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# Photosynthesis limitations during water stress acclimation and recovery in the drought-adapted *Vitis* hybrid Richter-110 (*V. berlandieri*×*V. rupestris*)

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## Abstract

**The hybrid Richter-110 (*Vitis berlandieri*×*Vitis rupestris*) has the reputation of being a genotype strongly adapted to drought. A study was performed with plants of R-110 subjected to sustained water-withholding to induce acclimation to two different levels of water stress, followed by rewatering to induce recovery. The goal was to analyse how photosynthesis is regulated during acclimation to water stress and recovery. In particular, the regulation of stomatal conductance ($g_s$), mesophyll conductance to $CO_2$ ($g_m$), leaf photochemistry (chlorophyll fluorescence and thermoluminescence), and biochemistry ($V_{c,max}$) were assessed. During water stress, $g_s$ declined to 0.1 and less than 0.05 mol $CO_2$ $m^{-2}$ $s^{-1}$ in moderately and severely water-stressed plants, respectively, and was kept quite constant during an acclimation period of 1-week. Leaf photochemistry proved to be very resistant to the applied water-stress conditions. By contrast, $g_m$ and $V_{c,max}$ were affected by water stress, but they were not kept constant during the acclimation period. $g_m$ was initially unaffected by water stress, and $V_{c,max}$ even increased above control values. However, after several days of acclimation to water stress, both parameters declined below ($g_m$) or at ($V_{c,max}$) control values. For the latter two parameters there seemed to be an interaction between water stress and cumulative irradiance, since both recovered to control values after several cloudy days despite water stress. A photosynthesis limitation analysis revealed that diffusional limitations and not biochemical limitations accounted for the observed decline in photosynthesis during water stress and slow recovery after rewatering, both in moderately and severely stressed plants. However, the relative contribution of stomatal (SL) and mesophyll conductance (MCL) limitations changes during acclimation to water stress, from predominant SL early during water stress to similar SL and MCL after acclimation. Finally, photosynthesis recovery after rewatering was mostly limited by SL, since stomatal closure recovered much more slowly than $g_m$.**

**Key words:** Drought, mesophyll conductance, photochemistry, photosynthetic limitations, stomatal conductance, Rubisco, thermoluminescence, water stress.

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Abbreviations: ABA, abscisic acid; $A_N$, light-saturated net photosynthesis; $C_a$, atmospheric $CO_2$ concentration; $C_c$, chloroplast $CO_2$ concentration; $C_i$, substomatal $CO_2$ concentration; $C_i^*$, $C_i$ at the $CO_2$ compensation point in the absence of mitochondrial respiration; $g_m$, mesophyll conductance to $CO_2$; $g_s$, stomatal conductance; HTL, high temperature thermoluminescence; $J_{flu}$, electron transport rate determined by chlorophyll fluorescence; $J_{max}$, maximum rate of electron transport; R-110, Richter-110 (a hybrid of *Vitis berlandieri*×*Vitis rupestris*); $R_n$, leaf respiration in the dark or night respiration; $R_d$, leaf respiration in the light or day respiration; Rubisco, ribulose-1,5-*bis*phosphate carboxylase/oxygenase; RuBP, ribulose-1,5-*bis*phosphate; TL, thermoluminescence; τ, Rubisco specificity factor; Γ*, $C_c$ at the $CO_2$ compensation point in the absence of mitochondrial respiration; $V_{c,max}$, maximum rate of carboxylation; VPD, vapour pressure deficit.

## Introduction

Low water availability is the main environmental factor limiting plant growth and yield worldwide, and global change will probably make water scarcity an even greater limitation to plant productivity across an increasing amount of land (Chaves *et al.*, 2008). The limitation of plant growth imposed by low water availability is mainly due to reductions of plant carbon balance, which is largely dependent on photosynthesis. For this reason, photosynthesis responses to water stress have been the subject of study and debate for decades, in particular, concerning which are the most limiting factors for photosynthesis under water stress (Flexas and Medrano, 2002; Lawlor and Cornic, 2002).

Reduced $CO_2$ diffusion from the atmosphere to the site of carboxylation is the main cause for decreased photosynthesis under most water-stress conditions (Centritto *et al.*, 2003; Flexas *et al.*, 2004; Grassi and Magnani, 2005; Chaves *et al.*, 2008; Erismann *et al.*, 2008; Peeva and Cornic, 2009). Although, in many water-stress situations, photosynthetic reductions cannot be fully explained by stomatal closure alone, early experiments by Kaiser and others (reviewed in Kaiser, 1987; Cornic *et al.*, 1992) showed total restoration of photosynthesis when very high $CO_2$ concentrations was applied. Moreover, Cornic *et al.* (1989), using chlorophyll fluorescence emission, suggested the importance of changes under drought of the $CO_2$ resistance path from ambient air to carboxylating sites. Therefore, reduced leaf diffusive capacity is not only due to stomatal closure, but also to reduced mesophyll conductance to $CO_2$ ($g_m$). Both the physiological bases and the role of $g_m$ remain elusive, although there is now evidence that $g_m$ can vary at least as fast as stomatal conductance (Flexas *et al.*, 2007*a*, 2008), and it has been suggested that some aquaporins are involved in its regulation (Hanba *et al.*, 2004; Flexas *et al.*, 2006*a*), particularly under water stress (Miyazawa *et al.*, 2008). Regardless of the mechanisms for regulation of $g_m$, the response of photosynthesis to soil water shortage can be divided into two distinct phases: during the first stage, characterized by a daily maximum stomatal conductance ($g_s$) above 0.05–0.10 mol $H_2O$ $m^{-2}$ $s^{-1}$, photosynthesis is mostly limited by restricted $CO_2$ diffusion (decreased $g_s$ plus decreased $g_m$); during the second stage, characterized by stomatal conductance below that threshold, a general metabolic impairment can eventually occur, particularly under conditions of high light intensity that favour oxidative stress (Flexas *et al.*, 2004, 2006*b*; Zhou *et al.*, 2007).

The photosynthetic responses described above proceed, in general, from studies in which water stress was applied to plants over relatively short periods, and in which measurements are taken on specific, often few days during the experimental period. However, under natural conditions, water stress normally develops much more gradually, over periods comprising weeks or months, and hence it is possible that some acclimation occurs, in addition to day-to-day variations in response to variable environmental conditions (Flexas *et al.*, 2006*b*). Acclimation to water stress may comprise responses involving gene expression and modification of plant physiology and morphology, taking place in days to weeks, which lead to a homeostatic compensation for the initial negative effects of water stress on photosynthesis. In leaves acclimated to water stress during their development, a higher photosynthesis rate than in non-acclimated leaves is often associated with morphological adaptations and higher electron transport rates (Maury *et al.*, 1996; Kitao *et al.*, 2003; Galmés *et al.*, 2006). However, less is known about short-term acclimation to water stress in already developed leaves. Although the general idea is that morphological changes and osmotic adjustments may be a long-term acclimation, recent studies on transcriptomics and proteomics in plants subjected to water stress showed that the genes or proteins associated with metabolism display acclimation responses less than a week after drought stress imposition (Watkinson *et al.*, 2003; Bogeat-Triboulot *et al.*, 2007). In such studies, photosynthetic pathways are, in general, not among the most altered by the stress (reviewed in Chaves *et al.*, 2009). Even in those photosynthetic genes responding to stress, the most common trend is a down-regulation, i.e. they would not contribute to acclimation of photosynthesis, but rather to its further decline. In addition, the alterations found at transcriptomic level are larger (5–10%) than at protein level (usually less than 1%). In summary, there is little evidence for the acclimation of photosynthesis to water-stress conditions in the short term, but studies that specifically address this issue are needed. Moreover, day-to-day variations of photosynthesis during an acclimation period may lead to erroneous conclusions when measurements are taken on a single day during the period.

Besides acclimation, the carbon balance of a plant during a period of water stress and recovery may depend as much on the velocity and degree of photosynthetic recovery as it depends on the degree and velocity of photosynthesis decline during water depletion (Flexas *et al.*, 2006*b*). In general, plants subjected to severe water stress recover only 40–60% of the maximum photosynthesis rate during the day after rewatering, and recovery continues during the next days, but maximum photosynthesis rates are not always recovered (Kirschbaum, 1987, 1988; Sofo *et al.*, 2004). The strong influence of previous water stress severity in the velocity and extent of photosynthesis recovery has been illustrated in kidney bean by Miyashita *et al.* (2005) and has been suggested for *Vitis vinifera* as well (Gómez-del-Campo *et al.*, 2007). Over the last three years, many studies have addressed the response of photosynthesis to rewatering after water stress, which highlights the importance of the issue, but none of these studies have analysed all the potential physiological limitations to recovery (dos Santos *et al.*, 2006; Grzesiak *et al.*, 2006; Hura *et al.*, 2006; Bogeat-Triboulot *et al.*, 2007; Gallé and Feller, 2007; Gallé *et al.*, 2007; Gómez-del-Campo *et al.*, 2007; Montanaro *et al.*, 2007; Pérez-Pérez *et al.*, 2007; Gomes *et al.*, 2008; Luo *et al.*, 2008). Early works by Kirschbaum (1987, 1988) suggested that recovery after a severe drought was a

two-stage process, the first involving leaf rewatering and stomata reopening, and the second, long-lasting period requiring *de novo* synthesis of photosynthetic proteins to overcome biochemical limitations. However, it should be noticed that, at that time, $g_m$ was not considered a possible limitation to photosynthesis. Hence any mechanism not involving stomata was ascribed to a biochemical limitation, i.e. to impairment of the primary photosynthetic machinery of the plant. More recently, Gomes *et al.* (2008) applied a photosynthesis limitation analysis to show that, in general, mesophyll limitations were more important than stomatal limitations during recovery, but also in this study, the role of biochemistry and mesophyll diffusion conductance on mesophyll limitations were not separated. Galmés *et al.* (2007*a*) were the first to apply the photosynthesis limitation analysis proposed by Grassi and Magnani (2005) to ten different Mediterranean species, and showed that limited recovery of $g_m$ was the main limiting factor for photosynthesis recovery the day after rewatering in many of these plants. However, it would be necessary to apply this analysis in plants subjected to different water-stress intensities, and to span it to longer time periods. On the other hand, in some species including the *Vitis* hybrid R-110 (*Vitis berlandieri*×*V. rupestris*), a sustained down-regulation of stomatal conductance after rewatering imposes a substantial limitation to photosynthesis recovery, at the time that it increases the intrinsic water-use efficiency (Bogeat-Triboulot *et al.*, 2007; Gallé and Feller, 2007; Gallé *et al.*, 2007; Pou *et al.*, 2008).

