Foliar photochemical processes and carbon metabolism under favourable and adverse winter conditions in a Mediterranean mixed forest, Catalonia (Spain)

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Abstract:

Evergreen trees in the Mediterranean region must cope with a wide range of environmental stresses from summer drought to winter cold. The mildness of Mediterranean winters can periodically lead to favourable environmental conditions above the threshold for a positive carbon balance, benefitting evergreen woody species more than deciduous ones. The comparatively lower solar energy input in winter decreases the foliar light saturation point. This leads to a higher susceptibility to photoinhibitory stress especially when chilly (<12 °C) or freezing temperatures (<0°C) coincide with clear skies and relatively high solar irradiances. Nonetheless, the advantage of evergreen species that are able to photosynthesize all year round where a significant fraction can be attributed to winter months, compensates for the lower carbon uptake during spring and summer in comparison to deciduous species. We investigated the ecophysiological behaviour of three co-occurring mature evergreen tree species (*Quercus ilex* L.*, Pinus halepensis* Mill.*,* and *Arbutus unedo* L.). Therefore, we collected twigs from the field during a period of mild winter conditions and after a sudden

1 cold period. After both periods, the state of the photosynthetic machinery was tested in the

2 laboratory by estimating the foliar photosynthetic potential with CO_2 response curves in

3 parallel with chlorophyll fluorescence measurements. The studied evergreen tree species

4 benefited strongly from mild winter conditions by exhibiting extraordinarily high

5 photosynthetic potentials. A sudden period of frost, however, negatively affected the

6 photosynthetic apparatus, leading to significant decreases in key physiological parameters

7 such as the maximum carboxylation velocity ($V_{c,max}$), the maximum photosynthetic electron

8 transport rate (J_{max}), and the optimal fluorometric quantum yield of photosystem II (F_v/F_m).

9 The responses of $V_{c,max}$ and J_{max} were highly species-specific, where *Q. ilex* exhibited the

10 highest and *P. halepensis* the lowest reductions. In contrast, the optimal fluorometric quantum

11 yield of photosystem II (F_v/F_m) was significantly lower in *A. unedo* after the cold period. The

12 leaf position played an important role in *Q. ilex* showing a stronger winter effect on sunlit

13 leaves in comparison to shaded leaves. Our results generally agreed with the previous

14 classifications of photoinhibition-tolerant (*P. halepensis*) and photoinhibition-avoiding (*Q.*

15 *ilex*) species on the basis of their susceptibility to dynamic photoinhibition, whereas *A. unedo*

16 was the least tolerant to photoinhibition, which was chronic in this species. *Q. ilex* and *P.*

17 *halepensis* seem to follow contrasting photoprotective strategies. However, they seemed

18 equally successful under the prevailing conditions exhibiting an adaptive advantage over *A.*

19 *unedo*. These results show that our understanding of the dynamics of interspecific competition

20 in Mediterranean ecosystems requires consideration of the physiological behaviour during

21 winter which may have important implications for long-term carbon budgets and growth

22 trends.

1   Introduction

24 Mediterranean-type ecosystems are widely associated with broadleaved evergreen

25 sclerophyllous shrubs and trees, the classic vegetation types in climates where hot and dry

26 summers alternate with cool and wet winters (Aschmann, 1973; Blumler, 1991; Orshan, 1983;

27 Specht, 1969). In summer, water is undoubtedly the most important factor limiting growth

28 and survival in the Mediterranean region, whereas spring and autumn provide better growing

29 conditions (Gracia et al., 1999; Orshan, 1983; Sabaté and Gracia, 2011). In winter, the low

30 temperatures and solar radiation limit the amount of energy available for the vegetation,

31 although soil-water contents and water-pressure deficits are favourable. This highly dynamic

32 seasonality of favourable and unfavourable conditions produces a rich diversity of plants in

these regions (Cowling et al., 1996). In turn, this features a highly diverse range of traits and taxa that has produced multiple survival strategies which help to explain the abundance and distribution of species (Matesanz and Valladares, 2013). Nonetheless, the predicted reductions in annual precipitation, increases in mean temperature, and increases in the variability and occurrence of extreme droughts and heat waves in arid and semi-arid regions are likely to affect species abundance and distribution (Friend, 2010; IPCC, 2013; Somot et al., 2008). The battle for survival and dominance in plant communities facing these novel changes in their environments evokes great uncertainties and worries in the scientific community concerning the adaptive ability, distribution shifts, or, at worst local extinction of species especially in Mediterranean type ecosystems (Matesanz and Valladares, 2013; Peñuelas et al., 2013).

In this context, a pivotal role devolves on the winter period in Mediterranean type- climates as mild winter temperatures can suddenly provide potential periods of growth and recovery from stressful summer drought periods, above all for evergreen trees. Thus, the success in the future dynamics of competition and novel environmental conditions will not only depend upon the tolerance to withstand abiotic stresses, but also on their effectiveness to benefit rapidly from periods when environmental conditions may be favourable such as in winter. The effective acclimation of the photosynthetic apparatus during winter was hereby in the focus of interest for this study. This acclimation is particularly essential for evergreen tree species in order to compensate for their lower photosynthetic rates during the growth period, relative to deciduous species. Plants have evolved diverse adaptive mechanisms to cope with the consequences of stress and to acclimate to low temperatures (Blumler, 1991; Öquist and Huner, 2003).

Hereby, mixed forests provide us with an ideal test-bed for investigating the different ecophysiological strategies and their sensitivities to abiotic stresses, because all tree species have to contend equally with the yearly variability of environmental conditions. Nevertheless, most ecophysiological studies have been conducted in spring and summer, and winter has been surprisingly overlooked despite its importance for our understanding of the dominance of certain vegetation-types and of the responses of vegetation to stress, seasonality, and species composition (Oliveira and Peñuelas, 2004; Orshan, 1983; Tretiach et al., 1997). Even though efforts have recently been made to elucidate the behaviour of sclerophyllous ecosystems under variable winter conditions (e.g. García-Plazaola et al., 1999, 1997;

Kyparissis et al., 2000; Levizou et al., 2004; Martínez-Ferri et al., 2004; Oliveira and Peñuelas, 2004, 2000), the physiological behaviour of co-occurring species of evergreen trees in the Mediterranean region, including leaf gas exchange (GE) and chlorophyll fluorescence (CF) methods, have been insufficiently studied for understanding the dynamics of photoinhibitory stress and interspecific competition. Therefore, in our study we used an ample set of parameters from GE & CF measurements in order to provide a snapshot in the plant's physiology and in order to characterize in detail the effects on the photosynthetic light and carbon reactions during winter (Flexas et al., 2008; Guidi and Calatayud, 2014). This study was conducted on three species of evergreen trees (*Quercus ilex* L., *Pinus halepensis* Mill., *Arbutus unedo* L.) in northern Catalonia near Barcelona, Spain.

Our aims were to i) investigate the foliar physiology of these three species under mild winter conditions, ii) analyse the effect of sudden changes from favourable to unfavourable conditions on photochemical and non-photochemical processes associated with electron transport, CO_2 fixation, and heat dissipation, iii) determine if leaves exhibit distinct locational (sunlit or shaded) responses to winter stress, and iv) identify the species-specific strategies when coping with stress, induced by low temperatures and frost. These topics are of particular interest due to the recent report of an increased dominance of angiosperm trees and the negative impacts on pines over extensive areas of the Iberian Peninsula (Carnicer et al., 2013). Therefore, we must improve our understanding of the interactions among co-occurring tree species competing for scarce resources and trying to survive and tolerate novel environmental conditions to be able to predict ecosystem responses to global climate change.

2   Material and Methods

2.1   Field site

Our experiment was conducted at the field station of Can Balasc in Collserola Natural Park, a coastal massif (8500 ha) in the hinterlands of Barcelona, northeastern Spain (41° 25' N, 2° 04' E, 270 m a.s.l.). The forest stand at the study site has an area of 0.7 ha and is on a northeast-facing slope. The climate is characterised by typical Mediterranean seasonal summer droughts and warm temperatures, with a mean August temperature of 22.8 °C. The proximity to the Mediterranean Sea provides mild winters where frosts and snow are rare, as reflected in the mean January temperature of 7.9 °C. Mean annual precipitation and

1   temperature are 723 mm and 15.1 °C (1951-2010), respectively (Ninyerola et al., 2000). The

2   soils have predominantly developed above lithological strata of shales and granite (Sanchez-

3   Humanes and Espelta, 2011). Sensors for measuring air temperature (HMP45C, Vaisala Oyj,

4   Finland) and solar radiation (SP1110 Skye Instruments Ltd., Powys, UK) were installed at a

5   height of 3 m, in a clearing *ca.* 1 km from the plot.

6   ## 2.2   Stand history and composition of tree species

7   The history of Collserola Natural Park is typical for the area, being characterised by intensive

8   exploitation for charcoal in *Quercus*- coppice forests and for agricultural purposes such as

9   olive production until the 20th century. The abandonment of these practices at the beginning

10   of the 20th century led to forest succession and restoration with the early successional and fast

11   growing Aleppo Pine (*P. halepensis* Mill.). As in wide parts of the Mediterranean basin, this

12   tree species was favoured by forest management for its rapid growth rates and timber yields

13   (Maestre and Cortina, 2004). The cessation of forest practices in the early 1950s led to a

14   second wave of succession characterised by extensive regeneration of the evergreen Holm

15   Oak (*Q. ilex* L.) and the deciduous Pubescent Oak (*Q. pubescens* Willd.). As a result, many

16   mixed forest stands in Collserola are currently characterised by two-layered canopies

17   consisting of a dense layer from *Quercus* species surmounted by shelter trees of *P. halepensis*.

18   The forest stand at our experimental site has reached the next and final stage of forest

19   succession, where the dense *Quercus* canopy is out-competing the early successional *P.*

20   *halepensis*, simply by suppressing the growth of the light demanding pine seedlings and

21   saplings. This final stage of succession is typical of many pine-oak forest-type sites in the

22   Iberia Peninsula. *P. halepensis*. is dependent mainly on fire disturbances for natural

23   regeneration (Zavala et al., 2000). Interestingly, the diversity of tree species is enriched by the

24   scattered occurrence of Strawberry trees (*A. unedo*) in the forest canopy being usually more

25   characterised as a shrubby species widely abundant in the macchia ecosystems of the Iberian

26   peninsula (Beyschlag et al., 1986; Reichstein et al., 2002). Its existence adds an ecological

27   value to the forest due to its flowering and fruiting behaviour attracting insects and birds. It

28   raises questions about its performance as a mature tree within the interspecific competition of

29   this mixed forest. The forest diversity also encompasses a dense understory mainly consisting

30   of *Pistacia lentiscus* L., *Erica arborea* L., *Phillyrea latifolia* L., *Rhamnus alaternus* L., *Cistus*

31   spp, *Crataegus monogyna* Jacq., *Bupleurum fruticosum* L., and other less abundant species.

32   The stand at our study site has reached a highly diverse stage of forest succession and has

1   provided us with a rare set of some of the most important Mediterranean tree species growing

2   together naturally.

3   **2.3   Sampling**

4   The sampling of the mild winter period took place between 09.01.-19.01.12 (DOY 9-19). The

5   frosty/chilly period lasted from 19.01.-04.02.12 (DOY 21-35). The sampling period after the

6   frosty/chilly period took place between 14.02.-24.02.12 (DOY 45-55). We obtained sunlit

7   leaves for GE-analyses by sampling five twigs with a pruning pull from the outer part of the

8   upper third of the crown, and shaded leaves by sampling five twigs from the inner part of the

9   crown, optimally at similar heights. In the second field campaign after the frost occurrence,

10   however, we were constrained to sample shaded leaves only from *Q. ilex* due to limitation in

11   labour and equipment. The shaded leaves of *P. halepensis* and *A. unedo* could only be

12   sampled in the first, but not in the second field campaign. The twigs were immediately re-cut

13   under water in buckets in the field and transported to the laboratory retained in plastic bags to

14   minimise transpiration. Five replicates of each species were collected for the analysis of GE.

15   The twigs were pre-conditioned in the laboratory at a room temperature of 24-28 °C in dim

16   light for 1-3 d and freshly cut the following morning before the measurement of GE

17   (Niinemets et al., 1999, 2005). We intended to avoid the problems we had faced in the field,

18   such as the limited ability of the instruments to reach the standard operating temperature of 25

19   °C, which was hampered by low ambient temperatures or unpredictable plant responses such

20   as closed stomata or patchy stomatal conductance (Mott and Buckley, 1998, 2000). The pre-

21   conditioned twigs instead had a stable C_i and sufficiently high gs, which are required for

22   conducting a noise-free CO_2-response curve. The method of cutting twigs rehydrated stressed

23   leaves at optimum conditions and allowed us to analyse their long-term acclimation to the

24   environmental conditions from which they were derived. This method has been used in other

25   studies (Epron and Dreyer, 1992; Haldimann and Feller, 2004; Laisk et al., 2002; Niinemets

26   et al., 1999, 2005), and we confirmed that the leaves remained fresh and functional for several

27   days controlled by g_s and fluorescent signals (data not shown). Our ambient values of the GE-

28   and CF-derived parameters accordingly represented the "ambient capacity" of pre-conditioned

29   leaves under near-optimal ambient environmental conditions of CO_2 concentrations and

30   saturating light and at a room temperature of 20-25 °C (Reich et al., 1998).

