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2 **drought stress during *Quercus ilex* L resprouting**

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1 **Mesophyll conductance to CO₂ and leaf morphological characteristics under**
2 **drought stress during *Quercus ilex* L resprouting**

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5 **Isabel FLECK^{1, a*}, Karen PEÑA-ROJAS^{1,2a}, Xavier ARANDA^{1,3}**

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8 ¹Departament de Biologia Vegetal. Facultat Biologia. Universitat de Barcelona.

9 Diagonal 645, 08028 Barcelona, Spain

10 ²Present address: Facultad de Ingeniería Forestal. Universidad de Chile Santa

11 Rosa 11315 - La Pintana. Santiago, Chile.

12 ³Present address: Departament de Tecnologia Hortícola. Institut de Recerca i

13 Tecnologia Agroalimentàries (IRTA), Torre Marimon, Caldes de Montbui, 08140

14 Barcelona, Spain.

15 These authors contributed equally to this work

16 * For correspondence. Telephone: 34-934021468, Fax: 34-934112842,

17 E-mail ifleck@ub.edu

18

19 **Running title:** mesophyll conductance in holm-oak resprouts

20

21 **Key words:** carbon isotopic composition/ leaf structure/ mesophyll conductance/

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23

1 **Abstract**

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

24

1 1. INTRODUCTION

2 Holm-oak (*Quercus ilex* L.) is a deep-rooted, evergreen dominant species
3 in Mediterranean forests which has a great capacity for resprouting after fire,
4 clear-cut, grazing or other disturbances. Resprouts after any of these events show
5 decreased shoot/root ratios, which makes more water and nutrients available to the
6 shoot than in the original plants and favours photosynthesis stimulation and rapid
7 growth (Fleck et al., 1998). *Q. ilex* is exposed to multiple environmental stress
8 factors such as drought, heat shock, chilling, nutrient deprivation and high light
9 stress amongst others. Increased probability of drought, heat and rising
10 atmospheric CO₂ concentration during the coming decades may be particularly
11 important in the Mediterranean basin (Christensen et al. 2007). Moreover, the
12 expected increased risk of uncontrolled fire episodes could lead to the exhaustion
13 of several species, generating a decline in their resprouting capacity and recovery.
14 There has been a long-standing controversy as to whether drought limits
15 photosynthesis by stomatal closure, metabolic impairment or through diffusive
16 resistances (Lawlor and Tezara 2009). Of these resistances, CO₂ transfer
17 conductance inside the leaf or mesophyll conductance (g_{mes}) is considered relevant
18 to photosynthesis (Flexas et al. 2008). Metabolic photosynthesis limitations (e.g.
19 injuries to photosynthetic biochemistry and photochemistry) during drought may
20 only be apparent: drought produces low g_s , closely related to g_{mes} , resulting in a
21 decreased availability of CO₂ in the chloroplast, which down-regulates the
22 biochemical machinery of photosynthesis. g_{mes} can be affected by leaf
23 morphology (Terashima et al., 2001); in fact, previous results of our group (Peña-

1 Rojas et al., 2005) related changes in g_{mes} in nursery-grown holm-oak plants
2 submitted to water stress to variations in leaf anatomy and gas-exchange
3 parameters.

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

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

1

2 **2. MATERIALS AND METHODS**

3 **2.1. Experimental site and plant material**

4 The study was carried out at Can Coll, Serra de Collserola forest, Barcelona,
5 Spain; 41°28'28"N, 2°7'32"E. A plot (400 x 280m) at altitude of 140m and
6 oriented N-NE was selected. The climate is Mediterranean, with cold winters,
7 cool and wet springs and autumns, and hot dry summers (Tab.1). The 35-year old
8 forest is dominated by *Quercus ilex* and *Pinus halepensis*. In February, 25
9 *Quercus ilex* plants were selected (5.9 ± 0.3 cm mean diameter at breast height
10 (DBH), 4.7 ± 0.2 m mean height, 1.4 ± 0.2 Kg mean leaf biomass) and the shoots
11 of 10 randomly selected plants were completely excised 15cm above soil level.
12 Resprouts (R) after this date were designated as RW (winter resprouts). In August,
13 10 more plants were completely excised and resprouts after this were designated
14 as RS (summer resprouts). Five plants were kept undisturbed, as controls (C) of
15 the clear-cut site. Leaf gas exchange and chlorophyll fluorescence were measured
16 in fully expanded leaves of the same age: in the first winter (W; February-March),
17 only controls and RW leaves were analysed, as RS had resprouted badly in the
18 autumn; in the subsequent summer (S; July-August), all treatments could be
19 analysed. Samples were collected for ^{13}C composition ($\delta^{13}\text{C}$), leaf mass per area
20 (LMA), leaf density (D) and leaf thickness (T) determinations.

21

1 2.2. Leaf gas exchange

2 A portable gas exchange system LI-6200 (Li-Cor Inc., Lincoln, NE, USA) was
3 used for punctual measurements at midday on nine attached, fully expanded,
4 current-year leaves per treatment, season and leaf orientation. Leaf cuvette
5 conditions differed according to the season (Tab.1). Results were expressed per
6 leaf-projected area (LA), obtained with an Epson GT5000 scanner and processed
7 using image analyser software. In each season, ten CO₂ response curves of CO₂
8 assimilation vs. intercellular CO₂ concentration (A/C_i) were obtained per
9 treatment on attached leaves with a LI-6400 instrument (Li-COR, Lincoln,
10 Nebraska, USA). Leaf cuvette conditions were established according to the season
11 and time of the day to reproduce a typical day in every season.

12 For A/C_i curves, PPFD was established as 600 μmol·m⁻²·s⁻¹, which is saturating
13 under these conditions (Peña-Rojas et al., 2004); a range of ambient CO₂
14 concentration (C_a) from 50 to 800 μmol·mol⁻¹ was covered. Analyses of the curves
15 permitted the determination of: A_{max}, net photosynthesis at saturating C_i and
16 PPFD; V_{c,max}, maximum carboxylation velocity of Rubisco; J_{max}, maximum
17 electron transport contributing to RuBP regeneration; l_s, stomatal limitation to A
18 (l_s(%)=100x (1-(A/A_{sat}))); A_{sat}, net photosynthesis at saturating light and C_i =
19 350 μmol·mol⁻¹.

