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Original article

Mesophyll conductance to CO_2 and leaf morphological characteristics under drought stress during *Quercus ilex* L. resprouting

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Abstract

- *Quercus ilex* L., the dominant species in Mediterranean forests and one with a great capacity for resprouting after disturbances, is threatened by the expected increase in fire frequency and drought associated with climate change.
- The aim of this study was to determine the contribution of photosynthesis limitants, especially mesophyll conductance (g_{mes}) during this species' resprouting and under summer drought.
- Resprouts showed 5.3-fold increased g_{mes} and 3.8-fold increased stomatal conductance (g_s) at midday with respect to leaves of undisturbed individuals. With increased drought, structural changes (decreased density and increased thickness) in resprouts contributed to the observed higher photosynthesis and increased g_{mes}. However, g_{mes} only partially depended on leaf structure, and was also under physiological control. Resprouts also showed lower non-stomatal limitations (around 50% higher carboxylation velocity ($V_{c,max}$) and capacity for ribulose-1,5-bisphosphate regeneration (J_{max})). A significant contribution of g_{mes} to leaf carbon isotope discrimination values was observed.
- g_{mes} exhibits a dominant role in photosynthesis limitation in *Q. ilex* and is regulated by factors other than morphology. During resprouting after disturbances, greater capacity to withstand drought, as evidenced by higher g_{mes}, g_s and lower non-stomatal limitants, enables increased photosynthesis and rapid growth.

Mots-clés :
composition isotopique du carbone /
structure des feuilles /
conductance mésophyllienne /
limitation de la photosynthèse /
Quercus ilex

Résumé – Conductance mesophyllienne pour le CO_2 et caractéristiques morphologiques des feuilles sous stress hydrique pendant la repousse de *Quercus ilex* L.

- *Quercus ilex* L., l'espèce dominante dans les forêts méditerranéennes qui a une grande capacité de rejets après des perturbations, est menacée par l'augmentation prévue de la fréquence des incendies et de la sécheresse associées au changement climatique.
- Le but de cette étude était de déterminer, chez cette espèce, la contribution des limitations de la photosynthèse, en particulier de la conductance du mésophylle (g_{mes}) au cours de la repousse et sous sécheresse estivale.
- Les feuilles des rejets ont présenté une conductance mésophylienne (g_{mes}) 5,3 fois plus élevée et une conductance stomatique (g_s) à midi 3,8 fois plus élevée par rapport aux feuilles d'arbres non perturbés. Avec l'accroissement de la sécheresse, les changements de structures (diminution de la densité et épaisseur accrue) dans les rejets ont contribué à augmenter la photosynthèse et à accroître g_{mes}. Toutefois, g_{mes} dépendait partiellement de la structure des feuilles, et était également sous contrôle physiologique. Les rejets ont aussi montré une abscence de limitation stomatique (vitesse de carboxylation ($V_{c,max}$) environ 50% plus élevée et une capacité de régénération pour le ribulose-1,5-bisphosphate (J_{max}). Une contribution significative de g_{mes} à la discrimination isotopique du carbone dans les feuille a été observée.
- La conductance mésophylienne (g_{mes}) a présenté un rôle dominant dans la limitation de la photosynthèse chez *Q. ilex* et est régulée par des facteurs autres que la morphologie. Au cours de la repousse après des perturbations, une plus grande capacité à résister à la sécheresse, mise en évidence par une g_{mes} et une g_s plus élevées, et une diminution des limitations non stomatiques, permettent une augmentation de la photosynthèse et une croissance rapide.

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1. INTRODUCTION

Holm-oak (*Quercus ilex* L.) is a deep-rooted, evergreen dominant species in Mediterranean forests which has a great capacity for resprouting after fire, clear-cut, grazing or other disturbances. Resprouts after any of these events show decreased shoot/root ratios, which makes more water and nutrients available to the shoot than in the original plants and favours photosynthesis stimulation and rapid growth (Fleck et al., 1998). *Q. ilex* is exposed to multiple environmental stress factors such as drought, heat shock, chilling, nutrient deprivation and high light stress amongst others. Increased probability of drought, heat and rising atmospheric CO_2 concentration during the coming decades may be particularly important in the Mediterranean basin (Christensen et al., 2007). Moreover, the expected increased risk of uncontrolled fire episodes could lead to the exhaustion of several species, generating a decline in their resprouting capacity and recovery.

There has been a long-standing controversy as to whether drought limits photosynthesis by stomatal closure, metabolic impairment or through diffusive resistances (Lawlor and Tezara, 2009). Of these resistances, CO_2 transfer conductance inside the leaf or mesophyll conductance (g_{mes}) is considered relevant to photosynthesis (Flexas et al., 2008). Metabolic photosynthesis limitations (e.g. injuries to photosynthetic biochemistry and photochemistry) during drought may only be apparent: drought produces low g_{s}, closely related to g_{mes}, resulting in a decreased availability of CO_2 in the chloroplast, which down-regulates the biochemical machinery of photosynthesis. g_{mes} can be affected by leaf morphology (Terashima et al., 2001); in fact, previous results of our group (Peña-Rojas et al., 2005) related changes in g_{mes} in nursery-grown holm-oak plants submitted to water stress to variations in leaf anatomy and gas-exchange parameters.

Carbon isotope discrimination ($\Delta^{13}C$) is largely due to Rubisco (which discriminates against ^{13}C during RuBP carboxylation), with the amount of discrimination depending on the ratio of CO_2 partial pressure at the carboxylation site (C_C) to CO_2 partial pressure in the surrounding air (C_a), which is affected by g_{s} and g_{mes} (Farquhar et al., 1989). As described above, morphological characteristics can affect internal resistances; thus, leaf thickness and leaf density as components of the leaf mass per area parameter (LMA) (Niinemets, 1999), can be an important source of variation in $\Delta^{13}C$.

The aim of this study was to characterize the photosynthetic limitants during holm oak regrowth after a clear-cut, and especially the contribution of mesophyll conductance (g_{mes}) under drought conditions. Two kinds of resprout were used for this study, which differed in their cutting season: winter, when plants had a high availability of stored underground reserves, and summer, when part of the stored reserves had already been remobilized and used to support early growth. Other aims were to relate the morphological characteristics of resprouts to the observed g_{mes} and to examine the effect of g_{mes} on carbon isotope discrimination ($\Delta^{13}C$) values. The characterization of the photosynthetic and growth limitations during *Q. ilex* resprouting after disturbances would help us to establish the adaptation capacity of this plant in the context of global change and biodiversity conservation in Mediterranean forests.