Therefore, current knowledge about physiological limitations to photosynthesis during short-term acclimation to different water-stress intensities and recovery after rewatering is scarce, but crucial to improve the understanding of plant responses to drought and for the development of water-saving irrigation schedules in agriculture (Flexas *et al.*, 2006*b*). Of particular interest would be to analyse such responses in species adapted to water-stress conditions, such as those found in Mediterranean regions. Among well-adapted crops, grapevine (*Vitis vinifera* L.) is especially interesting since it performs most of its phenological cycle during summer under non-irrigation conditions. The hybrid *Vitis* Richter-110 (*Vitis berlandieri*×*Vitis rupestris*) is often used as a rootstock in semi-arid viticultural areas, and it is especially well adapted to water-stress conditions. In previous works, it has been shown that R-110 presents a completely isohydric behaviour under water stress, during acclimation and during recovery after rewatering, i.e. it is able to maintain homeostasis in its leaf water relations regardless of decreased soil water availability and replenishment (Pou *et al.*, 2008). This is achieved by keeping high root and stem hydraulic conductivities, which involve complex changes in the expression of all major putative aquaporins (Galmés *et al.*, 2007*b*), as well as a strong fine stomatal regulation in response to water stress and leaf-to-air vapour pressure deficit, which is achieved by a mechanism combining variations in leaf abscisic acid content and hydraulic conductivity, leading to high water-use-efficiency (Pou *et al.*, 2008). In summary, R-110 seems especially well adapted to drought with respect to other *Vitis* genotypes and, under the experimental stress conditions applied in the present study, it shows higher photosynthesis, growth, and water use efficiency than cultivars of *Vitis vinifera* such as Grenache and Syrah (A Pou *et al.*, unpublished results).

The aims of the present work were to analyse in the drought-adapted R-110 how photosynthesis is regulated by different physiological limitations under water stress imposition, acclimation, and recovery. Our hypotheses were: (i) that down-regulation of mesophyll conductance to $CO_2$ may impose a limitation to photosynthesis of similar magnitude to that imposed by stomatal closure during acclimation to water stress in such drought-adapted species, while impairment of leaf photochemistry and biochemistry may be low; (ii) that during acclimation, day-to-day variations in leaf photosynthetic properties may play a role in setting the overall photosynthesis limitations during the period; and (iii) that photosynthesis recovery after rewatering may be mostly limited by diffusional limitations, rather than by biochemical limitations.

## Materials and methods

### *Plant material and water stress treatments*

Plants of Richter-110 (*Vitis berlandieri*×*Vitis rupestris*) were subjected to water withholding followed by rewatering during summer 2005 at the Universitat de les Illes Balears (Mallorca, Spain), as described in detail by Pou *et al.* (2008). Briefly, one-year-old plants were used, growing outdoors in 30 l pots filled with a mixture of clay soil and organic substrate. Control plants were irrigated daily to field capacity, while plants in which irrigation was stopped were divided in two groups, corresponding to two different levels of water stress defined by the leaf maximum daily stomatal conductance ($g_s$), as suggested by Flexas *et al.* (2002): moderate water stress ($g_s$ near 0.15 mol $H_2O$ $m^{-2}$ $s^{-1}$) and severe water stress ($g_s$ near 0.05 mol $H_2O$ $m^{-2}$ $s^{-1}$). The first level of $g_s$ was reached 4 d after stopping irrigation, while the second was achieved 8 d after stopping irrigation. Once the desired water stress was obtained, plants were maintained at constant water stress for 1 week to assess possible acclimation. This was achieved by replacing the amount of water consumed daily, as determined by weighing of the pots every evening. After 1 week at the established soil water deficit, all plants were rewatered to field capacity and recovery was followed for several days.

### *Instantaneous gas exchange and chlorophyll fluorescence measurements, and corrections for $C_i$*

Instantaneous gas-exchange and chlorophyll fluorescence measurements were taken daily, between 12.00 h and 13.00 h local time, on 10–12 leaves from different plants per treatment, using an open gas-exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA) with an integrated fluorescence chamber head (Li-6400-40; Li-Cor Inc., Nebraska, USA). No measurements were taken by days 4 and 5 due to

rainfall, and by day 9 due to technical problems with the Li-6400. All measurements were made on young, fully expanded leaves, at 1500 μmol $m^{-2}$ $s^{-1}$, with a $CO_2$ concentration in the leaf cuvette of 400 μmol $CO_2$ $mol^{-1}$ air. Block temperature was kept at 30 °C during all measurements, and the registered leaf temperatures ranged between 30 °C and 34 °C (Fig. 1). Respiration in the light or 'day' respiration ($R_d$), the apparent $CO_2$ photocompensation point ($C_i$*), and photosynthesis responses to $CO_2$ ($A_N$–$C_i$ curves) were determined only on five specific sampling days for each treatment: the day the desired stomatal conductance was first achieved, 7 d after sustaining the plants at constant soil moisture, just before rewatering ('acclimation'), and then 1, 3 and 7 d after rewatering.

From instantaneous measurements, net $CO_2$ assimilation ($A_N$), stomatal conductance ($g_s$) and the substomatal $CO_2$ concentration ($C_i$) were recorded. However, $C_i$ values can be overestimated due to two main problems that have been described particularly under water stress: an increasing importance of the cuticular conductance to vapour pressure as stomata close (Boyer *et al.*, 1997) and heterogeneous ('patchy') stomata closure (Laisk, 1983; Buckley *et al.*, 1997). Leaf cuticular conductance was calculated and patchy stomatal closure was tested as detailed below.

*Vitis* is a hypostomatous species, and hence cuticular conductance was estimated in three different ways: (i) measuring it with the IRGA on leaves with the abaxial surface covered with silicone grease and a polyethylene filter to prevent stomatal gas exchange (Boyer *et al.*, 1997); (ii) measuring gas exchange of leaves at night (Kerstiens, 1996), although there is now evidence that this could overestimate cuticular conductance due to incomplete stomatal closure at night (Kerstiens, 2006); and (iii) by determining cuticular transpiration after turgor loss during the measurements of pressure–volume curves (Burghardt and Riederer, 2003). The three methods yielded similar values, of 0.007±0.001, 0.006±0.001, and 0.008±0.001 mol $H_2O$ $m^{-2}$ $s^{-1}$, respectively, which were similar to those found by Boyer *et al.*

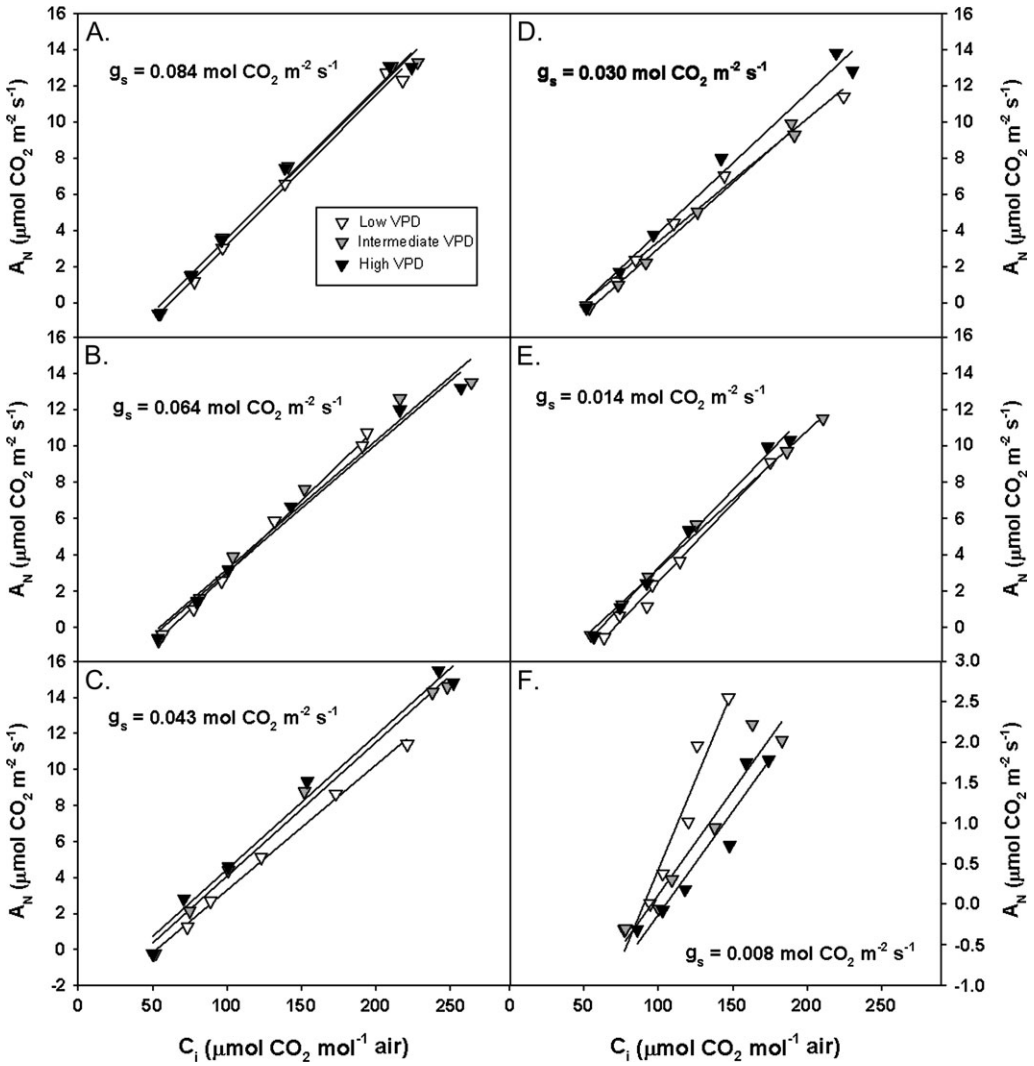


**Fig. 1.** The relationship between net photosynthesis ($A_N$) and substomatal $CO_2$ concentration ($C_i$) on leaves subject to increasing *VPD*. Representative examples of leaves differing in stomatal conductance are shown. *VPD* conditions: white triangles represent low *VPD* (typically 1.7–2.1 kPa), grey triangles represent intermediate *VPD* (typically 2.2–2.5 kPa), and black triangles represent high *VPD* (typically 2.6–3.6 kPa).