20 To assess the effect of heterogeneous stomatal conductance across the leaf
21 surface,, steady-state chlorophyll fluorescence was measured in six spots of 27
22 leaves of the same plants used in the experiment. Water potential (Ψ) of the same
23 leaves was also obtained with a Scholander-type pressure pump (Soil Moisture

1 3005, Soilmoisture Equipment Corp., Goleta, CA, USA). The coefficient of
2 variation of Φ_{PSII} (see below) was not statistically higher than system
3 repetitiveness (around 9%), indicating the absence of patchiness, and did not
4 correlate with Ψ .

5

6 **2.3. Chlorophyll fluorescence and calculation of mesophyll conductance and** 7 **CO₂ concentration in the chloroplast**

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

17 Mesophyll conductance (g_{mes}) and CO₂ concentration in the chloroplast
18 (C_c) were calculated from combined gas-exchange (LiCor 6400) and chlorophyll
19 fluorescence (Mini-PAM) measurements, as described by Epron et al. (1995), and
20 Galmés et al. (2007), except for respiration, which was calculated in the same
21 leaves at the end of an A/PPFD curve after a five min acclimatisation to darkness.
22 Galmés et al. (2007) showed that this method yields equivalent results to the
23 “constant J” method (Harley et al., 1992), which makes no *a priori* assumption

1 about the relationship between electron transport and fluorescence. Moreover,
2 Flexas et al. (2007) demonstrated that both methods gave results that were
3 comparable to Ethier and Livingston's findings (2004), which did not rely on
4 fluorescence measurements and to calculations by carbon isotope discrimination.
5 The rate of electron transport (ETR) was calculated as $ETR = \Phi_{PSII} \times PPDF \times 0.5$
6 $\times 0.82$, where 0.5 is a factor that assumes equal distribution of energy between the
7 two photosystems and 0.82 is the light absorptance we obtained on *Q. ilex* leaves
8 using an integrating sphere. According to the model of Epron et al. (1995), ETR
9 can be divided into two component fractions, $ETR_A + ETR_p$, used for CO_2
10 assimilation and for photorespiration, respectively. To calculate C_C , we used $S =$
11 $(ETR_A / ETR_p) / (C_C / O)$ (Laing et al., 1974), where S is the specificity factor of
12 Rubisco and O is the oxygen model fraction in the air. We used a value of $S = 93.3$
13 $mol\ mol^{-1}$ (Balaguer et al. 1996) that was corrected for leaf temperature according
14 to Brooks and Farquhar (1985). The ratio between mesophyll conductance to CO_2
15 and stomatal conductance (g_{mes} / g_s) was calculated at midday.

16

17 **2.4. Leaf Carbon Isotope composition**

18 Sixteen leaves per six plants per treatment and season were collected, oven-dried
19 at 65°C to constant dry weight and ground in a Mixer-Mill 8000 (Spex) in vials
20 with tungsten carbide balls. Water-soluble extracts were prepared as follows: 2g
21 of dry material per plant were suspended in 25ml water (3 replicates per plant)
22 and were heated to 100°C for 15min; after cooling to room temperature, samples
23 were filtered (Whatman nr.1), stored at -40°C and lyophilized. Approximately

1 4mg of the lyophilized water-soluble extract (WSE) and 4mg of dry mass (M_d)
2 were fed into a gas chromatograph (Carlo-Erba NA1500 Series II elemental
3 analyser, CE Elantech, Inc., Lakewood, NJ, USA), connected on-line to an isotope
4 ratio mass spectrometer (IRMS, Finnigan, Delta S; Thermo Finnigan, San Jose,
5 CA, USA) for $\delta^{13}\text{C}$ determination. $\delta^{13}\text{C}$ values were determined using a standard
6 calibrated against Pee Dee Belemnite (PDB) carbonate and used to estimate
7 carbon isotope discrimination ($\Delta^{13}\text{C}$) as: $\Delta^{13}\text{C} = 1000 \cdot (\delta_a - \delta_p) / (1 + \delta_p)$, where δ_a and
8 δ_p are values for air (-7.8‰) and the plant, respectively (Farquhar et al., 1989).

9

10 **2.5. Relative water content and leaf biomass parameters**

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

20 **2.6. Statistical analyses**

21 All statistical procedures were carried out through the SPSS in Windows (v. 11.0,
22 SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) tested the main
23 effects and interactions, against appropriate error terms. Main factors per

1 treatment and season for all variables were analysed. Leaf orientation was
2 included for gas exchange and chlorophyll fluorescence analyses. The kinds of
3 material analysed (WSE, M_D) were included in the analyses of parameters derived
4 from leaf $\delta^{13}\text{C}$. The *post-hoc* Duncan test was applied where suitable. Differences
5 were considered significant at $p \leq 0.05$. Only statistically significant differences
6 are described in the Results and Discussion that follow.

7

8 **3. RESULTS**

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

17 **3.1. Leaf gas exchange and chlorophyll fluorescence**

18 A , g_s and instantaneous water use efficiency ($\text{WUE}_i = A/g_s$) at midday (Tab. 2)
19 showed no difference between treatments in winter, whereas in summer, resprouts
20 gave higher values than C. Declines in A and g_s between winter and summer were
21 observed for all treatments, but were more pronounced in C, In all treatments
22 Φ_{PSII} and midday F_v/F_m values were lower in summer than in winter, whereas
23 NPQ were lower in winter than in summer (Tab. 2).