2. MATERIALS AND METHODS

2.1. Experimental site and plant material

The study was carried out at Can Coll, Serra de Collserola forest, Barcelona, Spain; 41° 28′ 28″ N, 2° 7′ 32″ E. A plot (400 × 280 m) at altitude of 140 m and oriented N-NE was selected. The climate is Mediterranean, with cold winters, cool and wet springs and autumns, and hot dry summers (Tab. I). The 35-year old forest is dominated by *Quercus ilex* and *Pinus halepensis*. In February, 25 *Quercus ilex* plants were selected (5.9 ± 0.3 cm mean diameter at breast height (DBH), 4.7 ± 0.2 m mean height, 1.4 ± 0.2 kg mean leaf biomass) and the shoots of 10 randomly selected plants were completely excised 15 cm above soil level. Resprouts (R) after this date were designated as RW (winter resprouts). In August, 10 more plants were completely excised and resprouts after this were designated as RS (summer resprouts). Five plants were kept undisturbed, as controls (C) of the clear-cut site. Leaf gas exchange and chlorophyll fluorescence were measured in fully expanded leaves of the same age: in the first winter (W; February–March), only controls and RW leaves were analysed, as RS had resprouted badly in the autumn; in the subsequent summer (S; July–August), all treatments could be analysed. Samples were collected for ^{13}C composition ($\delta^{13}C$), leaf mass per area (LMA), leaf density (D) and leaf thickness (T) determinations.

2.2. Leaf gas exchange

A portable gas exchange system LI-6200 (Li-Cor Inc., Lincoln, NE, USA) was used for punctual measurements at midday on nine attached, fully expanded, current-year leaves per treatment, season and leaf orientation. Leaf cuvette conditions differed according to the season (Tab. I). Results were expressed per leaf-projected area (LA), obtained with an Epson GT5000 scanner and processed using image analyser software. In each season, ten CO_2 response curves of CO_2 assimilation *vs*. intercellular CO_2 concentration (A/C_i) were obtained per treatment on attached leaves with a LI-6400 instrument (Li-COR, Lincoln, Nebraska, USA). Leaf cuvette conditions were established according to the season and time of the day to reproduce a typical day in every season.

For A/C_i curves, PPFD was established as 600 $\mu mol\ m^{-2}\ s^{-1}$, which is saturating under these conditions (Peña-Rojas et al., 2004); a range of ambient CO_2 concentration (C_a) from 50 to 800 $\mu mol\ mol^{-1}$ was covered. Analyses of the curves permitted the determination of: $A_{\max}$, net photosynthesis at saturating C_i and PPFD; $V_{c,\max}$, maximum carboxylation velocity of Rubisco; $J_{\max}$, maximum electron transport contributing to RuBP regeneration; l_s, stomatal limitation to A ($l_s(\%) = 100 \times (1 - (A/A_{\mathrm{sat}}))$; A_{sat}, net photosynthesis at saturating light and $C_i = 350\ \mu mol\ mol^{-1}$.

To assess the effect of heterogeneous stomatal conductance across the leaf surface, steady-state chlorophyll fluorescence was measured in six spots of 27 leaves of the same plants used in the experiment. Water potential (Ψ) of the same leaves was also obtained with a Scholander-type pressure pump (Soil Moisture 3005, Soilmoisture Equipment Corp., Goleta, CA, USA). The coefficient of variation of Φ_{PSII} (see below) was not statistically higher than system repetitiveness (around 9%), indicating the absence of patchiness, and did not correlate with Ψ.

Table I. Climatological data recorded at the forest site during the gas-exchange measurements of the different treatments (control, C; winter resprouts, RW; summer resprouts, RS); data are the mean ±SE of all measurements.

	Leaf Orientation	February–March		July–August		
		C	RW	C	RW	RS
PPFD (μmol m^{-2} s^{-1})	North	416 ± 150	508 ± 117	1393 ± 126	1559 ± 109	1708 ± 57
	South	1134 ± 98	1047 ± 171	1463 ± 136	1239 ± 159	1669 ± 34
VPD (KPa)	North	2.2 ± 0.1	1.9 ± 0.1	4.5 ± 0.2	4.2 ± 0.2	4.0 ± 0.3
	South	2.2 ± 0.2	2.0 ± 0.1	4.5 ± 0.3	3.7 ± 0.3	3.8 ± 0.2
Air temperature (°C)	North	22.2 ± 0.36	21.9 ± 0.4	36.2 ± 0.4	35.6 ± 0.4	35.6 ± 0.8
	South	22.4 ± 0.3	22.8 ± 0.7	35.9 ± 0.7	34.4 ± 0.8	35.2 ± 0.6
Leaf temperature (°C)	North	22.9 ± 0.8	22.4 ± 0.6	39.3 ± 1.1	38.6 ± 0.6	38.9 ± 0.8
	South	24.3 ± 0.4	24.8 ± 1.1	39.7 ± 0.9	35.6 ± 0.7	37.6 ± 0.6

2.3. Chlorophyll fluorescence and calculation of mesophyll conductance and CO$_2$ concentration in the chloroplast

Chlorophyll fluorescence parameters were quantified with a portable modulated fluorometer (Mini-PAM Photosynthesis Yield Analyzer, Walz, Effeltrich, Germany) on the same leaves used for gas-exchange measurements. Fluorescence parameters (F_m, F'_m, F_o and F_v), photochemical PSII efficiency (Φ_{PSII}) and the maximum quantum yield at midday (F_v/F_m) were determined as described (Fleck et al., 1998). Non-photochemical quenching (NPQ) was calculated using the Stern-Volmer equation: NPQ = $((F_m/F'_m) - 1)$. Adaptation took at least 20 min, after which F_v/F_m values reached about 95% of the pre-dawn values in *Q. ilex* (Fleck et al., 1998).

