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Effects of drought on photosynthesis in grapevines under field conditions: an evaluation of stomatal and mesophyll limitations

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Abstract. The effect of diffusional and photochemical limitations to photosynthesis was assessed in field-grown water-stressed grapevines (*Vitis vinifera* L.) by combined measurements of gas exchange and chlorophyll fluorescence. Drought was slowly induced, and the progressive decline of photosynthesis was examined in different grapevine cultivars along a continuous gradient of maximum mid-morning values of stomatal conductance (g), which were used as an integrative indicator of the water-stress conditions endured by the leaves.

Initial decreases of g were accompanied by decreases of substomatal CO_2 concentration (Ci), the estimated chloroplastic CO_2 concentration (Cc) and net photosynthesis (A_N), while electron transport rate (ETR) remained unaffected. With increasing drought, g, A_N, Ci and Cc further decreased, accompanied by slight decreases of ETR and of the estimated mesophyll conductance (g_{mes}). Severe drought led to strong reductions of both g and g_{mes}, as well as of ETR. The apparent carboxylation efficiency and the compensation point for CO_2 remained unchanged under severe drought when analysed on a Cc, rather than a Ci, basis, suggesting that previously reported metabolic impairment was probably due to decreased g_{mes}.

Keywords: drought, grapevines, mesophyll limitations, photosynthesis, *Vitis vinifera*.

Introduction

Drought is the main environmental factor limiting plant photosynthesis, growth and yield, even in plants well adapted to arid conditions, such as grapevine (*V. vinifera*; Chaves 1991; Lawlor 1995; Cornic and Massacci 1996). Stomatal closure is among the earliest responses to soil drying, and it is generally assumed to be the main cause of drought-induced decreases in photosynthesis, since stomatal closure decreases CO_2 availability in the mesophyll (Chaves 1991; Cornic and Massacci 1996). However, there is strong evidence that drought also affects mesophyll metabolism, reducing photosynthetic capacity (Quick *et al.* 1992; Lawlor 1995). Unfortunately, most of the studies related to the photosynthetic response to water stress have been performed under growth chamber conditions, which makes the extrapolation of results to field conditions difficult. Under field conditions, water stress is usually accompanied by high temperature and irradiance, which leads to different consequences due to multiple stress (Ort *et al.* 1994; Correia *et al.* 1999).

Stomatal limitations have been frequently described in field-grown water-stressed grapevines (Kriedemann and Smart 1969; Correia *et al.* 1995; Flexas *et al.* 1998). It has also been suggested, based on the analysis of the photosynthesis response to varying Ci, that water stress leads to metabolic impairment in field-grown grapes. It has been specifically shown that mild drought leads to limited ribulose-1,5-bisphosphate (RuBP) regeneration, and impaired Rubisco activity was the consequence of severe prolonged drought (Escalona *et al.* 1999). However, preliminary data on RuBP content and Rubisco activity of the same leaves suggest that metabolic impairment was not so important (J. Bota, J. Flexas, A. Keys, J. Loveland, M. Parry and H. Medrano, unpublished data). This would be in accordance with the previously described high stability of leaf photochemistry in water-stressed grapevines (Flexas *et al.* 1998).

Abbreviations used: A_G, gross CO_2 assimilation rate (calculated as the sum of A_N and R_D); A_G*, gross CO_2 assimilation rate (calculated as the sum of A_N and R_L); A_N, net CO_2 assimilation rate; Cc, chloroplastic CO_2 concentration; Ci, substomatal CO_2 concentration; ETR, electron transport rate; ETR/A_N, relative electron yield of net CO_2 assimilation; ETR/A_G and ETR/A_G*, relative electron yield of gross CO_2 assimilation; F_v/F_m, efficiency of excitation capture by open PSII in dark-adapted leaves; g, stomatal conductance; g_{mes}, mesophyll conductance; MN, Manto Negro; NPQ, non-photochemical quenching of chlorophyll fluorescence; PPFD, photosynthetic photon flux density; R_D, leaf mitochondrial respiration in the light, estimated from dark respiration measurements; R_L, total leaf respiration in the light, calculated from A_N-Ci curves; RuBP, ribulose-1,5-bisphosphate; RWC, leaf relative water content; T, Tempranillo; ε, carboxylation efficiency (initial slope of A_N-Ci curve); Ψ_{PD}, pre-dawn leaf water potential.

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These apparent discrepancies could arise from, at least, two main problems. First, it is possible that comparisons were made under different degrees of drought, since the assessment of drought severity is a complex matter. The two water-status parameters most commonly used to assess drought intensity are leaf water potential (Ψ) and relative water content (RWC). However, the precise response of stomatal conductance and photosynthesis to Ψ and RWC depends on the genotype (Kriedemann and Smart 1969; Tardieu and Simonneau 1998), the environmental conditions during drought (Schulze and Hall 1982), and the velocity of drought imposition (Flexas *et al.* 1999*a*), among other factors. Secondly, if g_{mes} decreases under drought, then Cc would become much lower than the calculated Ci. Thus, gas exchange analysis would limit the validity of assertions on photosynthetic metabolic impairment. Drought has been suggested to decrease g_{mes} (Beadle and Jarvis 1977; Cornic *et al.* 1989; Renou *et al.* 1990; Lal *et al.* 1996; Roupsard *et al.* 1996). Therefore, analysing the mesophyll conductance under drought would help to clarify the importance of metabolic impairment of photosynthesis in grapevines.

The aim of the present work is to analyse the progressive drought-induced downregulation of photosynthesis in field-grown grapevines along a continuous gradient of water-stress conditions. In particular, the contributions of stomatal- and mesophyll-diffusional limitations to photosynthesis are addressed.

Materials and methods

Plant material, treatments and water-stress assessment

Potted plants

An experiment was performed in 1-year old plants of 22 different Mediterranean grapevine cultivars, growing outdoors in 30-L pots, as described by Bota *et al.* (2001). Plants were irrigated daily during the summer of 1999. At the end of August, chlorophyll fluorescence and gas exchange of the 22 cultivars were measured. Irrigation was stopped then and, 6-d later, the plants were under water stress and again sampled.

The degree of water stress was assessed by pre-dawn leaf water potential (Ψ_{PD}), as determined with a Scholander chamber (Soilmoisture Equipment Corp., Goleta, CA, USA), and by pre-dawn RWC. The latter was determined as follows: RWC = (fresh weight – dry weight)/(turgid weight – dry weight). The turgid weight of the leaves was determined after 24 h of darkening in distilled water at 4°C (full turgor). Dry weight was obtained after 48 h at 70°C in an oven. Three replicates per cultivar and treatment were obtained. A continuous gradient of water stress was attained due to inter-variety differences in daily water consumption (Bota *et al.* 2001).

