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RESEARCH PAPER

The Mediterranean evergreen *Quercus ilex* and the semi-deciduous *Cistus albidus* differ in their leaf gas exchange regulation and acclimation to repeated drought and re-watering cycles

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Abstract

Plants may exhibit some degree of acclimation after experiencing drought, but physiological adjustments to consecutive cycles of drought and re-watering (recovery) have scarcely been studied. The Mediterranean evergreen holm oak (*Q. ilex)* and the semi-deciduous rockrose (*C. albidus*) showed some degree of acclimation after the first of three drought cycles (S1, S2, and S3). For instance, during S2 and S3 both species retained higher relative leaf water contents than during S1, despite reaching similar leaf water potentials. However, both species showed remarkable differences in their photosynthetic acclimation to repeated drought cycles. Both species decreased photosynthesis to a similar extent during the three cycles (20–40% of control values). However, after S1 and S2, photosynthesis recovered only to 80% of control values in holm oak, due to persistently low stomatal (g_s) and mesophyll (g_m) conductances to CO_2. Moreover, leaf intrinsic water use efficiency (*WUE*) was kept almost constant in this species during the entire experiment. By contrast, photosynthesis of rockrose recovered almost completely after each drought cycle (90–100% of control values), while the *WUE* was largely and permanently increased (by 50–150%, depending on the day) after S1. This was due to a regulation which consisted in keeping g_s low (recovering to 50–60% of control values after re-watering) while maintaining a high g_m (even exceeding control values during re-watering). While the mechanisms to achieve such particular regulation of water and CO_2 diffusion in leaves are unknown, it clearly represents a unique acclimation feature of this species after a drought cycle, which allows it a much better performance during successive drought events. Thus, differences in the photosynthetic acclimation to repeated drought cycles can have important consequences on the relative fitness of different Mediterranean species or growth forms within the frame of climate change scenarios.

Key words: Acclimation, drought–recovery cycles, mesophyll and stomatal conductance, osmotic adjustment, photosynthetic limitation analysis, water use efficiency.

Introduction

More frequent extreme drought events are expected within the next decades and, in particular, across the Mediterranean region (Schär *et al.*, 2004; Ciais *et al.*, 2005). Thus, acclimation and adaptation to limited water supply are of central importance for plant growth and survival. One of the most crucial physiological mechanisms involved in adaptation and/or survival is photosynthesis (Chaves *et al.*, 2002; Flexas *et al.*, 2004).

The most dominant growth forms in the Mediterranean ecosystem are evergreen and semi-deciduous plants. Several

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authors have addressed photosynthetic and growth form-related aspects among both groups (Margaris, 1981; Ehleringer and Mooney, 1983; Werner *et al.*, 2002; Galmes *et al.*, 2007*a*, *b*; Gulias *et al.*, 2009; Medrano *et al.*, 2009). It has been shown, that semi-deciduous species reach higher maximum photosynthetic rates than evergreens, and both groups present similar diurnal and seasonal variations (Ehleringer and Mooney, 1983; Medrano *et al.*, 2009). However, so far, hardly anyone has addressed (i) the velocity of photosynthetic decline during drought, (ii) the velocity of recovery from drought (re-watering) or (iii) the potential acclimation to consecutive drought–recovery cycles. The velocity of recovery has been suggested to be as much impaired as velocity and/or magnitude of decline in photosynthesis during drought (Niinemets, 2010), whereas repeated drought–recovery cycles are far more common than a single (prolonged) drought event. Thus, information is needed on how Mediterranean species of the two predominant growth forms (evergreen, semi-deciduous) respond to these environmental constraints. The aim of the present study was (i) to address the three points mentioned above and (ii) to evaluate the underlying limitations (stomatal, mesophyll or biochemical) related to such changes in photosynthesis.

Materials and methods

Plant material and experimental set-up

The experiments were carried out with two perennial species, the semi-deciduous rockrose (*Cistus albidus;* one year old) and the evergreen holm oak (*Quercus ilex;* two years old) during late spring (April to June). Saplings were grown outside in pots (15.0 l) under a roof of transparent plastic foil to protect them from rainfall. The roof absorbed less than 20% of the sunlight (i.e. in the visible range). Plant size and leaf area were similar in both species at the onset of the experiment.

Plants were divided in two groups of 5–10 individuals of each species and arranged in a randomized plot. One group was kept under well-watered conditions (control) throughout the entire experimental period. The other group was subjected to severe drought (stress) by withholding water until the stomatal conductance for water vapour (g_s) during mid-morning (at the peak photosynthetic activity) dropped below 50 mmol m^{-2} s^{-1} (Flexas and Medrano, 2002; Medrano *et al.*, 2002). This is a very convenient method for comparing different species under different conditions. There is a strong link between g_s and photosynthesis (perhaps co-regulation between them), so different relationships between *RWC* or water potential and photosynthetic rate and changes in metabolism in different species and studies may be 'normalized' by relating them to g_s. As stomatal adjustment (reducing g_s) is the first response to drought, severe drought can be considered when other limitations than stomata become dominant and impair photosynthetic activity. Accordingly, Flexas and co-workers defined this threshold of 50 mmol m^{-2} s^{-1}, because photosynthetic activity becomes predominantly inhibited by metabolic processes below this level of g_s—besides stomatal limitations (changes in g_s)—indicating a situation of severe drought stress experienced by the plant. When plants reached the desired drought intensity they were re-watered to field capacity in the evening and also during the following days until g_s and net photosynthesis (A_N) were almost restored to control values (recovery). Such a 'drought–recovery cycle' was repeated thereafter two more times. Measurements of leaf gas exchange and chlorophyll fluorescence parameters were taken almost every day throughout these three cycles, while leaf samples for biochemical analysis were taken at the peaks of severe drought and recovery.