1 Data derived from the A/C_i curves performed under midday conditions (Tab. 3)
 2 showed in winter no difference between R and C in A_{\max} , $V_{c,\max}$ or J_{\max} . There was
 3 a decrease from winter to summer, with R showing higher values than C (A_{\max} :
 4 66.1%, $V_{c,\max}$: 57.7%, J_{\max} : 59.3%, on average). Stomatal limitation (l_s) was
 5 higher in C than in R in both seasons, with l_s higher in summer than in winter for
 6 all treatments (52.1% higher on average). In summer, daily variations were
 7 observed for A_{\max} with the highest values in the morning, whereas no difference
 8 was found in $V_{c,\max}$, J_{\max} and l_s .

9 **3.2. Mesophyll conductance**

10 In winter, no difference between treatments was observed in midday g_{mes} . In
 11 summer, R showed higher daily values than C (Fig. 1). Morning values were
 12 36.1% higher than at midday and in the evening. g_{mes} values at midday declined
 13 by 97% in controls from winter to summer; whereas in R values declined by 76%.
 14 In both seasons, no significant difference in C_C values between treatments was
 15 found. (Tab. 4). At midday, the g_{mes}/g_s ratio was higher in winter than in summer
 16 in both kinds of plant, whereas no difference was found between treatments in the
 17 two seasons (Tab. 4).

18 **3.3. Leaf growth parameters**

19 | LMA showed no seasonal change. In winter, LMA, D and T were higher in C
 20 (Fig.2a, c, d), whereas in summer, R showed lower LMA and D but higher T. No
 21 seasonal difference in density and thickness was found in C. Mean leaf area (LA)
 22 was higher in R and decreased from winter to summer (Fig. 2b). LMA and D were

1 negatively related to g_{mes} ; whereas for T the relationship was positive (Fig. 3 a, b,
2 c).

3

4 **3.4. Leaf Carbon Isotope composition**

5 Isotope discrimination against ^{13}C ($\Delta^{13}\text{C}$), calculated from $\delta^{13}\text{C}$ data, was higher
6 in R than in C for both seasons (Tab. 5). Results for water-soluble extracts and dry
7 matter showed the same trends. $\Delta^{13}\text{C}$ showed a negative relationship with LMA
8 and a positive relationship with g_{mes} for both seasons (Fig. 4 a, b). $\Delta^{13}\text{C}$ showed a
9 negative relationship with WUE_i in the winter, whereas in the summer the
10 relationship became positive (Fig. 4 c).

11

12 **4. DISCUSSION**

13 In summer, higher temperatures, irradiance and VPD and lower precipitation than
14 in winter lead to increased drought in Mediterranean forests. In fact, g_s , used as an
15 integrative indicator for the degree of water stress (Galmés et al., 2007), showed
16 resprout values corresponding to moderate water stress ($g_s = 100\text{-}150 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)
17 ¹), whereas water stress was severe for undisturbed plants (g_s below $50 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).
18 Drought affected numerous measured parameters, declining by 20% in R
19 and by 50% in C: A , A_{max} , diffusive conductance (g_s and g_{mes}), $V_{c,max}$, J_{max} and
20 Φ_{PSII} . Higher values for R in the summer can be explained by the greater nutrient
21 and water availability for small resprouting shoots than for controls as reflected
22 both in higher g_s and RWC. Moreover, the larger photosynthetic sink for electrons
23 in R accounts for the lower thermal energy dissipation (estimated by the

1 chlorophyll fluorescence parameter, NPQ) observed in summer, as reported
2 elsewhere (Fleck et al. 1998). In contrast, environmental conditions in the winter
3 did not induce differences in resprouts from undisturbed plants.

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

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

20 The leaf structure of resprouts differed from controls and reflected their
21 higher water availability: in fact, R showed higher mean leaf area and lower
22 LMA, indicative of reduced water stress (Peña-Rojas et al., 2005). These structural
23 characteristics may be primarily responsible for changes in g_{mes} : the inverse

1 relationship between g_{mes} and LMA values (Fig. 3 a) has been also reported
2 (Niinemets et al., 2006). No seasonal change in LMA was observed in either kind
3 of plants. However, in resprouts, a decrease in density and an increase in
4 thickness were observed from winter to summer. These two components of LMA
5 are not necessarily interdependent, and may be controlled by different
6 environmental variables. However, high T is commonly associated with lower D
7 (Mediavilla et al., 2001). In accordance with our results (Fig. 3 b, c), g_{mes}
8 reduction has been related to increased D in peach (Syvertsen et al., 1995), and
9 decreased T in spinach leaves grown under salt conditions (Delfine et al., 1998).

10 Lower D and higher T in resprouts may also account for the observed
11 increased photosynthesis because they correlate with air space fraction in the
12 mesophyll (Niinemets, 1999) resulting in higher g_{mes} . Moreover, T is linearly
13 related to the surface area of cells exposed to intercellular air spaces per unit leaf
14 area (Hanba et al., 2002). As chloroplasts are usually distributed near the cell
15 surface, the T increase in R accounts for higher photosynthetic protein
16 accumulation per unit leaf area.

17 However, morphology is not the only factor determining g_{mes} since the
18 strong reduction in g_{mes} from winter to summer in controls was not paralleled by a
19 change in LMA, T or D; and daily changes in g_{mes} in the summer cannot be
20 attributed to changes in leaf morphology, either. g_{mes} responds not only in the
21 long term to environmental stress, but also changes within seconds to minutes
22 even faster than g_s does (Flexas et al., 2008). Short-term changes in g_{mes} have
23 been attributed to carbonic anhydrase (Gillon and Yakir, 2000) and chloroplast

1 aquaporin regulation (Terashima and Ono, 2002; ; Flexas et al., 2007). Thus, our
2 results are consistent with the idea that g_{mes} is grossly determined by leaf
3 structure, but is also the result of physiological control. In our study, the daily
4 variations in g_{mes} were of the same magnitude as the seasonal variations,
5 indicating that g_{mes} regulation might be as important as the constraints imposed by
6 morphology.