Field-grown plants

An additional field study was carried out in a commercial vineyard (Herederos de Ribas S.A., Mallorca, Spain) during summer 1999. Two cultivars of *V. vinifera* were studied; Tempranillo (T), commonly used in Spain, and Manto Negro (MN), a Mallorcan cultivar. Twenty-year old plants grafted on R-110 rootstock were used, either trained on a bilateral cordon (T) or on the traditional goblet (MN). The environmental conditions were similar to those previously described (Flexas *et al.* 1998; Escalona *et al.* 1999), except that rainfall was scarce in spring, and the potential evapotranspiration (ETP) was very high in June (*ca* 200 L m^{-2}). This induced an early drought, leading to lower values of *g* and photosynthesis in early July in non-irrigated plants, as well as at the end of summer, than had been previously found (Escalona *et al.* 1999). Two treatments were established, irrigation and rain-fed. The irrigation dosage was adjusted to about 40% ETP, as measured with an evaporimeter pan, and applied by a drip system (one drip per plant) twice a week from June to harvest.

In this experiment, the degree of water stress was assessed by the mid-morning light-saturated *g* value. In order to sample over a continuous gradient of water stress, sampling was made on different consecutive rows, where the plants displayed different degrees of water stress due to a border effect between irrigated and non-irrigated rows.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence parameters were measured on attached leaves with natural saturating light around mid-morning (11 am local time). Photon flux density (PPFD) incident on the leaves was always higher than 1000 μmol m^{-2} s^{-1}, which is known to be above photosynthesis saturation in these plants (Flexas *et al.* 1998; Escalona *et al.* 1999). In field-grown plants, measurements were taken once or twice a week, from 2 July to 4 August, with a total of eight samplings throughout the growing period. For each sampling time and treatment, six measurements were made on different plants. On potted plants, measurements were taken during the last weeks of August 1999. For each cultivar and treatment, three measurements were made on each of three different plants.

A portable pulse amplitude modulation fluorometer (PAM-2000; Walz, Effeltrich, Germany) was used. A measuring light of about 0.5 μmol photons m^{-2} s^{-1} was set at a frequency of 600 Hz to determine, at pre-dawn (6 am local time), the background fluorescence signal (F_{o}), the maximum fluorescence (F_{m}), and the maximum quantum efficiency of PSII [$F_{\mathrm{v}}/F_{\mathrm{m}} = (F_{\mathrm{m}} - F_{\mathrm{o}})/F_{\mathrm{m}}$]. The same measuring light was used to measure the steady-state fluorescence signal (F_{s}) under sunlight conditions at mid-morning, except that its frequency was increased to 20 kHz. To obtain the steady-state maximum fluorescence yield (F_{m}'), saturation pulses of about 10000 μmol photons m^{-2} s^{-1} and 0.8-s duration were applied. The PSII photochemical efficiency (F/F_{m}'; Genty *et al.* 1989) was then calculated as:

$$\Delta F/F_{\mathrm{m}}' = (F_{\mathrm{m}}' - F_{\mathrm{s}})/F_{\mathrm{m}}', \qquad (1)$$

and used for the calculation of ETR according to Krall and Edwards (1992):

$$\mathrm{ETR} = \Delta F/F_{\mathrm{m}}' \times \mathrm{PPFD} \times 0.5 \times 0.84, \qquad (2)$$

where PPFD is the photosynthetic photon flux density incident on the leaf, 0.5 is a factor that assumes equal distribution of energy between the two photosystems (the actual factor has been described to be between 0.4 and 0.6; Laisk and Loreto 1996; Albertsson 2001), and 0.84 is the assumed leaf absorptance (Flexas *et al.* 1998). NPQ at mid-morning was calculated as:

$$\mathrm{NPQ} = (F_{\mathrm{m}} - F_{\mathrm{m}}')/F_{\mathrm{m}}'. \qquad (3)$$

Gas exchange measurements

Immediately after chlorophyll fluorescence measurement (i.e. within the next 10 s), net CO$_2$ assimilation (A_{N}) and *g* were determined with a Li-6400 (Li-Cor Inc., Lincoln, NE, USA) on the same portion of the leaf without modifying leaf position, in order to maintain exactly the same light regime. A_{G} was calculated as the sum of A_{N} and the rate of mitochondrial respiration (R_{D}) in the light (Oberhuber and Edwards 1993), which was estimated from light response curves of a previous study on the same plants and environmental conditions (Escalona *et al.* 1999).

For field-grown plants, additional gas exchange measurements were performed on 21 and 22 July, and 3 and 4 August. On each of these two sampling times, three A_N-Ci curves were obtained for each treatment and cultivar as already described (Escalona *et al.* 1999). To perform these A_N-Ci curves, PPFD was set at 1000 μmol photons m^{-2} s^{-1} using a light-emitting diode lamp provided by the instrument, and the temperature inside the Li-6400 chamber was maintained at about 30°C during the measurements.

Calculation of Ci

Calculation of Ci was made according to the Li-6400 set of equations, which are ultimately based on the model proposed by Farquhar and co-workers (Farquhar *et al.* 1980; von Caemmerer and Farquhar 1981). Ci is an important parameter for determining whether non-stomatal limitations to CO_2 assimilation occur in the leaf. However, two main problems have been described relating to Ci calculations under drought; patchy stomata closure (Laisk 1983; Buckley *et al.* 1997), and changes in the cuticular conductance to vapour pressure (Boyer *et al.* 1997).

Heterogeneous stomatal closure would lead to an overestimation of Ci (Laisk 1983). To test if stomata closure was patchy, steady-state chlorophyll fluorescence intensity was sampled over the leaf blade prior to measurements. Five to six patches were measured over each leaf, and the differences were usually lower than 10%, except over leaf veins. Accordingly, no correction to Ci was made to account for patchy stomatal closure.

Boyer *et al.* (1997) described another important cause of overestimation of Ci due to a higher conductance of the leaf cuticle to H_2O than to CO_2. Such an overestimation was especially important when stomata were completely closed, that is, when cuticular conductance was the main determinant of leaf CO_2 uptake and transpiration. Thus, it is likely that such effects are important under severe water stress. To account for this problem, all Ci data were re-calculated according to the formula given by Boyer *et al.* (1997):

$$\mathrm{Ci} = \mathrm{Ca} - 1.6A/[(\mathrm{w_i} - \mathrm{w_a})/(E_\mathrm{l} - 2E_\mathrm{c})], \qquad (4)$$

where Ca is the CO_2 concentration of the atmosphere surrounding the leaf, w_i and w_a are the mole fractions of water inside the leaf and in the atmosphere, respectively, E_l is the leaf transpiration, and E_c is the cuticular transpiration. We used the maximum cuticular conductance given by Boyer *et al.* (1997) for grapevines (5 mmol H_2O m^{-2} s^{-1}) to calculate E_c for each measurement obtained. Although Boyer *et al.* (1997) showed that E_c decreased with leaf water potential, we assumed it to be constant, as the leaf water potentials used in their study are 2–3-fold lower than those observed in the field (Escalona *et al.* 1999). The calculated Ci was then corrected, taking into account the boundary layer conductance. Figure 1 shows the quotient between re-calculated Ci ('Ci Boyer') and the Ci given by the Li-6400 program ('Ci usual') plotted against g for all the data used in the present study. A single rectangular hyperbola satisfactorily fitted the data [Ci Boyer/Ci usual = $(1.02\ g)/(0.0037 + g)$; r^2=0.74]. Thus, when stomatal conductance is higher than 0.03 mol m^{-2} s^{-1}, the error in the calculation of Ci by the classical method is lower that 10%. Nevertheless, in the present study, all Ci values have been re-calculated to account for this effect.