31

2.4 GE and CF analyses

GE and CF were measured with a Li-Cor LI-6400XT Portable Photosynthesis System equipped with a LI-6400-40 Leaf Chamber Fluorometer (Li-Cor, Inc., Lincoln, NE, USA). Response curves for foliar net assimilation versus CO_2 concentration were recorded from five apparently healthy leaves per tree species and leaf position. CF was measured in parallel. *A. unedo* leaves were sufficiently large to cover the leaf cuvette (2 cm^2), whereas sunlit leaves of *Q. ilex* were in some cases too small, and the area of the leaves had to be adjusted after the measurements. For the leaves of *P. halepensis*, we positioned a layer of needles (appr. 10-15) on the leaf cuvette, avoiding gaps and overlays. The putty-like adhesive 'Blu-tack' (Bostik SA, La Plaine St Denis, France) was also used to seal the gaskets and to keep the needles in position.

2.4.1 Preparation and acclimation

Prior to recording the response curves, the temperature of the clamped leaves (T_{Leaf}) was adjusted to 25 °C, and the flow of ambient CO_2 in the leaf chamber (C_a) was set to 400 μmol CO_2 m^{-2} s^{-1} (controlled with a CO_2 mixer). The leaves were dark-adapted for 15-20 min before the measurements, and the data were logged when the GE-derived parameters such as stomatal conductance (g_s), stomatal internal CO_2 concentration (C_i) and mitochondrial respiration in darkness (R_n) had stabilised. For our purposes, dark-adaption did not necessarily mean strict prolonged darkness but referred to a sufficiently low level of ambient background light that did not cause an accumulation of reduced photosystem II (PSII) acceptors, which could be detected as an increase in fluorescence. The leaves were also pre-darkened with special leaf clips or a dark cloth to save time. The chamber light was then turned on at a saturating quantum flux density of 1000 μmol photons m^{-2} s^{-1} (20% blue LED, 80% red LED). The relatively high percentage of blue light stimulated the stomata to open (Farquhar and Sharkey, 1982; Kang et al., 2009; Niinemets et al., 2005). The relative humidity was maintained at 50% ($\pm$10%), and the air flow was maintained at 500 μmol s^{-1}. The above conditions were maintained for approximately 20-30 min until the net rate of carbon assimilation (A_{net}), g_s, and C_i of the leaf stabilised.

The GE-derived parameters A_{net}, g_s, and C_i likely require less time to stabilize, especially in healthy and unstressed leaves, but this minimum time range was necessary for the CF-derived parameters to ensure accurate measurement of the efficiency of harvesting light energy by

maximal oxidation and therefore open PSII reaction centres under ambient conditions of CO_2 and saturating light, which can be monitored by observing the stability of steady-state fluorescence (F_s). If this stability is not achieved, the effective quantum yield of PSII (Φ_{PSII}) and subsequent calculations of important parameters such as the rate of electron transport based on the CF measurement (J_{CF}) could be underestimated. After all parameters had stabilised, the steady-state GE-derived parameters and several CF-derived parameters in the light-adapted state were recorded simultaneously. F_s followed shortly afterwards by the maximum fluorescence yield in the light-adapted state (F_m') were logged by the emission of a pulse of white light at $10\,000$ mmol m^{-2} s^{-1} to close all PSII reaction centres, followed by a so-called 'dark pulse' for measuring the minimal fluorescence (F_o') of a light-adapted leaf that has been momentarily darkened. The measurement of CO_2 began after the completion of the preparation and acclimation, which required approximately 30 min in unstressed leaves and up to 2 h in stressed leaves.

2.4.2 CO_2 experiments

The CO_2-response curves were recorded at a T_{Leaf} of 25 °C and a quantum flux density of 1000 μmol photons m^{-2}s^{-1}. The values of C_a used to generate the response curves were 400 → 300 → 200 → 150 → 100 → 50 → 400 → 400 → 600 → 800 → 1200 → 2000 (in μmol CO_2 m^{-2} s^{-1}). The minimum and maximum times for stabilising A_{net}, g_s, and C_i for each log were set to 4 and 6 min, respectively.

2.4.3 Light experiments

Light-response curves (A/PPFD) were generated at a C_a of 400 μmol CO_2 m^{-2} s^{-1} by automatically applying changes in the photosynthetically active radiation with the LI-6400XT light source. To obtain precise responses at the low range of the light gradient for estimating the daily mitochondrial respiration by the Kok effect (Kok, 1948), we used the following PPFD sequence: 2500 → 2000 → 1500 → 1000 → 800 → 600 → 500 → 400 → 300 → 200 → 150 → 125→ 100 → 75 → 50 → 40 → 30 → 20 → 10 → 5 → 0 (in μmol photons m^{-2} s^{-1}). The minimum and maximum times between each light level for the generation of the A/PPFD curves were set to 1 and 2 min, respectively. The gradient from high to low light during an A/PPFD curve led to a drop in T_{Leaf} as the light decreased. The rapid changes in the light levels prevented the adjustment of T_{Leaf} while guaranteeing stable air and water fluxes and avoiding noisy measurements of C_i and g_s. We thus decided to maintain a stable Peltier-

1   block temperature (T_{block}) in the leaf cuvette. Hence, T_{block} was first adjusted so that T_{leaf} was

2   25 °C at the beginning of the A/PPFD curve and then kept stable throughout the experiment.

3   T_{Leaf} had dropped by approximately 1-3 °C by the completion of the A/PPFD curve.

4   The calculation of the parameters Fv/Fm, NPQ, qp, and temperature functions, in

5   supplementary material.

6   **2.5   Calculation of CF-derived parameters**

7   The maximum efficiency of PSII was calculated by:

8
$$\frac{F_v}{F_m} = \frac{(F_m - F_o)}{F_m} \tag{1}$$

9   where F_v is the variable fluorescence of a dark-adapted sample, F_m is the maximal

10   fluorescence measured after a saturating light pulse, and F_o is the minimal fluorescence

11   measured under darkness. These parameters were obtained from dark-adapted leaves with

12   closed PSII reaction centres as described in the previous sections. The F_v/F_m ratio describes

13   the fraction of photochemically absorbed photons under dark conditions. Typical values range

14   between 0.75 and 0.85, depending on age, health, and preconditioning. The F_v/F_m ratio

15   provides information about the maximum or optimum quantum yield and serves as indicator

16   of stress in the photosystems (Buschmann, 2007). Ratios below 0.80 are indicative of induced

17   photoprotection and sustained energy dissipation (Maxwell and Johnson, 2000; Verhoeven,

18   2014), whereas leaves with ratios below 0.74 are considered to be below the recovery

19   threshold (Björkman and Demmig, 1987). The effective quantum yield of PSII was estimated

20   by:

21
$$\Phi_{PSII} = \frac{(F_m' - F_s)}{F_m'} \tag{2}$$

22   where F_s is the steady-state fluorescence in a fully light-adapted sample, and F_m' is the

23   maximal fluorescence yield after a pulse of high light. The Φ_{PSII} is the counterpart of the

24   optimum quantum yield and represents the fraction of photochemically absorbed photons in a

25   light-adapted leaf (Maxwell and Johnson, 2000).

26
$$J_{CF} = \varepsilon * \Phi_{PSII} * \alpha_L \tag{3}$$

27   where ε is a scaling factor for the partitioning of intercepted light between photosystems I and

28   II. We assumed that light was equally distributed between both photosystems ($\varepsilon = 0.5$)

1 (Bernacchi et al., 2002; Niinemets et al., 2005). α_L is the foliar absorbance determined in

2 separate measurements of foliar reflectance and transmittance. The following values of α_L

3 were determined: 0.932 for *Q. ilex* and 0.912 for *P. halepensis*, with no differences between

4 sunlit and shaded leaves of these two species, and 0.935 for sunlit leaves of *A. unedo*, and

5 0.917 for shaded leaves of *A. unedo*. For the determination of these leaf absorptances (α_L),

6 foliar reflectance and transmittance were measured at midday in August 2012 using a

7 spectroradiometer UniSpec Spectral Analysis System (PP Systems, Haverhill, MA, USA).

8 The value of J_{CF} at a CO_2 concentration of 400 μmol CO_2 m^{-2} s^{-1} and a PPFD of 1000 μmol

9 photons m^{-2} s^{-1} was termed ambient photosynthetic electron transport (J_{amb}). Its relationship

10 with the net assimilation rate (J_{amb}/A_{net}) was used for the analyses of alternative electron sinks

11 beside carbon metabolism.

2.6 Estimation of light respiration and calculation of the effective quantum yield of CO_2 (Φ_{CO2})

14 In the literature, the term R_d was sometimes used for dark respiration (Farquhar et al., 1980;

15 Turnbull et al., 2003), but also for day respiration (Flexas et al., 2012; Yin et al., 2011). We

16 will use R_d to represent mitochondrial respiration during the day or under lighted conditions

17 and R_n to represent mitochondrial respiration at night or under dark-adapted conditions. We

18 estimated R_n during the day after darkening the leaf for at least 30 min. R_d was estimated from

19 the light-response curves with the combined GE and CF measurements proposed by Yin et al.

20 (2009), named the CF method. This method amended the Kok method (Kok 1948) by

21 substituting the A/PPFD relationship with A/PPFD * Φ_{PSII} (Yin et al., 2009). See reference for

22 details.

23 The effective quantum yield of CO_2 (Φ_{CO2}, unitless) can be calculated using the estimated α_L,

24 R_d, together with A_{net} and PPDF as follows

25 $$\Phi_{CO_2} = \frac{(A_{net} - R_d)}{PPFD * \alpha_L} \qquad (4)$$

2.7 The Farquhar, von Caemmerer, and Berry (1980) photosynthesis model (FvCB)

27 The FvCB photosynthesis model was employed on the assumption that foliar carbon

28 assimilation was limited either by Rubisco activity (A_c) or by ribulose-1,5-bisphosphate

29 (RuBP) regeneration (A_j) and was driven by light, temperature, and CO_2. The model was

1 further complemented with a third limitation: the photosynthetic rate limited by triose-
2 phosphate use (A_p) (Sharkey, 1985). A_{net} can then be determined by the minimum of these
3 three potential rates from an A/C_c curve:

4 $$A_{net} = min\{A_c, A_j, A_p,\}$$ (5)

5 where

6 $$A_c = V_{cmax} * \left[\frac{C_c - \Gamma^*}{C_c + K_c\left(1 + \frac{O}{K_O}\right)} \right] - R_d$$ (6)

7 where $V_{c,max}$ represents the maximum rate of Rubisco carboxylation, K_c is the Michaelis-
8 Menten constant of Rubisco for CO_2, O is the partial pressure of O_2 at Rubisco, and K_o is the
9 Michaelis-Menten constant of Rubisco for O_2 (Table C1, see Appendix C) and C_c determined
10 with the variable J method (Equ. A7 and A8, see Appendix A). The equation representing
11 photosynthesis limited by RuBP regeneration is:

12 $$A_j = J * \left[\frac{C_c - \Gamma^*}{4C_c + 8\Gamma^*} \right] - R_d$$ (7)

13 where J is the rate of electron transport. The denominator of the above equation represents the
14 stoichiometry of the number of electrons required to regenerate ATP and NADP; we have
15 used four for C_c and eight for Γ^* (Flexas et al., 2012). J becomes J_{max} under light and CO_2
16 saturation when the maximum possible rate of electron transport is theoretically achieved.

17 The photosynthetic rate limited by triose-phosphate use is estimated by:

18 $$A_p = \frac{3TPU * C_c}{\Gamma^*\left[C_c - \left(\frac{1 + 3\alpha_{TPU}}{2}\right)\right]} - R_d$$ (8)

19 where TPU is the rate of triose-phosphate use at saturating CO_2 concentrations, and α_{TPU} is
20 the proportion of glycerate not returned to the chloroplasts. This equation fits the A/C_c curve
21 plateau at high concentrations of CO_2 when a further increase in C_c no longer increases A_{net}
22 or, in some cases, decreases A_{net}.

23 These three estimated parameters ($V_{c,max}$, J_{max}, and TPU) define the biochemical capacity to
24 drive the photosynthetic assimilation of CO_2 but are defined here as the photosynthetic
25 potential (Niinemets et al., 2006). The term photosynthetic capacity is here dismissed, despite
26 its frequent use in the literature, to avoid confusion with studies that have used this term for

1   the maximum rate of assimilation under saturating light conditions (e.g. Bertolli and Souza,

2   2013).

3   2.8   Curve fitting

4   The procedure for fitting the curves to estimate the photosynthetic parameters $V_{c,max}$, J_{max}, and

5   TPU applied the least square fit method using the SOLVER estimator tool in Excel. In this

6   procedure, the squared errors of the observed points on the A/C_c curve and the modelled

7   points of Eq.(s) 6, 7, and 8 were calculated and summed. Prior to the fitting procedure, the

8   user must assess the limiting factors, i.e. which points are allocated to which Eq. (6 or 7 or 8).

9   The initial slope of the A/C_c curve is attributed to non-saturating CO_2 conditions when

10   Rubisco activity limits A_{net} (Eq. 6), while the slope of the curve is smoothed at higher CO_2

11   conditions (usually > 35 Pa), representing the limitation of the regeneration of ribulose-1,5-

12   biphosphate (RuPb) (and hence light is a limiting factor) (Eq. 7). The transition zone

13   (approximately at 25-35 Pa of C_i), however, is a grey zone where one point can be attributed

14   to either one or another limitation. These points can also introduce noise in the estimations in

15   cases of doubt and are best discarded. Moreover, unusual points with evidence of an error

16   during the measurements were not included in the curve-fitting procedure. At very high CO_2

17   concentrations, the A/C_c curve plateaus or even decreases slightly. In this case, these points

18   can be attributed to the limitation of triose-phosphate use (Eq. 8). The CO_2 response curves,

19   however, rarely exhibit such a plateau or decrease at high CO_2 concentrations when working

20   on a C_c rather than a C_i basis, so TPU could seldom be estimated in our study. Finally, when

21   attributing all observed points to one or another limitation, we could then estimate the values

22   of $V_{c,max}$ and J_{max} (and possibly TPU) with the SOLVER Excel tool, which iteratively changes

23   the three parameters to minimise the sum of squares of deviation from the observation.