7 g_{mes} variations in both kinds of plant paralleled changes in A and A_{max} ,
8 which may indicate a reduction of photosynthesis in response to sustained low
9 chloroplast CO_2 levels (Flexas et al., 2006). However, a limitation of
10 photosynthesis not directly related to CO_2 diffusion is suggested by the analysis of
11 A/C_i curves. The decreases in A and A_{max} in all treatments from winter to summer
12 were paralleled by those of $V_{c,max}$ and J_{max} , indicating a non-stomatal limitation of
13 photosynthesis. In R, this limitation was lower; they showed around 50% higher
14 $V_{c,max}$ and J_{max} . These results are compatible with a down-regulation of CO_2
15 assimilation to adjust mesophyll capacity to the decreased CO_2 supply due to g_s
16 and g_{mes} effects (Flexas et al., 2006). This adjustment of the mesophyll capacity
17 would result in maintenance of C_c as observed (Tab. 4), in the same way that C_i
18 (Tab. 2) tends to remain constant, as reported by Wong et al. (1979).

19 Morphological and physiological changes during drought can be
20 reflected in $\Delta^{13}C$ values:. We observed a negative relationship between $\Delta^{13}C$
21 and LMA values (Fig. 4a), as reported (Fleck et al., 1996). This trend may be a
22 consequence of a g_s decline, but can also be due to a g_{mes} decline. A significant
23 contribution of internal resistances to foliar $\Delta^{13}C$ has been proposed for other

1 species owing to its effect on CO₂ partial pressure at the carboxylation site
2 (Vitousek et al., 1990) and is reflected in Fig. 4 b. The expected, negative
3 relationship between WUE_i and D¹³C observed in winter values reflects a
4 similar contribution of g_s and g_{mes} to A and Δ¹³C, resulting in WUE_i increasing
5 as g_s decreases (A decreases less than g_s because of the sustained consumption
6 by RuBisCO) and D¹³C decreasing as g_s decreases (because of C_i decline).

7 The positive relationship observed in summer, (Fig. 4c) can be explained
8 by a dominant role of g_{mes}, mainly in C: here, A declines more than g_s, especially
9 in some plants, because of the strong reduction in g_{mes}, resulting in a decrease, and
10 not the expected increase, in WUE_i (Table 2) as g_s decreases; meanwhile, the sum
11 of reduced g_s and g_{mes}, caused the expected decrease in Δ¹³C, resulting in the
12 observed positive relationship with WUE_i. Interestingly, g_{mes} reduction has been
13 proposed as an explanation for the inability of typical gas exchange models to
14 predict WUE in Mediterranean ecosystems (Reichstein et al., 2002). In fact,
15 Warren and Adams (2006) proposed, from a theoretical point of view, that g_{mes}
16 may affect the relationship between Δ¹³C and WUE. Such a disagreement was not
17 found by Roussel et al. (2009) in *Quercus robur*, but Flexas et al. (2008) already
18 found a discrepancy between WUE and δ¹³C that could be attributed to changes in
19 g_{mes}, between transgenic tobacco plants, but ours is the first report of a clear
20 mismatch between Δ¹³C and WUE in forest growing plants that can be attributed
21 to g_{mes}. The original Δ¹³C model (Farquhar et al., 1982) already included a term
22 for g_{mes} that is often ignored in typical models, but should be included for
23 prediction of the absolute value of leaf Δ¹³C.

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

7

1

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1 **Table 1.** Climatological data recorded at the forest site during the gas-exchange
 2 measurements of the different treatments (control, C; winter resprouts, RW;
 3 summer resprouts, RS); data are the mean \pm S.E of all measurements