Parameters derived from A_N*-Ci curves*

After correcting Ci values to account for the cuticular effect, the initial slopes of A_N-Ci curves (ε) were taken as an estimate of the leaf carboxylation efficiency, which is usually related to Rubisco activity (von Caemmerer and Farquhar 1981). Extrapolation of the curve to Ci = 0 (R_L) was used as an estimate of the sum of photorespiration and mitochondrial respiration in the light (Escalona *et al.* 1999).

From the A_N-Ci curves, a strong second-order relationship was found between A_N, measured at ambient CO_2 concentration, and R_L. This correlation was used to estimate R_L for every situation in which only A_N was measured. Another estimate of gross CO_2 assimilation rate (A_G*) was then calculated as $A_G^* = A_N + R_L$. Although A_G is typically calculated as $A_G = A + R_D$, we believe that A_G* is a better estimation, since most of the CO_2 released in photorespiration is re-fixed by Rubisco (Takeba and Kozaki 1998; Loreto *et al.* 1999), especially under water stress (Stuhlfauth *et al.* 1990).

Calculation of Cc and g_{mes}

From combined gas-exchange and chlorophyll fluorescence measurements, Cc can be estimated (Di Marco *et al.* 1990; Harley *et al.* 1992; Loreto *et al.* 1994; Epron *et al.* 1995). We have used the method of Epron *et al.* (1995) for our estimations. According to this model, the ETR measured by chlorophyll fluorescence can be divided in two components: $\mathrm{ETR} = \mathrm{ETR}_A + \mathrm{ETR}_P$, where ETR_A is the fraction of ETR used for CO_2 assimilation, and ETR_P is the fraction of ETR used for photorespiration. ETR_A and ETR_P can be solved from data of A_N, R_D and ETR, and from the known stoichiometries of electron use in photosynthesis and photorespiration (see Epron *et al.* 1995 for details). To calculate Cc, a formula of Laing *et al.* (1974) is then used: $S = (\mathrm{ETR}_A/\mathrm{ETR}_P)/(\mathrm{Cc}/O)$, where S is the specificity factor of Rubisco, and O is the oxygen mole fraction at the oxygenation site, assumed to be equal to the mole fraction in the air. S was previously determined *in vitro* for MN and T plants, using the method of Parry *et al.* (1989). An S value of 100 mol mol^{-1} was observed at 25°C, with no difference between the two cultivars (J. Bota, J. Flexas, A. Keys, J. Loveland, M. Parry and H. Medrano, unpublished data).

This method works on the assumption that all the reducing power generated by the electron transport chain is used for photosynthesis plus photorespiration. We previously observed a good agreement between ETR and photosynthesis under non-photorespiratory conditions in grapevines, and it was further shown that the rates of the Mehler reaction were always low, being detectable only under extreme desiccation (Flexas *et al.* 1999*b*). In addition to this previous evidence, an estimation of photosynthesis plus photorespiration was made with an independent method (see previous section) to test if the sum of the two processes accounted for the total ETR under our experimental conditions.

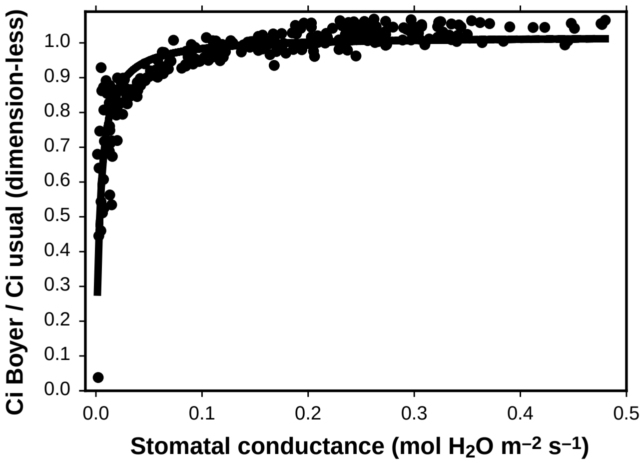

Fig. 1. Ratio between the internal CO_2 concentration calculated after accounting for cuticular diffusivities to H_2O and CO_2 (Ci Boyer) and that calculated according to the Li-6400 instructions (Ci usual) *vs* the stomatal conductance to water vapour (g). For details see text. The plot fitted a single rectangular hyperbola:
Ci Boyer/Ci usual = $(1.02 \times g)/(0.0037 + g)$; r^2=0.74.

After estimation of Cc, the apparent carboxylation efficiency (ε) was re-calculated using Cc instead of Ci. Also, from Ci and Cc values, g_{mes} was approximated as $g_{mes} = (A_N + R_D)/(Ci - Cc)$ (Epron *et al.* 1995).

Results

Stomatal conductance as a reference water-stress parameter in relation to photosynthesis

Figure 2 shows the relationship between g and net CO_2 assimilation for field-grown MN and T plants, as well as for the 22 varieties studied in pots. All the data best fitted a hyperbolic function, almost identical to that reported by Escalona *et al.* (1999). Large scattering in data from the 22 potted varieties was due mainly to inter-variety differences (Bota *et al.* 2001).