24   2.9   Correction for diffusion leakage

25   Large gradients between the ambient air and the CO2 concentrations inside the chamber are

26   created during the generation of a carbon-response curve. This leakage is particularly

27   important at the high and low ends of the carbon-response curve when a large CO2-

28   concentration gradient exists between the leaf chamber and the surrounding ambient

29   concentration. Based on the findings by Flexas et al. (2007a), we corrected Anet by

30   subtracting the diffusion leakage for each step of the A/Cc curve obtained from separate

31   response curves with leaves thermally killed in hot water.

2.10 Statistical Analyses

All statistical analyses were performed using the R software package, version 3.0.2 (http://www.r-project.org/). Differences in the parameters between the mild and cold winters were determined with Student's t-tests ($P \leq 0.05$). Shapiro-Wilk tests of normality tested for normality of the data. Data were normalised at $P \leq 0.1$. One-factorial analyses of variance (ANOVAs) with tree species as the main factor tested for differences between tree species of the parameters in the sampling periods. Significant differences were determined at $P \leq 0.05$ with Tukey's HSD tests. Regression analyses were conducted to study the relationship between J_{max} and $V_{c,max}$ and between J_{amb} and A_{net}. Analyses of covariance (ANCOVAs) tested for differences in slopes and intercepts.

3 Results

3.1 Environmental Variables

Collserola Natural Park experienced extremely mild winter conditions in November and December 2011 and January 2012, when average minimum temperatures (10.4 °C in November, 5 °C in December, and 3.4 °C in January) remained above 0 °C and no frosts occurred. Average maximum temperatures were 16.3 °C in November, 12.2 °C in December, and 11.4 °C in January. All species had considerable shoot growth of up to 15 cm during this mild period. Sudden low temperatures, however, led to frost on six consecutive days and a minimum average temperature of -2.3 °C (Day of the year (DOY) 21-26) followed by eight days of cool temperatures averaging +2.6 °C (DOY 27-35) (Figure 1). The average radiation during first field campaign (DOY 9-19) was 46 and during the period of frost 58 W m^{-2}.

3.2 Photosynthetic potentials

Of the three photosynthetic parameters describing the photosynthetic potential, $V_{c,max}$ and J_{max}, and TPU, only the first two could be satisfactorily estimated from the A/C$_c$-response curves. The leaves were only occasionally limited by TPU (6 out of 42), despite the excessive CO_2 concentrations in the higher section of the CO_2-response curve. TPU was therefore discarded from further analysis. $V_{c,max}$ and J_{max} were highest in *Q. ilex* but more importantly also decreased most strongly after the period of frost by nearly 50% ($P \leq 0.05$; Fig. 2). The photosynthetic potential of *P. halepensis* was affected the least, reflected by moderate decreases in $V_{c,max}$ and J_{max} (16% and 19%), which were not significant. $V_{c,max}$ and J_{max} were

lowest in *A. unedo* during the mild winter period and decreased by approximately 33% after the period of frost. This decrease, however, was not significant due to a large standard error.

3.3 GE-derived parameters under ambient conditions

The period of frost had a strong effect on several GE-derived parameters in *Q. ilex* leaves. The cold temperatures decreased R_n in *Q.ilex* leaves, but the effect was much weaker than for R_d and was not significant (Fig. 3). These parameters responded very weakly to the cold and frost in the leaves of *A. unedo* and *P. halepensis*. A_{net} and Φ_{CO2} were also reduced in *Q. ilex* leaves by approximately 50%. This was significant for the A_{net} (Fig. 4A) and low significant for Φ_{CO2} (Fig. 4B). Further differences were only significant for Φ_{CO2} in *P. halepensis* leaves being reduced by 12 % ($P \leq 0.05$). The CO_2 conductance was more strongly reduced in g_m than in g_s for *Q. ilex* and *A. unedo* leaves which was only significant for the former whereas these parameters seemed unaffected in *P. halepensis* leaves (Fig. 5A and 5B). As a consequence, we observed a tendency of a C_i- increase in parallel with a C_c- decrease in *Q. ilex* and *A. unedo* leaves due to a lower CO_2 uptake in carbon metabolism, but not in *P. halepensis* (Fig. 6A and 6B). The differences observed were not significant ($P \leq 0.05$).

3.4 CF-derived parameters under ambient conditions

The GE-derived parameters enabled us to study the immediate responses, but several CF-derived parameters allowed us to determine in more depth the physiological changes in parts of the light-harvesting apparatus, namely PSII. F_v/F_m estimates the maximum quantum yield of PSII and serves as a stress indicator (Fig. 7B). *A. unedo* leaves were most strongly affected by the period of frost, followed by *Q .ilex* leaves, whereas *P. halepensis* leaves were only marginally affected. The changes were not statistically significant in the latter two species ($P \leq 0.05$). Φ_{PSII} tended to decrease in all species but most strongly in *Q. ilex* leaves (42 %), however insignifcantly (Fig. 7A). NPQ responded very differently in the three species. NPQ did not change much between the two sampling periods in the leaves of *P. halepensis* (6%) but decreased significantly by 25% ($0.05 \leq P \leq 0.1$) in *A. unedo* leaves and tended to increase in *Q. ilex* leaves by 31% ($P \geq 0.05$), however insignificantly (Fig. 8).

3.5   Relationships of foliar photosynthetic variables

The covariance of several relationships of the foliar photosynthetic variables were analysed in an ANCOVA to test for differences in the slopes and intercepts in these relationships. The ANCOVA for the relationship between $V_{c,max}$ and J_{max} in *Q. ilex* leaves indicated a highly significant ($P \leq 0.01$) reduction in the slope and also intercept showing a similar strong effect on J_{max} than on $V_{c,max}$ due to the change in weather (Fig. 9A and Table 2). In P. halepensis, the slope was significantly and the intercept marginal significantly reduced (Fig. 9B and Table 2). This shows a comparatively stronger effect on $V_{c,max}$ than on J_{max} by the cold period. The sunlit leaves of A. unedo and the shaded leaves of Q. ilex did not show any significant changes in the relationship of $V_{c,max}$ and J_{max} (Fig. 9A, 9C and Table 2). The relationship between the rate of electron transport at ambient conditions derived from CF and the CO_2 assimilation at ambient CO_2 concentrations (J_{amb}/A_{net}) was similar in all tree species (Fig. 10A,B,C and Table 2). The slopes were higher in response to the stress imposed by the low temperatures but were not significant. When all species were combined the change of the slope was marginally significant, indicating a possible increased alternative electron sink other than carbon metabolism (Table 2).

3.6   Role of leaf position

Under mild conditions, the leaves of *Q. ilex* showed the most strongly pronounced differences in the leaf position (data of *P. halepensis* Mill. & *A. unedo* L. not shown). Leaves of *Q. ilex* growing under high irradiances had a more active carbon metabolism (A_{net}, R_d, R_n, and Φ_{CO2}), photochemical efficiency (Φ_{PSII}), and photosynthetic potential (high J_{max} and $V_{c,max}$) in all tree species. As described in *Material and Methods* the effect of the leaf position after the sudden cold period was only studied for *Q. ilex*. After the sudden frost period, the photosynthetic potential was much higher in sunlit than in shaded leaves of *Q. ilex*, with both J_{max} and $V_{c,max}$ being highly significant (Fig. 2 and Table 1). These differences disappeared after the cold period, because J_{max} and $V_{c,max}$ in the shaded leaves remained unaffected by the frost. F_v/F_m was generally higher in the shaded leaves, but not significantly ($P \leq 0.05$) (Fig. 8 and Table 1). The photosynthetic parameters under ambient conditions, such as A_{net}, g_s, C_i, C_c, and g_m, were not affected much by the leaf position (Fig. 4, 5, 6 and Table 1). Although not significant, the effects of the cold period on these parameters were stronger in the sunlit leaves. In comparison to these parameters, the leaf position had more pronounced effects on R_n and R_d (Fig. 3 and Table 1). The response of respiration to winter stress, however, differed

1 depending on the location of the leaves. R_n maintained the same balance between sunlit and

2 shaded leaves before and after the cold period, but R_d decreased comparatively more in sunlit

3 leaves due to the period of frost. This pattern was also reflected in Φ_{CO2} (Fig. 4B and Table 1)

4 and in the CF-derived parameters Φ_{PSII} and NPQ, (Fig. 7A, 8 and Table 1) indicating a

5 stronger effect on the photochemical machinery of sunlit leaves than on shaded leaves.

6 Shaded leaves also exhibited a lower J_{amb}/A_{net} ratio, but the ratio increased equally in both leaf

7 positions after the cold period, indicating a similar behaviour of dissipating energy by

8 alternative electron sinks (Fig. 10A and Table 1).

9 **4   Discussion**

10 **4.1   Winter in the Mediterranean region**

11 Mediterranean-type ecosystems are exposed to stress from summer droughts but also from

12 low temperatures in winter (Mitrakos, 1980). Less attention, however, has been paid to the

13 degree and extent as well as the wide variation among years and regions of these stress

14 periods, in response to which Mediterranean evergreen species have developed a dynamic

15 photoprotective ability in order to withstand these stressors (Kyparissis et al., 2000; Martínez-

16 Ferri et al., 2004). Despite the occurrence of lower temperatures than in spring conditions, in

17 winter the photosynthetic potential recovered once the leaves became acclimated to the new

18 conditions (Dolman et al., 2002; Hurry et al., 2000). This is important for the plants overall

19 performance because the photosynthetic exploitation of favourable conditions in winter is

20 crucial for achieving a positive carbon balance in Mediterranean evergreen tree species

21 (García-Plazaola et al., 1999b; Martínez-Ferri et al., 2004). We showed how a long lasting

22 comfortable winter period without frost lead to notably high photosynthetic potentials and

23 carbon assimilation in winter being equal to or partly even exceeding spring values (Sperlich

24 et al, unpublished data). As a result, increased winter temperatures influenced phenological

25 responses, advanced winter cambium activation, spring bud burst and leaf unfolding which

26 has been reported in an increasing number of studies (Peñuelas and Filella, 2001). These

27 observations were also reflected in the high sap flow per tree (J_t), ranging for all tree species

28 on average between 5 and 10 kg d^{-1} during the mild winter period (Sánchez et al., unpublished

29 results). Whereas sudden frosts have often been attributed to higher altitudes of the

30 Mediterranean region (Blumler, 1991; Tretiach et al., 1997), we showed that it can also be an

31 important factor for plant growth and distribution in other areas such as the sub-humid

32 Mediterranean climate of our study site (Garcia-Plazaola et al., 2003a). At night when frosts

16

1 are more likely to occur, we observed the lowest temperatures whereas at daytime the

2 temperatures were often above zero degrees. However, as we showed, not only cool daytime

3 but also cool nighttime temperatures or frosts can affect subsequent daytime photosynthesis

4 and induce photoprotective processes (see also Flexas et al., 1999). In our study, the sudden

5 occurring low temperatures affected strongly the photosynthetic apparaturs, although the

6 responses were highly species specific. We will elucidate the physiological mechanism in the

7 following.

8 **4.2   PSII – primary target of stress induced by low temperatures**

9 Typically in winter there is an imbalance between light energy absorbed in photochemistry

10 and light energy used in metabolism. This is shown in our data by increased thermal energy

11 dissipation (NPQ) and reduced PSII efficiency (Φ_{PSII}) in order to reduce the harmful effects of

12 excess energy reflecting an inactivation and damage of PSII reaction centres, more precisely,

13 the reaction-centre protein D1 (Aro et al., 1993; Demmig-Adams and Adams, 1992; Mulo et

14 al., 2012). More precise information about the underlying processes that have altered this

15 efficiency is provided by the F_v/F_m ratio. Chronic changes occurring in the F_v/F_m ratio can be

16 related to a cascade of processes which are induced to protect the photosynthetic apparatus

17 including i) re-organisation of the thylakoid membrane, ii) closure of reaction centres, iii)

18 and/or reduced antennal size (Ensminger et al., 2012; Huner et al., 1998; Maxwell and

19 Johnson, 2000; Verhoeven, 2014). The small changes in the F_v/F_m ratio observed in the leaves

20 of *Q. ilex* and *P. halepensis* reflected photoprotective responses without any photodamage.

21 The significantly decline of F_v/F_m in *A. unedo*, however, indicated strong chronic

22 photoinhibition and is an indication of severe photodamage (Martínez-Ferri *et al.*, 2004). We

23 conclude that *A. unedo* suffered most notably from the low temperatures whereas *Q. ilex* and

24 *P. halepensis* were equipped with a good photoprotective capacity able to keep the

25 photosynthetic apparatus intact (Öquist and Huner, 2003). *Q. ilex* showed the most dynamic

26 responses, negating the harmful excitation stress by lowering the photochemical operating

27 efficiency (Φ_{PSII}) and increasing the use of alternative thermal-energy pathways (NPQ). This

28 photoprotective capability represented by a higher NPQ is usually linked to the xanthophyll

29 cycle that responds to environmental factors such as temperature, water deficit, and nutrient

30 availability (Demmig-Adams and Adams, 1996; García-Plazaola et al., 1997). Inter-

31 conversions of the cycle and pool sizes occur following the need to dissipate excess excitation

32 energy in response to summer drought (García-Plazaola et al., 1997; Munné-Bosch and

Peñuelas, 2004), but also to winter stress (Corcuera et al., 2004; Garcia-Plazaola et al., 2003a; Kyparissis et al., 2000; Oliveira and Penuelas, 2001). The implicit interpretation of being equipped with a high capacity of photoprotection when NPQ increases was recently questioned by Lambrev et al. (2012). This study reported that quenching and photoprotection were not necessarily linearly related and stated that several possibilities of photoprotective responses other than NPQ of CF existed, such as antennal detachment that could possibly vary with species and growth conditions. The highly dynamic and photoprotective capability of *Q. ilex* leaves, however, was also demonstrated by several other photosynthetic parameters such as $V_{c,max}$, J_{max}, A_{net}, Φ_{CO2}, and R_d, which confirmed this trend and were in accord with the findings by Corcuera *et al.* (2004). Despite reports of several mechanisms of resistance to drought stress in *A. unedo*, including increased levels of zeaxanthin that indicates an enhanced thermal dissipation of excess excitation energy in periods of summer stress (Munné-Bosch and Peñuelas, 2004), we found that *A. unedo* leaves had a lower capacity of photoprotection in response to induced over-excitation of the photosystems by winter stress.