Plant water status

When stressed plants reached the desired drought level and after full recovery the relative water content (*RWC*) of four to six leaves per treatment was determined at midday (13.00 h) as

$$RWC(\%) = 100\times(FW - DW)/(TW - DW)$$

where *FW*, *TW*, and *DW* denote the weight of fresh, turgid, and dry leaf tissue, respectively. *FW* was determined immediately after sampling, while *TW* was obtained after incubating the leaf discs in distilled water for 48 h in the dark at 4 °C. *DW* was determined from oven-dried material after 72 h at *c*. 70 °C.

In parallel to the sampling for *RWC*, the leaf water potential (*LWP*) was determined with a Scholander pressure chamber in leaves of both treatments (n=3–5) at midday (Galle *et al.*, 2007).

Leaf gas-exchange and chlorophyll a *fluorescence*

Throughout the experiments, maximum net CO_2 assimilation (A_N), g_s, and chlorophyll *a* fluorescence were measured simultaneously with an open infrared gas-exchange analyser system (Li-6400; Li-Cor Inc., Lincoln, NE, USA) equipped with a leaf chamber fluorometer (Li-6400-40, Li-Cor Inc.). At least four measurements on the youngest fully expanded, sun-exposed leaves of control and stressed plants were carried out each day in the late morning (11.00–12.30 h) under light-saturating photosynthetic photon flux density (PPFD) of 1500 μmol m^{-2} s^{-1} (provided by the light source of the Li-6400 with 10% blue light). With regard to the prevailing ambient temperature and to provide a comparable database all records were taken at 28 °C (fixed block temperature of the Li-6400). The CO_2 concentration in the Li-6400 leaf chamber (C_a) was set to 400 μmol CO_2 mol^{-1} air and the relative humidity of the incoming air ranged between 40% and 60%. The CO_2 response curves ('A_N–C_i curves') were performed in watered, non-watered, and re-watered plants by varying the CO_2 concentration around leaves that have n previously beeacclimated to saturating light conditions (*c*. 15–20 min at a PPFD of 1500 μmol m^{-2} s^{-1}). The A_N–C_i curves were started at a C_a of 400 μmol CO_2 mol^{-1} air and then stepwise reduced (by 50 μmol CO_2 mol^{-1} air) until 50 μmol CO_2 mol^{-1} air was reached, thereafter CO_2 was stepwise increased by 100 μmol CO_2 mol^{-1} air until 2000 μmol CO_2 mol^{-1} air was reached.

From the fluorescence measurements, the actual quantum efficiency of the photosystem II (PSII)-driven electron transport (Φ_{PSII}) was determined according to Genty *et al.* (1989) as

$$\Phi PSII = \left(F_m^{'} - F_s\right)/F_m^{'}$$

where F_s is the steady-state fluorescence in the light (here PPFD 1500 μmol m^{-2} s^{-1}) and F_m the maximum fluorescence obtained with a light-saturating pulse (~8000 μmol m^{-2} s^{-1}). As Φ_{PSII} represents the number of electrons transferred per photon absorbed by PSII, the rate of electron transport (J) can be calculated as

$$J\left(\mu mole^{-}m^{-2}s^{-1}\right) = \Phi_{PSII}\times PPFD\times\alpha$$

where the term α includes the product of leaf absorptance and the partitioning of absorbed quanta between photosystems I and II. The α was determined for each treatment from the slope of the relationship between Φ_{PSII} and (Φ_{CO2}) (i.e. the quantum efficiency of gross CO_2 fixation), which was obtained by varying light

intensity under non-photorespiratory conditions in an atmosphere containing <1% O_2 (Valentini *et al.*, 1995).

From combined gas-exchange and chlorophyll *a* fluorescence measurements, the mesophyll conductance for CO_2 (g_m) was estimated according to Harley *et al.* (1992) as

$$g_m = A_N/(C_i - (\Gamma^*(J + 8(A_N + R_d)))/(J - 4(A_N + R_d)))$$

where Γ^* is the O_2 photo-compensation point and R_d is day respiration. A_N and C_i were obtained from gas-exchange measurements. A value of 37 μmol mol^{-1} for the CO_2 compensation point under non-respiratory conditions (Γ^*) in *Q. ilex*, and a Γ^* of 40.6 in *C. albidus* was used as calculated from their Rubisco specificity factor 'τ' (Balaguer *et al.*, 1996; Galmes *et al.*, 2006) and according to Brooks and Farquhar (1985): $\tau = 0.5O/\Gamma^*$ (O denotes for the oxygen molar fraction at the oxygenation site). All Rubisco kinetics and their temperature dependencies were taken from Bernacchi *et al.* (2002).

In the experiments, night respiration (R_n) was used as a proxy for R_d by dividing R_n by 2 (Villar *et al.*, 1995; Niinemets *et al.*, 2005). R_n was determined by gas-exchange (Li-6400) and oxygen electrode (Rank Brothers) measurements ($n \geq 4$) at 28 °C, after plants had been dark-adapted for more than half an hour during the afternoon.

Calculated values of g_m were used to convert A_N–C_i curves into A_N–C_c curves according to the following equation:

$$C_c = C_i - (A_N/g_m)$$

Maximum velocity of carboxylation ($V_{c,max}$) was derived from A_N–C_c curves according to Bernacchi *et al.* (2002). Estimates of $V_{c,max}$ from A_N–C_i curves according to Ethier and Livingston (2004) were similar (data not shown) to those derived from A_N–C_c curves, hence only A_N–C_c derived values are presented here.

Corrections for the leakage of CO_2 into and out of the leaf chamber of the Li-6400 have been applied to all gas-exchange data, as described by Flexas *et al.* (2007).

Quantitative limitation analysis

To assess the limitations imposed by water stress and recovery on photosynthesis, a quantitative limitation analysis of photosynthesis was conducted for all three data sets according to Grassi and Magnani (2005) with modifications. According to their approach measurements of A_N, g_s, g_m, and $V_{c,max}$ were used to calculate the proportion of the three major components of total limitation for CO_2 assimilation: stomatal (SL) and mesophyll conductance (ML), as well as biochemical processes (BL). Since actual electron transport rate (i.e. fluorescence derived J) is tightly coupled with $V_{c,max}$ (Galmes *et al.*, 2007*b*) and should indeed reflect gross photosynthesis (Genty *et al.*, 1989; Valentini *et al.*, 1995), BL was calculated using J instead of $V_{c,max}$ as a surrogate for leaf biochemistry. Thus, possible errors in the determination of $V_{c,max}$, which have been updated recently (Patrick *et al.*, 2009; Gu *et al.*, 2010), can be avoided (as $V_{c,max}$ values are derived from A_N–C_c curves and depend on the validity of Rubisco kinetics as estimated by Bernacchi *et al.*, 2002),. The validity of using of J instead of $V_{c,max}$ for calculations of BL has been verified and confirmed by Galle *et al.* (2009).