In a previous study, the relationship between ETR and Ψ_{PD} from field-grown plants was found to be different from that of potted plants. It was stated that the relationship between ETR and plant water status needed to be elucidated in order to confirm the responsiveness of ETR to water stress (Flexas *et al.* 1999*a*). However, plotting ETR *vs* g eliminated these discrepancies. A single hyperbolic correlation was found for both field- and potted-vines (Medrano *et al.* 2002). This evidence suggested that g could be the best indicator of water stress intensity in relation to photosynthesis, serving to unify data from different experiments and allowing the examination of a continuous gradient of water-stress conditions. This was confirmed by regression analysis in the experiment with potted plants. Figure 3 shows the correlation between ETR and RWC, Ψ_{PD}, and g, as well as the curves of best fit determined for each variable. Clearly, there was no correlation between ETR and RWC (Fig. 3*A*). A negative, slightly curvilinear correlation was observed between ETR and the absolute values of Ψ_{PD} (Fig. 3*B*). Very high values of Ψ_{PD}, even under severe

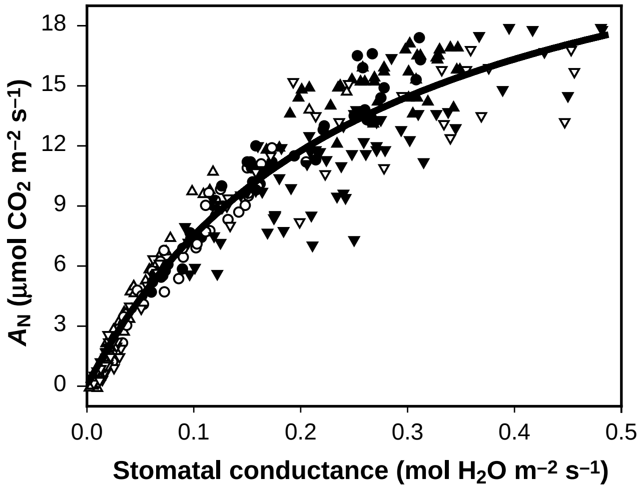

Fig. 2. Relationship between net CO_2 assimilation (A_N) and stomatal conductance (g) in field-grown MN (upward triangles), field-grown T (circles), and the 22 potted varieties (downward triangles). Closed symbols, irrigated plants; open symbols, non-irrigated plants. The best-fit adjustment is shown as a single rectangular hyperbola (r^2=0.92).

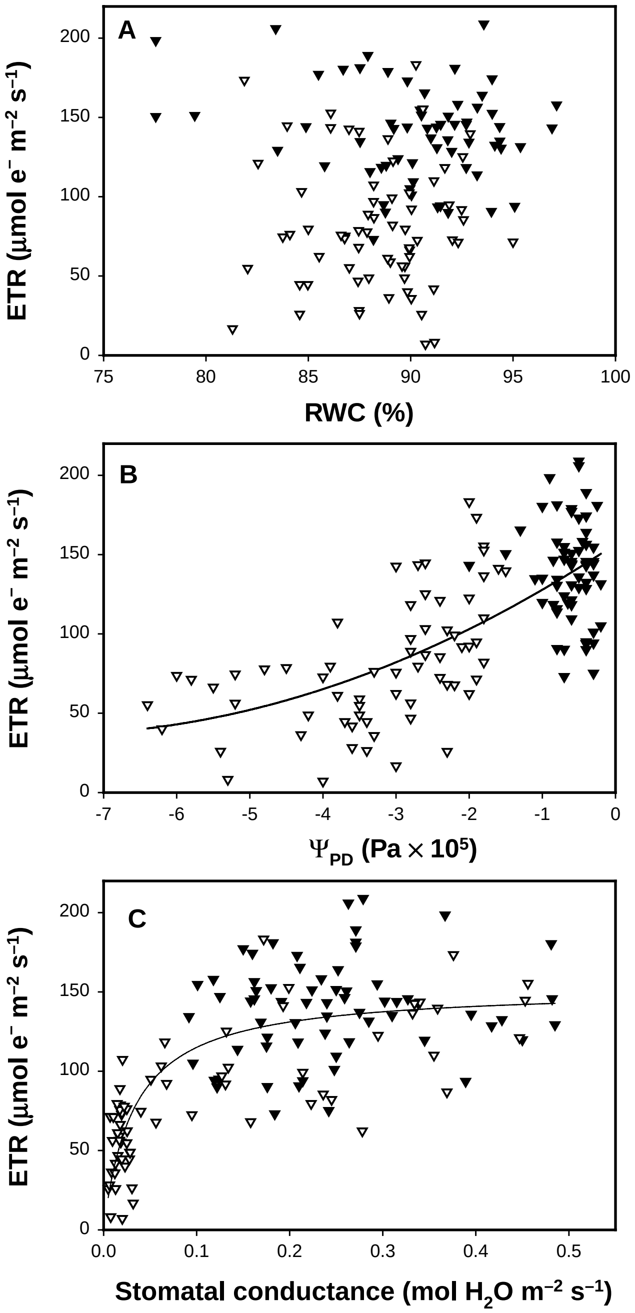

Fig. 3. Relationship between electron transport rate (ETR) and (*A*) pre-dawn leaf relative water content (RWC), (*B*) pre-dawn leaf water potential (Ψ_{PD}), and (*C*) stomatal conductance (g). Data from the experiment with potted plants of 22 grapevine varieties. Closed symbols, irrigated plants; open symbols, non-irrigated plants. No relationship was found between ETR and RWC. A second-order linear regression was found to be the best fit between ETR and Ψ_{PD} (r^2=0.50). The curve of best fit for the ETR-g plot was a single rectangular hyperbola (r^2=0.60).

drought, were due to the age of plants and the absence of rootstock, as already discussed (Bota *et al.* 2001). Although significant, this correlation presented a very large scattering both for irrigated and water-stressed plants, as well as two clearly separated regions for irrigated and non-irrigated plants. The correlation between ETR and *g* was higher than between ETR and either Ψ_{PD} or RWC (Fig. 3*C*), and eliminated the marked separation between irrigated and non-irrigated plants observed when Ψ_{PD} was used as a reference. Large scattering was present mainly at high *g*, as a consequence of large inter- and intra-variety heterogeneity (Bota *et al.* 2001).

On the basis of this evidence, in the present study, the variations of any photosynthetic parameter along a drying treatment will be referred to the corresponding mid-morning *g* value.

Drought and downregulation of PSII

As in potted plants, ETR from field-grown plants was shown to correlate hyperbolically to *g* (Fig. 4*A*). Contrary to the relationship between A_N and *g*, a plateau was clearly observed for ETR. This was reached at *g* values above 0.1–0.15 mol H_2O m^{-2} s^{-1}. Thus, as *g* decreased from about 0.5 to 0.1 mol H_2O m^{-2} s^{-1}, A_N decreased continuously with almost no change in ETR. The NPQ response to *g* almost mirrored that of ETR (Fig. 4*B*), as expected from the well-known co-regulation of both parameters. In spite of ETR decreases at low *g*, pre-dawn F_v/F_m remained near 0.8, only eventually dropping down to 0.74 at low *g* (not shown). This supports the view that permanent photoinhibition is rare in grapevines, even under drought (Flexas *et al.* 1998).