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

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1 **Table 2.** Midday values of net photosynthesis (A), stomatal conductance (g_s),
 2 intercellular CO₂ concentration (C_i), instantaneous water use efficiency ($WUE_i =$
 3 A/g_s), relative water content (RWC), PSII efficiency (Φ_{PSII}), non-photochemical
 4 quenching of fluorescence (NPQ) and potential quantum yield of PSII at midday
 5 (F_v/F_m). Data are presented according to treatment (control, C; winter resprouts,
 6 RW; summer resprouts, RS), leaf orientation (north, south) and season (winter,
 7 summer). Values are mean \pm S.E. of nine replicates. Significant differences across
 8 rows or columns ($p \leq 0.05$) are indicated by different letters (treatment (a, b, c),
 9 season (A, B) and leaf orientation (α, β)).
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	Season	Leaf Orientation	Treatments		
			C	RW	RS
A ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Winter	North	4.47 \pm 0.32 ^{aAα}	4.84 \pm 0.38 ^{aAα}	---
		South	5.67 \pm 0.66 ^{aAβ}	6.82 \pm 0.77 ^{aAβ}	---
	Summer	North	1.20 \pm 0.20 ^{aBα}	3.28 \pm 0.39 ^{bBα}	4.03 \pm 0.81 ^{bα}
		South	0.87 \pm 0.09 ^{aBβ}	4.55 \pm 0.69 ^{bBβ}	5.35 \pm 0.33 ^{bβ}
g_s ($\text{mmol m}^{-2} \text{s}^{-1}$)	Winter	North	272.1 \pm 34.3 ^{aAα}	300.6 \pm 27.6 ^{aAα}	---
		South	253.1 \pm 46.6 ^{aAα}	263.7 \pm 40.0 ^{aAα}	---
	Summer	North	41.8 \pm 2.9 ^{aBα}	123.9 \pm 12.1 ^{bBα}	144.0 \pm 12.2 ^{bα}
		South	37.6 \pm 5.8 ^{aBα}	144.7 \pm 21.9 ^{bBα}	165.3 \pm 22.1 ^{bα}
C_i ($\mu\text{mol} \cdot \text{mol}^{-1}$)	Winter	North	291 \pm 10 ^{Aaα}	278 \pm 6 ^{aAα}	---
		South	241 \pm 13 ^{aAβ}	240 \pm 11 ^{aAβ}	---
	Summer	North	276 \pm 8 ^{aAα}	248 \pm 13 ^{aBα}	247 \pm 8 ^{aα}
		South	283 \pm 4 ^{aBα}	254 \pm 12 ^{bAα}	240 \pm 4 ^{bα}
WUE_i ($\mu\text{mol mmol}^{-1}$)	Winter	North	19.78 \pm 3.4 ^{aAα}	17.95 \pm 2.0 ^{aAα}	---
		South	26.13 \pm 4.7 ^{aAβ}	29.44 \pm 4.0 ^{aAβ}	---
	Summer	North	28.58 \pm 3.8 ^{aBα}	26.82 \pm 2.1 ^{aBα}	28.35 \pm 5.1 ^{aα}
		South	24.14 \pm 2.6 ^{aAβ}	32.63 \pm 5.6 ^{bAβ}	33.95 \pm 3.1 ^{bβ}
RWC (%)	Winter	North	75.5 \pm 2.8 ^{aAα}	85.3 \pm 1.4 ^{bAα}	---
		South	73.7 \pm 1.5 ^{aAα}	83.0 \pm 1.7 ^{bAα}	---
	Summer	North	67.8 \pm 2.2 ^{aBα}	78.6 \pm 2.1 ^{bAα}	77.6 \pm 1.9 ^{bα}
		South	65.2 \pm 1.2 ^{aBα}	77.9 \pm 1.1 ^{bAα}	77.7 \pm 1.4 ^{bα}
Φ_{PSII}	Winter	North	0.71 \pm 0.01 ^{aBβ}	0.64 \pm 0.03 ^{aBα}	---
		South	0.52 \pm 0.07 ^{aBα}	0.56 \pm 0.04 ^{aBα}	---
	Summer	North	0.09 \pm 0.02 ^{aAα}	0.09 \pm 0.01 ^{aAα}	0.08 \pm 0.03 ^{aα}
		South	0.04 \pm 0.02 ^{aAα}	0.086 \pm 0.01 ^{bAα}	0.08 \pm 0.01 ^{bα}
NPQ	Winter	North	0.17 \pm 0.05 ^{aAα}	0.26 \pm 0.09 ^{aAα}	---
		South	0.83 \pm 0.25 ^{aAβ}	0.60 \pm 0.13 ^{aAβ}	---
	Summer	North	2.36 \pm 0.29 ^{aBα}	1.88 \pm 0.20 ^{aBα}	1.93 \pm 0.04 ^{aα}
		South	2.72 \pm 0.22 ^{bBα}	2.01 \pm 0.18 ^{aBα}	2.32 \pm 0.26 ^{aα}
F_v/F_m	Winter	North	0.77 \pm 0.01 ^{aBα}	0.75 \pm 0.02 ^{aBα}	---
		South	0.76 \pm 0.01 ^{aBα}	0.75 \pm 0.01 ^{aBα}	---
	Summer	North	0.68 \pm 0.03 ^{aAα}	0.68 \pm 0.01 ^{aAα}	0.70 \pm 0.02 ^{aα}
		South	0.65 \pm 0.01 ^{aAα}	0.67 \pm 0.03 ^{aAα}	0.66 \pm 0.03 ^{aα}

1 **Table 3.** Net CO₂ assimilation at saturating C_i and light (A_{max}), maximum
 2 carboxylation velocity of Rubisco (V_{c,max}), maximum potential rate of electron
 3 transport contributing to RuBP regeneration (J_{max}) and stomatal limitation (I_s)
 4 from A/C_i curves for the different treatments (control, C; winter resprouts, RW;
 5 summer resprouts, RS) and season (winter, summer). In summer, the time of day
 6 was also considered. Each value represents the mean ± S.E. of ten replicates.
 7 Significant differences across rows or columns (p ≤ 0.05) are indicated by
 8 different letters (treatment (a, b, c), season (A, B) and time of the day (α, β, γ)).
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	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 ± 0.3 ^{aβ}	6.1 ± 0.6 ^{bβ}	6.5 ± 0.9 ^{bβ}
	Summer	Midday	1.1 ± 0.2 ^{aαA}	3.4 ± 0.5 ^{bαA}	4.8 ± 1.3 ^{bα}
		Evening	1.1 ± 0.3 ^{aα}	3.6 ± 0.6 ^{bα}	3.4 ± 0.2 ^{bα}
V _{c,max} (μmol · m ⁻² · s ⁻¹)	Winter	Midday	30.7 ± 2.8 ^{aB}	31.8 ± 1.6 ^{aA}	---
		Morning	11.8 ± 2.1 ^{aα}	26.6 ± 3.5 ^{bα}	27.3 ± 4.3 ^{bα}
	Summer	Midday	13.5 ± 4.0 ^{aαA}	29.9 ± 2.3 ^{bαA}	25.1 ± 5.7 ^{bα}
		Evening	11.8 ± 2.4 ^{aα}	27.8 ± 6.3 ^{bα}	23.7 ± 3.0 ^{bα}
J _{max} (μmol · m ⁻² · s ⁻¹)	Winter	Midday	63.4 ± 4.9 ^{aB}	66.8 ± 2.8 ^{aB}	---
		Morning	20.0 ± 2.9 ^{aα}	42.5 ± 4.8 ^{bα}	46.2 ± 6.7 ^{bα}
	Summer	Midday	17.1 ± 4.1 ^{aαA}	34.9 ± 4.3 ^{bαA}	40.0 ± 9.1 ^{bα}
		Evening	16.1 ± 3.3 ^{aα}	37.8 ± 8.2 ^{bα}	32.2 ± 4.5 ^{bα}
I _s (%)	Winter	Midday	26.2 ± 1.5 ^{bA}	21.9 ± 1.1 ^{aA}	---
		Morning	58.9 ± 6.3 ^{bα}	39.9 ± 2.5 ^{aα}	43.9 ± 1.5 ^{aα}
	Summer	Midday	51.6 ± 3.9 ^{bαB}	37.5 ± 3.0 ^{aαB}	42.2 ± 2.1 ^{aα}
		Evening	51.5 ± 3.8 ^{bα}	41.8 ± 1.0 ^{aα}	37.1 ± 4.3 ^{aα}