4.3 High photosynthetic potentials and strong effects of low temperatures

$V_{c,max}$ and J_{max} were strongly correlated (Wullschleger, 1993), being regulated in a coordinated manner above all in *Q. ilex*. Interestingly, the ANCOVAs indicated that J_{max} decreased more strongly than did $V_{c,max}$. This is because the above described photoprotective adjustments lead to a lower energy-use efficiency in the reaction centres and consequently also to a downregulation of the photosynthetic electron transport J_{max}. The larger decrease of J_{max} relative to $V_{c,max}$ indicated that low temperature stress became manifest first in a hampered pathway of photochemical energy, because PSII complexes are primarily affected by light-induced damage (Maxwell and Johnson, 2000; Taz and Zeiger, 2010; Vass, 2012). Hence, the limitations of the photosynthetic rate by RuBP regeneration are stronger affected by frost and cold induced stress than those by RuBP carboxylation. The relative amounts of photosynthetic proteins can probably explain the differences observed in the $J_{max}/V_{c,max}$ ratio (Hikosaka et al., 1999; Onoda et al., 2005).

The physiological responses were highly species-specific. *Q. ilex* leaves responded with significant decreases (approximately 50%) in their photosynthetic potentials (both $V_{c,max}$ and J_{max}). In contrast, $V_{c,max}$ and J_{max} decreased in *P. halepensis* leaves by only 16 and 19%, respectively, and in *A. unedo* leaves by approximately 30% (for both parameters).

4.4 Inhibition of carbohydrate metabolism

As demonstrated above, adjustments to the frost event took place via the energy flow in the antennal systems and a downregulation of photosynthetic electron transport as well as regulatory mechanisms including the inhibition of Rubisco activity, but also via stomatal and mesophyllic diffusion behaviour (Ensminger et al., 2012; Gratani et al., 2000; Taz and Zeiger, 2010). Interestingly, the mesophyllic diffusion resistance was stronger pronounced as a response to low temperatures, especially in *Q. ilex* reducing the CO_2 available for fixation in the chloroplasts. This underlines the recently growing awareness in the scientific community about the important role of g_m as an additional regulating parameter as response to stress, above all in sclerophyllic species (Flexas et al., 2008; Niinemets et al., 2011). In general, our results demonstrated that the efficiency of carbon use in the photosynthetic metabolism and foliar respiratory responses were highly species dependant (Zaragoza-Castells et al. 2007, 2008). For instance, *P. halepensis* and *Q. ilex* leaves depicted extraordinarily high values of A_{net}, R_d, R_n, and Φ_{CO2} in the mild winter period, but only *Q. ilex* exhibited a significant downregulation after the frost event. The downregulation of photosynthesis, the most efficient process to get rid of excess energy, suggests alternative energy pathways such as photorespiration. We did not measure photorespiration directly, but we could infer some of its characteristics by studying the relationship between J_{amb} and A_{net}. All tree species had a relatively higher proportion of electron flux during the period that can be explained by utilization in the carbon metabolism. This has been mainly attributed to photorespiration, but also to the Mehler reaction that protects plants from photodamage in bright light (Allen and Ort, 2001; D'Ambrosio et al., 2006; Flexas et al., 1998, 1999; Fryer et al., 1998; Huner et al., 1998).

4.5 Leaf position specific responses to abiotic stress in winter

It is well know that leaves growing under high irradiances have a more active carbon metabolism (A_{net}, R_d, R_n, and Φ_{CO2}), photochemical efficiency (Φ_{PSII}), and photosynthetic potential (high J_{max} and $V_{c,max}$) (Taz and Zeiger, 2010). Hereby, *Q. ilex* showed the most strongly pronounced differences between sunlit and shaded leaves. Plants develop leaves with a highly specialised anatomy and morphology for the absorption of the prevailing light in their local environments resulting generally in smaller but also thicker sunlit leaves (Kull and Niinemets, 1993; Terashima and Hikosaka, 1995). Nevertheless, the higher carbon metabolism and photochemical activity of sunlit leaves decreased strongly, partly below the

level of shaded leaves, whereas shaded leaves showed little sign of any downregulation but maintained a relatively stable effective quantum yield of CO_2 assimilation in both periods. Furthermore, the photosystems showed no sign of photodamage and generally maintained a higher maximum efficiency than did sunlit leaves. We concluded that foliar-level physiology during winter was better protected in the shaded crown of *Q. ilex* unexposed to the dramatic changes in radiation in the outer canopy, confirming the results by Valladares et al. (2008). We also concluded that *Q. ilex* is a highly dynamic species able to rapidly change its metabolism on the antioxidant and photoprotective level in dependence to its leaf position (García-Plazaola et al., 1997, 1999a; Martínez-Ferri et al., 2004). We show that the foliar plasticity in morphology and anatomy of *Q. ilex* (Bussotti et al., 2002; Valladares et al., 2000) can also be attributed to its biochemical metabolism. We stress that the solar environment of the leaves is a crucial factor when assessing tree performance, especially when comparing tree species in a competitive context.

4.6 Ecological context

Q. ilex had the most drastic photoprotective response to frost and cool temperatures, whereas *P. halepensis* exhibited a homeostatic behaviour with a very active carbon assimilatory and respiratory metabolism in both periods. *A. unedo* was intermediate, with large decreases in the parameters of carbon metabolism but also a high variability in its response to frost. *A. unedo* also had the lowest photoprotective capability, which might be explained by previous characterisations to be semi-deciduous to drought being at the borderline to evergreen sclerophyllous species (Gratani and Ghia, 2002a, 2002b). Moreover, *A. unedo* occurs naturally most commonly as a shrub and is less frequently found in the forest canopy of mixed forests growing up to 8-10 m tall as in our study site (Beyschlag et al., 1986; Reichstein et al., 2002). Investments in leaves are thus lower and leaf longevity shorter. Leaves of *A. unedo* are more rapidly replaced relative to more sclerophyllic leaves such as those of *Q. ilex*. We postulated that *A. unedo*, considered a relict of the humid-subtropical Tertiary tree flora, was more sensitive to winter stress, which is consistent with its presence mostly in the western Mediterranean basin and its frequent occurrence in coastal zones where humidity and temperature are the main factors determining its geographical distribution (Gratani and Ghia, 2002a and references therein). Our results suggested that *Q. ilex* could greatly benefit from favourable winter conditions exhibiting a high photosynthetic potential and carbon metabolism. Angiosperms are known to make efficiently use of favourable winter

periods to recover depleted carbon reserves and embolism induced loss of hydraulic capacity (Carnicer et al., 2013 and references therein). When these relatively favourable conditions changed, *Q. ilex* quickly re-adjusted the photosynthetic machinery to the prevailing conditions, as indicated by the largest decreases in photosynthetic potential and carbon metabolism. Some researchers have proposed the lutein-epoxy cycle in photoprotection of *Quercus* as a mechanism to maintain sustained energy dissipation (Garcia-Plazaola et al., 2003b), which could help to account for the higher tolerance to low temperatures in *Q. ilex* relative to other co-occurring Mediterranean trees or shrubs (Ogaya and Peñuelas, 2003, 2007). *P halepensis* did not suffer a pronounced chronic photoinhibition, confirming the results by Martínez-Ferri *et al.* (2004). Despite a pronounced downregulation of photosynthetic electron transport and an increase in alternative electron sinks, the light-saturated ambient photosynthesis and stomatal conductance remained surprisingly high and constant. *P. halepensis* thus exhibited a successful refinement of photosynthetic electron flow and possibly a successful repair of protein D1 in the PSII reaction centre. The strong downregulation in *Q. ilex* and the homogenous response of *P. halepensis* were possibly due to distinct, previously described strategies. *Q. ilex* has been characterised as a photoinhibition-avoiding species and *P. halepensis* as a photoinhibition-tolerant species (Martinez-Ferri et al., 2000). We have extended this categorisation for *A. unedo*, a less photoinhibition-tolerant tree species, which favoured carbon metabolic processes at the cost of chronic photoinhibition and photodamage. This strategy is similar to those in other semi-deciduous shrubs (Oliveira and Penuelas, 2001; Oliveira and Peñuelas, 2004). The physiological responses of *Q. ilex*, a slowly growing late-successional species, to environmental stressors are highly plastic (Zavala *et al.*, 2000) due to its vegetative activity in a wide range of temperatures and high stomatal control in stressful conditions (Gratani et al., 2000; Savé et al., 1999), high plasticity index and resprouting dynamics (Espelta et al., 1999; Gratani et al., 2000), deep rooting system and large carbohydrate pools (Canadell and Lopez-Soria, 1998; Canadell et al., 1999), and high adaptive variability in foliar phenomorphology (Sabaté et al., 1999). Our findings showed the intra-crown variability in *Q. ilex*, where shaded leaves were widely unaffected by the inhibitory cold stress (Oliveira and Penuelas, 2001). The ability of *Q. ilex* to perform rapid metabolic changes in the antioxidant and photoprotective mechanisms could be of adaptive importance (García-Plazaola et al., 1999a). In contrast, *P. halepensis* is a fast growing conifer that quickly occupies open spaces after disturbances such as fires (Zavala *et al.*, 2000). *P. halepensis*, as do all pines, has a low ability to store carbohydrates and therefore follows a

strategy of water conservation and embolism avoidance (Meinzer et al., 2009). High rates of photosynthesis and growth require high concentrations of carboxylation enzymes in the carbon cycle that have high maintenance costs (Valladares and Niinemets, 2008), perhaps accounting for the high respiration rates found in *P. halepensis* leaves. Moreover, differences among the species are also likely to be the result of distinct foliar morphologies and crown architectures. Pine trees are characterised by a relatively low exposure of foliar surface area to direct sunlight due to the cylindrical shape and steep angles of their needles but at the same time are able to exploit a wider range of incident light angles than broadleaved trees. Despite reported flexible adjustments in the orientation of the leaves in several Mediterranean broadleaved sclerophyllic species (Oliveira and Peñuelas, 2000; Vaz et al., 2011; Werner et al., 2002), needle leaves probably still confer some benefits to attain near-saturated photosynthetic rates over a wider range of diurnal and seasonal variation in sun angles (Jordan and Smith, 1993; Lusk et al., 2003), while at the same time showing a high tolerance to photoinhibition. This might account for the good performance of *P. halepensis* under mild winter conditions with moderate abiotic stresses such as in our study. However, under more severe and re-occurring frost events, *P. halepensis* might reach the threshold of its tolerance and severe frost damage can occur. This explains also its absence in mountain regions with more severe winters where *Q .ilex* becomes more competitive. Despite following distinct physiological strategies, both *Q. ilex* and *P. halepensis* seem to cope equally well with the winter conditions they were exposed to whereas the foliar photosynthetic systems of *A. unedo* were more sensitive to sudden frost impacts. Thus, *A. unedo* might have been in a competitive disadvantage for the following growing season.

Overall, we conclude that the photosynthetic exploitation of relatively favorable winter conditions might be crucial for evergreen Mediterranean tree species for achieving a positive annual carbon balance. The winter period might give important insights helping to explain the dynamics of Mediterranean forest communities when withstanding increased novel environmental conditions projected in multiple climate change scenarios and benefitting from periods of potential recovery and growth in winter.

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3

1   **References**

2   Allen, D. J. and Ort, D. R.: Impacts of chilling temperatures on photosynthesis in warm-
3   climate plants., Trends Plant Sci., 6(1), 36–42 [online] Available from:
4   http://www.ncbi.nlm.nih.gov/pubmed/11164376, 2001.

5   Aro, E. M., Virgin, I. and Andersson, B.: Photoinhibition of photosystem. II. Inactivation,
6   protein damage and turnover, Biochim. Biophys. Acta - Bioenerg., 1143(2), 113–134, 1993.

7   Aschmann, H.: Distribution and Peculiarity of Mediterranean Ecosystems, in Mediterranean
8   Type Ecosystems, vol. 7, edited by F. Castri and H. A. Mooney, pp. 11–19, Springer Berlin
9   Heidelberg, Berlin, Heidelberg., 1973.

10   Bernacchi, C. J., Portis, A. R., Nakano, H., Caemmerer, S. Von and Long, S. P.: Temperature
11   Response of Mesophyll Conductance . Implications for the Determination of Rubisco Enzyme
12   Kinetics and for Limitations to Photosynthesis in Vivo, Plant Physiol., 130, 1992–1998,
13   doi:10.1104/pp.008250.water, 2002.

14   Bertolli, S. C. and Souza, G. M.: The level of environmental noise affects the physiological
15   performance of Glycine max under water deficit, Theor. Exp. Plant Physiol., 25(1), 36–45,
16   2013.

17   Beyschlag, W., Lange, O. L. and Tenhunen, J. D.: Photosynthesis und Wasserhaushalt der
18   immergrünen mediterranen Hartlaubpflanze Arbutus unedo L. im Jahresverlauf am
19   Freilandstandort in Portugal I. Tagesläufe von CO2-Gaswechsel und Transpiration unter
20   natürlichen Bedingungen, Flora, 178, 409–444, 1986.

21   Björkman, O. and Demmig, B.: Photon yield of O2 evolution and chlorophyll fluorescence
22   characteristics at 77 K among vascular plants of diverse origins, Planta, 170(4), 489–504,
23   1987.