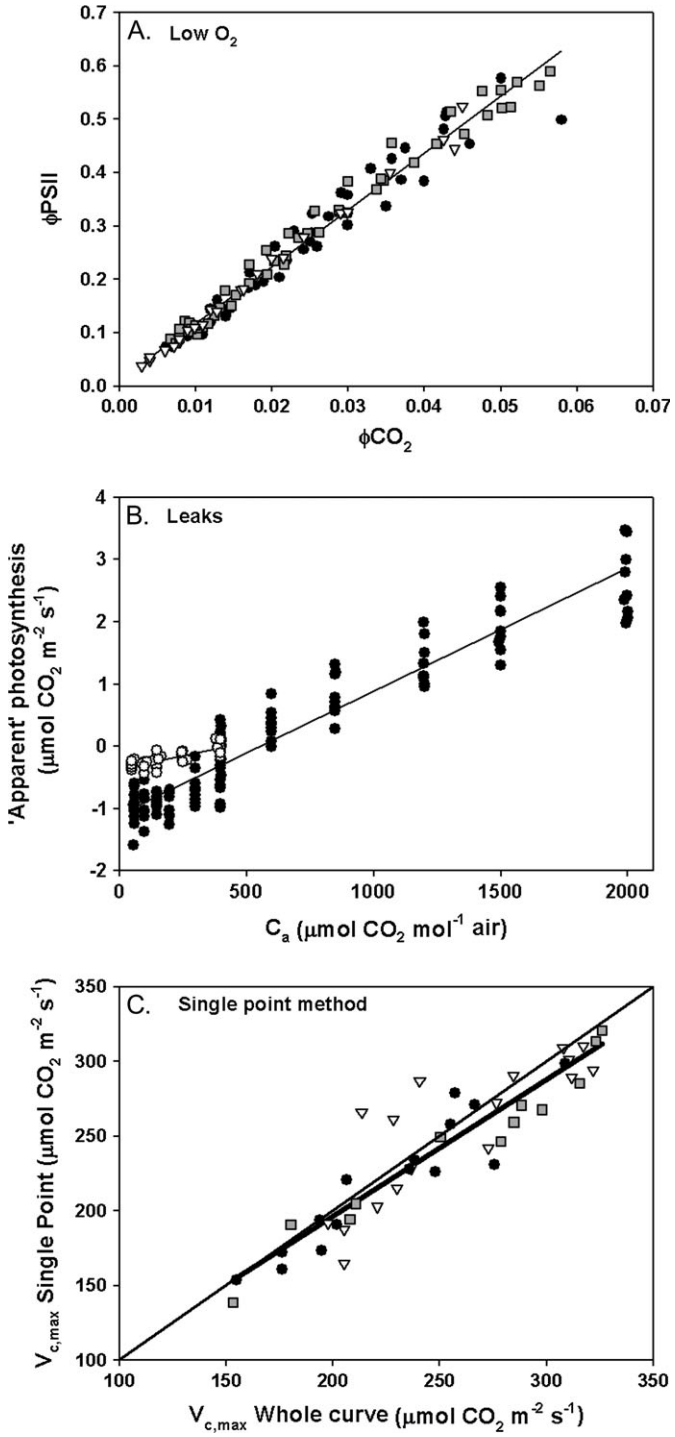


**Fig. 2.** (A) The relationship between photochemical efficiency of photosystem II ($\Phi_{PSII}$) and $\Phi_{CO2}$ [$(A_N + R_d)/PPFD$] under non-photorespiratory conditions (less than 1% $O_2$) in Richter-110 leaves under irrigation (black circles), moderate drought (grey triangles), and severe drought (white triangles). (B) The responses of leakage $CO_2$ flow from the gas exchange cuvette ('apparent net photosynthesis') to $CO_2$ concentration ($C_a$) in the 2 $cm^2$ chamber (filled circles) and the 6 $cm^2$ chamber (empty circles) filled with a dead leaf. Ranges of $C_a$ are different in each case because a 2 $cm^2$ chamber was used for entire $A_N$–$C_i$ curves, while a 6 $cm^2$ chamber was used for the 'Laisk method', with a more limited $C_a$ range (see Materials and methods). (C) The relationship between the maximum capacity for carboxylation ($V_{c,max}$) determined using the single point method and using the whole $A_N$–$C_i$ curve in single replicates for plants under irrigation (black circles), moderate water stress (grey squares), and severe water stress (white triangles). The thick line is the best-fit while the thin line represents the 1:1 relationship.

(1997) for another *Vitis* species but, contrary to Boyer *et al.* (1997) without any significant difference between treatments. Therefore, a value of 0.007 mol $H_2O$ $m^{-2}$ $s^{-1}$ was used to recalculate $g_s$ and $C_i$ as described previously (Boyer *et al.*, 1997; Flexas *et al.*, 2002).

In order to detect symptoms of stomatal patchiness, two different checks were performed. In the first, chlorophyll fluorescence was measured in different areas of the leaf blade (Flexas *et al.*, 2002). Five to six patches were measured over each leaf, and the differences in fluorescence parameters were usually lower than 10%, except over leaf veins (data not shown). In the second, the initial slope of several photosynthetic response curves to intercellular $CO_2$ concentration ($A_N$–$C_i$ curves) on the same leaf was determined under conditions of increasing vapour pressure deficit (*VPD*) and decreasing stomatal conductance, following Grassi and Magnani (2005). As can be observed (Fig. 1), when $g_s$ was above 0.06 mol $CO_2$ $m^{-2}$ $s^{-1}$ all three curves looked identical, which was interpreted as evidence of the absence of patchiness (Fig. 1A, B). For $g_s$ values ranging between 0.01 and 0.06 mol $CO_2$ $m^{-2}$ $s^{-1}$, some deviation was observed (Fig. 1C–E), but still very minor to consider it causing a significant bias in the value of $C_i$. Only when $g_s$ dropped below 0.01 mol $CO_2$ $m^{-2}$ $s^{-1}$ (Fig. 1F) was there clear evidence of impairment of the calculation of $C_i$. Since these low $g_s$ values were never averaged by any treatment during the experiment, no correction to account for patchiness was done in the calculation of $C_i$.

Concerning chlorophyll fluorescence, the actual photochemical efficiency of photosystem II ($\phi_{PSII}$) was determined by measuring steady-state fluorescence ($F_s$) and maximum fluorescence during a light-saturating pulse of *c.* 8000 µmol $m^{-2}$ $s^{-1}$ ($F'_m$) following the procedures of Genty *et al.* (1989):

$$\phi_{PSII} = \frac{F'_m - F_s}{F'_m} \quad (1)$$

The electron transport rate ($J_{flu}$) was then calculated as:

$$J_{flu} = \phi_{PSII} \times PPFD \times \alpha \times \beta \quad (2)$$

where *PPFD* is the photosynthetically active photon flux density, $\alpha$ is leaf absorptance and $\beta$ reflects the partitioning of absorbed quanta between photosystems II and I. The product $\alpha \times \beta$ was determined, following Valentini *et al.* (1995), from the relationship between $\phi_{PSII}$ and $\phi_{CO2}$ obtained by varying either light intensity under non-photorespiratory conditions in an atmosphere containing less than 1% $O_2$. The relationship obtained showed a slope of 10.6 with an almost zero intercept, and no significant differences were observed between treatments (Fig. 2A).

The maximum quantum efficiency of PSII ($F_v/F_m$=($F_m$–$F_o/F_m$)) was determined for two different days at pre-dawn (06.00 h local time). *NPQ* at mid-morning was calculated each day using the closest measured pre-dawn $F_m$ $\left[\mathrm{NPQ}=\frac{F_m-F'_m}{F'_m}\right]$.

### *Respiration in the light, apparent $CO_2$ photocompensation point and $A_N$–$C_i$ curves*

Respiration in the night ($R_n$) was determined on several days at pre-dawn using the Li-6400. Respiration in the light or 'day' respiration ($R_d$) and the apparent $CO_2$ photocompensation point ($C_i^*$) were determined according to the method of Laisk (1977) as described in von Caemmerer (2000). Briefly, $A_N$–$C_i$ curves were measured at three different PPFDs (50, 200, and 500 μmol $m^{-2}$ $s^{-1}$) at six different $CO_2$ levels ranging from 300 to 50 μmol $CO_2$ $mol^{-1}$ air, using the 6 $cm^2$ leaf chamber. The intersection point of the three $A_N$–$C_i$ curves was used to determine $C_i^-$ (*x*-axis) and $R_d$ (*y*-axis). $C_i^*$ was used as a proxy for the chloroplastic $CO_2$ photocompensation point (Γ*), according to Warren and Dreyer (2006). According to Galmés *et al.* (2006), only $C_i^*$ values for irrigated plants were considered, which averaged 42.0±0.9 μmol $CO_2$ $mol^{-1}$ air at a leaf temperature of 30 °C, i.e. a Γ* of 43.1 μmol $CO_2$ $mol^{-1}$ ($\Gamma^*=C_i^*+\frac{R_d}{g_m}$), corresponding to a Rubisco specificity factor of 90. Considering published Γ* response functions to temperature for several species (reviewed by Warren and Dreyer, 2006), this would correspond to a Rubisco specificity factor of about 100 at 25 °C, i.e. totally agreeing with the actually determined value for *Vitis* (Bota *et al.*, 2002).

