lower chlorophyll contents could be a strategy to avoid capture of excessive light at colder sites and at those with greater sunlight intensity (Núñez-Olivera et al. 1994) or could be an adaptive response against photo-inhibition as chlorophyll reduces the light harvesting capacity of the leaf (Kyparissis et al. 1995).

Foliar $\delta^{13}C$ and stomatal conductance responded to drought at the site- and species-level

Foliar $\delta^{13}C$ varied by site and species. Foliar $\delta^{13}C$ was greater at the drier SW site and lower in *Q. ilex* than in *A. unedo*. At the site-level, this result was due to the extra month of summer drought that affected the SW site. Air temperature and rainfall data showed an intensifying drought at both sites with respect to long-term averages, an extended drought period in spring and summer with less rainfall at the SW site (May to September) than at the NW site (June to September). This was supported by comparison of the three years mean spring rainfall (April to June) during the study period at both sites: the SW site recorded 134.6 mm, 46.1% less than the 249.7 mm at the NW site. Foliar $\delta^{13}C$ values at the NW site were lower in uncoppiced than in coppiced trees. At the SW site, all growth forms showed higher net $\delta^{13}C$ values than at the NW site (i.e., +2.45‰ for uncoppiced trees, +0.58 and +1.20‰ for coppiced trees). Since $\delta^{13}C$ is directly related to intrinsic water-use efficiency (iWUE= A/gs), $\delta^{13}C$ variations can be the result of changes in gs or A. For instance, an increase in $\delta^{13}C$, interpreted as a reduction in pi (intercellular partial pressures of CO_2 inside the leaf) in the Farquhar model (1982), can be the result of either (1) decreased gs (at constant A); or (2) increased A (at constant gs). In this study A was not measured directly, therefore we did not calculate iWUE, but seasonal gs measurements were carried out under constant PAR values (i.e., 1,000 and 1,200 $\mu E \cdot m^{-2} \cdot s^{-1}$), hence we can assume that the lower gs values at the SW site explained the foliar $\delta^{13}C$ increases (greater decrease in gs over A). At the same time, the higher gs values at the NW site explained the lower foliar $\delta^{13}C$ values. With regard to the slight differences in foliar $\delta^{13}C$ by growth form, trees naturally grow more slowly with increasing age, thus decreasing their carbon gain (Piper and Fajardo 2011). We assumed that the photosynthetic rate decreased with increasing age, therefore the lower A values probably explained the lower foliar $\delta^{13}C$ values in the uncoppiced trees at the NW site. However all growth forms at the SW site had higher foliar $\delta^{13}C$ values, rendering unlikely the hypothesis that an increase in $\delta^{13}C$ was due to increasing A.

Conclusions

Our comparatively dry SW site had higher foliar $\delta^{13}C$, lower stomatal conductance, lower carotenoid content, and similar

foliar nitrogen concentration and total chlorophyll. Similar results were observed by Van de Water et al. (2002) comparing foliar $\delta^{13}C$ values at dry and wet sites. The lower rainfall at the SW site was associated with lower gs and higher foliar $\delta^{13}C$ values, supporting the evidence that spring rainfall can markedly influence water-use efficiency in Mediterranean environments. Within the general frame of references, the paper's findings provide further knowledge of acclimation capacity of Mediterranean forest ecosystems.

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