24   Blumler, M. A.: Winter-Deciduous versus Evergreen Habit in Mediterranean Regions : A
25   Model, USDA For. Serv. Gen. Tech. Rep PSW-126, 194–197, 1991.

26   Buschmann, C.: Variability and application of the chlorophyll fluorescence emission ratio
27   red/far-red of leaves., Photosynth. Res., 92(2), 261–71, doi:10.1007/s11120-007-9187-8,
28   2007.

29   Bussotti, F., Bettini, D., Grossoni, P., Mansuino, S., Nibbi, R., Soda, C. and Tani, C.:
30   Structural and functional traits of Quercus ilex in response to water availability, Evironmental
31   Exp. Bot., 47(1), 11–23, 2002.

32   Canadell, J., Djema, A., López, B., Lloret, F., Sabaté, S., Siscart, D. and Gracia, C. A.:
33   Structure and Dynamics of the Root System, in Ecology of Mediterranean Evergreen Oak
34   Forests, edited by F. Rodà, J. Retana, C. A. Gracia, and J. Bellot, pp. 47–59, Springer Berlin
35   Heidelberg., 1999.

Canadell, J. and Lopez-Soria, L.: Lignotuber reserves support regrowth following clipping of two Mediterranean shrubs, Funct. Ecol., 12(1), 31–38, doi:10.1046/j.1365-2435.1998.00154.x, 1998.

Carnicer, J., Barbeta, A., Sperlich, D., Coll, M. and Peñuelas, J.: Contrasting trait syndromes in angiosperms and conifers are associated with different responses of tree growth to temperature on a large scale., Front. Plant Sci., 4(October), 409, doi:10.3389/fpls.2013.00409, 2013.

Corcuera, L., Morales, F., Abadia, a. and Gil-Pelegrin, E.: The effect of low temperatures on the photosynthetic apparatus of Quercus ilex subsp. ballota at its lower and upper altitudinal limits in the Iberian peninsula and during a single freezing-thawing cycle, Trees, 19(1), 99–108, doi:10.1007/s00468-004-0368-1, 2004.

Cowling, R. M., Rundel, P. W., Lamont, B. B., Arroyo, M. K. and Arianoutsou, M.: Plant diversity in Mediterranean-climate regions, Trends Ecol. Evol., 11(9), 362–366, 1996.

D'Ambrosio, N., Arena, C. and De Santo, A. V.: Temperature response of photosynthesis, excitation energy dissipation and alternative electron sinks to carbon assimilation in Beta vulgaris L., Environ. Exp. Bot., 55(3), 248–257, doi:10.1016/j.envexpbot.2004.11.006, 2006.

Demmig-Adams, B. and Adams, W. W.: Photoprotection and other responses of plants to high light stress, Annu. Rev. Pant Physiol. Plant Mol. Biol., 43, 599–626, 1992.

Demmig-Adams, B. and Adams, W. W.: The role of xantophyll cycle carotenoids in the protection of photosynthesis, Trends Plant Sci., 1(1), 21–26, 1996.

Dolman, a. J., Moors, E. J. and Elbers, J. a.: The carbon uptake of a mid latitude pine forest growing on sandy soil, Agric. For. Meteorol., 111(3), 157–170, doi:10.1016/S0168-1923(02)00024-2, 2002.

Ensminger, I., Berninger, F. and Streb, P.: Response of photosynthesis to low temperature, in Terrestrial Photosynthesis in a Changing Environment - A Molecular, Physiological and Ecological Approach, edited by J. Flexas, F. Loreto, and H. Medrano, pp. 272–289, Cambridge University Press, Cambridge., 2012.

Epron, D. and Dreyer, E.: Effects of severe dehydration on leaf photosynthesis in Quercus petruea (Matt .) Liebl.: photosystem II efficiency , photochemical and nonphotochemical fluorescence quenching and electrolyte leakage, Tree Physiol., 10, 273–284, 1992.

Espelta, J. M., Sabaté, S. and Retana, J.: Resprouting Dynamics, in Ecology of Mediterranean Evergreen Oak Forests, edited by F. Rodà, J. Retana, C. A. Gracia, and J. Bellot, pp. 61–71., 1999.

Farquhar, G. D., von Caemmerer, S. and Berry, J. A.: A Biochemical Model of Photosynthesis CO_2 Assimilation in Leaves of C3 Species, Planta, 149, 78–90, 1980.

Farquhar, G. D. and Sharkey, T. D.: Stomatal Conductance and Photosynthesis, Annu. Rev. Plant Physiol., 33(1), 317–345, doi:10.1146/annurev.pp.33.060182.001533, 1982.

Flexas, J., Badger, M., Chow, W., Medrano, H. and Osmond, C.: Analysis of the relative increase in photosynthetic O(2) uptake when photosynthesis in grapevine leaves is inhibited following low night temperatures and/or water stress, Plant Physiol., 121(2), 675–84 [online] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=59431&tool=pmcentrez&renderty pe=abstract, 1999.

Flexas, J., Diaz-Espejo, A., Galmés, J., Kaldenhoff, R., Medrano, H. and Ribas-Carbo, M.: Rapid variations of mesophyll conductance in response to changes in CO2 concentration around leaves., Plant. Cell Environ., 30(10), 1284–98, doi:10.1111/j.1365-3040.2007.01700.x, 2007.

Flexas, J., Escalona, J. M. and Medrano, H.: Down-regulation of photosynthesis by drought under field conditions in grapevine leaves, Aust. J. Plant Physiol., 25(8), 893, doi:10.1071/PP98054, 1998.

Flexas, J., Loreto, F. and Medrano, H.: Terrestrial Photosynthesis in a Changing Environment - A Molecular, Physiological and Ecological Approach, edited by J. Flexas, F. Loretto, and H. Medrano, Cambridge University Press, Cambridge., 2012.

Flexas, J., Ribas-Carbó, M., Diaz-Espejo, A., Galmés, J. and Medrano, H.: Mesophyll conductance to CO2: current knowledge and future prospects., Plant. Cell Environ., 31(5), 602–21, doi:10.1111/j.1365-3040.2007.01757.x, 2008.

Friend, A. D.: Terrestrial plant production and climate change., J. Exp. Bot., 61(5), 1293–309, doi:10.1093/jxb/erq019, 2010.

Fryer, M. J., Andrews, J. R., Oxborough, K., Blowers, D. A. and Baker, N. R.: Relationship between CO2 Assimilation, Photosynthetic Electron Transport, and Active O2 Metabolism in Leaves of Maize in the Field during Periods of Low Temperature, Plant Physiol., 116(2), 571–80 [online] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=35114&tool=pmcentrez&renderty pe=abstract, 1998.

García-Plazaola, J. I., Artetxe, U. and Becceril, María, J.: Diurnal changes in antioxidant and carotenoid composition in the Mediterranean schlerophyll tree Quercus ilex (L.) during winter, Plant Sci., 143, 125–133, 1999a.

García-Plazaola, J. I., Artetxe, U., Duñabeitia, M. K. and Becerril, J. M.: Role of Photoprotective Systems of Holm-Oak (Quercus ilex) in the Adaptation to Winter Conditions, J. Plant Physiol., 155(4-5), 625–630, doi:10.1016/S0176-1617(99)80064-9, 1999b.

García-Plazaola, J. I., Faria, T., Abadia, J., Abadia, A., Chaves, M. M. and Pereira, J. S.: Seasonal changes in xanthophyll composition and photosynthesis of cork oak (Quercus suber L.) leaves under mediterranean climate, J. Experimantal Bot., 48(314), 1667–1674, 1997.

Garcia-Plazaola, J. I., Olano, J. M., Hernandez, A. and Becerril, J. M.: Photoprotection in evergreen Mediterranean plants during sudden periods of intense cold weather, Trees, 17, 285–291, doi:10.1007/s00468-002-0234-y, 2003a.

1   Garcia-Plazaola, J. I., Olano, J. M., Hernandez, A. and Becerril, J. M.: Photoprotection in
2   evergreen Mediterranean plants during sudden periods of intense cold weather, TREES, 17,
3   285–291, doi:10.1007/s00468-002-0234-y, 2003b.

4   Genty, B., Briantais, J.-M. and Baker, N. R.: The relationship between the quantum yield of
5   photosynthetic electron transport and quenching of chlorophyll fluorescence, Biochim.
6   Biophys. Acta, 990(1), 87–92, 1989.

7   Gracia, C. A., Tello, E., Sabat, S. and Bellot, J.: GOTILWA: An Integrated Model of Water
8   Dynamics and Forest Growth. Ecology of Mediterranean Evergreen Oak Forests, in Ecology
9   of Mediterranean evergreen oak forests. Springer, edited by F. Rodà, J. Retana, C. A. Gracia,
10   and J. Bellot, pp. 163–178, Springer Berlin Heidelberg, Berlin., 1999.

11   Gratani, L. and Ghia, E.: Adaptive strategy at the leaf level of Arbutus unedo L . to cope with
12   Mediterranean climate, Flora, 197, 275–284, 2002a.

13   Gratani, L. and Ghia, E.: Changes in morphological and physiological traits during leaf
14   expansion of Arbutus unedo, Environ. Exp. Bot., 48(1), 51–60, 2002b.

15   Gratani, L., Pesoli, P., Crescente, M. F., Aichner, K. and Larcher, W.: Photosynthesis as a
16   temperature indicator in Quercus ilex L., Glob. Planet. Change, 24(2), 153–163,
17   doi:10.1016/S0921-8181(99)00061-2, 2000.

18   Guidi, L. and Calatayud, A.: Non-invasive tools to estimate stress-induced changes in
19   photosynthetic performance in plants inhabiting Mediterranean areas, Environ. Exp. Bot.,
20   103, 42–52, doi:10.1016/j.envexpbot.2013.12.007, 2014.

21   Haldimann, P. and Feller, U.: Inhibition of photosynthesis by high temperature in oak (
22   Quercus pubescens L .) leaves grown under natural conditions closely correlates with a
23   reversible heat- dependent reduction of the activation state of ribulose-1 , 5- bisphosphate
24   carboxylase / oxy, Plant Cell Environ., 27(9), 1169–1183, 2004.

25   Harley, P. C., Loreto, F., Di Marco, G. and Sharkey, T. D.: Theoretical Considerations when
26   Estimating the Mesophyll Conductance to CO2 Flux by Analysis of the Response of
27   Photosynthesis to CO2., Plant Physiol., 98(4), 1429–1436 [online] Available from:
28   http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1080368&tool=pmcentrez&rende
29   rtype=abstract, 1992.

30   Hikosaka, K., Murakami, a. and Hirose, T.: Balancing carboxylation and regeneration of
31   ribulose-1,5- bisphosphate in leaf photosynthesis: temperature acclimation of an evergreen
32   tree, Quercus myrsinaefolia, Plant, Cell Environ., 22(7), 841–849, doi:10.1046/j.1365-
33   3040.1999.00442.x, 1999.

34   Huner, N. P. A., Öquist, G. and Sarhan, F.: Energy balance and acclimation to light and cold,
35   Trends Plant Sci., 3(6), 224–230, doi:10.1016/S1360-1385(98)01248-5, 1998.

36   Hurry, V., Strand, a, Furbank, R. and Stitt, M.: The role of inorganic phosphate in the
37   development of freezing tolerance and the acclimatization of photosynthesis to low

temperature is revealed by the pho mutants of Arabidopsis thaliana., Plant J., 24(3), 383–96 [online] Available from: http://www.ncbi.nlm.nih.gov/pubmed/11069711, 2000.

IPCC: IPCC, 2013: Summary for Policymakers., in Climate change 2013: the physical science basis., edited by S. T.F., D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex, and P. M. Midgley, Cambridge University Press, Cambridge (UK) and New York (USA)., 2013.

Jordan, D. N. and Smith, W. K.: Simulated influence of leaf geometry on sunlight interception and photosynthesis in conifer needles., Tree Physiol., 13(1), 29–39 [online] Available from: http://www.ncbi.nlm.nih.gov/pubmed/14969899, 1993.

Kang, C.-Y., Lian, H.-L., Wang, F.-F., Huang, J.-R. and Yang, H.-Q.: Cryptochromes, phytochromes, and COP1 regulate light-controlled stomatal development in Arabidopsis., Plant Cell, 21(9), 2624–41, doi:10.1105/tpc.109.069765, 2009.

Kok, B.: A critical consideration of the quantum yield of Chlorella-photosynthesis, Enzymologia, 13, 1–56, 1948.

Kull, O. and Niinemets, U.: Variations in leaf morphometry and nitrogen concentration in Betula pendula Roth., Corylus avellana L. and Lonicera xylosteum L., Tree Physiol., 12(3), 311–8 [online] Available from: http://www.ncbi.nlm.nih.gov/pubmed/14969921, 1993.

Kyparissis, A., Drilias, P. and Manetas, Y.: Seasonal fluctuations in photoprotective xanthophyll cycle) and photoselective (chlorophylls) capacity in eight Mediterranean plant species belonging to two different growth forms, Aust. J. Plant Physiol., 27(3), 265–272, 2000.

Laisk, A., Oja, V., Rasulov, B., Rämma, H., Eichelmann, H., Kasparova, I., Pettai, H. and Padu, E.: A computer-operated routine of gas exchange and optical measurements to diagnose photosynthetic apparatus, Plant, Cell Environ., 25, 923–943, 2002.

Lambrev, P. H., Miloslavina, Y., Jahns, P. and Holzwarth, A. R.: On the relationship between non-photochemical quenching and photoprotection of Photosystem II., Biochim. Biophys. Acta, 1817(5), 760–9, doi:10.1016/j.bbabio.2012.02.002, 2012.