$CO_2$-response curves were performed in light-adapted leaves of different plants for each day and treatment, using two Li-6400 units simultaneously. Photosynthesis was induced with a *PPFD* of 1500 μmol $m^{-2}$ $s^{-1}$ (previously performed light-response curves had shown this to be sufficient light to saturate photosynthesis) and 400 μmol $mol^{-1}$ $CO_2$ surrounding the leaf ($C_a$). The amount of blue light was set to 10% *PPFD* to maximize stomatal aperture. Air temperature was kept at 30 °C, and leaf-to-air vapour pressure deficit was different depending on the day and treatment, but kept within a variation of 0.5 kPa during the performance of a single curve. Once steady-state was reached (usually 30 min after clamping the leaf), a $CO_2$-response curve was performed. Gas exchange and chlorophyll fluorescence were first measured at 400 μmol $mol^{-1}$, then $C_a$ was decreased stepwise until 50 μmol $mol^{-1}$. Upon completion of measurements at low $C_a$, this was returned to 400 μmol $mol^{-1}$ to restore the original $A_N$. Then $C_a$ was increased stepwise until 2000 μmol $mol^{-1}$ to complete the curve. The number of different $C_a$ values used for the curves was 12 and the time lag between two consecutive measurements at different $C_a$ was restricted to 2–4 min, so that each curve was completed in 30–40 min.

Leakage of $CO_2$ in and out of the leaf cuvette was determined as described for the range of $CO_2$ concentrations used in this study with photosynthetically inactive leaves (obtained by heating the leaves until no variable chlorophyll fluorescence was observed) enclosed in the leaf chamber (Flexas *et al.*, 2007*b*). The leakage values obtained did not differ significantly among days, so they were pooled together (Fig. 2B) and the average relationship was used to correct the measured leaf fluxes for the entire experiment. The higher number of points at the lower end of the relationship (Fig. 2B) was because test for leakage was also made for the 6 $cm^2$ leaf chamber used for the 'Laisk' method.

### *Estimation of photorespiration and $g_m$ by gas exchange and chlorophyll fluorescence*

From combined gas-exchange and chlorophyll fluorescence measurements, the photorespiration rate ($P_r$) was calculated according to Valentini *et al.* (1995). In their model, they assumed that all the reducing power generated by the electron transport chain is used for photosynthesis and photorespiration, and that chlorophyll fluorescence gives a reliable estimate of the quantum yield of electron transport. Thus, $P_r$ can be solved from data of $A_N$, $R_d$, and $J_{flu}$, and from the known stochiometries of electron use in photorespiration, as follows (Valentini *et al.*, 1995): $P_r$=1/12 [$J_{flu}$–4($A_N$+$R_d$)].

The method by Harley *et al.* (1992) was used to make estimations of $g_m$ as:

$$g_m=A_N/\left(C_i-\left(\Gamma^*\left(J_{flu}+8\left(A_N+R_d\right)\right)/\left(J_{flu}-4\left(A_N+R_d\right)\right)\right)\right) \quad (3)$$

where $A_N$ and $C_i$ are taken from gas exchange measurements at saturating light and Γ* and $R_d$ were estimated using the Laisk (1977) method (see previous section).

The calculated values of $g_m$ were used to convert $A_N$–$C_i$ curves into $A_N$–$C_c$ curves using the following equation:

$$C_c=C_i-(A_N/g_m) \quad (4)$$

From $A_N$–$C_c$ curves, the maximum carboxylation capacity ($V_{c,max}$) and the maximum capacity for electron transport rate ($J_{max}$) were calculated using the temperature dependence of kinetic parameters of Rubisco described on a $C_c$ basis by Bernacchi *et al.* (2002), whereby net assimilation rate is given as:

$$A_N=\min\{A_c,A_q\}-R_d \quad (5)$$

With:

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

$$A_q=\frac{J(C_c-\Gamma^*)}{4(C_c+2\Gamma^*)} \quad (7)$$

where $A_c$ and $A_q$ represent photosynthesis limited by carboxylation and RuBP regeneration, respectively, $K_c$ and $K_o$ are the Rubisco Michaelis–Menten constants for

carboxylation and oxygenation, respectively, and $o_i$ is the leaf internal oxygen concentration (assumed equal to the external).

Complete $A_N$–$C_i$ curves (and hence $A_N$–$C_c$ curves) were performed on several days. From them, it was confirmed that at ambient $CO_2$ concentration, net photosynthesis was always in the $V_{c,max}$ region and not in the $J_{max}$ region, regardless of the day and treatment. In order to estimate $V_{c,max}$ for each day of the experiment, even for days in which only instantaneous measurements at ambient $CO_2$ were available, the 'single point' method described by Wilson *et al.* (2000), modified by Grassi and Magnani (2005) to account for $g_m$, was used. This method consisted of an estimation of $V_{c,max}$ using a single value of $A_N$ and $C_c$ (at ambient $CO_2$) plus $R_d$. For cases where the whole $CO_2$ response curve was available, together with instantaneous measurements, it was verified that the single point method yielded results similar to those adjusting the entire curve (Fig. 2C).

### *Quantitative limitation analysis*

To partition photosynthesis limitations into components related to stomatal conductance ($S_L$), mesophyll conductance ($MC_L$), and leaf biochemical characteristics ($B_L$), a modification of the approach proposed by Grassi and Magnani (2005) was considered. At ambient $CO_2$ concentration, light-saturated photosynthesis is generally limited by substrate availability, which was verified by $A_N$–$C_i$ curves in the present data for each species and treatment (see previous section), i.e. photosynthesis can be expressed using equation 6 (Farquhar *et al.*, 1980). To compare their relative limitations to assimilation due to water stress, acclimation, and recovery, photosynthetic limitations were partitioned into their functional components following the approach proposed by Grassi and Magnani (2005), with some modifications. This approach which requires the measurement of $A_N$, $g_s$, $g_m$, and $V_{c,max}$, makes it possible to partition photosynthesis limitations into components related to stomatal conductance ($S_L$), mesophyll conductance ($MC_L$), and leaf biochemical characteristics ($B_L$), assuming that a reference maximum assimilation rate can be defined as a standard. In the current study, the maximum assimilation rate, concomitant with $g_s$, $g_m$, 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, presumably due to leaf ageing, the values for irrigated plants *for each day* were considered as the reference for the moderate and stressed plants determined on the same day. In doing so, photosynthesis limitations due to leaf ageing in irrigated plants were assessed by comparing the values along the experiment with the maximum values observed (obtained by the third day of experiment). On the other hand, 'pure' water stress limitations (i.e. without interaction with leaf ageing) were obtained for moderate and severely stressed plants. Whenever one of the involved parameters ($g_s$, $g_m$, and $V_{c,max}$) was higher in stressed than in irrigated plants, its corresponding limitation was set to zero, and the other limitations recalculated accordingly.

Finally, non-stomatal limitations were defined as the sum of the contributions of mesophyll conductance and leaf biochemistry ($NS_L=MC_L+B_L$), while diffusive limitations were the sum of stomatal and mesophyll conductance components ($D_L=S_L+MC_L$).

### *Thermoluminescence measurements*

Thermoluminescence (TL) glow curves of *Vitis* R-110 leaf discs were measured using a home-made apparatus (SBE-INRA/CEA-Saclay, France), as described in detail by Ducruet (2003), with modifications as in Sajnani *et al.* (2007). Data acquisition and signal analysis were performed using dedicated software developed in Saclay (see Ducruet, 2003, and references therein). The sample cuvette consists of a horizontal cylindrical chamber (2.5 cm diameter) with a copper film on the bottom, glued to a thermoelectric Peltier plate (model DT 1089-14; Marlow Industries, USA) below the chamber for temperature regulation. A thin thermocouple is placed under the copper film in the centre of the plate. The bottom face of the Peltier element is maintained at a constant temperature by a flow of water. A drop of water (100 µl) is placed on the centre and the leaf disc is pressed on the bottom by a washer. A circular Pyrex window between the leaf disc and the washer reduces water loss from the sample during warming (except for high temperature thermoluminescence, HTL, measurements). The common side of a 5-arms light guide (Walz, Effeltrich, Germany) was placed 5 mm above the sample, one arm being used for conveying the luminescence emission to a red-sensitive Hamamatsu H5701-50 analogue photomultiplier through a red filter (>670 nm) and the other arms for different types of illumination. In standard experiments, leaf discs, from plants dark-adapted for short (120 min: Day) or long periods (8 h: Night), were punched out under a dim green light then incubated in darkness for 2 min at 20 °C and cooled to 1 °C for 1 min. At the end of this period, leaf discs were subsequently illuminated with one or three saturating single-turnover flashes, of white xenon light XST-103 (Walz, Germany) separated by 1 s or alternatively with a Far Red light (FR) LED 102-FR (Walz) operated at setting 10 for 30 s. Luminescence emission was immediately recorded while warming the sample from 0 °C to 80 °C at a heating rate of 0.5 °C $s^{-1}$.

HTL measurements were carried out using the same set-up and a similar protocol, but without a covering window to allow drying of the sample during warming, in order to prevent hydrolysis of peroxides at high temperature (Ducruet and Vavilin, 1999). The heating rate of the sample was of 0.1 °C $s^{-1}$ and the measuring range from 10 °C to 160 °C. HTL bands reflect lipid peroxidation in stressed samples.