In the current study, the maximum assimilation rate, concomitantly with g_s, g_m, and J (and $V_{c,max}$), was generally reached under well-watered conditions, therefore the control treatment was used as a reference. However, since A_N of irrigated plants declined during the experiment, especially in *Cistus albidus*, presumably due to leaf ageing, the values for irrigated plants 'for each day' were considered as the reference for the stressed or recovering plants determined during the same day. In doing so, photosynthesis limitations due to leaf ageing were eliminated and, hence, 'pure' water stress limitations were obtained for stressed plants (Flexas *et al.*, 2009). Whenever one of the involved parameters (g_s, g_m, and J) was higher in stressed than in irrigated plants, its corresponding limitation was set to zero, and the other limitations re-calculated accordingly (Galle *et al.*, 2009).

Grassi and Magnani (2005) also defined a fourth photosynthesis limitation associated with leaf temperature (TL). However, this one is not considered here because: (i) photosynthesis limitations were calculated separately for each of the three experiments, and the differences in leaf temperature between irrigated and water stressed plants were small (i.e. <4 °C); (ii) leaf temperature was already considered in determining g_m; (iii) Grassi and Magnani (2005) already showed that TL was generally negligible, reaching maximum values as low as 4–7% even for leaf temperature differences between the reference value and the treatment of up to 20 °C.

Biochemical analysis

Leaf discs of at least four plants per treatment and species were snap-frozen in liquid N_2 at each drought and recovery cycle (S1–S3 and R1–R3) around midday and stored at –80 °C until analysis.

Total soluble carbohydrates (TSC) were determined from the soluble part of leaf extracts according to Stieger and Feller (1994). Glucose was used as reference standard.

The pool of ascorbic acid, as well as its oxidized (dehydroascorbate, DHA) and reduced (ascorbate, AA) form, was determined according to Law *et al.* (1983) and modified as described in Barth and Krause (1999).

Results

The climatic conditions during the experimental period were typical for a Mediterranean late spring. Air temperature ranged between 18 °C and 32 °C during the day (minimum temperatures at night were above 10 °C), while rainfall was less than 100 mm during April and May (data not shown). Leaf temperature of holm oak and rockrose reached maximal values of 34 °C and 32 °C, respectively.

The relative leaf water content (RWC) and the leaf water potential (Ψ) at midday decreased significantly in both species during the three drought events (S1, S2, S3) compared with the corresponding control plants (Fig. 1). However, RWC and Ψ were restored to control values after re-watering in all cases, except for rockrose at R3 where Ψ was only partially restored (Fig. 1d). At the minimum level, RWC and Ψ decreased to 75% (S1) and –2.9 MPa (S2) in stressed oaks, respectively. In rockrose, RWC and Ψ were as low as 57% (S1) and –2.6 MPa (S3), respectively. In general, and most likely related to the leaf structure, RWC was somewhat higher in the more sclerophyllous leaves of holm oak than of rockrose, with control values of around 87% (holm oak) and 76% (rockrose).

According to their growth form (Ehleringer and Mooney, 1983; Gulias *et al.*, 2009), higher photosynthetic rates (A_N), stomatal (g_s) and mesophyll (g_m) conductances were measured in the semi-deciduous rockrose than in the evergreen holm oak under well-watered conditions (Fig. 2). Net photosynthetic rates (A_N) of well-watered (control) rockrose and holm oak averaged around 15 μmol m^{-2} $s^{-1}$and 9 μmol m^{-2} s^{-1}, respectively (Fig. 2a, b). A_N declined with prolonged water deficit during all three drought cycles, reaching minimum values of around 5 μmol m^{-2} s^{-1} and 2 μmol m^{-2} s^{-1} in rockrose and holm oak, respectively. After re-watering, A_N

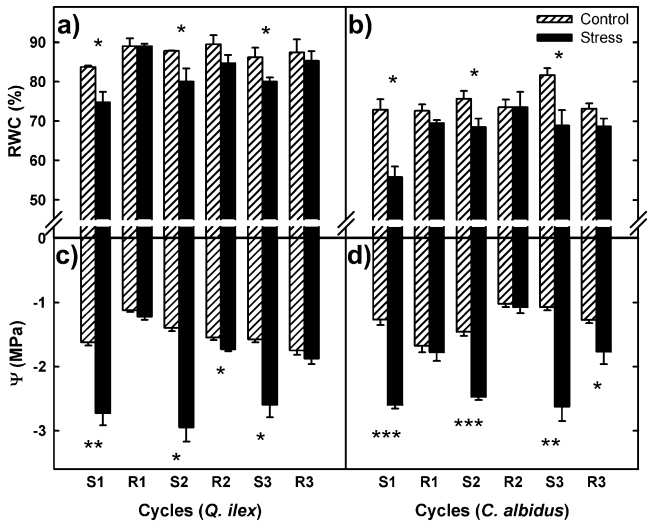

Fig. 1. Changes in relative water content (*RWC*) and water potential of leaves (Ψ) of *Q. ilex* (a, c) and *C. albidus* (b, d) at the days of severe drought (S) and recovery (R) in control (hatched bars) and stressed plants (black bars). The numbers of cycles are also indicated. Means and standard errors of at least four leaves of each species and treatment are presented. Significant differences in *RWC* and Ψ between corresponding control and stressed plants are indicated by asterisks (*$P \leqslant 0.05$, **$P \leqslant 0.01$, ***$P \leqslant 0.001$).

