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Limitations to leaf photosynthesis in field-grown grapevine under drought — metabolic and modelling approaches

João P. Maroco[AB], *M. Lucília Rodrigues*[C], *Carlos Lopes*[C] *and M. Manuela Chaves*[AC]

[A]Laboratório de Ecofisiologia Molecular, Instituto de Tecnologia Química e Biológica, Apartado 12, 2781–901 Oeiras, Portugal.
[B]Instituto Superior de Psicologia Aplicada, Rua Jardim do Tabaco 44, 1149–041 Lisboa, Portugal. Corresponding author; email: jpmaroco@ispa.pt
[C]Instituto Superior de Agronomia, Tapada da Ajuda, 1349–017 Lisboa, Portugal.

Abstract. The effects of a slowly-imposed drought stress on gas-exchange, chlorophyll *a* fluorescence, biochemical and physiological parameters of *Vitis vinifera* L. leaves (cv. Aragonez, syn. Tempranillo) growing in a commercial vineyard (South Portugal) were evaluated. Relative to well-watered plants (predawn water potential, Ψ_{PD} = –0.13 ± 0.01 MPa), drought-stressed plants (Ψ_{PD} = –0.97 ± 0.01 MPa) had lower photosynthetic rates (*ca* 70%), stomatal conductance, and PSII activity (associated with a higher reduction of the quinone A pool and lower efficiency of PSII open centres). Stomatal limitation to photosynthesis was increased in drought-stressed plants relative to well-watered plants by *ca* 44%. Modelled responses of net photosynthesis to internal CO_2 indicated that drought-stressed plants had significant reductions in maximum Rubisco carboxylation activity (*ca* 32%), ribulose-1,5-bisphosphate regeneration (*ca* 27%), and triose phosphate (triose-P) utilization rates (*ca* 37%) relative to well-watered plants. There was good agreement between the effects of drought on modelled biochemical parameters, and *in vitro* activities of key enzymes of carbon metabolism, namely Rubisco, glyceraldehyde-3-phosphate dehydrogenase, ribulose-5-phosphate kinase and fructose-1,6-bisphosphate phosphatase. Quantum yields measured under both ambient (35 Pa) and saturating CO_2 (100 Pa) for drought-stressed plants were decreased relative to well-watered plants, as well as maximum photosynthetic rates measured at light and CO_2 saturating conditions (three times ambient CO_2 levels). Although stomatal closure was a strong limitation to CO_2 assimilation under drought, comparable reductions in electron transport, CO_2 carboxylation, and utilization of triose-P capacities were also adaptations of the photosynthetic machinery to dehydration that slowly developed under field conditions. Results presented in this study confirm that modelling photosynthetic responses based on gas-exchange data can be successfully used to predict metabolic limitations to photosynthesis.

Keywords: drought, enzymes of carbon metabolism, gas-exchange, modelling, photosynthesis, *Vitis vinifera*.

Introduction

There is ongoing discussion on how drought affects photosynthesis, namely on the relative roles of restricted diffusion of CO_2 into the leaf due to stomata closure and inhibition of CO_2 metabolism (Tezara *et al.* 1999; Cornic 2000). Recent studies have shown that both the photochemical apparatus and CO_2 assimilation capacity are quite resistant to drought stress, and that stomatal closure, with a reduction in mesophyll CO_2 availability, is the main factor responsible for reductions in CO_2 assimilation (stomatal effects) under mild drought (Cornic and Massacci 1996; Chaumont *et al.* 1997; Correia *et al.* 1999). However, other studies suggest a non-stomatal limitation of CO_2 assimilation via a direct effect of drought on ATP synthase, with a reduction of ATP production (Lawlor 1995; Tezara *et al.* 1999) and ribulose-1,5-bisphosphate (RuBP) regeneration (Gunasekera and Berkowitz 1993). The reduction in the maximum photosynthetic capacity under long-term drought allows photosynthesis to operate near break point of the RuBP- and CO_2-limited regions of the A/C_i (where A is net CO_2