Mesophyll conductance (g_{mes}) and CO$_2$ concentration in the chloroplast (C_{C}) were calculated from combined gas-exchange (LiCor 6400) and chlorophyll fluorescence (Mini-PAM) measurements, as described by Epron et al. (1995), and Galmés et al. (2007), except for respiration, which was calculated in the same leaves at the end of an A/PPFD curve after a five min acclimatisation to darkness. Galmés et al. (2007) showed that this method yields equivalent results to the "constant *J*" method (Harley et al., 1992), which makes no *a priori* assumption about the relationship between electron transport and fluorescence. Moreover, Flexas et al. (2007) demonstrated that both methods gave results that were comparable to Ethier and Livingston's findings (2004), which did not rely on fluorescence measurements and to calculations by carbon isotope discrimination.

The rate of electron transport (ETR) was calculated as ETR $= \Phi_{\text{PSII}} \times \text{PPDF} \times 0.5 \times 0.82$, where 0.5 is a factor that assumes equal distribution of energy between the two photosystems and 0.82 is the light absorptance we obtained on *Q. ilex* leaves using an integrating sphere. According to the model of Epron et al. (1995), ETR can be divided into two component fractions, ETR$_A$+ETRp, used for CO$_2$ assimilation and for photorespiration, respectively. To calculate C_{C}, we used $S = (\text{ETR}_A/\text{ETR}_P)/(C_{\text{C}}/\text{O})$ (Laing et al., 1974), where S is the specificity factor of Rubisco and O is the oxygen model fraction in the air. We used a value of $S = 93.3$ mol mol^{-1} (Balaguer et al., 1996) that was corrected for leaf temperature according to Brooks and Farquhar (1985). The ratio between mesophyll conductance to CO$_2$ and stomatal conductance ($g_{\text{mes}}/g_{\text{s}}$) was calculated at midday.

2.4. Leaf carbon isotope composition

Sixteen leaves per six plants per treatment and season were collected, oven-dried at 65 °C to constant dry weight and ground in a Mixer-Mill 8000 (Spex) in vials with tungsten carbide balls. Water-soluble extracts were prepared as follows: 2 g of dry material per plant were suspended in 25 mL water (3 replicates per plant) and were heated to 100 °C for 15 min; after cooling to room temperature, samples were filtered (Whatman nr. 1), stored at −40 °C and lyophilized. Approximately 4 mg of the lyophilized water-soluble extract (WSE) and 4 mg of dry mass (M_{d}) were fed into a gas chromatograph (Carlo-Erba NA1500 Series II elemental analyser, CE Elantech, Inc., Lakewood, NJ, USA), connected on-line to an isotope ratio mass spectrometer (IRMS, Finnigan, Delta S; Thermo Finnigan, San Jose, CA, USA) for δ^{13}C determination. δ^{13}C values were determined using a standard calibrated against Pee Dee Belemnite (PDB) carbonate and used to estimate carbon isotope discrimination (Δ^{13}C) as: $\Delta^{13}\text{C} = 1000 \cdot (\delta_{\text{a}} - \delta_{\text{p}})/(1 + \delta_{\text{p}})$, where δ_{a} and δ_{p} are values for air (−7.8‰) and the plant, respectively (Farquhar et al., 1989).

2.5. Relative water content and leaf biomass parameters

Relative water content (RWC) was measured at midday in five young leaves of five plants per treatment. RWC was calculated as $[(M_{\text{f}} - M_{\text{d}})/(M_{\text{fs}} - M_{\text{d}}) \times 100]$, with M_{f} being plant fresh mass; M_{FS}, plant fresh saturated mass (after rehydrating samples for 24 h in the dark); and M_{D}, plant dry mass (after oven-drying samples at 65 °C until constant weight). Leaf mass per area, LMA, was determined (M_{d}/LA), and its components (M_{f}/LA) and $[(M_{\text{d}}/M_{\text{f}}) \times 100]$, as indicators of leaf thickness (T) and leaf density (D), respectively (Niinemets, 1999), were calculated on the same plants as for gas-exchange measurements (30 leaves per treatment) in winter and summer.

2.6. Statistical analyses

All statistical procedures were carried out through the SPSS in Windows (v. 11.0, SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) tested the main effects and interactions, against appropriate error terms. Main factors per treatment and season for all variables were analysed. Leaf orientation was included for gas exchange and chlorophyll fluorescence analyses. The kinds of material analysed (WSE, *DM*) were included in the analyses of parameters derived from leaf δ^{13}C. The *post-hoc* Duncan test was applied where suitable. Differences were considered significant at $p \leq 0.05$. Only statistically significant differences are described in the Results and Discussion that follow.

Table II. Midday values of net photosynthesis (A), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), instantaneous water use efficiency ($WUE_i = A/g_s$), relative water content (RWC), PSII efficiency (ΦPSII), non-photochemical quenching of fluorescence (NPQ) and potential quantum yield of PSII at midday (F_v/F_m). Data are presented according to treatment (control, C; winter resprouts, RW; summer resprouts, RS), leaf orientation (north, south) and season (winter, summer). Values are mean ±SE of nine replicates. Significant differences across rows or columns ($p \leq 0.05$) are indicated by different letters (treatment (a, b, c), season (A, B) and leaf orientation (α, β)).