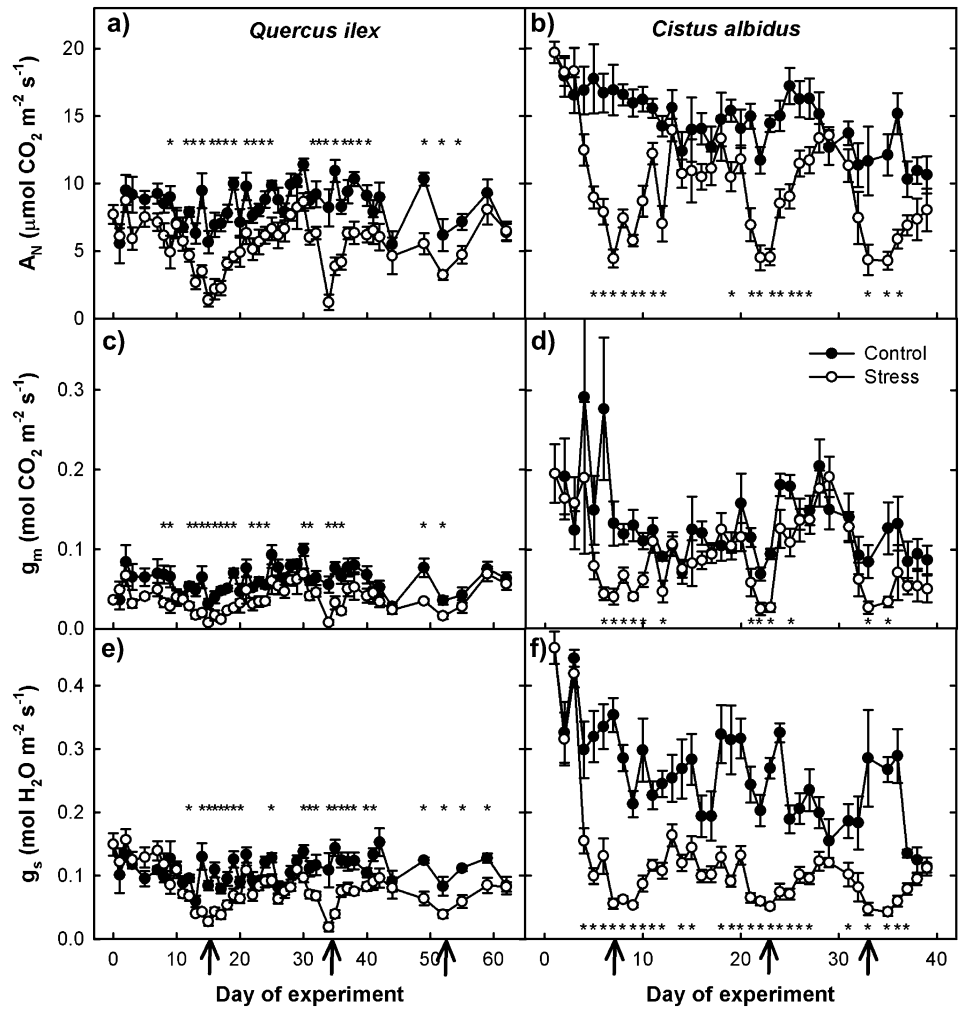

Fig. 2. Variation of net photosynthetic rates (A_N), mesophyll conductance (g_m), and stomatal conductance (g_s) in control (filled circles) and stress plants (open circles) of *Q. ilex* (a, c, e) and *C. albidus* (b, d, f) during the three drought and recovery cycles. Means and errors of at least four plants per treatment and species are shown. Arrows indicate the day when desired drought intensity was reached and re-watering phase started that evening. Asterisks indicate significant difference between control and stressed plants ($P \leqslant 0.05$).

was restored to control values in all three cycles. Similarly, stomatal (g_s) and mesophyll (g_m) conductance declined during drought stress and recovered after re-watering (Fig. 2c–f). The desired drought level was reached in all three cycles and species, as indicated by the drop of g_s below 50 mmol H_2O m^{-2} s^{-1} (see the Materials and methods). A slight but continuous decrease in A_N and g_s and a slight decline in g_m was observed in rockrose leaves throughout the experiment period. Notably, g_s of stressed rockrose remained mostly and markedly below control values after the first drought event, indicating constitutive modifications of stomatal opening or of leaf diffusion components (see also g_m/g_s ratio; see Supplementary Fig. S2 at *JXB* online), while g_m only differed significantly during the periods of severe drought among drought and control plants. In addition to the profound changes of g_s during the first drought cycle, rockrose's leaf mass per unit area (LMA; see Supplementary Fig. S1 at *JXB* online) dropped significantly during the first drought and was restored slowly thereafter, while the clerophyllous leaves of holm oak maintained their high LMA, irrespective of drought. Overall, higher LMA in holm oak than in rockrose represent common characteristics of evergreen and deciduous species (Ehleringer and Mooney, 1983). Although being considered a summer-deciduous perennial, rockrose did not undergo early senescence or show wilting of leaves throughout the entire experiment (neither did holm oak).

When expressing the above- described data of leaf gas exchange of stressed plants as a percentage of the corresponding control values, the following patterns and motifs of responses could be revealed (Fig. 3). A_N, g_s, and g_m of stressed holm oak responded similarly during the drought–recovery-cycles (except for the first drought event, until day 10), thus following the same changes in amplitude/percentage (Fig. 3a). By contrast, A_N and g_m of stressed rockrose were less affected than g_s (they always presented higher percentages than g_s; Fig. 3b), which resulted in steadily higher intrinsic water use efficiency (*WUE*) during the experiment with particularly high values of *WUE* during severe drought events. However, during the last recovery from drought (R3) this relationship changed and g_s exceeded A_N and g_m, resulting in a breakdown of the previously improved *WUE*. Overall, g_m presented the greatest variations in rockrose, markedly exceeding control values during recovery (Fig. 3b; R1 and R2), while g_s displayed the smallest range of variation during the entire experiment.