Levizou, E., Drilias, P. and Kyparissis, A.: Exceptional photosynthetic performance of Capparis spinosa L . under adverse conditions of Mediterranean summer, Photosynthetica, 42(2), 229–235, 2004.

Lusk, C. H., Wright, I. and Reich, P. B.: Photosynthetic differences contribute to competitive advantage of evergreen angiosperm trees over evergreen conifers in productive habitats, New Phytol., 160, 329–336, 2003.

Maestre, F. T. and Cortina, J.: Are Pinus halepensis plantations useful as a restoration tool in semiarid Mediterranean areas?, For. Ecol. Manage., 198(1-3), 303–317, doi:10.1016/j.foreco.2004.05.040, 2004.

1   Martinez-Ferri, E., Balaguer, L., Valladares, F., Chico, J. M. and Manrique, E.: Energy
2   dissipation in drought-avoiding and drought-tolerant tree species at midday during the
3   Mediterranean summer, Tree Physiol., 20(2), 131–138, doi:10.1093/treephys/20.2.131, 2000.

4   Martínez-Ferri, E., Manrique, E., Valladares, F. and Balaguer, L.: Winter photoinhibition in
5   the field involves different processes in four co-occurring Mediterranean tree species., Tree
6   Physiol., 24(9), 981–90 [online] Available from:
7   http://www.ncbi.nlm.nih.gov/pubmed/15234895, 2004.

8   Matesanz, S. and Valladares, F.: Ecological and evolutionary responses of Mediterranean
9   plants to global change, Environ. Exp. Bot., doi:10.1016/j.envexpbot.2013.09.004, 2013.

10   Maxwell, K. and Johnson, G. N.: Chlorophyll fluorescence — a practical guide, J. Exp. Bot.,
11   51(345), 659–668, 2000.

12   Meinzer, F. C., Johnson, D. M., Lachenbruch, B., McCulloh, K. a. and Woodruff, D. R.:
13   Xylem hydraulic safety margins in woody plants: coordination of stomatal control of xylem
14   tension with hydraulic capacitance, Funct. Ecol., 23(5), 922–930, doi:10.1111/j.1365-
15   2435.2009.01577.x, 2009.

16   Mitrakos, K.: A theory for Mediterranean plant life, Acta Oecologica/Oecologia Plant., 1,
17   245–252, 1980.

18   Mott, K. A. and Buckley, T. N.: Stomatal heterogeneity, J. Experimantal Bot., 49(March),
19   407–417, 1998.

20   Mott, K. A. and Buckley, T. N.: Patchy stomatal conductance: emergent collective behaviour
21   of stomata, Trends Plant Sci., 1385(1997), 258–262, 2000.

22   Mulo, P., Sakurai, I. and Aro, E.-M.: Strategies for psbA gene expression in cyanobacteria,
23   green algae and higher plants: from transcription to PSII repair., Biochim. Biophys. Acta,
24   1817(1), 247–57, doi:10.1016/j.bbabio.2011.04.011, 2012.

25   Munné-Bosch, S. and Peñuelas, J.: Drought-induced oxidative stress in strawberry tree
26   (Arbutus unedo L.) growing in Mediterranean field conditions, Plant Sci., 166(4), 1105–1110,
27   doi:10.1016/j.plantsci.2003.12.034, 2004.

28   Niinemets, Ü., Cescatti, A., Rodeghiero, M. and Tosens, T.: Leaf internal diffusion
29   conductance limits photosynthesis more strongly in older leaves of Mediterranean evergreen
30   broad-leaved species, Plant, Cell Environ., 28(12), 1552–1566, doi:10.1111/j.1365-
31   3040.2005.01392.x, 2005.

32   Niinemets, Ü., Cescatti, A., Rodeghiero, M. and Tosens, T.: Complex adjustments of
33   photosynthetic potentials and internal diffusion conductance to current and previous light
34   availabilities and leaf age in Mediterranean evergreen species Quercus ilex, Plant, Cell
35   Environ., 29(6), 1159–1178, doi:10.1111/j.1365-3040.2006.01499.x, 2006.

36   Niinemets, U., Flexas, J. and Peñuelas, J.: Evergreens favored by higher responsiveness to
37   increased CO2, Trends Ecol. Evol., 26(3), 136–42, doi:10.1016/j.tree.2010.12.012, 2011.

Niinemets, Ü., Oja, V. and Kull, O.: Shape of leaf photosynthetic electron transport versus temperature response curve is not constant along canopy light gradients in temperate deciduous trees, Plant. Cell Environ., 22(12), 1497–1513, doi:10.1046/j.1365-3040.1999.00510.x, 1999.

Ninyerola, M., Pons, X. and Roure, J. M.: A Methodological Approach of Climatological Modelling of Air Temperature and Precipitation, Int. J. Climatol., 20, 1823–1841, 2000.

Ogaya, R. and Peñuelas, J.: Comparative seasonal gas exchange and chlorophyll fluorescence of two dominant woody species in a Holm Oak Forest, Flora, 198, 132–141, 2003.

Ogaya, R. and Peñuelas, J.: Leaf mass per area ratio in Quercus ilex leaves under a wide range of climatic conditions. The importance of low temperatures, Acta Oecologica, 31(2), 168–173, doi:10.1016/j.actao.2006.07.004, 2007.

Oliveira, G. and Penuelas, J.: Allocation of absorbed light energy into photochemistry and dissipation in a semi-deciduous and an evergreen Mediterranean woody species during winter, Aust. J. Plant Physiol., 28(6), 471–480, doi:Doi 10.1071/Pp00150, 2001.

Oliveira, G. and Peñuelas, J.: Comparative photochemical and phenomorphological responses to winter stress of an evergreen (Quercus ilex L.) and a semi-deciduous (Cistus albidus L.) Mediterranean woody species, Acta Oecologica, 21(2), 97–107, 2000.

Oliveira, G. and Peñuelas, J.: Effects of winter cold stress on photosynthesis and photochemical efficiency of PSII of the Mediterranean Cistus albidus L . and Quercus ilex L ., Plant Ecol., 175, 179–191, 2004.

Onoda, Y., Hikosaka, K. and Hirose, T.: Seasonal change in the balance between capacities of RuBP carboxylation and RuBP regeneration affects CO2 response of photosynthesis in Polygonum cuspidatum., J. Exp. Bot., 56(412), 755–63, doi:10.1093/jxb/eri052, 2005.

Öquist, G. and Huner, N. P. A.: Photosynthesis of overwintering evergreen plants., Annu. Rev. Plant Biol., 54, 329–55, doi:10.1146/annurev.arplant.54.072402.115741, 2003.

Orshan, G.: Approaches to the Definition of Mediterranean Growth Forms, in Mediterranean type-ecosystems - The role of nutrients, edited by F. J. Kruger, D. T. Mitchell, and J. U. M. Jarvis, pp. pp. 86–100, Springer-Verlag, Berlin - Heidelberg., 1983.

Peñuelas, J. and Filella, I.: Responses to a Warming World, Science (80-.)., 294, 794–795, doi:10.1126/science.1066860, 2001.

Peñuelas, J., Sardans, J., Estiarte, M., Ogaya, R., Carnicer, J., Coll, M., Barbeta, A., Rivas-Ubach, A., Llusià, J., Garbulsky, M., Filella, I. and Jump, A. S.: Evidence of current impact of climate change on life: a walk from genes to the biosphere., Glob. Chang. Biol., 19(8), 2303–38, doi:10.1111/gcb.12143, 2013.

Reich, P. B., Ellsworth, D. S. and Walters, M. B.: Leaf structure (specific leaf area) modulates photosynthesis-nitrogen relations: evidence from within and across species and functional groups, Funct. Ecol., 12(6), 948–958, doi:10.1046/j.1365-2435.1998.00274.x, 1998.

Reichstein, M., Tenhunen, J. D., Roupsard, O., Ourcival, J.-M., Rambal, S., Dore, S. and Valentini, R.: Ecosystem respiration in two Mediterranean evergreen Holm Oak forests: drought effects and decomposition dynamics, Funct. Ecol., 16(1), 27–39, doi:10.1046/j.0269-8463.2001.00597.x, 2002.

Sabaté, S. and Gracia, C. A.: Water Processes in Trees: Transpiration and Photosynthesis, in Water for Forests and People in the Mediterranean – What Science Can Tell Us, edited by Y. Birot, C. A. Gracia, and M. Palahí, pp. 72–75, European Forest Institut, Joensuu. [online] Available from: http://www.efi.int/portal/virtual_library/publications/what_science_can_tell_us/, 2011.

Sabaté, S., Sala, A. and Gracia, C. A.: Leaf Traits and Canopy Organisation, in Ecology of Mediterranean Evergreen Oak Forests, edited by F. Rodà, J. Retana, C. A. Gracia, and J. Bellot, pp. 121–131, Springer Berlin Heidelberg., 1999.

Sanchez-Humanes, B. and Espelta, J. M.: Increased drought reduces acorn production in Quercus ilex coppices: thinning mitigates this effect but only in the short term, Forestry, 84(1), 73–82, doi:10.1093/forestry/cpq045, 2011.

Savé, R., Castell, C. and Terrades, J.: Gas Exchange and Water Relations, in Ecology of Mediterranean Evergreen Oak Forests, edited by F. Rodà, J. Retana, C. A. Gracia, and J. Bellot, pp. 135–144, Springer Berlin Heidelberg., 1999.

Sharkey, T. D.: Photosynthesis in intact leaves of C3 plants: Physics, physiology and rate limitations., Bot. Rev., 51, 53–105, 1985.

Somot, S., Sevault, F., Déqué, M. and Crépon, M.: 21st century climate change scenario for the Mediterranean using a coupled atmosphere–ocean regional climate model, Glob. Planet. Change, 63(2-3), 112–126, doi:10.1016/j.gloplacha.2007.10.003, 2008.

Specht, R. L.: A comparison of the sclerophyllous vegetation characteristics of Mediterranean type climates in France, California, and southern Australia. I. Structure, morphology, and succession., Aust. J. Bot., 17(2), 277–292, 1969.

Taz, L. and Zeiger, E.: Plant Physiology - 5th Edition, edited by L. Taz and E. Zeiger, Sinauer Associates, Inc., Sunderland., 2010.

Terashima, I. and Hikosaka, K.: Comparative ecophysiology of leaf and canopy photosynthesis, Plant Cell Environ., 18, 1111–1128, 1995.

Tretiach, M., Bolognini, G. and Rondi, A.: Photosynthetic activity of Quercus ilex at the extremes of a transect between Mediterranean and submediterranean vegetation (Trieste-NE Italy)., Flora, 192, 369–378, 1997.

Turnbull, M. H., Whitehead, D., Tissue, D. T., Schuster, W. S. F., Brown, K. J. and Griffin, K. L.: Scaling foliar respiration in two contrasting forest canopies, Funct. Ecol., 17(1), 101–114, doi:10.1046/j.1365-2435.2003.00713.x, 2003.

Valladares, F., Martinez-Ferri, E., Balaguer, L., Perez-Corona, E. and Manrique, E.: Low leaf-level response to light and nutrients in Mediterranean evergreen oaks: a conservative resource-use strategy ?, New Phytol., 148(1), 79–91, 2000.

Valladares, F. and Niinemets, Ü.: Shade Tolerance, a Key Plant Feature of Complex Nature and Consequences, Annu. Rev. Ecol. Evol. Syst., 39(1), 237–257, doi:10.1146/annurev.ecolsys.39.110707.173506, 2008.

Valladares, F., Zaragoza-Castells, J., Sánchez-Gómez, D., Matesanz, S., Alonso, B., Portsmuth, A., Delgado, A. and Atkin, O. K.: Is shade beneficial for mediterranean shrubs experiencing periods of extreme drought and late-winter frosts?, Ann. Bot., 102(6), 923–33, doi:10.1093/aob/mcn182, 2008.

Vass, I.: Molecular mechanisms of photodamage in the Photosystem II complex., Biochim. Biophys. Acta, 1817(1), 209–17, doi:10.1016/j.bbabio.2011.04.014, 2012.

Vaz, M., Maroco, J., Ribeiro, N., Gazarini, L. C., Pereira, J. S. and Chaves, M. M.: Leaf-level responses to light in two co-occurring Quercus (Quercus ilex and Quercus suber): leaf structure, chemical composition and photosynthesis, Agrofor. Syst., 82(2), 173–181, doi:10.1007/s10457-010-9343-6, 2011.

Verhoeven, A.: Sustained energy dissipation in winter evergreens, New Phytol., 201(1), 57–65, doi:10.1111/nph.12466, 2014.

Werner, C., Correia, O. and Beyschlag, W.: Characteristic patterns of chronic and dynamic photoinhibition of different functional grops in a Mediterranean ecosystem, Funct. Plant Biol., 29, 99–1011, 2002.

Wullschleger, S. D.: Biochemical Limitations to Carbon Assimilation in C 3 Plants — A Retrospective Analysis of the j Curves from 109 Species, J. Experimantal Bot., 44(262), 907–920, 1993.

Yin, X., Struik, P. C., Romero, P., Harbinson, J., Evers, J. B., VAN DER Putten, P. E. L. and Vos, J.: Using combined measurements of gas exchange and chlorophyll fluorescence to estimate parameters of a biochemical C photosynthesis model: a critical appraisal and a new integrated approach applied to leaves in a wheat (Triticum aestivum) canopy., Plant. Cell Environ., 32(5), 448–64, doi:10.1111/j.1365-3040.2009.01934.x, 2009.

Yin, X., Sun, Z., Struik, P. C. and Gu, J.: Evaluating a new method to estimate the rate of leaf respiration in the light by analysis of combined gas exchange and chlorophyll fluorescence measurements., J. Exp. Bot., 62(10), 3489–99, doi:10.1093/jxb/err038, 2011.