Abbreviations: A, net CO_2 assimilation; A_{max}, maximum net CO_2 assimilation; C_a, external CO_2 partial pressure; C_i, intercellular CO_2 partial pressure; ETp, potential evapotranspiration; FruBPase, fructose-1,6-bisphosphate phosphatase; F_v'/F_m', efficiency of PSII open centres; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; g_s, stomatal conductance; J_{max}, maximum electron transport rate; PPFD, photosynthetic photon flux density; Q_A, primary quinone receptor of PSII; 1-q_P, reduction state of the Q_A pool; RuBP, ribulose-1,5-bisphosphate; Ru5PK, ribulose-5-phosphate kinase; TPU, triose-P utilization; V_{Cmax}, maximum Rubisco activity; Φ_{PSII}, quantum yield of PSII; Ψ_{PD}, predawn water potential.

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assimilation and C_i is the intercellular partial pressure of CO_2) curve (Lambers *et al.* 1998). This adaptation mechanism maximizes the effectiveness of both light and dark reactions under stress. However, this downregulation of photosynthesis in response to water stress is not fully understood (Lambers *et al.* 1998). Differences among species and in the rates of imposition of water deficits, as well as the superimposition of other environmental stresses, may also play a role in the relative importance of stomatal *vs* non-stomatal limitations under drought (Chaves 1991). In grapevine (*Vitis vinifera* L.), water stress aggravates photoinhibition of the photochemical apparatus under elevated irradiances, causing stomatal closure and downregulation of carbon assimilation (Correia *et al.* 1990; Quick *et al.* 1992; Rodrigues *et al.* 1993; Chaumont *et al.* 1997; Flexas *et al.* 1998, 1999*b*). Non-stomatal effects have been suggested for grapevine responses to drought under field conditions, as estimated from coupled gas-exchange and fluorescence measurements (Flexas *et al.* 1998, 1999*b*; Escalona *et al.* 1999). However, unresponsiveness of net CO_2 assimilation relative to C_i suggests that the occurrence of non-stomatal effects under drought stress may be, to some degree, attributable to patchy stomatal aperture in heterobaric leaves, although these effects may be minimized when water deficits are imposed slowly (Mott and Buckley 2000). The increasing importance of cuticular transpiration under water deficit may also add to the erroneous overestimation of C_i (Downton *et al.* 1988; Raschke *et al.* 1990; Meyer and Genty 1998; Cornic 2000).

Our hypothesis in the present study is that by measuring the activities of key enzymes of the CO_2 assimilation pathway (involved in carboxylation, regeneration of RuBP and utilization of triose-P) we may override the ambiguity in estimating non-stomatal effects of water stress, by using net CO_2 assimilation responses to internal CO_2. We therefore used both biochemical and modelling approaches to differentiate between stomatal *vs* metabolic responses of grapevine to water stress driven by a slow and seasonal drought under field conditions.

Materials and methods

Plant material and growth conditions

Fourteen-year old grapevine plants (*Vitis vinifera* L. cv. Aragonez syn. Tempranillo) were selected from an irrigation experiment in a commercial vineyard in the South of Portugal (Alentejo). Vine spacing was 1.2 m within rows and 2.5 m between rows. Plants were trained on a vertical trellis with three fixed wires (50, 90 and 130 cm above the ground) and a pair of movable foliage wires for upwards shoot positioning. The vines were spur pruned on a bilateral Royat Cordon (16 buds per vine). Well-watered plants were drip irrigated from the end of May onwards every 3–4 d with 80% of potential evapotranspiration (ETp, as estimated by the Penman-Monteith method), while drought-stressed plants were rain fed, suffering progressive soil water depletion during the growing season as described by Lopes *et al.* (2001). All measurements were performed in July (mid-Mediterranean summer).

Plant water status

Ψ_{PD} was measured in 4–6 individual mature leaves using a pressure chamber (Model 1000; PMS Instrument Co., Corvallis, OR, USA).