Drought and increased electron transport to O_2 and/or other acceptors

The ratio ETR/A_N has been used previously as an indicator of electron transport to acceptors different to CO_2, of which O_2 is thought to be the most important (Krall and Edwards 1992; Flexas *et al.* 1998, 1999*a*, *b*). However, this ratio must be strongly influenced by respiration, particularly under conditions of low *A* (Krall and Edwards 1991, 1992) as occurs under water stress. To account for respiration rates, we have also calculated the ratio of ETR to gross CO_2 assimilation (ETR/A_G and ETR/A_G*). Figure 5 shows ETR/A_N, ETR/A_G and ETR/A_G* with varying *g* in field-grown MN plants. Identical relationships were observed for field-grown T plants and for the 22 potted varieties (not shown). ETR/A_N increased exponentially as *g* decreased, reaching very high ratios (up to 180) at low *g* (Fig. 5*A*). ETR/A_G showed a similar variation with respect to *g*, with only slightly lower values (Fig. 5*B*).

To support the applicability of the method by Epron *et al.* (1995) to the estimation of Cc, the sum of photosynthesis and photorespiration should account for the whole ETR. Since A_G* is the sum of photosynthesis, photorespiration and dark respiration, the ratio ETR/A_G* was taken as an indicator of the presence of other processes consuming electrons. As shown in Fig. 5*C*, ETR/A_G* was lower than ETR/A_G, and almost constant along the whole gradient of *g* (about 6–7).

Drought and mesophyll limitations to photosynthesis

Variations in Ci were used as a first indicator of mesophyll limitations to photosynthesis. Ci initially decreased in parallel with *g* (Fig. 6*A*). When *g* declined below about 0.05 mol H_2O m^{-2} s^{-1}, the declining tendency of Ci disappeared, and the plot presented large scattering in this region, with some values still decreasing while many others increased steeply. However, the estimated Cc declined continuously along the whole *g* gradient, reaching values

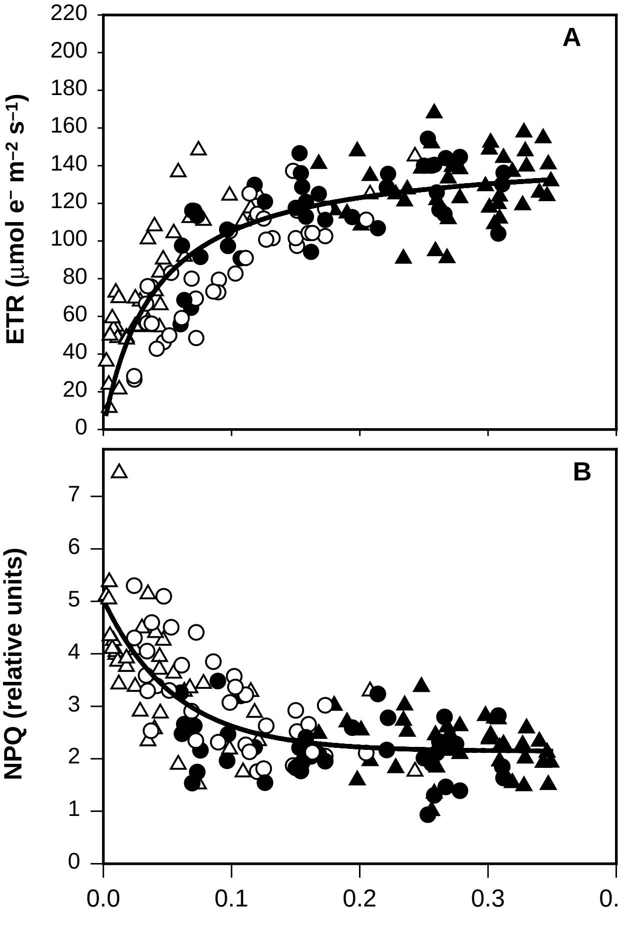

Fig. 4. (*A*) Relationship between electron transport rate (ETR) and stomatal conductance (*g*), and (*B*) between non-photochemical quenching of chlorophyll fluorescence (NPQ) and stomatal conductance (*g*), in field-grown MN (upward triangles) and T (circles). Closed symbols, irrigated plants; open symbols, non-irrigated plants. The best-fit regression for the ETR plot was as a single rectangular hyperbola (r^2=0.73), and that for the NPQ plot was a single exponential decay (r^2=0.56).

close to 50 μmol mol^{-1} when stomata were completely closed (Fig. 6*B*).

From Ci and Cc values, the mesophyll conductance (g_{mes}) was calculated along a *g* gradient for field-grown MN and T plants (Fig. 7). Similar results were found for the other 22 varieties (not shown). The large scattering of data for *g* above 0.15 mol H_2O m^{-2} s^{-1} was likely due to the effects of leaf ontogeny (Escalona *et al.* 1999), and to larger errors of the method as g_{mes} increases (Harley *et al.* 1992). Even under irrigation, Cc was only about 50–60% of Ci (i.e. g_{mes} was about 0.15–0.3 mol m^{-2} s^{-1}). In spite of the scattering, g_{mes} clearly tended to decline as *g* decreased. When *g* = 0.15 mol m^{-2} s^{-1}, g_{mes} was still high (between 0.1 and 0.2 mol m^{-2} s^{-1}). When *g* dropped further, g_{mes} also decreased strongly in all the studied cases.

Data from A_N-Ci curves showed that the apparent ε was approximately constant, although scattered, around 0.06 at high *g*, then decreased sharply when *g* decreased below 0.15 mol H_2O m^{-2} s^{-1} (Fig. 8, open symbols). However, using Cc instead of Ci for the estimation of ε, different results were obtained (Fig. 8, closed symbols). Absolute values of ε increased to 0.11, and some eventual drought-induced decline was apparent only when *g* was below 0.05 mol H_2O m^{-2} s^{-1}.