Zaragoza-Castells, J., Sánchez-Gómez, D., Valladares, F., Hurry, V. and Atkin, O. K.: Does growth irradiance affect temperature dependence and thermal acclimation of leaf respiration? Insights from a Mediterranean tree with long-lived leaves., Plant. Cell Environ., 30(7), 820–33, doi:10.1111/j.1365-3040.2007.01672.x, 2007.

1   Zavala, M. A., Espelta, J. M. and Retana, J.: Constraints and Trade-Offs in Mediterranean
2   Plant Communities: The Case of Holm Oak-Aleppo Pine Forests, Bot. Rev., 66(1), 119–149,
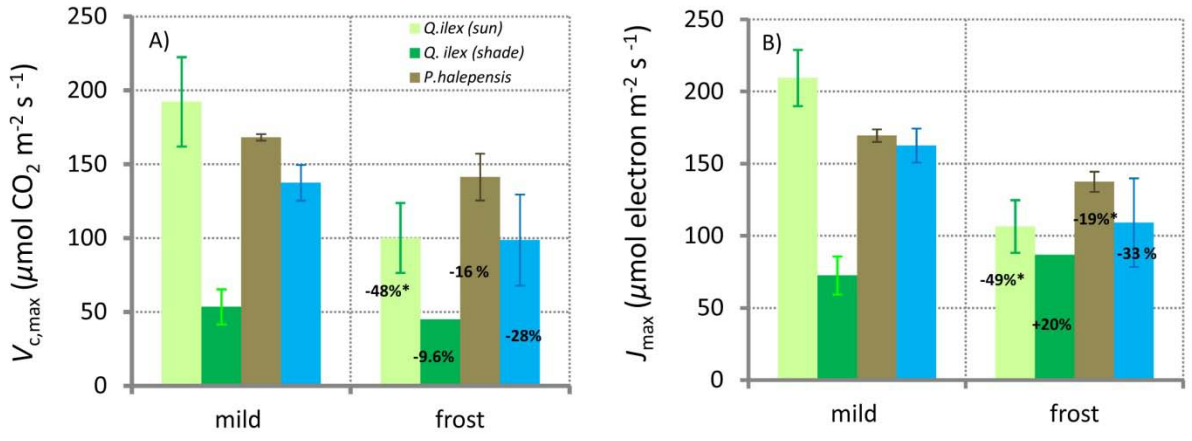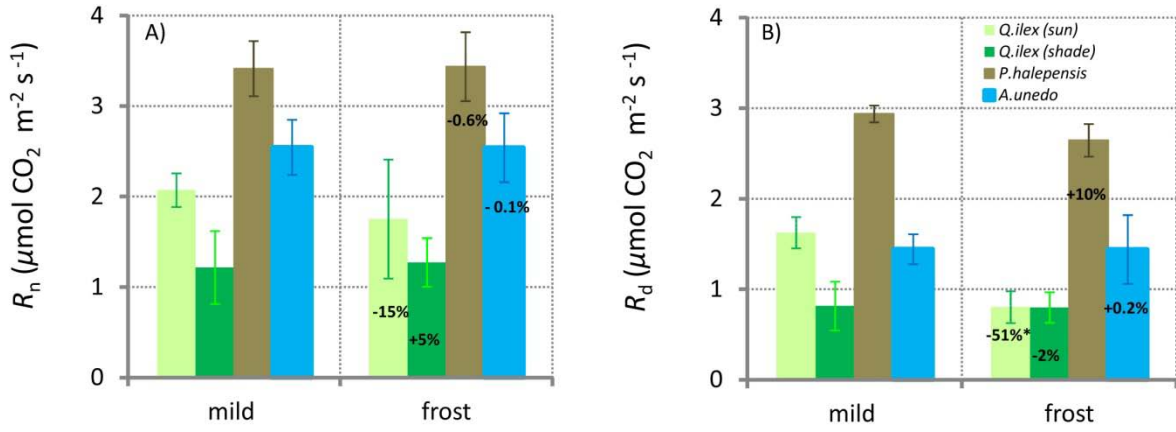3   2000.
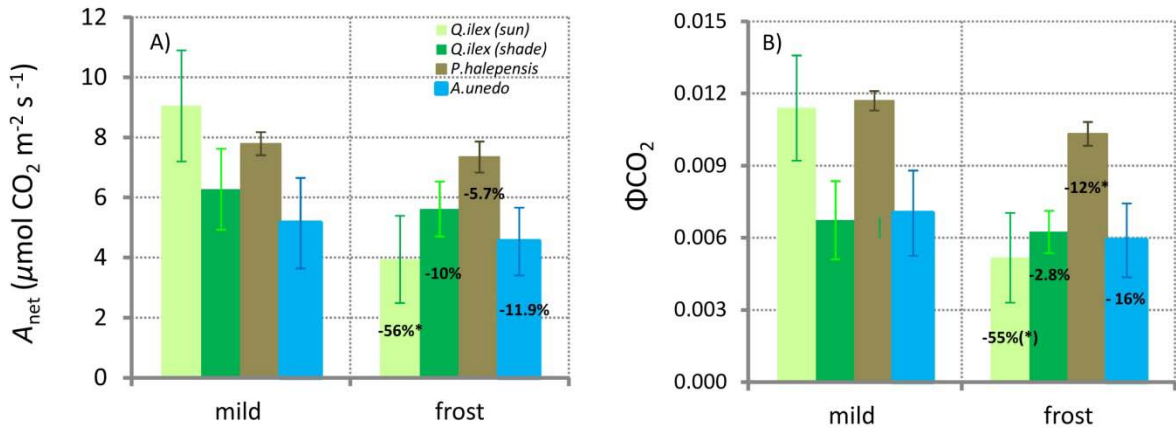
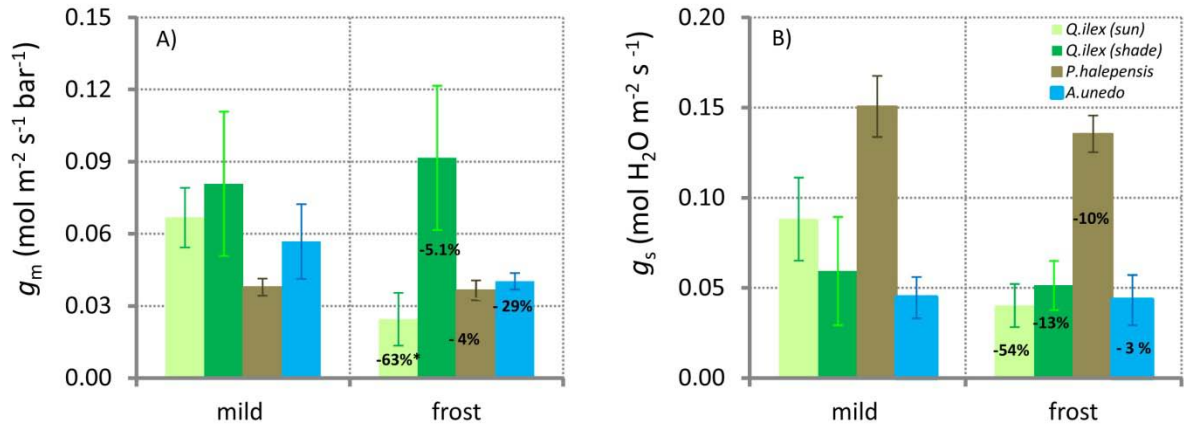4

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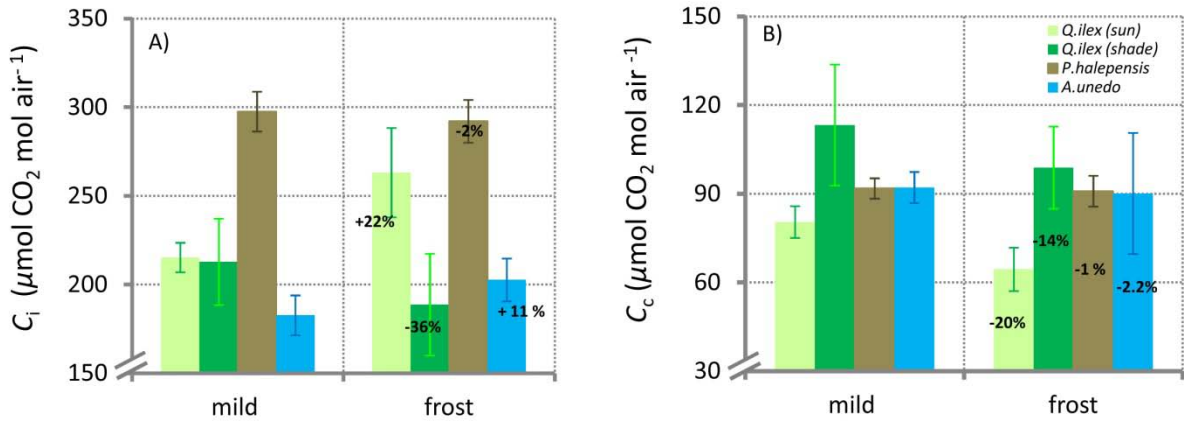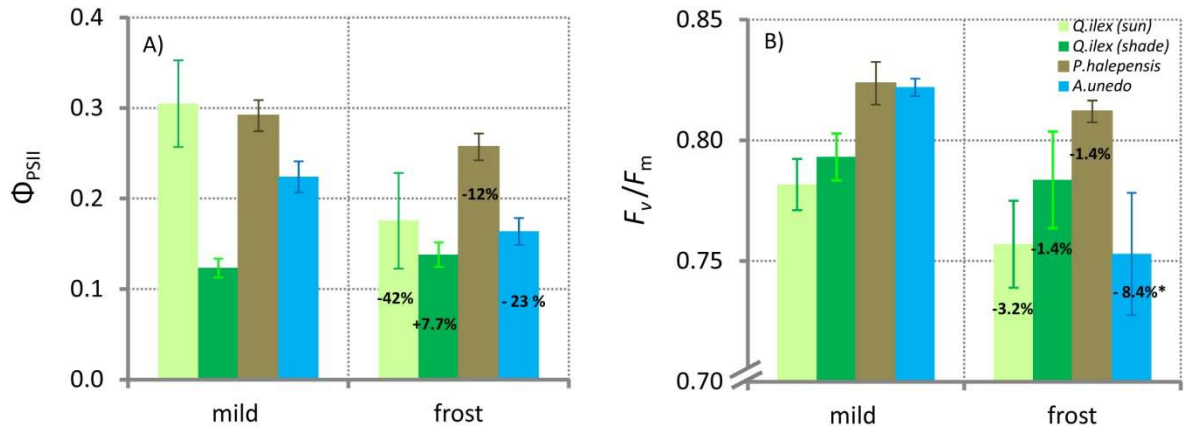2
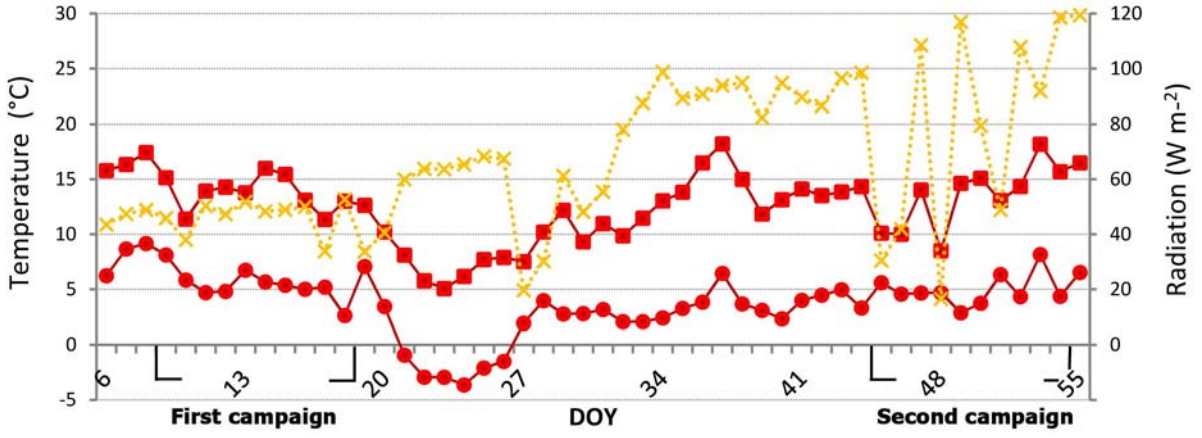
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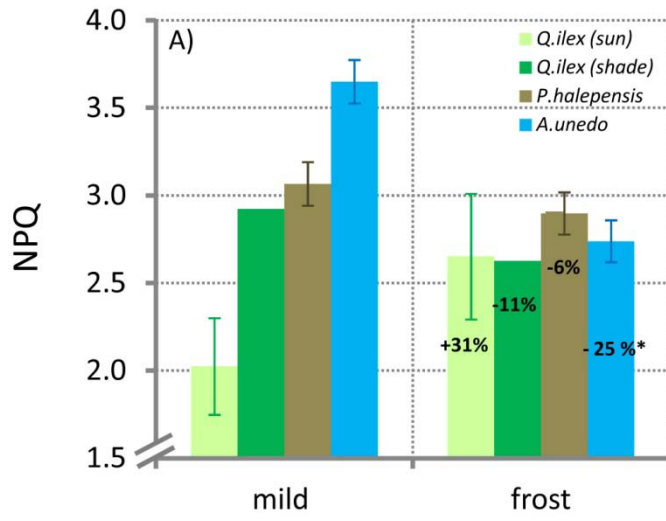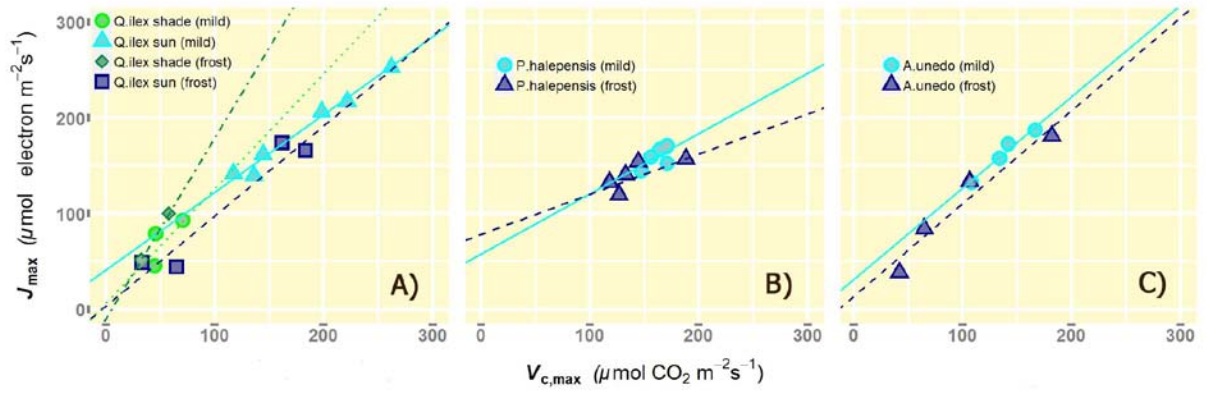1   **Fig.6**
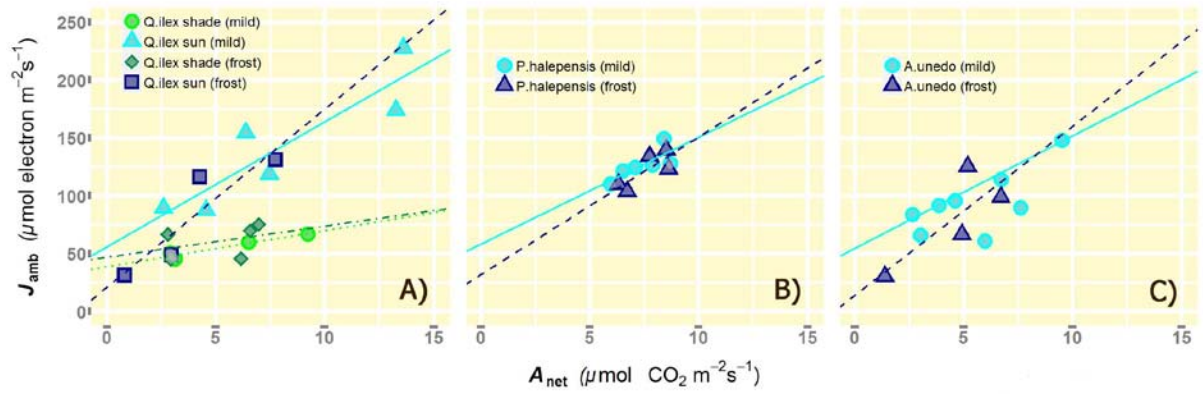