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Table 4. CO₂ 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 ± S.E. of 10 replicates. Significant differences (p ≤ 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}	---
		Morning	76.9 ± 4.0 ^{aα}	78.5 ± 2.9 ^{aα}	84.4 ± 5.9 ^{aβ}
	Summer	Midday	66.1 ± 8.0 ^{aαA}	90.7 ± 8.4 ^{aαA}	96.3 ± 11.5 ^{aβ}
		Evening	60.6 ± 0.8 ^{aα}	88.2 ± 9.9 ^{aα}	67.8 ± 3.2 ^{aα}
g _{mes} /g _s	Winter	Midday	0.18 ± 0.03 ^{aB}	0.17 ± 0.3 ^{aB}	---
	Summer	Midday	0.03 ± 0.01 ^{aA}	0.04 ± 0.01 ^{aA}	0.06 ± 0.02 ^a

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1 **Table 5.** Isotope discrimination against ^{13}C ($\Delta^{13}\text{C}$), calculated from isotope
 2 composition data ($\delta^{13}\text{C}$). Data are shown according to treatments (control, C;
 3 winter resprouts, RW; summer resprouts, RS), material analysed (dry matter, DM;
 4 water-soluble extract, WSE) and season (winter and summer). Values are mean \pm
 5 S.E. of 6 replicates. Significant differences across rows or columns ($p \leq 0.05$) are
 6 indicated by different letters: treatment (a, b, c), season (A, B) and material
 7 analysed (α , β).
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	Season	Material Analysed	Treatments		
			C	RW	RS
$\Delta^{13}\text{C}$ (‰)	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	$18.5 \pm 0.46^{aA\alpha}$	$21.3 \pm 1.42^{bA\alpha}$	$19.8 \pm 0.66^{b\alpha}$

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1 **Figure legends**

2 **Figure 1.** Mesophyll conductance to CO₂ (g_{mes}), per treatment (control, C; winter
3 resprouts, RW; summer resprouts, RS) and season (winter, summer). In summer,
4 the time of day was included (morning, midday, evening). Values are mean \pm S.E.
5 of 10 replicates. Significant differences ($p \leq 0.05$) are indicated by different letters
6 (treatment (a, b, c), season (A, B) and, in summer, time of day (α , β , γ)).

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

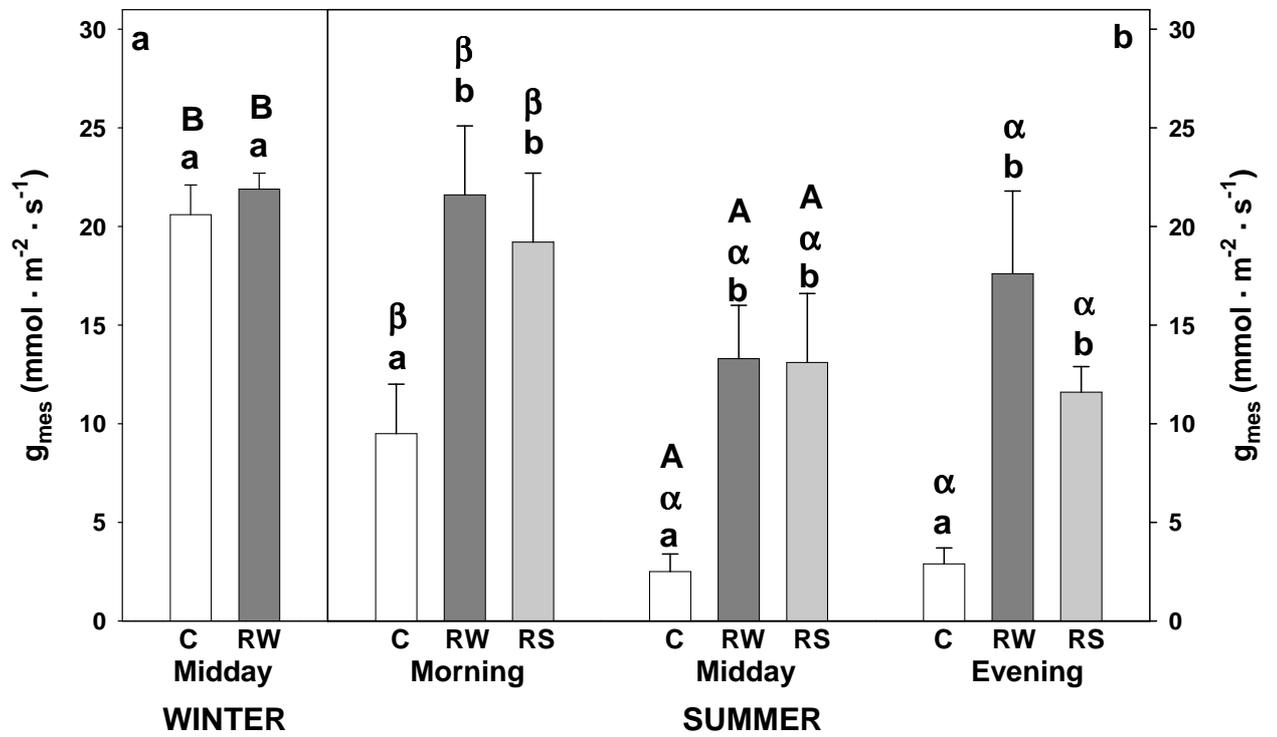
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14 **Figure 3.** Mesophyll conductance to CO₂ (g_{mes}) vs. leaf mass per area (LMA)
15 (a), leaf density (D) (b) and leaf thickness (T) (c) per season (winter, black;
16 summer, white). Symbols represent single measurements of: control (C; ●, ○);
17 winter resprouts, (RW; ▲, Δ); summer resprouts, (RS; ▽). Asterisks indicate
18 statistically significant correlations (* $p \leq 0.05$).