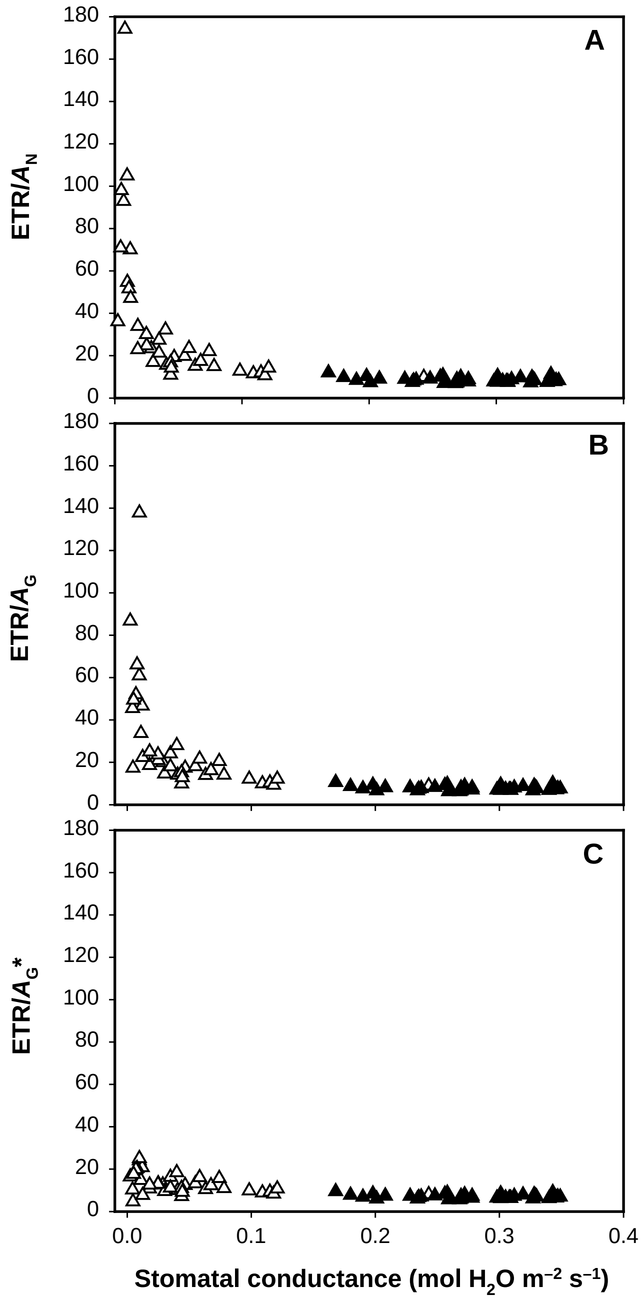

Fig. 5. Ratio of electron transport rate (ETR) to CO_2 assimilation *vs* stomatal conductance (*g*) in field-grown MN. As a measure of CO_2 assimilation, we used either net CO_2 assimilation, A_N (*A*), gross CO_2 assimilation, A_G (*B*), or gross CO_2 assimilation accounting for photorespiration, A_G* (*C*). Closed symbols, irrigated plants; open symbols, non-irrigated plants.

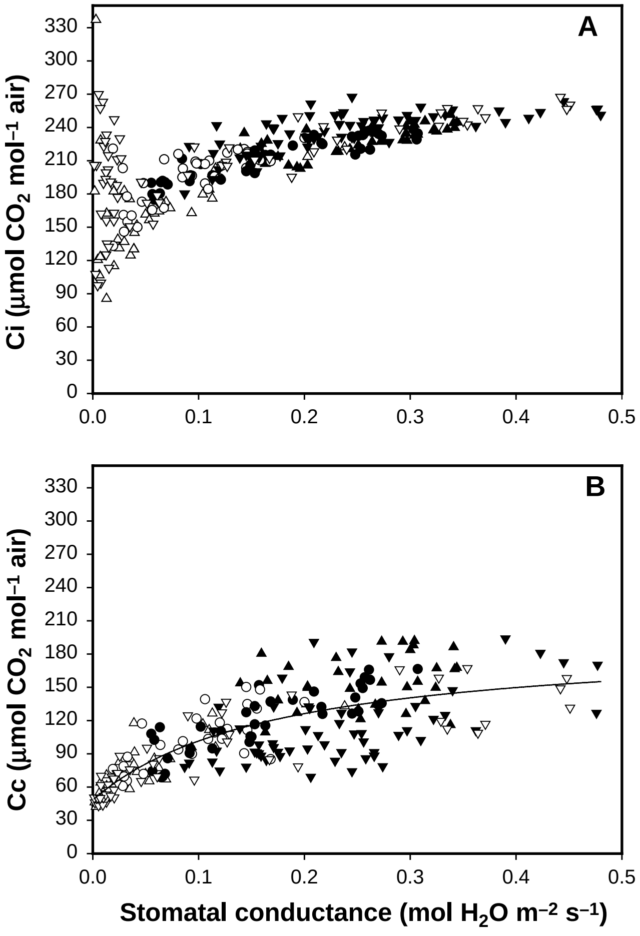

Fig. 6. Relationship between either substomatal CO_2 concentration (*A*) or chloroplastic CO_2 concentration (*B*), and stomatal conductance (*g*), in field-grown MN (upward triangles), T (circles), and the 22 potted varieties (downward triangles). Closed symbols, irrigated plants; open symbols, non-irrigated plants.

Discussion

Stomatal conductance as a unifying parameter for assessing photosynthetic regulation in response to drought

Luo (1991) and Brodribb (1996) already proposed the use of *g* as an indicator to assess the inflexion point between stomatal and non-stomatal limitations to photosynthesis under drought. The present results show that the most common parameters reflecting photosynthetic activity show a more comparable response when *g* is taken as an indicator of water stress. In previous studies, other parameters in relation to photosynthesis, such as pre-dawn F_v/F_m (Flexas *et al.* 1998) and Ci (Escalona *et al.* 1999), were also more dependent on *g* than on Ψ_{PD}. A striking inherent implication of these correspondences is that, under water stress, down-regulation of photosynthesis should depend more on CO_2 availability in the chloroplast (i.e. stomatal closure and mesophyll resistance) than on leaf water potential or leaf water content, as already suggested (Sharkey 1990). This could be understood as a direct adjustment of photosynthetic metabolism to CO_2 availability, which is known to act as a regulator of Rubisco (Perchorowicz and Jensen 1983) and other enzymes (Sharkey 1990; Cornic and Massacci 1996).

Accuracy of gas exchange and chlorophyll fluorescence determinations

Since the present results rely on many assumptions with respect to the accuracy of gas exchange and chlorophyll fluorescence determinations along a *g* gradient, it would be necessary to address the question before extracting conclusions from the data.

Two main assumptions are made in respect to gas exchange data. First, that estimation of Ci is accurate. Second, that R_D and R_L do accurately reflect mitochondrial respiration in the light and photorespiration, respectively. With respect to the first assumption, heterogeneous stomatal closure and cuticular conductance are the two main problems invalidating Ci calculation under drought (Laisk 1983; Boyer *et al.* 1997; Buckley *et al.* 1997). Both have been taken into account for the calculations here (see 'Materials and methods'). Sampling chlorophyll fluorescence variations along the leaf blade generally revealed low variations. Therefore, in principle, no incidence of patchy stomatal closure was observed. These results are in agreement with Gunasekera and Berkowitz (1992), who suggested that patchy incidence is probably larger in experiments of rapid dehydration than in experiments performed under field conditions. Moreover, Buckley *et al.* (1997) found that the patchy-induced error in Ci calculation was not large until *g* was lower than 0.03 mol H_2O m^{-2} s^{-1}, a low value reached only by a few leaves in the present study. Calculation errors due to cuticular conductance were corrected according to Boyer *et al.* (1997; see also Fig. 1). Therefore, the Ci values presented here are, in principle, free of any large error. Nevertheless, any conclusion based on Ci estimations when *g* was lower than 0.03–0.04 mol H_2O m^{-2} s^{-1} must be viewed with some care (Buckley *et al.* 1997; Laisk and Oja 1998).