With regard to the main limiting processes of photosynthesis during drought and recovery, the predominant role of the diffusion processes was evident in both species (Fig. 4). In holm oak, the contribution of the mesophyll (ML) to the limitations of photosynthesis were greatest during the first two drought events, while stomatal limitations (SL) became more dominant during the third drought event. By contrast, SL was mostly similar to ML during the repeated drought stress in rockrose (Fig. 4c). Most remarkably, even negative values of SL were determined during recovery due to reduced g_s and elevated g_m (exceeding control values, Fig. 3b). Overall, total limitation (ML+SL+BL) reached more than 70% during each drought event and in both species (Fig. 4),

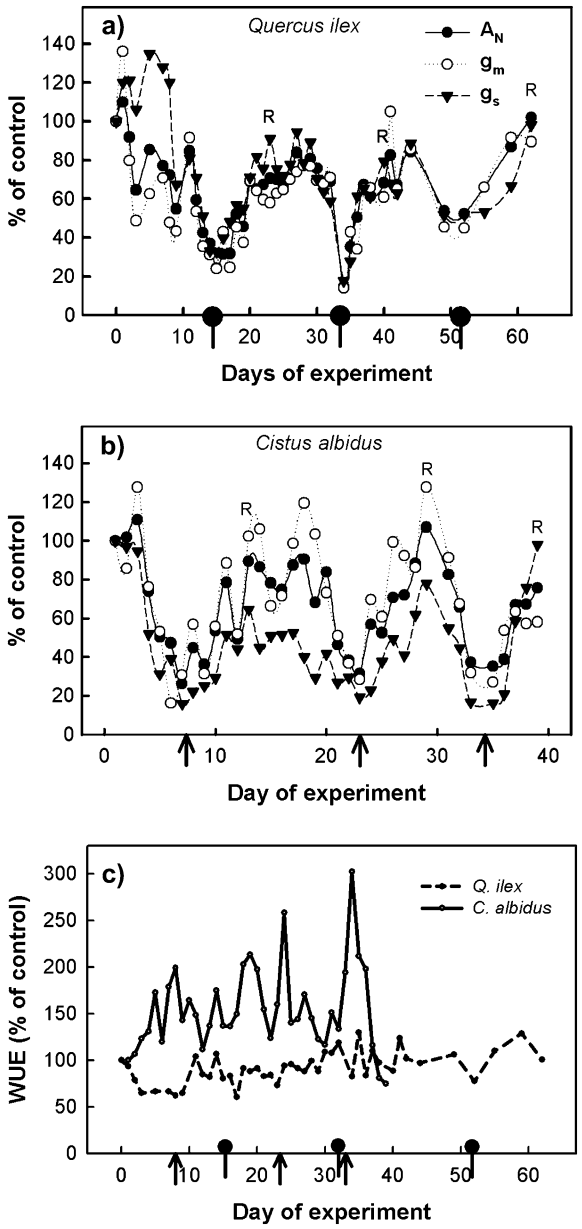

Fig. 3. Deviation from the control values during the drought–recovery cycles are shown for net photosynthetic rates (A_N), mesophyll conductance (g_m), and stomatal conductance (g_s), as well as for intrinsic water use efficiency (*WUE*; A_N/g_s) of *Q. ilex* (a, c) and *C. albidus* (b, c). All data points represent means of at least four leaves. Errors are not shown to provide better visibility. Arrows and bullets at the *x*-axis indicate the day when desired drought intensity was reached for *C. albidus* (arrows) and *Q. ilex* (bullets), respectively. R indicates the day of full recovery.

while it always dropped below 20% after recovery, even reaching negative values in the case of rockrose. Thus, all limitation factors were considerably reduced after the onset of re-watering in all three cycles. Limitation of photosynthesis by biochemical processes (BL) was of minor relevance for both species, as BL reached maximum values of only 10–15% during severe drought.

Although the species-specific response of leaf gas exchange parameters seemed to follow a similar pattern

during the three drought–recovery cycles, the period of time for each cycle varied among the species (Table 1). Most notably, recovery from drought was always faster than drought establishment in both species, in which holm oak displayed the quickest recovery of both species (i.e. in terms of recovery versus drought establishment). Moreover, both species recovered the slowest after the second drought cycle, when referring to the lowest ratio between the days of recovery and the days of drought stress (R_i/S_i). Accordingly, the fastest recovery from drought was thus detected during the third cycle for holm oak and during the first cycle for rockrose. In absolute numbers, the shortest drought and recovery phase of holm oak was in S2 (11 d) and R2 (6 d) and of rockrose in S3 (6 d) and R3 (5d).

Beside great changes in photosynthetic parameters during drought and recovery (Fig. 2), the maximum carboxylation efficiency of Rubisco ($V_{c,max}$) in holm oak and rockrose was not affected throughout the experiment, as no significant changes could be detected (Table 1). Maximum photosynthetic electron transport efficiency (J_{max}) remained almost unaltered during the entire experimental period, with only one exception for rockrose in S3, where a significant ($P \leq 0.05$) decrease of J_{max} was observed. Overall, rockrose presented higher $V_{c,max}$ and J_{max} than holm oak.

During the three drought–recovery cycles, adjustments of the total soluble carbohydrates (TSC), the ascorbate pool (AA+DHA) and its redox state (DHA/AA+DHA) were observed in both species (Fig. 5). Repeated drought cycles resulted in an increasing amount of TSC in leaves of holm oak (Fig. 5a) as well as causing increased oxidation of the ascorbate pool (DHA; Fig. 5e; S2, S3). However, after recovery (R1, R2) the redox state of ascorbate (DHA/

Table 1. Changes in the maximum rates of Rubisco carboxylation ($V_{c,max}$; μmol e^- m^{-2} s^{-1}), of electron transport (J_{max}; μmol e^- m^{-2} s^{-1}) and in the time period until desired drought or recovery was reached (days) in *Q. ilex* and *C. albidus* during the three drought-recovery cycles. C, S, and R denote the control, severe drought, and recovery treatments, respectively, whereas the number indicates the cycle of drought and recovery