	Season	Leaf orientation	Treatments		
			C	RW	RS
A ($\mu mol\ m^{-2}\ s^{-1}$)	Winter	North	$4.47 \pm 0.32^{aA\alpha}$	$4.84 \pm 0.38^{aA\alpha}$	—
		South	$5.67 \pm 0.66^{aA\beta}$	$6.82 \pm 0.77^{aA\beta}$	—
	Summer	North	$1.20 \pm 0.20^{aB\alpha}$	$3.28 \pm 0.39^{bB\alpha}$	$4.03 \pm 0.81^{b\alpha}$
		South	$0.87 \pm 0.09^{aB\beta}$	$4.55 \pm 0.69^{bB\beta}$	$5.35 \pm 0.33^{b\beta}$
g_s ($mmol\ m^{-2}\ s^{-1}$)	Winter	North	$272.1 \pm 34.3^{aA\alpha}$	$300.6 \pm 27.6^{aA\alpha}$	—
		South	$253.1 \pm 46.6^{aA\alpha}$	$263.7 \pm 40.0^{aA\alpha}$	—
	Summer	North	$41.8 \pm 2.9^{aB\alpha}$	$123.9 \pm 12.1^{bB\alpha}$	$144.0 \pm 12.2^{b\alpha}$
		South	$37.6 \pm 5.8^{aB\alpha}$	$144.7 \pm 21.9^{bB\alpha}$	$165.3 \pm 22.1^{b\alpha}$
C_i ($\mu mol\ mol^{-1}$)	Winter	North	$291 \pm 10^{Aa\alpha}$	$278 \pm 6^{aA\alpha}$	—
		South	$241 \pm 13^{aA\beta}$	$240 \pm 11^{aA\beta}$	—
	Summer	North	$276 \pm 8^{aA\alpha}$	$248 \pm 13^{aB\alpha}$	$247 \pm 8^{a\alpha}$
		South	$283 \pm 4^{aB\alpha}$	$254 \pm 12^{bA\alpha}$	$240 \pm 4^{b\alpha}$
WUE_i ($\mu mol\ mmol^{-1}$)	Winter	North	$19.78 \pm 3.4^{aA\alpha}$	$17.95 \pm 2.0^{aA\alpha}$	—
		South	$26.13 \pm 4.7^{aA\beta}$	$29.44 \pm 4.0^{aA\beta}$	—
	Summer	North	$28.58 \pm 3.8^{aB\alpha}$	$26.82 \pm 2.1^{aB\alpha}$	$28.35 \pm 5.1^{a\alpha}$
		South	$24.14 \pm 2.6^{aA\beta}$	$32.63 \pm 5.6^{bA\beta}$	$33.95 \pm 3.1^{b\beta}$
RWC (%)	Winter	North	$75.5 \pm 2.8^{aA\alpha}$	$85.3 \pm 1.4^{bA\alpha}$	—
		South	$73.7 \pm 1.5^{aA\alpha}$	$83.0 \pm 1.7^{bA\alpha}$	—
	Summer	North	$67.8 \pm 2.2^{aB\alpha}$	$78.6 \pm 2.1^{bA\alpha}$	$77.6 \pm 1.9^{b\alpha}$
		South	$65.2 \pm 1.2^{aB\alpha}$	$77.9 \pm 1.1^{bA\alpha}$	$77.7 \pm 1.4^{b\alpha}$
Φ_{PSII}	Winter	North	$0.71 \pm 0.01^{aB\beta}$	$0.64 \pm 0.03^{aB\alpha}$	—
		South	$0.52 \pm 0.07^{aB\alpha}$	$0.56 \pm 0.04^{aB\alpha}$	—
	Summer	North	$0.09 \pm 0.02^{aA\alpha}$	$0.09 \pm 0.01^{aA\alpha}$	$0.08 \pm 0.03^{a\alpha}$
		South	$0.04 \pm 0.02^{aA\alpha}$	$0.086 \pm 0.01^{bA\alpha}$	$0.08 \pm 0.01^{b\alpha}$
NPQ	Winter	North	$0.17 \pm 0.05^{aA\alpha}$	$0.26 \pm 0.09^{aA\alpha}$	—
		South	$0.83 \pm 0.25^{aA\beta}$	$0.60 \pm 0.13^{aA\beta}$	—
	Summer	North	$2.36 \pm 0.29^{aB\alpha}$	$1.88 \pm 0.20^{aB\alpha}$	$1.93 \pm 0.04^{a\alpha}$
		South	$2.72 \pm 0.22^{bB\alpha}$	$2.01 \pm 0.18^{aB\alpha}$	$2.32 \pm 0.26^{a\alpha}$
F_v/F_m	Winter	North	$0.77 \pm 0.01^{aB\alpha}$	$0.75 \pm 0.02^{aB\alpha}$	—
		South	$0.76 \pm 0.01^{aB\alpha}$	$0.75 \pm 0.01^{aB\alpha}$	—
	Summer	North	$0.68 \pm 0.03^{aA\alpha}$	$0.68 \pm 0.01^{aA\alpha}$	$0.70 \pm 0.02^{a\alpha}$
		South	$0.65 \pm 0.01^{aA\alpha}$	$0.67 \pm 0.03^{aA\alpha}$	$0.66 \pm 0.03^{a\alpha}$

3. RESULTS

Although the two kinds of resprout used for this study differed in their cutting season, the only difference found between them was the time the resprouts took to appear: RW resprouted in the following spring, 2–3 months after cutting, whereas RS resprouted badly in the autumn and were suitable for photosynthesis measurements only from the next spring onwards (7–8 months after cutting). Since from this moment on they showed no difference from RW plants in the parameters analysed, all kinds of resprouts will be considered as R in the Discussion section.

3.1. Leaf gas exchange and chlorophyll fluorescence

A, g_s and instantaneous water use efficiency ($WUE_i = A/g_s$) at midday (Tab. II) showed no difference between treatments in winter, whereas in summer, resprouts gave higher values than C. Declines in A and g_s between winter and summer were observed for all treatments, but were more pronounced in C,

Table III. Net CO_2 assimilation at saturating C_i and light (A_{max}), maximum carboxylation velocity of Rubisco ($V_{c,max}$), maximum potential rate of electron transport contributing to RuBP regeneration (J_{max}) and stomatal limitation (l_s) from A/C_i curves for the different treatments (control, C; winter resprouts, RW; summer resprouts, RS) and season (winter, summer). In summer, the time of day was also considered. Each value represents the mean ±SE of ten replicates. Significant differences across rows or columns ($p \leq 0.05$) are indicated by different letters (treatment (a, b, c), season (A, B) and time of the day (α, β, γ)).