### *Determination of ascorbate*

Ascorbate (Asc) and dehydroascorbate (DHAsc) were determined using the modified bipyridyl methods of Okamura (1980) and Knörzer *et al.* (1996). 0.3–0.4 g of fresh leaf

tissue was ground to a fine powder in liquid nitrogen, and then homogenized by adding 2 ml of cold metaphosphoric acid 5% (w/v). The homogenate was clarified by centrifuging at 13 200 rpm for 15 min at 4 °C. 125 µl supernatant aliquots were used for subsequent determinations. Sample A, for the determination of reduced ascorbate (Asc), contained 270 µl of the extract, neutralized with 27 µl 1 N NaOH, 300 µl 150 mM sodium phosphate buffer (pH 7.4) and 200 µl $H_2O$. Sample B, for the determination of total ascorbate (Asc+DHAsc), consisted of 270 µl of the extract neutralized as above, mixed with 300 µl 150 mM sodium phosphate buffer (pH 7.4) and 100 µl 0.1 M dithiothreitol (DTT) to reduce the DHAsc present in the extract. After 15 min incubation at room temperature, the excess DTT was removed by the addition of 75 µl of 0.5% (w/v) *N*-ethylmaleimide, and the sample was incubated for a further 30 s. Subsequently, samples A and B were treated identically. The samples were mixed with 300 µl of 10% (w/v) trichloroacetic acid, 300 µl of 44% (v/v) phosphoric acid, 200 µl of 4% 2,2-bipyridyl (w/v in 70% ethanol), and 150 µl of 3% (w/v) $FeCl_3$, incubated for 1 h at 37 °C, and the $A_{525}$ was recorded. In parallel, standard samples with known amounts of ascorbate, treated identically as the extract probes, were measured. Ascorbate concentration was calculated on the basis of the standard curve. The DHAsc concentration was calculated by subtracting the Asc concentration measured in sample A from the total Asc determined in sample B.

## Results

### *Experimental conditions and plant water status*

Climate conditions during the experiment (July–August 2005) were those typical for Mediterranean regions, with midday air temperatures usually above 30 °C and relative humidity below 60% (Pou *et al.*, 2008), leading to leaf-to-air vapour pressure deficits (*VPD*) typically above 2 kPa and as high as 3.5 kPa when plants were water-stressed (Fig. 3A), and to leaf temperatures between 31 °C and 34 °C, the highest corresponding to the most stressed plants (Fig. 3B). There were two days (4 and 5 after the onset of the experiment) that were completely cloudy and rainy, with decreased day temperatures, during which no measurements could be taken, and a second period of only partially cloudy days (8 to 12 after the onset of the experiment), during which measurements were preformed normally. During this period, leaf-to-air *VPD*s were the lowest and all leaf temperatures remained close to 30 °C, regardless of the treatments.

There was a gradient of substrate water availability from the highest values in irrigated plants to the lowest in severely stressed plants. During the acclimation period, substrate water availability was maintained constant. The 2 d rainy period did not affect substrate water content because all the plants had been placed inside a greenhouse. Despite differences in substrate water availability, pre-dawn leaf water potential was always kept above –0.2 MPa, and midday water potential between –1.0 and –1.4 MPa, the lowest values not corresponding to the most severely water-stressed plants (Pou *et al.*, 2008).

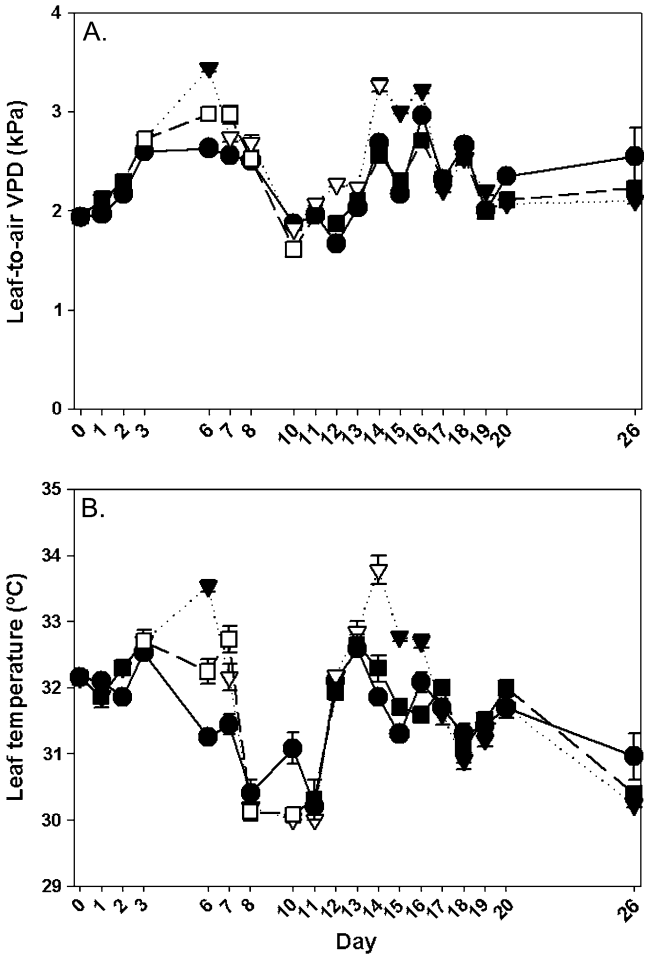


**Fig. 3.** Evolution during the experiment of (A) leaf-to-air vapour pressure deficit (*VPD*) and (B) leaf temperature. Values represent means ±SE of 10–12 replicates per treatment. The treatments were irrigation (circles), moderate water stress (squares), and severe water stress (triangles). Day 0 corresponds to the first day of water stress application. Empty symbols correspond to the days when plants were at the desired water stress level (acclimation period), filled symbols represent the days previous to reach this level as well as the days of recovery.

### *Evolution of photosynthetic parameters*

Net photosynthesis ($A_N$) of irrigated plants progressively declined during the experiment, from about 18 µmol $CO_2$ $m^{-2}$ $s^{-1}$ by day 0 to values slightly above 15 µmol $CO_2$ $m^{-2}$ $s^{-1}$ by day 26 (Fig. 4A). However, water stress imposition resulted in larger reductions, to 12–13 µmol $CO_2$ $m^{-2}$ $s^{-1}$ under moderate stress and to less than 5 µmol $CO_2$ $m^{-2}$ $s^{-1}$ under severe stress (Fig. 4A). During acclimation to water stress, $A_N$ oscillated within the range, particularly for severely stressed plants. Upon rewatering, recovery of $A_N$ was almost complete in about 3 d, both in moderately and severely stressed plants (Fig. 4A).

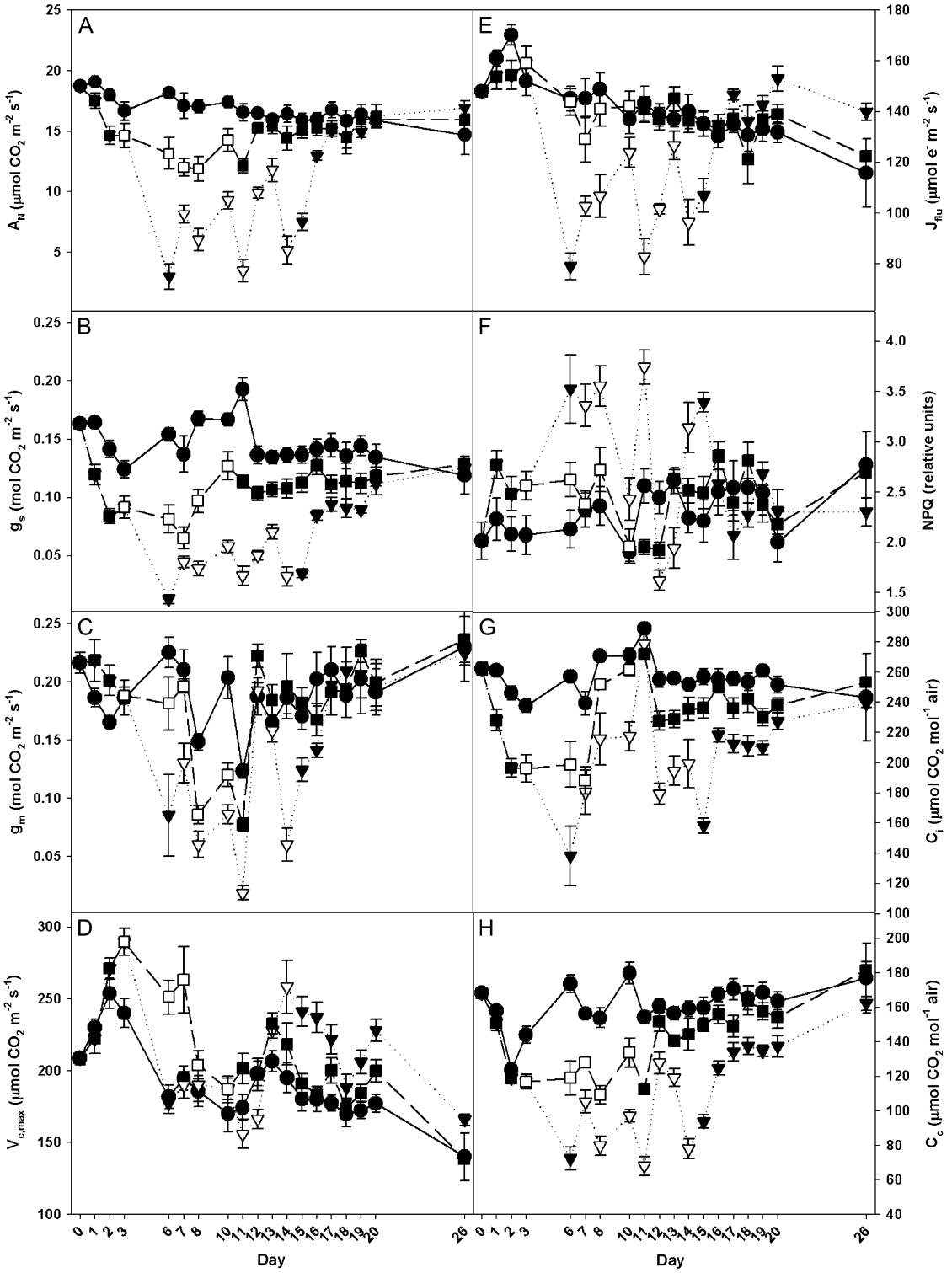


**Fig. 4.** Evolution during the experiment of (A) net photosynthesis ($A_N$), (B) stomatal conductance ($g_s$), (C) mesophyll conductance ($g_m$), (D) maximum capacity for carboxylation ($V_{c,max}$), (E) electron transport rate ($J_{flu}$), (F) non-photochemical quenching of chlorophyll fluorescence (*NPQ*), (G) substomatal $CO_2$ concentration ($C_i$), and (H) chloroplast $CO_2$ concentration ($C_c$). Values represent means ±SE of 10–12 replicates per treatment. Symbols as in Fig. 3.