19

20 **Figure 4.** Isotope discrimination against ¹³C ($\Delta^{13}C$) of water-soluble extracts vs.
21 leaf mass per area (LMA) (a), g_{mes} (b) and instantaneous water use efficiency
22 ($WUE_i = A/g_s$) (c) per season (winter, black; summer, white). Symbols represent
23 single measurements of: control (C; ●, ○); winter resprouts, (RW; ▲, Δ); summer

1 resprouts, (RS; ∇). Asterisks indicate statistically significant correlations (* $p \leq 0.05$).

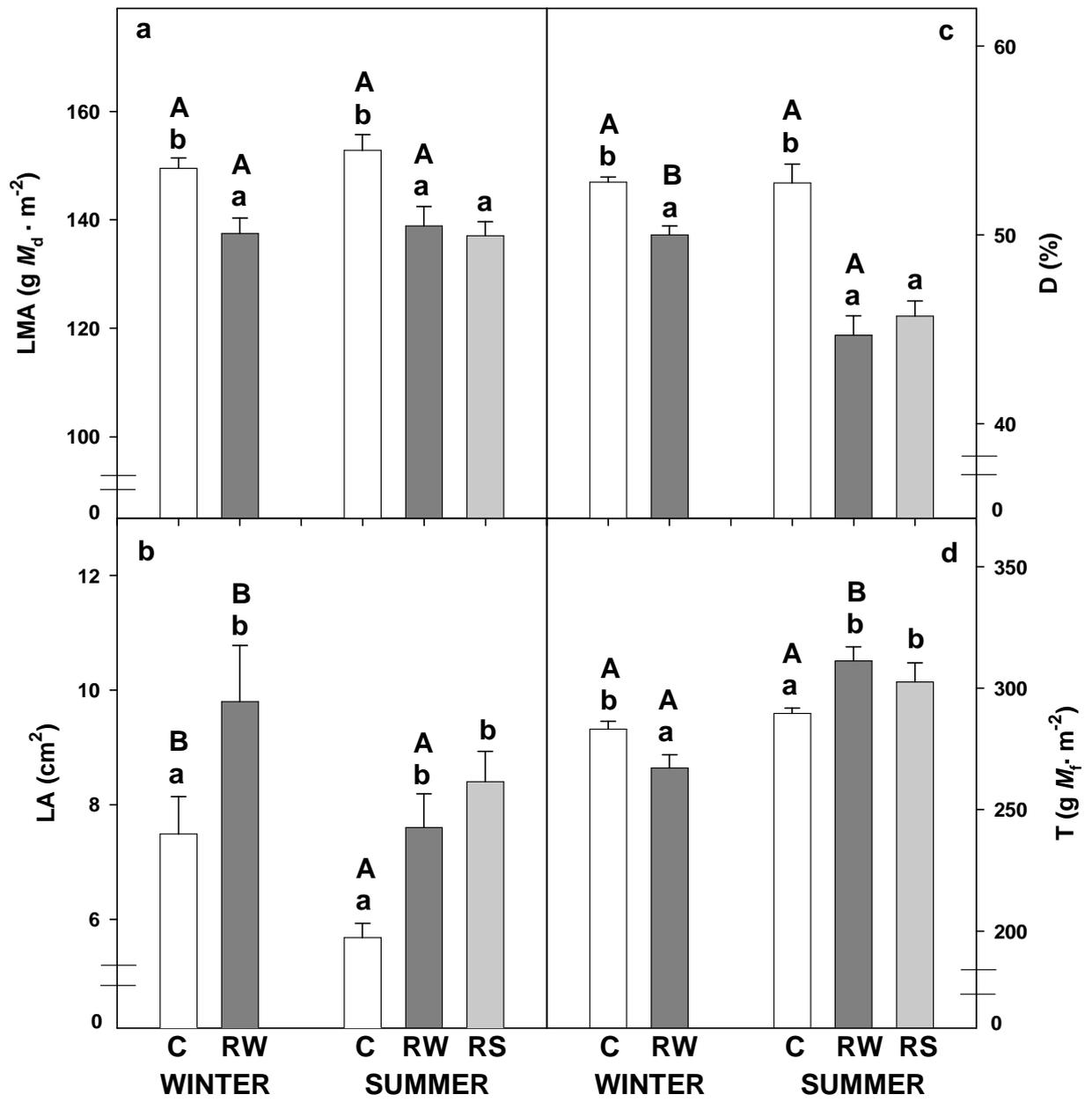


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4 Fig.1.