	Season	Time of the day	Treatments		
			C	RW	RS
A_{max} (μmol m^{-2} s^{-1})	Winter	Midday	6.6 ± 0.5^{aB}	7.2 ± 0.3^{aB}	—
		Morning	$2.7 \pm 0.3^{a\beta}$	$6.1 \pm 0.6^{b\beta}$	$6.5 \pm 0.9^{b\beta}$
	Summer	Midday	$1.1 \pm 0.2^{a\alpha A}$	$3.4 \pm 0.5^{b\alpha A}$	$4.8 \pm 1.3^{b\alpha}$
		Evening	$1.1 \pm 0.3^{a\alpha}$	$3.6 \pm 0.6^{b\alpha}$	$3.4 \pm 0.2^{b\alpha}$
$V_{c,max}$ (μmol m^{-2} s^{-1})	Winter	Midday	30.7 ± 2.8^{aB}	31.8 ± 1.6^{aA}	—
		Morning	$11.8 \pm 2.1^{a\alpha}$	$26.6 \pm 3.5^{b\alpha}$	$27.3 \pm 4.3^{b\alpha}$
	Summer	Midday	$13.5 \pm 4.0^{a\alpha A}$	$29.9 \pm 2.3^{b\alpha A}$	$25.1 \pm 5.7^{b\alpha}$
		Evening	$11.8 \pm 2.4^{a\alpha}$	$27.8 \pm 6.3^{b\alpha}$	$23.7 \pm 3.0^{b\alpha}$
J_{max} (μmol m^{-2} s^{-1})	Winter	Midday	63.4 ± 4.9^{aB}	66.8 ± 2.8^{aB}	—
		Morning	$20.0 \pm 2.9^{a\alpha}$	$42.5 \pm 4.8^{b\alpha}$	$46.2 \pm 6.7^{b\alpha}$
	Summer	Midday	$17.1 \pm 4.1^{a\alpha A}$	$34.9 \pm 4.3^{b\alpha A}$	$40.0 \pm 9.1^{b\alpha}$
		Evening	$16.1 \pm 3.3^{a\alpha}$	$37.8 \pm 8.2^{b\alpha}$	$32.2 \pm 4.5^{b\alpha}$
l_s (%)	Winter	Midday	26.2 ± 1.5^{bA}	21.9 ± 1.1^{aA}	—
		Morning	$58.9 \pm 6.3^{b\alpha}$	$39.9 \pm 2.5^{a\alpha}$	$43.9 \pm 1.5^{a\alpha}$
	Summer	Midday	$51.6 \pm 3.9^{b\alpha B}$	$37.5 \pm 3.0^{a\alpha B}$	$42.2 \pm 2.1^{a\alpha}$
		Evening	$51.5 \pm 3.8^{b\alpha}$	$41.8 \pm 1.0^{a\alpha}$	$37.1 \pm 4.3^{a\alpha}$

In all treatments Φ_{PSII} and midday F_v/F_m values were lower in summer than in winter, whereas NPQ were lower in winter than in summer (Tab. II).

Data derived from the A/C_i curves performed under midday conditions (Tab. III) showed in winter no difference between R and C in A_{max}, $V_{c,max}$ or J_{max}. There was a decrease from winter to summer, with R showing higher values than C (A_{max}: 66.1%, $V_{c,max}$: 57.7%, J_{max}: 59.3%, on average). Stomatal limitation (l_s) was higher in C than in R in both seasons, with l_s higher in summer than in winter for all treatments (52.1% higher on average). In summer, daily variations were observed for A_{max} with the highest values in the morning, whereas no difference was found in $V_{c,max}$, J_{max} and l_s.

3.2. Mesophyll conductance

In winter, no difference between treatments was observed in midday g_{mes}. In summer, R showed higher daily values than C (Fig. 1). Morning values were 36.1% higher than at midday and in the evening. g_{mes} values at midday declined by 97% in controls from winter to summer; whereas in R values declined by 76%. In both seasons, no significant difference in C_C values between treatments was found (Tab. IV). At midday, the g_{mes}/g_s ratio was higher in winter than in summer in both kinds of plant, whereas no difference was found between treatments in the two seasons (Tab. IV).

3.3. Leaf growth parameters

LMA showed no seasonal change. In winter, LMA, D and T were higher in C (Figs. 2a, 2c, 2d), whereas in summer, R showed lower LMA and D but higher T. No seasonal difference in density and thickness was found in C. Mean leaf area (LA) was higher in R and decreased from winter to summer (Fig. 2b). LMA and D were negatively related to g_{mes}; whereas for T the relationship was positive (Figs. 3a–3c).

3.4. Leaf carbon isotope composition

Isotope discrimination against ^{13}C ($\Delta^{13}C$), calculated from $\delta^{13}C$ data, was higher in R than in C for both seasons (Tab. V). Results for water-soluble extracts and dry matter showed the same trends. $\Delta^{13}C$ showed a negative relationship with LMA and a positive relationship with g_{mes} for both seasons (Figs. 4a, 4b). $\Delta^{13}C$ showed a negative relationship with WUE_i in the winter, whereas in the summer the relationship became positive (Fig. 4c).

4. DISCUSSION

In summer, higher temperatures, irradiance and VPD and lower precipitation than in winter lead to increased drought in Mediterranean forests. In fact, g_s, used as an integrative indicator for the degree of water stress (Galmés et al., 2007), showed

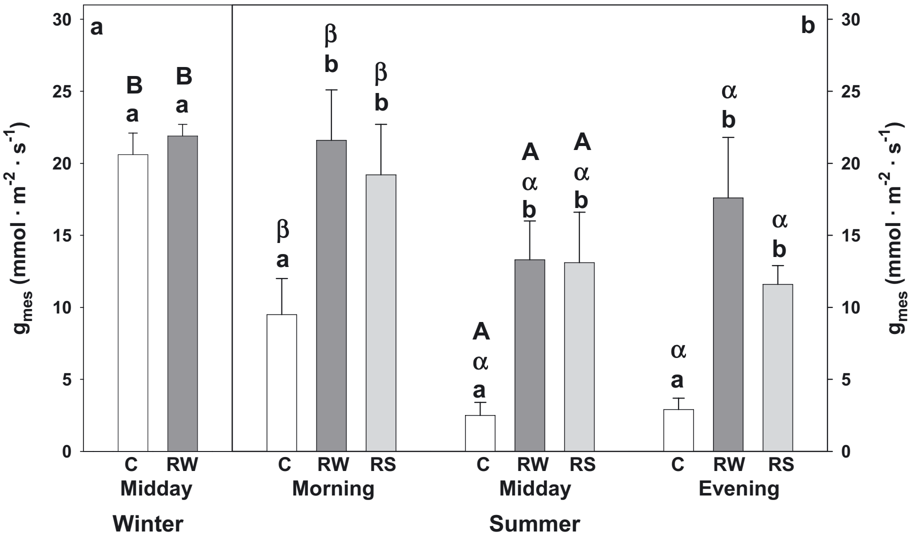

Figure 1. Mesophyll conductance to CO_2 (g_{mes}), per treatment (control, C; winter resprouts, RW; summer resprouts, RS) and season (winter, summer). In summer, the time of day was included (morning, midday, evening). Values are mean ±SE of 10 replicates. Significant differences ($p \leq 0.05$) are indicated by different letters (treatment (a, b, c), season (*A*, *B*) and, in summer, time of day (α, β, γ).