These variations in photosynthesis were accompanied by similar variations in stomatal conductance ($g_s$), which, in irrigated plants, was kept around 0.15 mol $CO_2$ $m^{-2}$ $s^{-1}$ (i.e. 0.24 mol $H_2O$ $m^{-2}$ $s^{-1}$), while in moderately and severely stressed plants it declined to the levels established in the Materials and methods, i.e. to 0.1 and below 0.05 mol $CO_2$ $m^{-2}$ $s^{-1}$ in moderately and severely water-stressed plants, respectively (Fig. 4B). Oscillations of $g_s$ among days during the acclimation period were also present, although less marked than for $A_N$. Unlike $A_N$, recovery of $g_s$ after

rewatering was slow, taking about 2 weeks to restore the values found in irrigated plants (Fig. 4B). Mesophyll conductance to $CO_2$ ($g_m$) followed a somewhat different pattern (Fig. 4C). In moderately stressed plants, there was no decline in $g_m$ after 5 d of acclimation, but it suddenly halved during the last 2 d of acclimation. During rewatering, $g_m$ stayed low for the first day and fully recovered to control values by day 2. On the other hand, $g_m$ was strongly reduced during the first days of acclimation to severe water stress (Fig. 4C). However, during acclimation to severe water stress, $g_m$ totally recovered during the two semi-cloudy and more humid days (Fig. 3; see also Pou *et al.*, 2008), coincident with the highest $A_N$ determined in severely stressed plants during the acclimation period (Fig. 4A). One day later (i.e. the day after rewatering), low values were again observed. Upon rewatering, the $g_m$ of severely stressed plants recovered to control values within 3 d.

During the first 3 d of the experiment, $V_{c,max}$ of irrigated plants slightly increased, and then progressively declined during the rest of the experiment (Fig. 4D), similar to $A_N$ (Fig. 4A) and $J_{flu}$ (Fig. 4E). For water-stressed plants, the initial period of increase was extended, so that the maximum values achieved were higher than in irrigated plants. However, prolonged water stress induced some decrease of $V_{c,max}$ (Fig. 4D). For moderately stressed plants, $V_{c,max}$ was depressed only during the last 2 d of acclimation (i.e. the same days in which $g_m$ was depressed), being restored to values *above* irrigated plants 3 d after rewatering. For severely stressed plants, $V_{c,max}$ was depressed early in the acclimation period, but was restored *above* irrigated plants before rewatering (Fig. 4D), at the end of the semi-cloudy period but 2 d later than $g_m$ restoration (Fig. 4C). $J_{max}$ could be determined only in those days in which complete $A_N$–$C_i$ curves were performed. Similarly to $V_{c,max}$, $J_{max}$ decreased from values of *c.* 200 μmol $m^{-2}$ $s^{-1}$ by day 3 in both irrigated and moderately stressed plants, to rates ranging from 130 to 170 μmol $m^{-2}$ $s^{-1}$ after day 7, with no significant differences between treatments (data not shown). The rate of chloroplast electron transport ($J_{flu}$) in moderately stressed plants did not differ from that of irrigated plants during the experiment. In severely stressed plants $J_{flu}$ was generally lower during water stress, totally recovering after 2 d of rewatering (Fig. 4E). During acclimation to water stress, non-photochemical quenching of chlorophyll fluorescence (*NPQ*) was slightly higher and substantially higher than in irrigated plants in moderately and severely stressed plants, respectively, but recovery was fast after rewatering (Fig. 4F).

As a consequence of decreased $g_s$ during water stress, the substomatal $CO_2$ concentration ($C_i$) was also depressed (Fig. 4G). The depression was more marked for the chloroplast $CO_2$ concentration ($C_c$), which was more differentiated between treatments (Fig. 4H). It is worth noting that some changes in $C_i$ did not simply follow those changes in $g_s$. For instance, in moderately stressed plants, during the last 2 d of acclimation, $C_i$ increased to control values (Fig. 4G) due to a simultaneous slight increase in $g_s$ (Fig. 4B) and a decrease of $g_m$ (Fig. 4C) and $V_{c,max}$ (Fig. 4D). Similarly, in severely stressed plants, by day 14 (i.e. after the semi-cloudy period) $C_i$ was increased to control values because low $g_m$ was restored after a temporary increase (Fig. 4C) but $V_{c,max}$ was still kept high (Fig. 4D). These particular changes of $C_i$ were not observed for $C_c$. After rewatering, due to a slow recovery of $g_s$, $C_i$ and $C_c$ were maintained lower in the previously stressed than in the irrigated plants (Fig. 4G, H).

As a consequence of reduced $C_c$, $A_N$ was lower under water stress. However, $V_{c,max}$ was kept at or above control values, and $J_{flu}$ was not decreased in moderately stressed plants and was less affected than $A_N$ in severely stressed plants. This was due to increased (moderately stressed plants) or sustained (severely stressed plants) photorespiration ($P_r$) during the acclimation period, which was not fully reversed after rewatering (Fig. 5A). Indeed, $P_r$ was strongly correlated with $V_{c,max}$ during the entire experiment (Fig. 5B).

In addition to $J_{flu}$ and *NPQ*, leaf primary photochemistry was assessed independently by measuring (i) pre-dawn maximal photochemical efficiency of PSII ($F_v/F_m$), (ii)

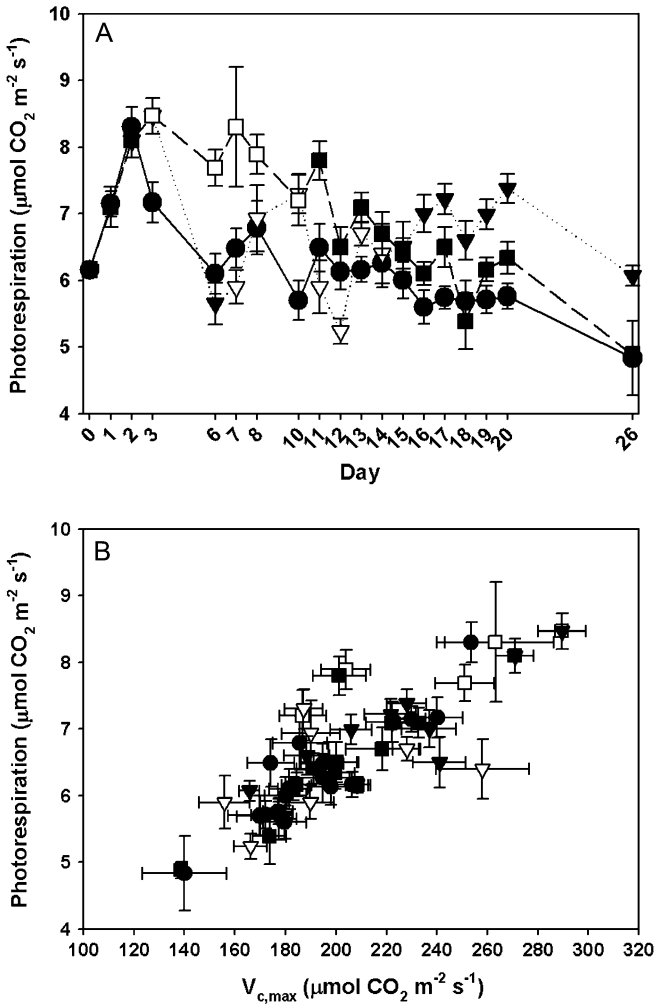


**Fig. 5.** Evolution of photorespiration during the experiment (A) and the relationship between photorespiration and the maximum capacity for carboxylation (B). Values represent means ±SE of 10–12 replicates per treatment. Symbols as in Fig. 3.

thermoluminescence, and (iii) determining ascorbate pools as indicative of possible oxidative stress. $F_v/F_m$ was kept above 0.8 during the entire experiment (Table 1), and, by the end of acclimation period it was significantly higher in moderately (0.82) and severely stressed plants (0.83) than in irrigated plants (0.80). Similarly, the total pool of ascorbate did not change significantly during the experiment (data not shown), and the ratio of reduced to total ascorbate was kept between 0.95 and 0.97 regardless of the treatment (Table 2).

**Table 1.** Pre-dawn $F_v/F_m$ in leaves by the day each treatment was achieved (Day 1) and 7 d after acclimation to each treatment (Day 7)

Values are means ±SE of 10–15 replicates per treatment.

| | Well irrigated | Moderate stress | Severe stress |
|---|---|---|---|
| Day 1 | 0.819±0.002 | 0.820±0.002 | 0.826±0.003 |
| Day 7 | 0.800±0.003 | 0.817±0.003 | 0.828±0.001 |

**Table 2.** Reduced/total ascorbate in leaves by the day each treatment was achieved (Day 1), 7 d after acclimation to each treatment (Day 7) and the first day upon rewatering (Day 8)

Values are means ±SE of six replicates.

| | Well irrigated | Moderate stress | Severe stress |
|---|---|---|---|
| Day 1 | 0.96±0.02 | 0.95±0.03 | 0.96±0.02 |
| Day 7 | 0.97±0.01 | 0.96±0.01 | 0.95±0.02 |
| Day 8 (1st recovery) | 0.97±0.01 | 0.95±0.01 | 0.97±0.01 |

TL curves were measured in leaves with a dark adaptation period of 8 h, under three flashes (3F), one flash (1F), and far red light (FR). 3F measurements induced a B band corresponding to 'pure' S3 states, which are more sensitive than S2 (usually induced with 1F) to lumen acidity, i.e. the peak temperature of the B band Tm(B) under 3F was lower than Tm(B) under 1F. Tm(B) under 3F was about 25 °C (solid line Fig. 6A) in irrigated plants, whereas under 1F the Tm was around 30 °C (dashed line Fig. 6A). The differences observed in intensity between young and mature leave were simply due to the total chlorophyll content of the leaves. FR light produced a curve with a B band with a Tm near 25 °C and an AG band near 45 °C (Fig. 6B). 3F did not induce an AG band in *Vitis* (Fig. 6A), even under water stress (Fig. 6C, D). However, water stress induced differences in TL curves obtained under 3F, consisting in a downshift of the B band from around 28 °C to 17 °C, although these differences were similar regardless of stress intensity (Fig. 6C, D). During the recovery phase the down-shift of the B band under 3F persisted for at least 7 d after rewatering, which was the last day TL was measured. HTL measurements were also obtained during the experiment, and no differences were observed between control and treated plants (data not shown).