Gas exchange and fluorescence

A/C_i response curves were generated using a portable LI 6400 infra-red gas analyser [IRGA (Li-Cor, Lincoln, NE, USA)] with a constant leaf temperature of 25°C and 1200 μmol photons m^{-2} s^{-1} supplied by the LI 6400-02B light system (Li-Cor). Relative humidity inside the cuvette was kept at 70 ± 2%. Daily courses of gas exchange between the leaf and the atmosphere were performed with the LI 6400 under naturally occurring photosynthetic photon flux density (PPFD) and air temperature. Maximum net CO_2 assimilation and incident light quantum yield rates were calculated from light response curves measured at 35 and 100 Pa ambient CO_2 using the portable LI 6400 IRGA. Chlorophyll *a* fluorescence was measured with a PAM 2000 fluorometer (Walz, Effeltrich, Germany), and the quantum yield of PSII (Φ_{PSII}), reduction state of the primary acceptors ($1\text{-}q_P$), and efficiency of PSII open centres (F_v'/F_m') were calculated as described in Maroco *et al.* (1998).

Biochemical modelling and relative stomatal limitation

The net CO_2 assimilation biochemical model for C_3 plants of Farquhar *et al.* (1980) can be written as:

$$A = V_C - 0.5V_O - R_d = V_C\left(1 - \frac{0.5O}{\tau C_i}\right) - R_d \quad (1)$$

where V_C and V_O are the rates of carboxylation and oxygenation of Rubisco, respectively, R_d is the mitochondrial respiration, O and C_i are the oxygen and CO_2 partial pressures in intercellular spaces, respectively, and τ is the specificity of Rubisco for CO_2/O_2. According to Farquhar *et al.* (1980), with modifications by Sharkey (1985), V_C is a minimum function of the carboxylation rates, limited by either (i) kinetic properties and amount of Rubisco (Wc), (ii) the rate of RuBP regeneration (Wj), or (iii) the availability of inorganic phosphate (Wp). That is V_C = minimum(Wc, Wj, Wp).

The rate of carboxylation limited by the amount and kinetic properties of Rubisco is given by:

$$Wc = \frac{V_{Cmax} \cdot C_i}{C_i + K_C(1 + O/K_O)} \quad (2)$$

where V_{Cmax} is the maximum rate of carboxylation, and K_C and K_O are the Michaelis-Menten constants for the carboxylation and oxygenation processes, respectively.

The rate of carboxylation limited by the regeneration of RuBP is a function of the rate of electron transport, and is given by:

$$Wj = \frac{J \cdot C_i}{4(C_i + O/\tau)} \quad (3)$$

where it is assumed that four electrons are enough to generate the three ATP and two NADPH required in the Calvin cycle to regenerate RuBP, and J is the rate of electron transport through PSII given by:

$$J = \frac{\alpha \cdot I}{\sqrt{\left[1 + \frac{\alpha^2 I^2}{J_{max}^2}\right]}} \quad (4)$$

where α is the fraction of incident-light photons that are converted into electrons, and J_{max} is the maximum, light saturated, rate of electron transport.

Finally, the rate of carboxylation limited by inorganic phosphate availability inside the chloroplast is given by:

$$Wp = 3TPU + \frac{V_C \cdot O}{2 \cdot C_i \cdot \tau} \quad (5)$$

where TPU is the rate of triose-P utilization (for sucrose and starch synthesis) (Sharkey, 1985).

Michaelis-Menten constants (K_C and K_O), α, τ, and temperature-dependence of the model parameters were corrected as described by Harley *et al.* (1992). According to these authors, the temperature dependence of K_C, K_O, α, and τ is described by an exponential function of the type Parameter = exp[$c - \Delta H_a/(RT)$] where c is a scaling constant characteristic for each parameter, ΔH_a is the activation energy for the parameter, R is the ideal gas constant, and T is the leaf temperature in degrees Kelvin [see Harley *et al.* (1992) for the values of the constants used]. V_{Cmax} and R_d were estimated by fitting the model to measured A/C_i data for C_i less than 20 Pa because, in this C_i range, V_C is limited only by Wc if light is saturating, as it was in this case. Finally, J_{max} and TPU were estimated by fitting the model, with previously estimated V_{Cmax} and R_d, to the complete A/C_i data using a non-linear curve fitting routine from SAS (version 6.12; SAS Institute, Cary, NC, USA).