Although errors in the determination of R_D are important for the estimation of A_G and g_{mes} (Harley *et al.* 1992), the method used here for the determination of R_D is commonly used and assumed to be accurate (Oberhuber and Edwards 1993; Loreto *et al.* 1994; Delfine *et al.* 1998; Earl and Tollenaar 1998). Moreover, the results obtained are in agreement with the values given by these authors. The use of R_L as an indicator of mitochondrial respiration plus photorespiration is more controversial. Measurements of CO_2 efflux into CO_2-free air have been shown to be an erroneous

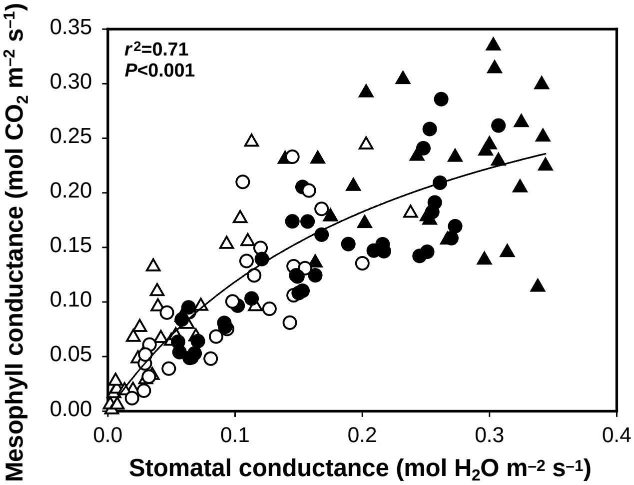

Fig. 7. Mesophyll conductance (g_{mes}) as a function of stomatal conductance (*g*) in field-grown MN (triangles) and T (circles). Closed symbols, irrigated plants; open symbols, non-irrigated plants. The best-fit regression for the plot was as a single rectangular hyperbola (r^2=0.71).

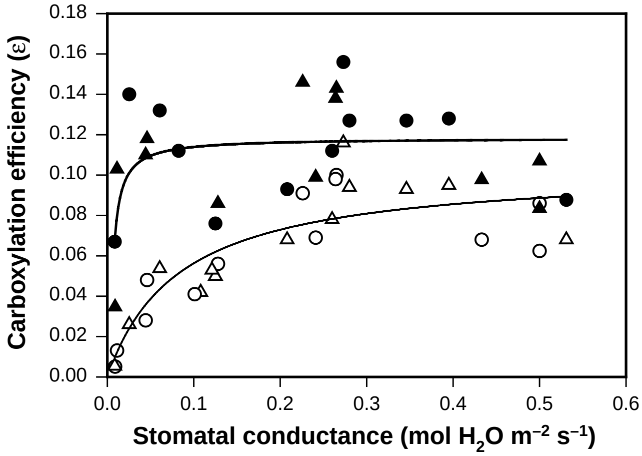

Fig. 8. Relationship between the carboxylation efficiency (ε), estimated either as the initial slope of A_N *vs* Ci (empty symbols), or A_N *vs* Cc curves (closed symbols), and stomatal conductance (*g*) in field-grown plants.

way of estimating photorespiration, due to enhancement of photorespiration in CO_2-free air and inactivation of Rubisco by low CO_2 (Sharkey 1988). However, it must be noticed that here we do not measure CO_2 efflux into CO_2-free air, but simply extrapolate the initial slope of A_N-Ci curves to a hypothetical Ci = 0, from a point where Ci is still high enough to avoid Rubisco inhibition. In grapevines, this method yields estimates of photorespiration that differ by less than 10% from those obtained by the methods of Sharkey (1988) and Valentini *et al.* (1995) (Regina and Carbonneau 1995; J. Flexas, unpublished data). In the present study we preferred the method of extrapolating A_N to Ci = 0 over that of Sharkey (1988), which is based on the kinetic properties of Rubisco, due to the suspected drought-induced variation in the Cc/Ci relationship (see 'Results'). We preferred it to that of Valentini *et al.* (1995), based on combined gas exchange and chlorophyll fluorescence measurements, since this assumes that other processes, such as the Mehler reaction, do not contribute substantially to electron consumption. To test the validity of the latter assumption was of great importance in the present study, because the estimation of Cc and g_{mes} also rely on its validity. Therefore, estimation of the rate of photorespiration by an independent method was needed.

With respect to chlorophyll fluorescence data, the main assumption made is that ETR values estimated by this method accurately reflect the actual values of electron transport in the leaves. The leaf absorptance value of 0.84 has been contrasted in different grapevine varieties (Schultz 1996), so its use is probably accurate here. The 0.5 ratio that accounts for the balance between PSII and PSI light absorption is more doubtful. However, Laisk and Loreto (1996) determined that this ratio varied only between 0.45 and 0.6 among different species, so the use of 0.5 seems accurate as well. Moreover, it was shown in grapevines that the method used here yields ETR values that are in agreement with measurements of gross O_2 evolution using a mass spectrometer (Flexas *et al.* 1999*b*). Therefore, it is assumed that our ETR calculations are quite accurate. For this reason, no calibration was performed under low-O_2 conditions, as recommended (Genty *et al.* 1989), since this kind of calibration is difficult to perform under field conditions.

Overall, the validity of all the assumptions discussed can be tested by the values of the ETR/A_G* ratio (see Earl and Tollenaar 1998 for a similar comparison in C_4 plants). We observed values of *ca* 6–7. These were close, although not identical, to the minimum value (5.6) obtained under non-photorespiratory conditions (Flexas *et al.* 1999*b*). Moreover, the ratio was almost constant along the whole *g* gradient, although a high scattering was observed at very low *g*. These results supported the validity of the method by Epron *et al.* (1995) under our experimental conditions. Even if the small deviation of the ETR/A_G* ratio from 5.6, the minimum value observed in grapevines, means that a small part of ETR is consumed in alternative processes, the constancy of the ratio means that the error would be constant along the studied gradient of *g*. Therefore, although the absolute values of Cc and g_{mes} should be viewed with care, comparisons under different stress conditions are acceptable.

Effects of stomatal closure on leaf photochemistry and photorespiration

When water stress induced decreases in *g* from a maximum to about 0.15 mol H_2O m^{-2} s^{-1}, stomatal closure appeared to be the main limitation to photosynthesis, as deduced from decreases of A_N accompanied by parallel decreases of Ci and Cc. Both F_v/F_m and ETR remained quite unaffected within this range of *g*, as did the carboxylation efficiency estimated from either A_N-Ci or A_N-Cc curves. These results suggest that photorespiration progressively replaced photosynthesis as an electron-consuming process. Indeed, ETR/A_G showed a slow tendency to increase within this range of *g*. In field-grown MN, for instance, ETR/A_G increased by 27% from *g* = 0.35 to *g* = 0.15 mol H_2O m^{-2} s^{-1}. This corresponded exactly with a decrease of 27% Ci within the same range, which strongly suggests increased photorespiration. As reflected by an almost constant ETR/A_G*, most of the thylakoid electron transport was used for carboxylation plus oxygenation of Rubisco under these conditions, the other electron acceptors being negligible. Increased photorespiration as stomata close is a direct consequence of the increase in the O_2/CO_2 ratio inside the chloroplast. This may be the cause of the maintenance of a high ETR, thus affording photoprotection (Takeba and Kozaki 1998; Flexas *et al.* 1999*a*, *b*; Wingler *et al.* 1999).