### *Photosynthesis limitations*

In irrigated plants, the total photosynthesis limitation increased from 0% at the beginning of the experiment to

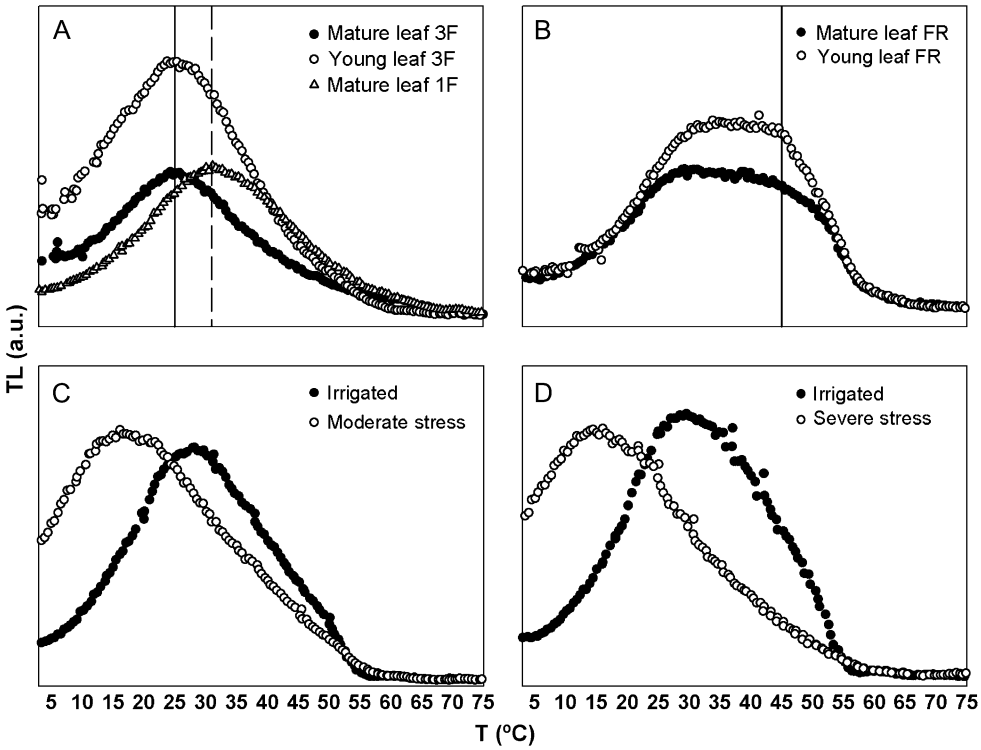


**Fig. 6.** TL curves obtained in 8 h dark-adapted mature leave from hybrid Richter-110 under 1F and in mature and young leaves under 3F (A) and under FR light (B). TL curves obtained after 1 week from irrigated plants, plants with moderate (C) and severe water stress (D) under 3F after an 8 h dark adaptation period.

about 20% at the end, about 1 month later (Fig. 7A). This limitation was mostly due to a biochemical limitation ($B_L$, i.e. decreased $V_{c,max}$), while stomatal and mesophyll conductance limitations were of minor importance. In contrast, although moderate water stress led to a similar total limitation (25–30%) during the acclimation period (Fig. 7B), this was mostly due to diffusional limitations (stomatal, $S_L$ plus mesophyll conductance, $MC_L$). The same occurred in severely stressed plants, in which a total limitation as high as >80% was fully accounted for by the sum of $S_L$ and $MC_L$, with no incidence of $B_L$ (Fig. 7C).

Among the two diffusional limitations, $S_L$ appeared early during the imposition of water stress, and was of higher importance than $MC_L$ during the first days of acclimation to both moderate and severe water stress (Fig. 7B, C). However, $MC_L$ increased during the acclimation period, being of similar magnitude under moderate stress or even higher under severe stress than $S_L$ before rewatering. However, $MC_L$ was rapidly reversed after rewatering, totally disappearing in two (moderate stress) or three (severe stress) days, while $S_L$ lasted for at least 1 week after rewatering (Fig. 7B, C).

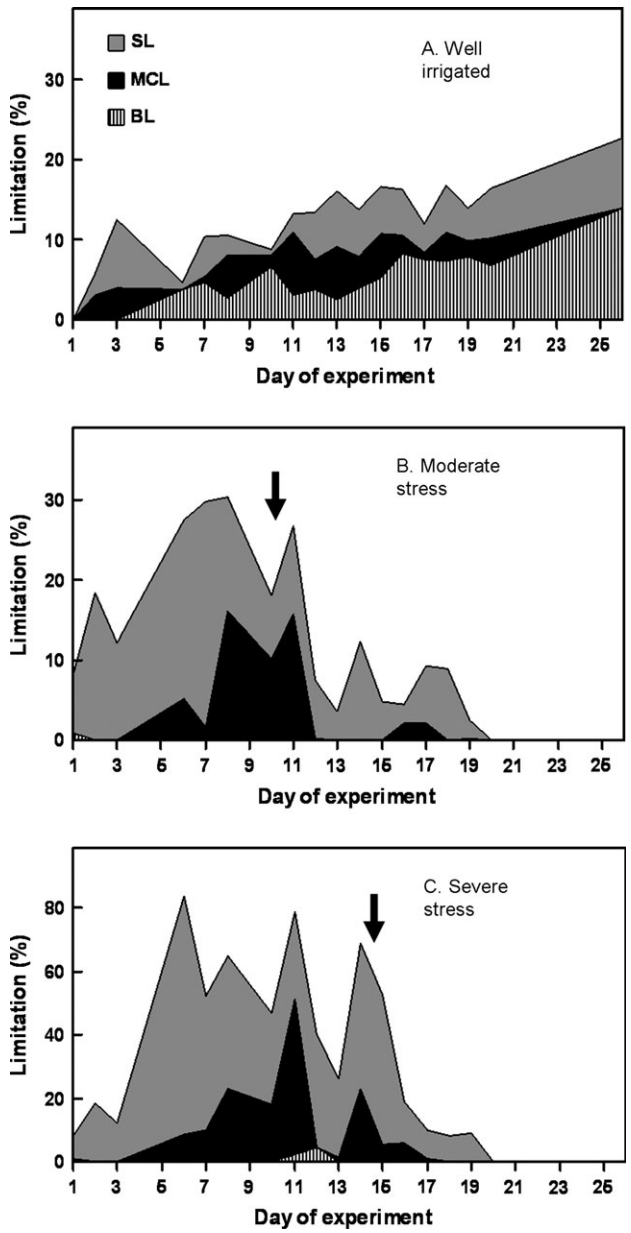


**Fig. 7.** Quantitative limitation of photosynthesis in well-irrigated (A), moderately (B), and severely (C) water-stressed grapevines. SL, MCL, and BL denote for stomatal, mesophyll, and biochemical limitations, respectively.

## Discussion

Regulation of photosynthesis under water-stress imposition, short-term acclimation and recovery, was analysed in a 28 d experiment using plants of a drought-adapted *Vitis* hybrid R-110, growing outdoors under typical Mediterranean conditions (i.e. high temperature and irradiance). During this period, photosynthesis progressively declined in continuously irrigated plants, which was not due to diffusional limitations, but rather to decreased photosynthetic capacity, as reflected by both decreased $V_{c,max}$ and $J_{flu}$. These symptoms are typical of leaf ageing, particularly in deciduous species like *Vitis* (Grassi and Magnani, 2005) but also in evergreens (Niinemets *et al.*, 2005). Once this ageing effect was considered or removed (limitation analysis), the effects of both moderate and severe water stress on photosynthesis were consistent with previous reports, i.e. the decrease in $A_N$ was mostly due to diffusional limitations, consisting of decreased stomatal and mesophyll conductance to $CO_2$ (Flexas *et al.*, 2004, 2006*b*). Also in *Vitis* sp. it has been verified many times that moderate water stress decreased photosynthesis by diffusional limitations only, and irrigated and water-stressed plants often show similar $V_{c,max}$, $J_{flu}$, and $F_v/F_m$ (Flexas *et al.*, 1998, 1999*a*, 2002; De Souza *et al.*, 2003, 2005). However, under more severe water stress and in some cultivars, decreased $V_{c,max}$ (Maroco *et al.*, 2002), $J_{max}$ (De Souza *et al.*, 2003, 2005), $J_{flu}$ (Flexas *et al.*, 1998, 1999*a*, *b*, 2002), and even $F_v/F_m$ (Flexas *et al.*, 1998, 2002) have been observed.