Table IV. CO_2 concentration in the chloroplast (C_C) and midday mesophyll conductance and stomatal conductance ratio (g_{mes}/g_s) at C_a = 350 µmol mol^{-1} from A/C_i curves for different treatments (control, C; winter resprouts, RW; summer resprouts, RS) and season (winter, summer). In summer, the time of day was also considered for C_C values. Each value represents the mean ±SE of 10 replicates. Significant differences ($p \leq 0.05$) are indicated by different letters (treatment (a, b, c), season (*A*, *B*) and time of the day (α, β, γ)).

	Season	Time of the day	Treatments		
			C	RW	RS
C_C (µmol m^{-2} s^{-1})	Winter	Midday	75.3 ± 4.3^{aA}	75.8 ± 4.0^{aA}	—
	Summer	Morning	$76.9 \pm 4.0^{a\alpha}$	$78.5 \pm 2.9^{a\alpha}$	$84.4 \pm 5.9^{a\beta}$
		Midday	$66.1 \pm 8.0^{a\alpha A}$	$90.7 \pm 8.4^{a\alpha A}$	$96.3 \pm 11.5^{a\beta}$
		Evening	$60.6 \pm 0.8^{a\alpha}$	$88.2 \pm 9.9^{a\alpha}$	$67.8 \pm 3.2^{a\alpha}$
g_{mes}/g_s	Winter	Midday	$0.18 \pm 0.03^{a\mathrm{B}}$	$0.17 \pm 0.3^{a\mathrm{B}}$	—
	Summer	Midday	$0.03 \pm 0.01^{a\mathrm{A}}$	$0.04 \pm 0.01^{a\mathrm{A}}$	0.06 ± 0.02^{a}

Table V. Isotope discrimination against ^{13}C ($\Delta^{13}C$), calculated from isotope composition data ($\delta^{13}C$). Data are shown according to treatments (control, C; winter resprouts, RW; summer resprouts, RS), material analysed (dry matter, DM; water-soluble extract, WSE) and season (winter and summer). Values are mean ±SE of 6 replicates. Significant differences across rows or columns ($p \leq 0.05$) are indicated by different letters: treatment (a, b, c), season (*A*, *B*) and material analysed (α, β).

	Season	Material analysed	Treatments		
			C	RW	RS
$\Delta^{13}C$ (‰)	Winter	DM	$20.2 \pm 0.25^{aB\alpha}$	$21.5 \pm 0.15^{bB\beta}$	—
		WSE	$19.9 \pm 0.30^{aB\alpha}$	$21.0 \pm 0.14^{bA\alpha}$	—
	Summer	DM	$18.8 \pm 0.18^{aA\alpha}$	$20.2 \pm 0.13^{bA\alpha}$	$20.9 \pm 0.30^{b\alpha}$
		WSE	$18.5 \pm 0.46^{aA\alpha}$	$21.3 \pm 1.42^{bA\alpha}$	$19.8 \pm 0.66^{b\alpha}$

resprout values corresponding to moderate water stress (g_s = 100−150 mmol m^{-2} s^{-1}), whereas water stress was severe for undisturbed plants (g_s below 50 mmol m^{-2} s^{-1}). Drought affected numerous measured parameters, declining by 20% in R and by 50% in C: A, A_{max}, diffusive conductance (g_s and g_{mes}), $V_{c,max}$, J_{max} and Φ_{PSII}. Higher values for R in the summer can be explained by the greater nutrient and water availability for small resprouting shoots than for controls as reflected both in higher g_s and RWC. Moreover, the larger photosynthetic sink for electrons in R accounts for the lower thermal energy dissipation (estimated by the chlorophyll fluorescence parameter, NPQ) observed in summer, as reported elsewhere (Fleck et al., 1998). In contrast, environmental conditions in the winter did not induce differences in resprouts from undisturbed plants.

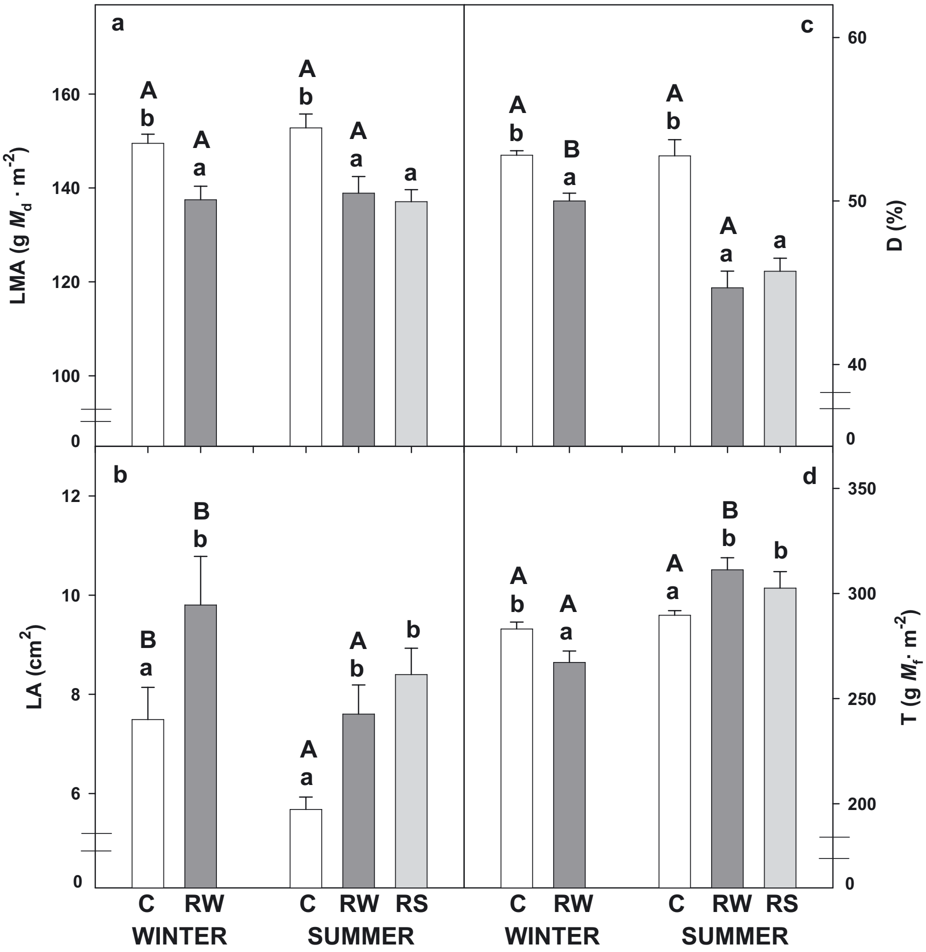

Figure 2. Leaf mass per area (LMA), mean area of a leaf (LA), leaf density (*D*) and leaf thickness (*T*) per treatment (control, C; winter resprouts, RW; summer resprouts, RS) in winter and summer. Values are mean ±SE of 30 replicates. Significant differences ($p \leq 0.05$) are indicated by different letters (treatment (a, b, c); season (*A*, *B*)).