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1 **Tables**

2 **Table 1**: P values of Student's t-tests for the differences between sunlit and shaded leaves of

3 *Q. ilex.*

	Both periods	Mild period	Frost period
$V_{c,max}$	0.001	0.002	0.172
J_{max}	0.006	0.002	0.553
J/V	0.279	0.797	0.249
F_v/F_m	0.611	0.533	0.535
A_{net}	0.546	0.594	0.745
g_s	0.156	0.791	0.127
C_i	0.151	0.326	0.154
g_m	0.041	0.066	0.107
C_c	0.138	0.364	0.203
CUE	0.151	0.728	0.439
R_n	0.061	0.470	0.356
R_l	0.016	0.004	0.577
J_{amb}/A_{net}	0.052	0.014	0.203
Φ_{PSII}	0.290	0.315	0.825
Φ_{CO2}	0.750	0.886	0.497
qp	0.195	0.045	0.882
NPQ	0.192	0.903	0.126
$\Delta(C_a\text{-}C_i)$	0.037	0.321	0.068
$\Delta(C_i\text{-}C_c)$	0.043	0.073	0.113
$\Delta(C_a\text{-}C_c)$	0.023	0.006	0.122

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1 **Table 2**. Regression coefficients and results from ANCOVA analyses of the J_{amb}/A_{net} and $J_{max}/V_{c,max}$ relationships.

Regression analyses of J_{max} & $V_{c,max}$

tree species	Q. ilex			Q. ilex			P. halepensis			A. unedo			all species		
leaf position	sunlit			shaded			sunlit			sunlit			sunlit		
	reg. line	R^2	P	reg. line	R^2	P	reg. line	R^2	P	reg. line	R^2	P	reg. line	R^2	P
mild	y = 0.81 x + 41.6	0.97	2E-04	y = 1.2 x + 6.1	0.48	0.193	y = 115.9 x + 148.8	0.04	0.32	y = 0.954 x + 31.5	0.95	0.017	y = 50.2 x + 0.77	0.94	1.4E-07
frost	y = 0.94 x + 3.6	0.89	0.035	y = 1.89 x - 9.19			y = 971 x + 9.9	0.53	0.1	y = 0.97 x + 13.7	0.91	0.029	y = 10.5 x + 0.93	0.90	7.2E-05
p (slope)	5.76E-02			0.83			0.058			0.69			0.072		
p (intercept)	8.91E-09			0.3			0.022			0.28			0.008		

Regression analyses of J_{amb} & A_{net}

tree species	Q. ilex			Q. ilex			P. halepensis			A. unedo			all species		
leaf position	sunlit			shaded			sunlit			sunlit			sunlit		
	reg. line	R^2	P	reg. line	R^2	P	reg. line	R^2	P	reg. line	R^2	P	reg. line	R^2	P
mild	y = 10.8 + 56.1	0.76	0.014	y = 3.1 x + 39	0.91	0.029	y = 9.22 x + 58.3	0.51	0.068	y = 9.7 x + 54.9	0.96	0.005	y = 10.9 x + 51.9	0.84	7.1E-06
frost	y = 15.4 x + 21.1	0.73	0.093	y = 2.7 x + 46.8	-0.13	0.52	y = 11.9 x + 31.9	0.52	0.105	y = 14.6 x + 14.2	0.46	0.200	y = 13.5 x + 22.3	0.76	1.7E-04
p (slope)	0.337			0.72			0.59			0.322			0.098		
p (intercept)	0.51			0.45			0.31			0.29			0.071		

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Figure 1. Maximum and minimum temperatures on the primary y-axes (in red squares and circles, respectively) and radiation (in yellow crosses) on the secondary y-axes are presented for the mild and frost winter period for the day of the year (DOY) in January and February 2012.

Figure 2. Bar plot of the effect of a sudden period of frost following a mild winter period in 2012 on A) the maximum velocity of carboxylation ($V_{c,max}$) and B) the maximum rate of electron transport (J_{max}) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar), and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \geq P \leq 0.1$).

Figure 3. Bar plot of the effect of a sudden period of frost following a mild winter period on A) nighttime respiration (R_n) and B) daytime respiration (R_d) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar), and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \geq P \leq 0.1$).

Figure 4. Bar plot of the effect of a sudden period of frost following a mild winter period on A) net assimilation (A_{net}) and B) the effective quantum yield of net CO_2 assimilation (ΦCO_2) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar), and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \geq P \leq 0.1$).

Figure 5. Bar plot of the effect of a sudden period of frost following a mild winter period on A) mesophyllic conductance (g_m) and B) stomatal conductance (g_s) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar), and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \geq P \leq 0.1$).

Figure 6. Bar plot of the effect of a sudden period of frost following a mild winter period on A) the stomatal internal CO_2 concentration (C_i) and B) the chloroplastic CO_2 concentration

1   (C_c) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P.*

2   *halepensis* (beige bar), and *A. unedo* (blue bar). The error bars represent the standard error,

3   and the percentages indicate the change between periods where significance is indicated with

4   an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \geq P \leq 0.1$).

5   **Figure 7.** Bar plot of the effect of a sudden period of frost following a mild winter period on

6   A) the effective quantum yield of photosystem II (Φ_{PSII}) and B) the maximum efficiency of

7   photosystem II (F_v/F_m) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex*

8   (dark green bar), *P. halepensis* (beige bar), and *A. unedo* (blue bar). The error bars represent

9   the standard error, and the percentages indicate the change between periods where

10 significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk

11 in brackets ($0.05 \geq P \leq 0.1$).

12 **Figure 8.** Bar plot of the effect of a sudden period of frost following a mild winter period on

13 non-photochemical quenching (NPQ) in sunlit leaves of *Q. ilex* (light green bar), in shaded

14 leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar), and *A. unedo* (blue bar). The error

15 bars represent the standard error, and the percentages indicate the change between periods

16 where significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an

17 asterisk in brackets ($0.05 \geq P \leq 0.1$).

18 **Figure 9.** Relationship between the maximum velocity of carboxylation ($V_{c,max}$) and the

19 maximum rate of electron transport (J_{max}) in *Q. ilex* (A), *P. halepensis* (B), *A. unedo* (C),

20 leaves. Leaves measured under mild conditions are indicated by green circles and cyan

21 triangles in shaded and sunlit locations, respectively. Leaves measured after the period of

22 frost are indicated by green diamonds and blue squares in shaded and sunlit locations,

23 respectively.

24 **Figure 10.** Relationship between the rate electron transport from chlorophyllic fluorescence

25 (J_{amb}) and net assimilation (A_{net}) at ambient CO_2 concentrations and saturating light (A_{net}) in

26 *Q. ilex* (A), *P. halepensis* (B), *A. unedo* (C), leaves. Leaves measured under mild conditions

27 are indicated by green circles and cyan triangles in shaded and sunlit locations, respectively.

28 Leaves measured after the period of frost are indicated by green diamonds and blue squares in

29 shaded and sunlit locations, respectively.

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Appendix A

Temperature functions

The effective Michaelis-Menten constants K_c and K_o and the photorespiratory compensation point, Γ^*, were taken from (Bernacchi et al., 2002) and are summarized in Table 3. The following generic temperature response functions were used to adjust these parameters to the prevailing T_{Leaf} during the experiments

$$K_c = e^{\left(c - \left(\frac{\Delta Ha}{R \times \left(273.15 + T_{Leaf}\right)}\right)\right)} \tag{A1}$$

$$\text{(13)}$$

and

$$K_o = e^{\left(c - \left(\frac{\Delta Ha}{R \times \left(273.15 + T_{Leaf}\right)}\right)\right)} \tag{A2}$$

$$\text{(14)}$$

and

$$\Gamma^* = e^{\left(c - \left(\frac{\Delta Ha}{R \times \left(273.15 + T_{Leaf}\right)}\right)\right)} \times \frac{O_2}{20.9} \tag{A3}$$

$$\text{(15)}$$

where R is a unitless gas constant (0.008314), c is a scaling constant, ΔHa represents the activation energy and O_2 is the oxygen concentration of the ambient air assumed to be 20.9 kPa.

CF- parameters

$$NPQ = \frac{(F_m - F_m\prime)}{F_m\prime} \tag{A4}$$

Photochemical quenching (qP) indicates the proportion of open PSII reaction centres and tends to be highest in low light when leaves use light most efficiently (Maxwell and Johnson, 2000). qP was estimated by:

$$qP = \frac{F_m' - F_s}{F_m' - F_o'} \qquad (A5)$$

where F_o' is the minimum fluorescence in a light-adapted leaf after a pulse of darkness. The rate of photosynthetic electron transport, J_{CF}, is calculated by (Genty et al., 1989):

Estimation of mesophyll conductance

The CO_2 pathway leads from the atmosphere to the intercellular air spaces through the stomata and from there diffuses through the air spaces of the mesophyll, cell walls, cytosol, and chloroplastic envelopes and finally reaches the sites of CO_2 fixation in the chloroplastic stroma where it is fixed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). In this study, we call this pathway the internal mesophyll diffusion conductance (g_m) and estimate it with the variable-J method by Harley et al. (1992):

$$g_m = \frac{A_{net}}{C_i - \frac{[\Gamma^* * J_{CF} + 8(A_{net} + R_d)]}{J_{CF} - 4(A_{net} + R_d)}} \qquad (A7)$$

where Γ^* is the CO_2 concentration at which the photorespiratory efflux of CO_2 equals the rate of photosynthetic uptake of CO_2 (Table 3). Similarly to g_s, g_m is defined as a unitless molar fraction, rendering the units for conductance the same as those for photosynthesis. Nonetheless, the drawdown of CO_2 from the intercellular airspaces to the sites of carboxylation is thought to be dominated by the liquid phase of the chloroplast and is hence dependent on the partial pressure of the gas according to Henry's law (Harley et al., 1992). The units for conductance (mol m^{-2} s^{-1} bar^{-1}) are thus directly comparable to g_s when the atmospheric pressure is 1 bar. We assumed normal pressure (1.01325 bar) in our experiments that were conducted in Barcelona, which is close to sea level. The variable-J method accounts for the variation in g_m with C_i and provides more accurate estimates of photosynthetic parameters than do A/C_c curves that assume a constant g_m, especially during episodes of water stress (Flexas et al., 2007). The chloroplastic CO_2 concentration can then be determined using C_i, A_{net}, and g_m:

$$C_c = C_i - \frac{A_{net}}{g_m} \qquad (A8)$$

where C_c is the chloroplastic CO_2 concentration.

1   **Appendix C**

2

3   **Table C1**. The scaling constant (c) and energies of activation (ΔHa) describing the

4   temperature responses for Rubisco enzyme kinetic parameters K_c, K_o and Γ^*. Taken from

5   Bernacchi et al., (2002).

6

	25°C	c	ΔHa	unit
K_c	27.24	35.98	80.99	Pa
K_o	16.58	12.38	23.72	kPa
Γ^*	3.74	11.19	24.46	Pa

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