*In vitro* studies have shown that this is mostly due to the reduced activity of fructose-1,6-biphosphate phosphatase and eventually some other enzymes involved in the Calvin cycle (Maroco *et al.*, 2002; De Souza *et al.*, 2005), as well as to the decreased activity of Rubisco (Maroco *et al.*, 2002) due to both decreased concentration and activation state (Bota *et al.*, 2004). However, transcriptomic analysis in *Vitis* has also shown that some photosynthetic genes, like that of Rubisco activase, some Calvin cycle enzymes, and some PSI and PSII-related genes are conversely up-regulated during the acclimation to water stress (Cramer *et al.*, 2007). Although proteomic analysis showed that some photosynthetic proteins were down-regulated during water stress, it also confirmed that some – notably Rubisco and sedoheptulose-1,5-bisphosphatase – were indeed up-regulated (Vincent *et al.*, 2007). In the present study, by determining day-to-day $V_{c,max}$, both effects were observed: on the one hand, early acclimation to water stress increased $V_{c,max}$ as

compared to irrigated plants, and on the other hand, under prolonged water stress $V_{c,max}$ declined (Fig. 4D). Cramer *et al.* (2007) proposed the following explanation: as stomatal conductance decreases with water deficit, internal $CO_2$ concentrations in the leaf are predicted to be reduced, thus causing a slower rate of photosynthesis. Under these conditions, increases in Rubisco activase could improve photosynthetic efficiency by increasing the amount of Rubisco that is activated for $CO_2$ fixation, thus compensating for the reduced stomatal conductance. The present results are in accordance with this hypothesis: increased $V_{c,max}$ in response to decreased $C_c$ improved photosynthetic efficiency during the early days of water stress and, most notably, during recovery. We suggest that the decline of $V_{c,max}$ during water stress was due to oxidative stress affecting Rubisco, as demonstrated by Zhou *et al.* (2007) and suggested by the presence of degradation products of Rubisco during water stress (Vincent *et al.*, 2007). Indirect evidence for this comes from the fact that, in moderately stressed plants, decreased $V_{c,max}$ did not occur during the first days but only 3 d after the first cloudy period, i.e. after several days of substantial irradiance in addition to water scarcity. Moreover, in severely stressed plants $V_{c,max}$ was totally restored prior to rewatering, in coincidence with the end of the second cloudy period. Although in this study, Rubisco activity was not measured with biochemical methods, and $V_{c,max}$ determinations could be questioned; previous work with several species including *Vitis* showed good agreement between the initial Rubisco activity determined *in vitro* and $V_{c,max}$ derived from $A_N$–$C_c$ curves, but not $V_{c,max}$ derived from $A_N$–$C_i$ curves (Bota *et al.*, 2004). Therefore, we feel confident that the estimations of $V_{c,max}$ (and $J_{max}$) presented in this research are reliable and reflect the mechanisms discussed.

Contrary to carboxylation, leaf photochemistry was more stable during the experiment and in response to water stress, in contrast to previous reports (Maroco *et al.*, 2002; Xu and Baldocchi, 2003; Misson *et al.*, 2006). While $J_{max}$ did not show any difference between treatments, decreased $J_{flu}$ occurred only in severely stressed plants and can be interpreted in terms of dynamic down-regulation due to increased *NPQ* (Flexas *et al.*, 2002). Indeed, $F_v/F_m$ was even higher in the most-stressed plants than in irrigated plants, with moderately stressed plants showing intermediate values. This has already been shown in some species, particularly in those showing increased paraheliotropism in response to water stress (Kao and Tsai, 1998; Pastenes *et al.*, 2005), including *Vitis californica* (Gamon and Pearcy, 1990). That leaf photochemistry was resistant to water stress was confirmed by thermoluminescence analysis. The downshift of the B band during water stress was expected (Miranda and Ducruet, 1995). The lower Tm(B) could be due a residual pH gradient (ΔpH) following proton pumping under light, which has been suggested to be enhanced by water stress-induced cyclic electron transport (Golding and Johnson, 2003) or to a dark stable pH gradient maintained by chlororespiration (Rumeau *et al.*, 2007). However, 3F did not induce an AG band in *Vitis* (Fig. 6A), even under water stress (Fig. 6C, D), unlike pea, tobacco or barley but, similar to maize, i.e. this flash-induced AG band is dependent on species (J-M Ducruet, unpublished results). The increased pH gradient could be due to increased photorespiration and/or electron transport to alternative sinks under water stress (Flexas *et al.*, 1999*b*), which were apparent during water stress (Fig. 5), and/or to impaired proton pumping during photophosphorylation (Tezara *et al.*, 2008). Regardless of its nature, an increased pH gradient producing a downshift of the B band is consistent with the increase of *NPQ* (Fig. 4F), particularly in severely stressed plants, confirming that electron transport is resistant to water stress and that the electron-proton system is not uncoupled, as previously suggested by Kaiser *et al.* (1981*a*) working with intact chloroplasts subjected to osmotic stress. However, the downshift of the B band persisted during recovery (not shown), which could contrast with the very fast recovery of *NPQ* (Fig. 4F). This difference can be explained by the fact that active *NPQ* requires a lumen pH of about 6 (Kramer *et al.*, 1999) while the downshift of the B band appears even with a lumen pH of 7 to 8 (Miranda and Ducruet, 1995). Finally, the leaves measured did not show HTL bands suggesting that no lipid peroxidation (i.e oxidative stress) was present in stressed *Vitis* plants (Ducruet, 2003), in agreement with a constant reduction state of the ascorbate pool during the experiment.

In summary, although some acclimation of $V_{c,max}$ occurred, as well as some inhibition of $V_{c,max}$ itself and, to a lesser extent, of photochemistry (Fig. 7), stomatal closure and down-regulation of mesophyll conductance to $CO_2$ during water stress were the main photosynthesis limitations in such drought-adapted species. However, the importance of these two limitations varied during the period of acclimation to water stress, as well as during recovery after rewatering. When measured only on specific days during a water-stress experiment, typically all sunny days, $g_s$ and $g_m$ changed almost in parallel in response to water stress, i.e. the correlation between both is very high (Flexas *et al.*, 2002; Warren, 2008). The present daily results show that the two parameters did not correlate so well. Hence, during the early days of water stress only $g_s$ declined while $g_m$ remained constant. Both under moderate and severe water stress, $MC_L$ increased later during the acclimation period while $S_L$ decreased, so that both achieved a similar magnitude by the end of the period. Therefore, it will appear that acclimation to water stress involves balancing stomatal and non-stomatal limitations. Although the function of such balancing is unknown, it has been suggested that it may help to keep $C_i$ sufficiently high so as not to induce a feedback stomatal reopening (Flexas *et al.*, 2008; Peeva and Cornic, 2009). $C_i$ indeed increased during the acclimation period, from the lowest value on the day water stress was achieved (both moderate and severe) to values closer to the irrigated plants by the end of acclimation (Fig. 4G). Despite the present results, the opposite seems to happen in other species (i.e. $g_m$ tends to recover during the acclimation period, lowering $MC_L$) such as tobacco or holm oak (A Gallé, unpublished results). Hence more studies are needed

to achieve a general view of how diffusion limitations interact during the acclimation to water stress.

On the other hand, once water stress was established and maintained, $g_s$ was much more stable than $g_m$. An intriguing behaviour of the latter was a total recovery of $g_m$ during the severe water stress period, coincident with a 2 d cloudy period. This recovery preceded by 1 d that of $V_{c,max}$, and was fully reversible within 1 d. Gallé *et al.* (2009) have shown in tobacco that, while the response of $g_s$ to water stress, acclimation, and recovery is similar under several light conditions, $g_m$ declines the most and recovers the least under high light conditions, while under low light conditions it does not decrease under water stress. Considered together with the present results, it appears that the response of $g_m$ to water stress may be dependent on the prevailing light conditions, similar to what was demonstrated for Rubisco activity and photochemistry (Zhou *et al.*, 2007). The mechanistic basis for this differential response remains unknown. Clearly, further studies are needed to understand the interactions between water stress and other environmental variables on $g_m$.

Finally, stomatal limitations appeared to be the most important in delaying photosynthesis recovery after rewatering. This is in agreement with reports by Gallé and Feller (2007) and Gallé *et al.* (2007), who showed a sustained reduction of $g_s$ lasting for weeks after rewatering in some tree species. Sustained stomatal limitations in this genotype do not appear to be related to abscisic acid (ABA) metabolism, since leaf xylem ABA levels were fully restored to control values immediately after rewatering (Pou *et al.*, 2008). On the other hand, ABA has been shown to have a similar effect on $g_s$ and $g_m$ (Flexas *et al.*, 2006*c*), while only $g_s$ showed delayed recovery in the present study. Most likely, hydraulic limitations are responsible for sustained low $g_s$ (Pou *et al.*, 2008) although the development of organic structures occluding stomatal pores, such as those described by Gallé and Feller (2007), cannot be ruled out.

The present results are not in complete disagreement with those of Galmés *et al.* (2007*a*), who showed that $g_m$ was the most limiting factor for photosynthesis recovery in most species analysed. This is because, in their report, only recovery after 24 h of rewatering was analysed. In the present results, mesophyll conductance limitations the day after rewatering were still substantial (in moderately stressed plants even higher than stomatal limitations), but they vanished in 2–3 d while stomatal limitations lasted for at least 1 week after rewatering. Early suggestions that photosynthesis recovery after water stress was mostly limited by sustained impairment of several different components of leaf biochemistry (Kaiser *et al.*, 1981*a*, *b*; Kirschbaunn, 1987; Ehnnali and Earl, 2005) and disruption of chloroplast membrane integrity (Kaiser *et al.*, 1981*a*, *b*) do not seem to apply in drought-adapted species like *Vitis* sp.

In conclusion, the present results reinforce the idea that, at least in drought-adapted species, diffusional limitations account for most of the observed water stress-induced depression of photosynthesis. However, the relative contribution of stomatal and non-stomatal limitations changes during acclimation to water stress, which also involves the up-regulation of photosynthetic capacity. Moreover, the intense campaign of measurements revealed that $g_s$, $g_m$, and $V_{c,max}$ are not as closely regulated under water stress as is often reported. The former appears to be more independent of environmental conditions except *VPD* (Pou *et al.*, 2008), while for the latter two there seems to be an interaction between water stress and cumulative irradiance, although this remains to be confirmed. Finally, it is shown that photosynthesis recovery after rewatering is mostly limited by diffusional limitations rather than by biochemical limitations, and particularly by sustained stomatal closure, which recovers much more slowly than $g_m$.

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