Resprout g_{mes} was markedly higher (3.75-fold) than in C during summer drought. A decline in g_{mes} with changes in plant water availability has been observed for other species (Roupsard et al., 1996). The obtained g_{mes} values were lower than those reported for *Q. ilex* well-watered plants (Loreto et al., 1992). The absolute g_{mes} values obtained in our study may be under-estimated as some parameters used in the calculations were not measured but assumed from the literature (leaf absorptance, light partition between photosystems I and II) or substituted by approximations (use of dark respiration instead of light respiration). However, our results are in the range obtained by Niinemets et al. (2005) for the same species in a forest study.

The 6-fold decrease in the ratio $g_{\text{mes}}/g_{\text{s}}$ from winter to summer in controls and resprouts suggests a stronger photosynthesis limitation by g_{mes} in *Q. ilex* than in previously published for other species (Niinemets et al., 2005). During water stress, C_i may be overestimated because of patchy stomatal closure, and consequently g_{mes} would be underestimated. However, patchiness was not detected in this study (Materials and Methods, Leaf gas exchange).

The leaf structure of resprouts differed from controls and reflected their higher water availability: in fact, R showed higher mean leaf area and lower LMA, indicative of reduced water stress (Peña-Rojas et al., 2005).These structural characteristics may be primarily responsible for changes in g_{mes}: the inverse relationship between g_{mes} and LMA values (Fig. 3a) has been also reported (Niinemets et al., 2006). No seasonal change in LMA was observed in either kind of plants. However, in resprouts, a decrease in density and an increase in thickness were observed from winter to summer. These two components of LMA are not necessarily interdependent, and may be controlled by different environmental variables. However, high *T* is commonly associated with lower *D* (Mediavilla et al., 2001). In accordance with our results (Figs. 3b, 3c), g_{mes} reduction has been related to increased *D* in peach (Syvertsen et al., 1995), and decreased *T* in spinach leaves grown under salt conditions (Delfine et al., 1998).

Lower *D* and higher *T* in resprouts may also account for the observed increased photosynthesis because they correlate with air space fraction in the mesophyll (Niinemets, 1999) resulting in higher g_{mes}. Moreover, *T* is linearly related to the

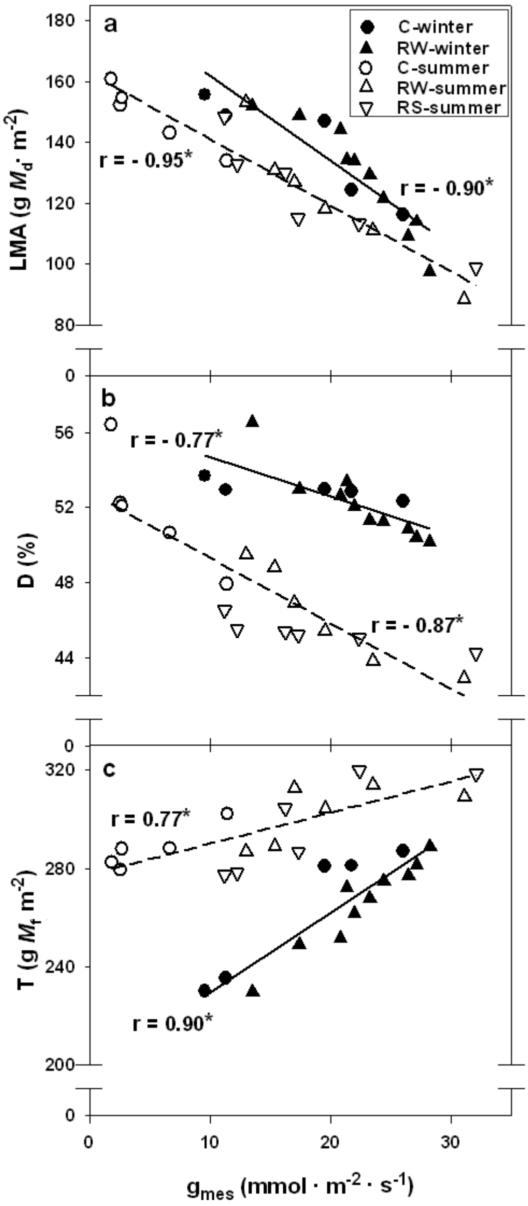

Figure 3. Mesophyll conductance to CO_2 (g_{mes}) vs. leaf mass per area (LMA) (a), leaf density (D) (b) and leaf thickness (T) (c) per season (winter, black; summer, white). Symbols represent single measurements of: control (C; •, ○); winter resprouts, (RW; ▲, △); summer resprouts, (RS; ∇). Asterisks indicate statistically significant correlations (* $p \leq 0.05$).