When *g* dropped from 0.15 to 0.05 mol H_2O m^{-2} s^{-1}, Ci and Cc further decreased, indicating that stomatal limitations to photosynthesis were still dominant. Within this range of *g*, ETR started to decrease, paralleled by increases of NPQ. However, ETR was maintained at values close to 75% of the maximum. Indeed, photosynthesis plus photorespiration would be able to maintain ETR at 75% of control values at the CO_2 compensation point if no Rubisco was impaired (Takeba and Kozaki 1998). The ratio ETR/A_G increased sharply within this range of *g*, suggesting that photorespiration was still increasing, at least in relation to photosynthesis. The constancy of ETR/A_G* within this range of *g* supported the idea that the sum of photosynthesis and photorespiration fully accounted for the consumption of ETR (Valentini *et al.* 1995).

When *g* dropped below 0.05 mol H_2O m^{-2} s^{-1}, A_N and ETR became very low, while NPQ attained maximum values. Still, F_v/F_m was maintained at a high level. Thus, it seems that permanent photoinhibition was not a primary cause for declining photosynthesis under drought, as already shown in grapevines (Flexas *et al.* 1998, 1999*a*, *b*). ETR/A_G* was still low, although with scattering, due to low accuracy in the determination of both ETR and A_G* at such very low *g*. These results suggest that alternative sinks for electrons, such as the Mehler reaction, were still low.

Mesophyll limitations to photosynthesis

When working under field conditions, it is difficult to obtain unequivocal, fine relationships between the different parameters measured. Large scattering of ETR and Ci, for instance, show the existence of important inter-individual variability, and are translated into very large scattering of Cc and g_{mes}. The fact that gas exchange and chlorophyll fluorescence are not measured exactly at the same time adds some more uncertainty. Therefore, when combining these distinct measurements to calculate, for instance, the mesophyll resistance, the results must be viewed with some care. Nevertheless, in spite of large scattering, clear correspondences have been shown between these different photosynthetic parameters and *g*. This is also true for the estimated g_{mes}, which decreased as drought induced *g* to decline (Fig. 7). As discussed, at *g* lower than 0.03–0.04 mol H_2O m^{-2} s^{-1}, the estimations of g_{mes} must be viewed with some care, due to the low accuracy of ETR and Ci determinations at low photosynthetic rates. However, g_{mes} consistently declined, even at higher *g*, particularly when *g* dropped below 0.15 mol m^{-2} s^{-1}. Within this range of *g*, A_N was still substantial, patchiness was unlikely, and the accuracy of ETR determination was high. Thus, it appears that an important effect of progressive water stress might be increasing the resistance of mesophyll to CO_2 diffusion, as already suggested (Beadle and Jarvis 1977; Renou *et al.* 1990; Tourneux and Peltier 1995; Roupsard *et al.* 1996). Reduced g_{mes} has been ascribed to a drought-induced collapse of parts of the mesophyll, due to loss of turgor (Cornic *et al.* 1989; Renou *et al.* 1990). However, other explanations would be required to justify g_{mes} reductions in the present study, since turgor loss was not likely in view of the almost constant RWC (see Fig. 3). g_{mes} involves a complexity of processes, including diffusion of CO_2 in the gas phase, its dissolution in the liquid phase, conversion to HCO_3^- catalysed by carbonic anhydrase, and diffusion in the liquid phase. Carbonic anhydrase, for instance, was shown to be inhibited by progressive drought (Jones 1973). In any case, the precise steps and mechanisms affected by water stress remain to be elucidated.

Irrespective of the precise nature of diffusional limitations, Cc appears to be much lower than estimated by Ci, the difference increasing under drought (see Cornic and Massacci 1996 for a similar conclusion). Therefore, A_N-Ci curves should be re-analysed. When analysed on a Ci basis, the apparent ε progressively decreased when *g* dropped below 0.15–0.2 mol m^{-2} s^{-1}, suggesting reduced Rubisco activity (von Caemmerer and Farquhar 1981). However, when analysed on a Cc basis, ε was maintained at an almost constant level through the entire *g* gradient. Only when *g* dropped below 0.05 mol m^{-2} s^{-1} did ε decrease somewhat, although these results must be viewed with care at such low *g* (see previous discussion). At these low *g* values, Cc was lower than 100 µmol mol^{-1}, reaching 50 µmol mol^{-1} when *g* was close to zero (see Fig. 6). CO_2 concentrations below 100 µmol mol^{-1} have been shown to inhibit Rubisco activity (Perchorowicz and Jensen 1983). *In vitro*-determined Rubisco activity in field-grown, severely water-stressed grapevines decreased by 15–20% relative to irrigated plants (J. Bota, J. Flexas, A. Keys, J. Loveland, M. Parry and H. Medrano, unpublished data). Thus, it is likely that decreased ε at very low *g* was reflecting a decrease in Rubisco activity. The strong ETR reductions observed at this low *g* also support the possibility of impaired Rubisco.

In summary, estimation of Cc has suggested that diffusional limitations are the predominant factor limiting grapevine photosynthesis under mild to moderate drought (i.e. when *g* was decreased from a maximum to around 50 mmol H_2O m^{-2} s^{-1}). Photochemistry was progressively decreased to adjust to the balance between photosynthesis and photorespiration. Decreased Rubisco activity would be only an eventual, secondary effect of very severe drought. Therefore, previously observed non-stomatal limitations on photosynthesis were likely due to an overestimation of Ci by commonly used gas exchange methods, and to increased mesophyll resistance. This pattern of responses is very similar to that described by Delfine *et al.* (1998) in salt-stressed spinach. However, further studies are needed to confirm these findings, since they are raised by the indirect method of applying a gas exchange-chlorophyll fluorescence model, which would imply that there are many assumptions that need testing.

Note added in proof: A possible role of aquaporins in determining the mesophyll conductance to CO_2 has been suggested very recently [Terashima and Ono (2002) *Plant and Cell Physiology* **43**, 70–78]. The possibility of these water channels being on the basis of g_{mes} downregulation under drought is very attractive, and deserves further study since it would represent a mechanism for coregulation of photosynthesis and water losses at the cellular level.

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