	C_{start}	S1	R1	S2	R2	S3	R3	C_{end}
Q. ilex								
$V_{c,max}$	190.0	163.2	216.7	200.0	212.9	219.3	234.3	219.1
	±11.2	±24.6	±10.6	±5.6	±8.5	±5.3	±16.4	±15.4
J_{max}	154.4	155.6	161.4	140.6	169.1	165.1	175.4	167.2
	±6.1	±8.8	±8.9	±8.0	±5.1	±11.4	±10.8	±24.4
Days		14	9	11	6	12	10	
C. albidus								
$V_{c,max}$	274.4	297.6	305.9	313.9	291.4	236.0	265.3	249.5
	±15.0	±22.5	±12.8	±19.3	±7.4	±16.3	±14.9	±10.0
J_{max}	211.3	201.3	237.4	197.7	225.0	149.1	204.0	188.7
	± 9.3	±15.2	±12.3	±17.9	±4.0	±12.5	±10.3	±7.4
Days		7	6	13	10	6	5	

Means and standard errors of at least four plants are shown. Significant differences between control (C_s+C_e) and stressed plants ($P \leq 0.05$) are indicated by underlined values.

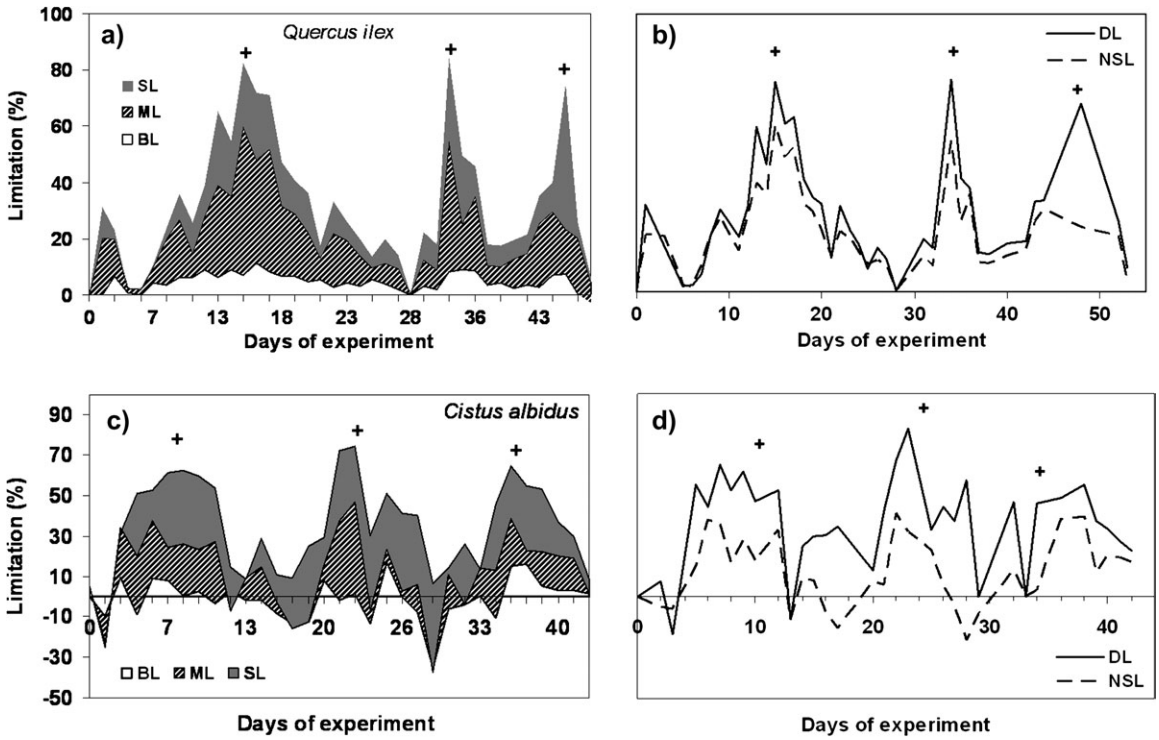

Fig. 4. Quantitative limitation analysis of *Q. ilex* (a, b) and *C. albidus* (c, d) during the three drought and recovery cycles. Total limitation of photosynthesis is represented by the sum of stomatal (SL), mesophyll (ML), and biochemical (BL) limitations (a, c), while ML+SL represent diffusional limitations (DL) and ML+BL represent non-stomatal limitations (NSL) (b, d). Crosses indicate the day when desired drought intensity was reached.

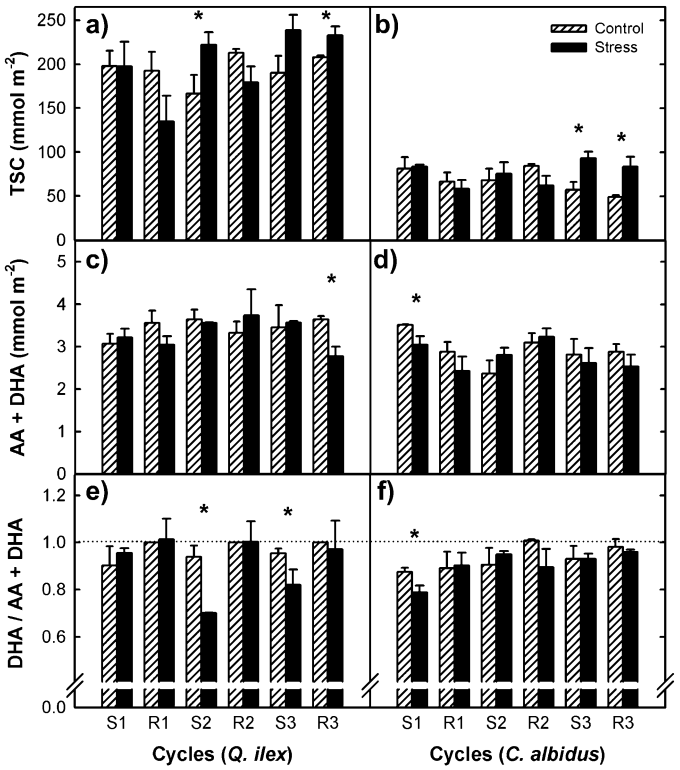

Fig. 5. Changes of total soluble carbohydrates (TSC), the ascorbate pool (reduced+oxidized ascorbate: AA+DHA) as well as its oxidation status (DHA/AA+DHA) during the three drought–recovery cycles in *Q. ilex* (a, c, e) and *C. albidus* (b, d, f). Means and standard errors of at least four control (hatched bars) and stress plants (black bars) for each cycle are shown. Asterisks indicate significant differences between corresponding control and stress plants ($P \leqslant 0.05$).