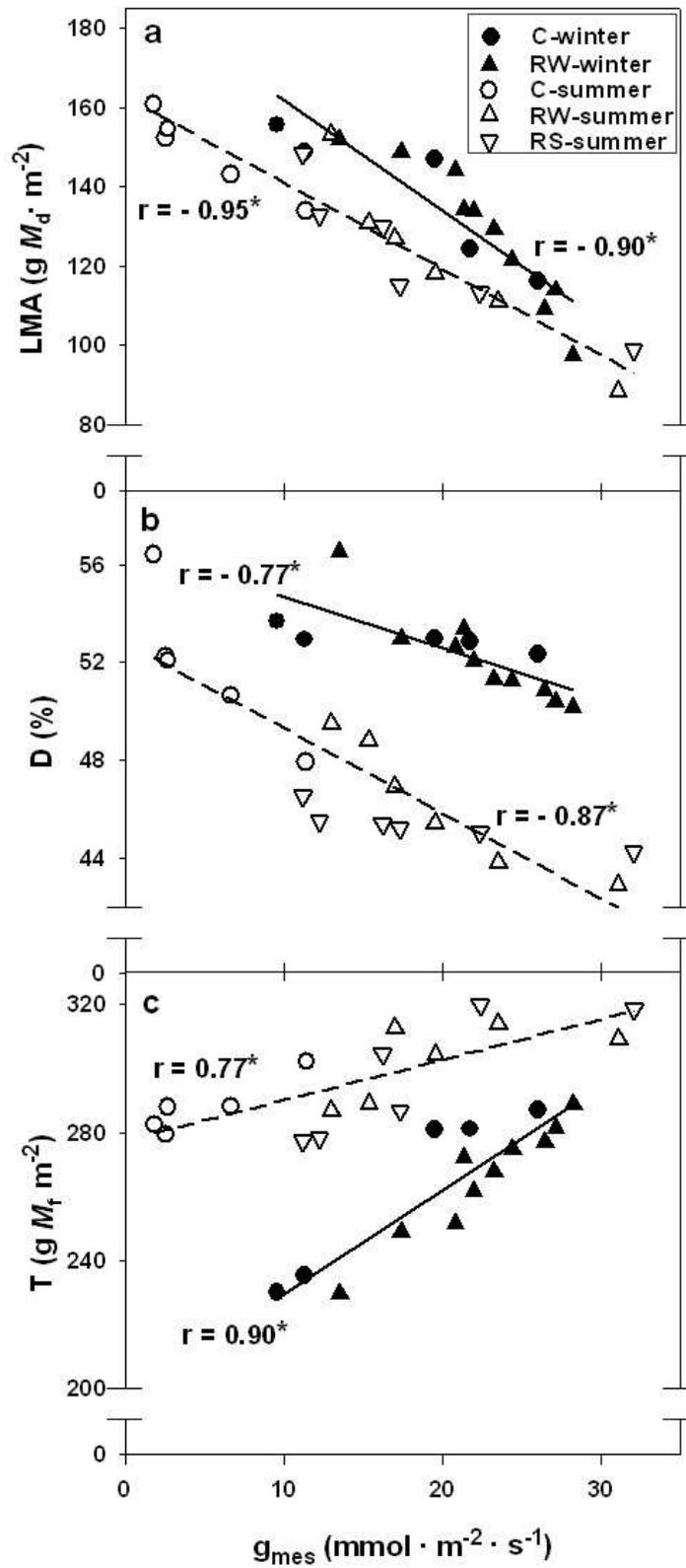
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4 Fig. 2.

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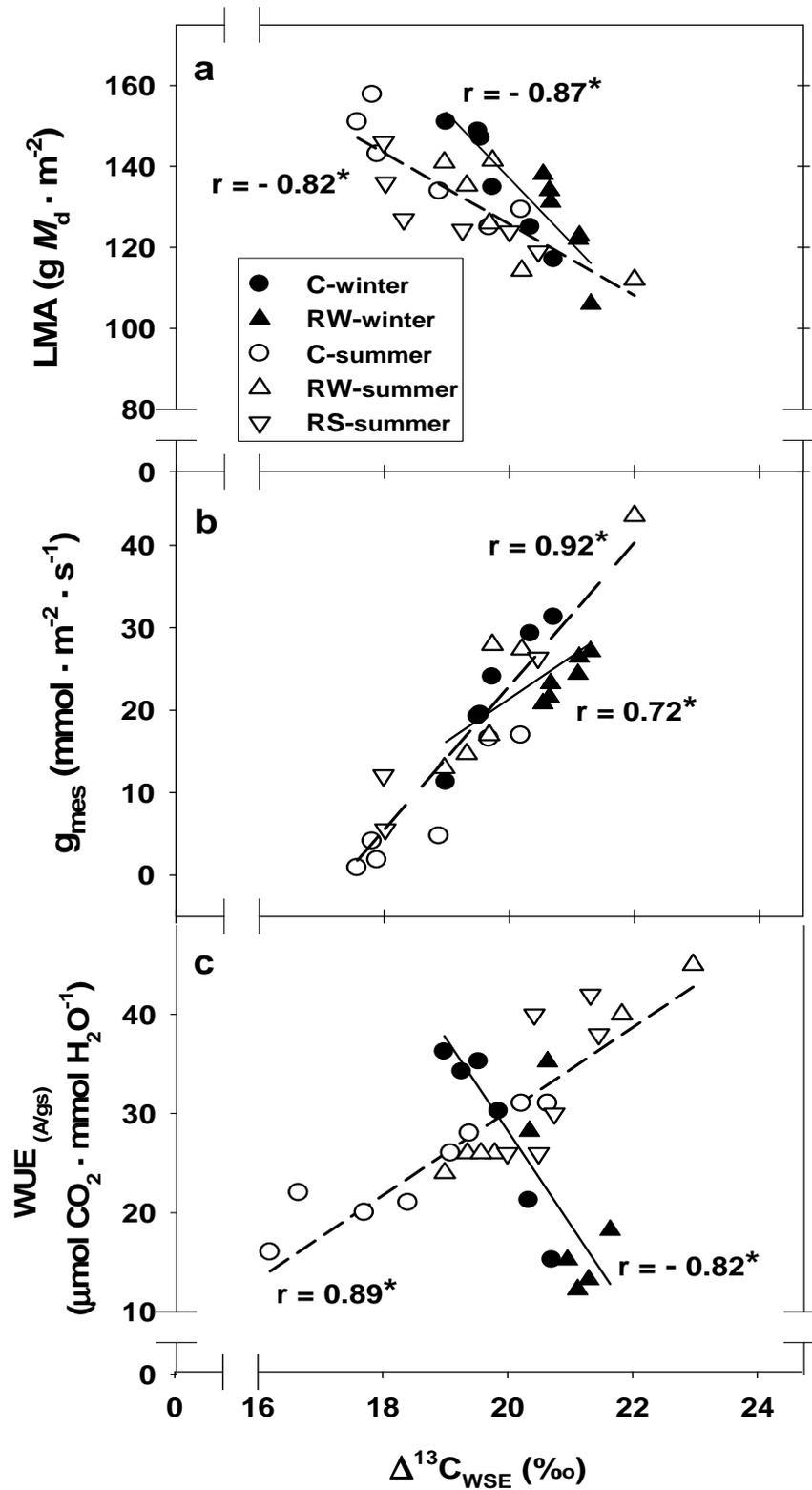


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3 Fig.4.