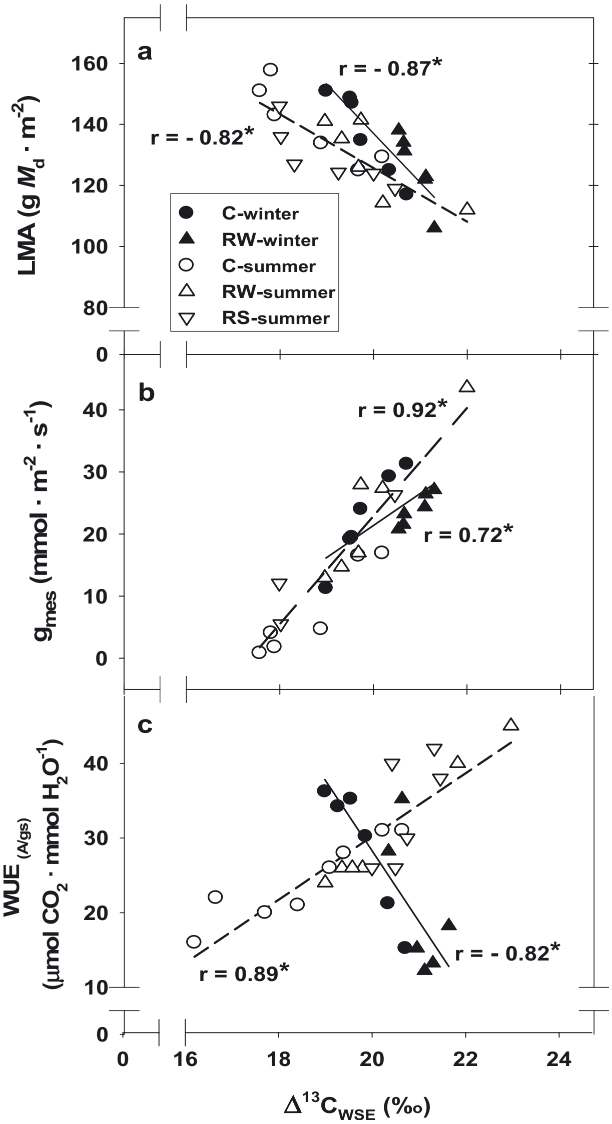

Figure 4. Isotope discrimination against^{13}C (Δ^{13}C) of water-soluble extracts vs. leaf mass per area (LMA) (a), g_{mes} (b) and instantaneous water use efficiency ($WUE_i = A/g_s$) (c) per season (winter, black; summer, white). Symbols represent single measurements of: control (C; •, ○); winter resprouts, (RW; ▲, △); summer resprouts, (RS; ∇). Asterisks indicate statistically significant correlations (* $p \leq 0.05$).

surface area of cells exposed to intercellular air spaces per unit leaf area (Hanba et al., 2002). As chloroplasts are usually distributed near the cell surface, the T increase in R accounts for higher photosynthetic protein accumulation per unit leaf area.

However, morphology is not the only factor determining g_{mes} since the strong reduction in g_{mes} from winter to summer in controls was not paralleled by a change in LMA, T or D; and daily changes in g_{mes} in the summer cannot be attributed to changes in leaf morphology, either. g_{mes} responds not only in the long term to environmental stress, but also changes within seconds to minutes even faster than g_s does (Flexas et al., 2008). Short-term changes in g_{mes} have been attributed to carbonic anhydrase (Gillon and Yakir, 2000) and chloroplast aquaporin regulation (Terashima and Ono, 2002; Flexas et al., 2007). Thus, our results are consistent with the idea that g_{mes} is grossly determined by leaf structure, but is also the result of physiological control. In our study, the daily variations in g_{mes} were of the same magnitude as the seasonal variations, indicating that g_{mes} regulation might be as important as the constraints imposed by morphology.

g_{mes} variations in both kinds of plant paralleled changes in A and $A_{\max}$, which may indicate a reduction of photosynthesis in response to sustained low chloroplast CO_2 levels (Flexas et al., 2006). However, a limitation of photosynthesis not directly related to CO_2 diffusion is suggested by the analysis of A/C_i curves. The decreases in A and $A_{\max}$ in all treatments from winter to summer were paralleled by those of $V_{c,\max}$ and $J_{\max}$, indicating a non-stomatal limitation of photosynthesis. In R, this limitation was lower; they showed around 50% higher $V_{c,\max}$ and $J_{\max}$. These results are compatible with a down-regulation of CO_2 assimilation to adjust mesophyll capacity to the decreased CO_2 supply due to g_s and g_{mes} effects (Flexas et al., 2006). This adjustment of the mesophyll capacity would result in maintenance of C_c as observed (Tab. IV), in the same way that C_i (Tab. II) tends to remain constant, as reported by Wong et al. (1979).

Morphological and physiological changes during drought can be reflected in $\Delta^{13}C$ values: we observed a negative relationship between $\Delta^{13}C$ and LMA values (Fig. 4a), as reported (Fleck et al., 1996). This trend may be a consequence of a g_s decline, but can also be due to a g_{mes} decline. A significant contribution of internal resistances to foliar $\Delta^{13}C$ has been proposed for other species owing to its effect on CO_2 partial pressure at the carboxylation site (Vitousek et al., 1990) and is reflected in Figure 4b. The expected, negative relationship between WUE_i and $\Delta^{13}C$ observed in winter values reflects a similar contribution of g_s and g_{mes} to A and $\Delta^{13}C$, resulting in WUE_i increasing as gs decreases (A decreases less than g_s because of the sustained consumption by RuBisCO) and in $\Delta^{13}C$ decreasing as g_s decreases (because of C_i decline).

The positive relationship observed in summer, (Fig. 4c) can be explained by a dominant role of g_{mes}, mainly in C: here, A declines more than g_s, especially in some plants, because of the strong reduction in g_{mes}, resulting in a decrease in WUE_i, and not in its increase, as expected as g_s decreases (Tab. II). Meanwhile, the sum of reduced g_s and g_{mes}, caused the expected decrease in $\Delta^{13}C$, resulting in the observed positive relationship with WUE_i. Interestingly, g_{mes} reduction has been proposed as an explanation for the inability of typical gas exchange models to predict WUE in Mediterranean ecosystems (Reichstein et al., 2002). In fact, Warren and Adams (2006) proposed, from a theoretical point of view, that g_{mes} may affect the relationship between $\Delta^{13}C$ and WUE. Such a disagreement was not found by Roussel et al. (2009) in *Quercus robur*, but Flexas et al. (2008) already found a discrepancy between WUE and $\delta^{13}C$ that could be attributed to changes in g_{mes}, between transgenic tobacco plants, but ours is the first report of a clear mismatch between $\Delta^{13}C$ and WUE in forest growing plants that can be attributed to g_{mes}. The original $\Delta^{13}C$ model (Farquhar et al., 1982) already included a term for g_{mes} that is often ignored in typical models, but should be included for prediction of the absolute value of leaf $\Delta^{13}C$.

We conclude that g_{mes} exerts a dominant role in photosynthesis limitation in *Q. ilex.* A regulation of g_{mes} exists beyond the morphological constraints, and both factors may well be of a similar magnitude. The greater capacity of resprouts to withstand drought that implied lower photosynthetic limitants (both diffusive and non-stomatal) will permit their growth and recovery after increased fire episodes associated with the climate change.

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