AA+DHA) was similar in both control and stressed oak leaves, whereas a significant reduction of the ascorbate pool was observed after the last drought event (Fig. 5c; R3). Apart from this, the ascorbate pool remained almost unaltered at about 3.5 mmol m^{-2} in control and stressed oak leaves. Notably, elevated TSC values remained during the last cycle (S3, R3) compared with the corresponding controls (Fig. 5a).

Such a marked increase in TSC during the last cycle was also found in stressed rockrose leaves (Fig. 5b; S3, R3), while little variation was detected during the two cycles before. In contrast to what has been observed in holm oak, the ascorbate pool (AA+DHA) and its redox state were only significantly affected during the first drought event (Fig. 5d, f; S1) but did not differ from controls during the rest of the experiment. In general, the pool of TSC was smaller in leaves of rockrose than of holm oak, while their ascorbate pools were very similar. Moreover, rockrose leaves presented somewhat more oxidized ascorbate levels than holm oak leaves (Fig. 5e, f).

The relationship between A_N and g_m as well as between A_N and g_s represent hyperbolic regression functions with good correlation among both species (Fig. 6). However, carbon fixation and photochemistry (A_N versus J) were less correlated in rockrose than in holm oak. The regression function of A_N versus g_m saturates above a g_m of 0.1 mol CO_2 m^{-2} s^{-1} for rockrose and a g_m of 0.05 mol CO_2 m^{-2} s^{-1} for holm oak, thus representing growth form-related differences (Niinemets *et al.*, 2009). Also data on A_N versus g_s indicate an earlier saturation of A_N in terms of g_s for holm oak (<0.2 mol H_2O m^{-2} s^{-1}) than for rockrose (>0.3 mol H_2O m^{-2} s^{-1}). Overall, A_N correlated best with g_s in rockrose, while in holm oak A_N correlated best with g_m.

Discussion

Leaf photosynthetic responses to repeated drought cycles have been examined on the evergreen holm oak and the semi-deciduous rockrose. These two species were selected as belonging to the two major growth forms of the Mediterranean climate, although a single species may not be fully representative for its growth form. By keeping this in mind, the response of leaf photosynthetic traits to three drought–recovery cycles have been studied in order to assess the underlying physiological processes of photosynthetic acclimation.

Maximum rates of net photosynthesis (A_N), stomatal (g_s) and mesophyll conductance (g_m) under well-watered conditions were typically higher in leaves of rockrose than of holm oak (Fig. 2), according to their respective growth form and leaf longevity (Ehleringer and Mooney, 1983; Reich *et al.*, 1992; Gulias *et al.*, 2009). Furthermore, somewhat higher maximum rates of Rubisco carboxylation ($V_{c,max}$) and photosynthetic electron transport (J_{max}) were determined in rockrose than in holm oak. In both species, these two parameters remained almost unaltered throughout all three drought cycles, indicating preserved and functional photosynthetic machinery among the species and treatments.

Differences among both species were also observed during the progression of drought (withholding water) and upon recovery in terms of velocity of decline and increase, respectively, in A_N, g_s, and g_m (Fig. 3; Table 1). For all three parameters, the drought-induced decline was always faster than the recovery after re-watering, whereas A_N, g_s; and g_m declined in a concerted manner during each drought cycle in both species. Moreover, A_N, g_s; and g_m of holm oak seemed to be strictly co-regulated during drought and recovery, as indicated by the variation of these parameters in the percentage of control values (Fig. 3a) and by the recurrent variations of the limiting factors to photosynthesis (Fig. 4). By contrast, in rockrose, g_s and g_m responded differently after the first drought cycle. Most remarkably, g_s recovered only partly although A_N was fully restored to control values after re-watering (Figs 2, 3), thus leading to a higher water use efficiency (*WUE*) of rockrose leaves (Fig. 3c). Interestingly, g_s remained on a somewhat lower level than well-watered control plants after this first drought cycle, suggesting some internal adjustments towards minimized loss of water and/or improved water use for carbon gain (*WUE*). In addition to that, g_m always exceeded control values during re-watering,

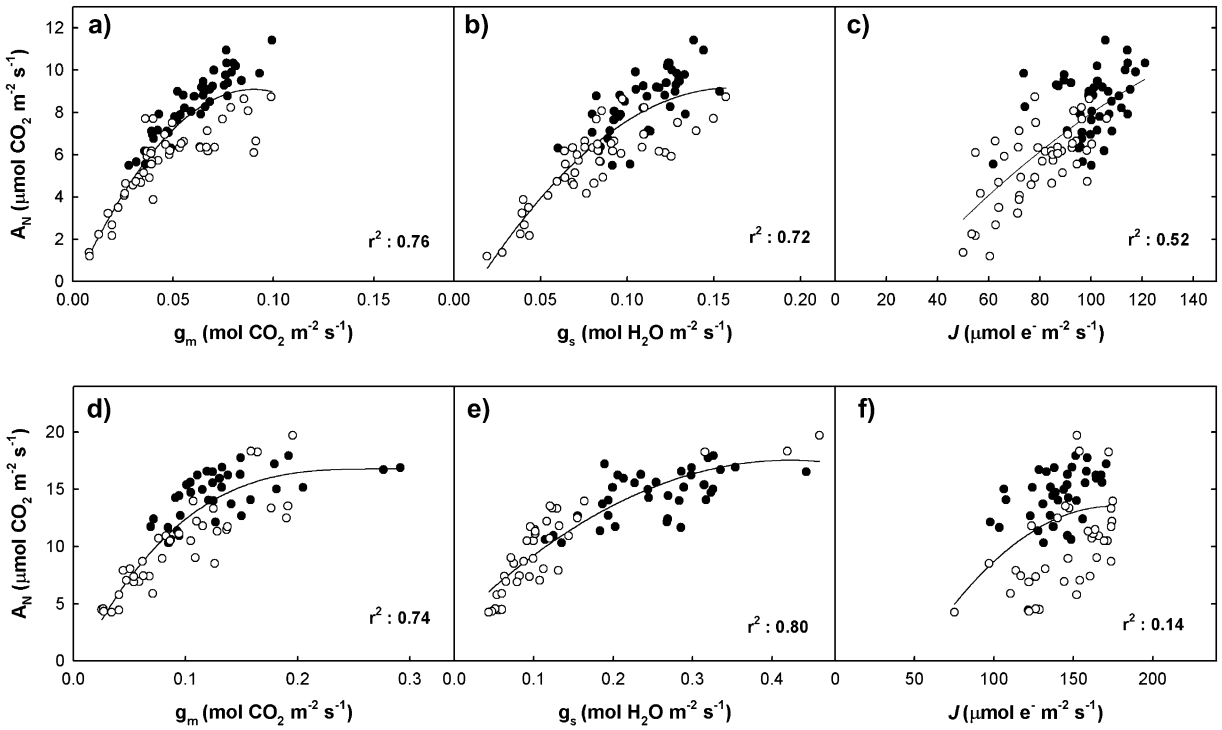

Fig. 6. The relationship of net photosynthesis (A_N) to mesophyll conductance (g_m), stomatal conductance (g_s) and photosynthetic electron transport (J) in *Q. ilex* (a, b, c) and *C. albidus* (d, e, f) under stress and well-watered conditions. Open circles and closed circles represent single measurements on stress and control plants throughout the entire experiment, respectively.

thereby improving the diffusion and availability of CO_2 across the leaf (i.e. in chloroplasts), which, in turn, facilitated rapid photosynthetic recovery and similar rates of A_N than in control plants but with lower g_s. Therefore, the ratio g_m/g_s was increased in rockrose as a consequence of the drought-re-watering cycles, leading to general increases in *WUE*, as already shown in other species increasing this ratio (Duan *et al.*, 2009; Flexas *et al.*, 2010). The underlying mechanisms for these relatively rapid leaf-internal adjustments (few days) still remain unclear, but are unlikely to be related to changes in leaf structure/morphology. One may speculate that such modifications took place at the cellular level (within the mesophyll) through changes in aquaporins or carbonic anhydrases, which have been shown to play a role in the diffusion of CO_2 across the mesophyll (Gillon and Yakir, 2000; Uehlein *et al.*, 2003, 2008; Heckwolf *et al.*, 2011). With regard to these leaf-intrinsic adjustments in rockrose it became evident that stomata posed the predominant limitation to photosynthesis during drought and initial recovery (Fig. 4a), whereas g_m (ML) predominantly limited photosynthesis in holm oak (Fig. 4c). Thus, diffusional limitations largely affected the photosynthesis of rockrose during most of the stress and recovery periods, whereas in holm oak an almost equal contribution of both diffusional and non-diffusional limitations was detected. It should be noted that the maximum limitations to photosynthesis were similar among both species and that, in the case of rockrose, even negative values have been obtained (Fig. 4c, d), which was due to the improved g_m during recovery exceeding the control values.

From accompanying measurements related to oxidative stress, no change of the pool and redox state of ascorbate—the major and most abundant antioxidant in leaves (Smirnoff, 2000)—was detected in rockrose after the first drought cycle (Fig. 5), suggesting that oxidative stress/damage was of minor relevance or not present after experiencing the first drought event. By contrast, some variation during drought was detected in holm oak, whereas this range of reduction was most likely not sufficient to affect cell homeostasis or cause irreversible damage. Total soluble carbohydrates accumulated during repeated drought events in both species (Fig. 5), which may suggest either restricted sugar exportation from leaves through the xylem (Bota *et al.*, 2004) or, most likely, some kind of osmotic adjustment (Epron and Dreyer, 1996). In addition, relative leaf water content (*RWC*) was less reduced during the second and third drought cycle whereas leaf water potential (ψ) was more reduced (S2 for holm oak, S3 for rockrose), possibly indicating that such adjustment took place (Larcher *et al.*, 1981). However, further studies are needed to confirm the presence of osmotic adjustment during consecutive drought cycles.

Concluding remarks

The response of photosynthetic traits to repeated drought and recovery cycles differed among holm oak and rockrose with regard to their leaf physiology and ontogeny. In general, the velocity of photosynthetic changes in response to both water stress imposition and recovery were faster in rockrose than in holm oak, although of similar magnitude. Moreover, profound adjustments of leaf diffusion components consistent in adjusting g_s low and g_m high (i.e.

increasing g_m/g_s) have been detected in rockrose during the first drought cycle, which resulted in improved *WUE* and most likely in more rapid recovery after drought. Moreover, g_s remained on a lower level thereafter, while g_m exceeded control values during re-watering, thus optimizing carbon gain per unit water used through the successive drought cycles.

Here, the shorter life-span of rockrose leaves might be an important aspect, as rockrose has to adjust and optimize its photosynthetic activity during its short life-span and under varying environmental conditions (Reich *et al.*, 1992; Gulias *et al.*, 2009). A rapid or flexible adjustment of leaf-intrinsic parameters is not necessary for maintained growth or possibly too costly for holm oak, due to its long-living leaves and the strategy of long-term maximization of carbon gain relative to construction and maintenance costs (Ehleringer and Mooney, 1983).

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Changes of leaf mass per unit area (*LMA*) during the three drought-recovery cycles in holm oak and rockrose.

Supplementary Fig. S2. Changes of the ratio of stomatal to mesophyll conductances for CO_2.

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