The limitation to photosynthesis imposed by closed stomatal conductance, both under well-watered and drought conditions, was estimated through the relative stomatal limitation (RSL) calculated from A/C_i response curves using the equation:

$$RSL = \frac{A_{C_i=35Pa} - A_{C_a=35Pa}}{A_{C_i=35Pa}} \times 100\%. \quad (6)$$

Estimates of net CO_2 assimilation at C_i = 35 Pa and at C_a = 35 Pa (A_{Ci} and A_{Ca}, respectively) were obtained from previously fitted Eqn 1 to A/C_i (or C_a) data.

Protein, sugar and enzyme assays

Six leaf discs (5.22 cm^2) from two different leaves per plant were harvested in the light, frozen in liquid N_2 and stored at –80°C until assay. For protein determination and enzyme activities, the frozen leaf discs were ground to a fine slurry in 1.5 mL of an extraction solution containing 200 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2.6H_2O$, 10 mM $NaHCO_3$, 10 mM β-mercaptoethanol, 2 mM dithiothreitol, 2% Triton X-100, 4% (v/v) 'Complete-protease inhibitor cocktail with EDTA', 10% polyvinylpolypyrrolidone, and 10% glycerol. The extract was centrifuged at 16000 *g* for 4 min at 4°C, and the supernatant collected. The pellet was resuspended in 0.2 mL of the extraction solution, and after a 16000 *g* centrifugation at 4°C for 2 min, both supernatants were combined and used for total soluble protein determination and enzyme activity measurements. Solubilized protein was measured using Bio-Rad's protein assay kit according to the manufacturers instructions (Bio-Rad, Hercules, CA, USA), and protein integrity was followed by SDS-PAGE.

Enzyme activities were measured spectrophotometrically by following the oxidation of NADH at 340 nm and 21°C as described by Leegood (1993) for Rubisco (EC 4.1.1.39), and by Maroco *et al.* (1999) for glyceraldehyde-3-phosphate dehydrogenase (G3PDH; EC 1.2.1.13), ribulose-5-phosphate kinase (Ru5PK; EC 2.7.1.19) and fructose-1,6-bisphosphate phosphatase (FruBPase; EC 3.1.3.11). All chemicals and coupling enzymes were from Sigma (St Louis, MO, USA) except for the 'Complete' cocktail, which was from Roche (Mannheim, Germany). The presence of very acidic vacuoles as well as an abundance of phenolic compounds, which increase in concentration under drought stress conditions (data not shown), made the extraction of active enzymes from the leaves of grapevines very difficult. Rubisco solubilization required a high percentage (up to 2%) of a mild detergent (suggesting that Rubisco is somehow associated with the insoluble thylakoid membranes) and several protease inhibitors, especially for drought-stressed leaves (data not shown, but see, for example, Kanna-Chopra *et al.* 1999). Soluble sugars and starch were measured enzymatically as described in Stitt *et al.* (1983, 1989).

Sampling and statistical analysis

All measurements and sample collections were carried out on the sun-exposed side of the vines, in two recently fully-expanded leaves per plant and four plants per treatment. Data are shown as means ± s.e. in both tables and figures. Statistically significant differences between treatments were analysed by Student *t*-tests with α = 0.05.

Results

Plant water status

Well-watered plants maintained Ψ_{PD} at about –0.2 MPa throughout the growing season, while in drought-stressed plants, Ψ_{PD} decreased from mid-May to the end of July at a rate of 0.01 MPa d^{-1}. At the time the experiments were performed (mid-summer; at veraison), Ψ_{PD} was –0.13 ± 0.01 MPa for well-watered plants, and –0.97 ± 0.01 MPa for drought-stressed plants. At this date, the accumulated ETp was around 600 mm, and accumulated irrigation was around 200 mm (Fig. 1).

Net CO_2 assimilation, stomatal conductance and PSII yield

Relative to well-watered controls, drought-stressed plants had lower CO_2 exchange rates [A (Fig. 2*a*; around 70% less throughout the day)] which were associated with lower

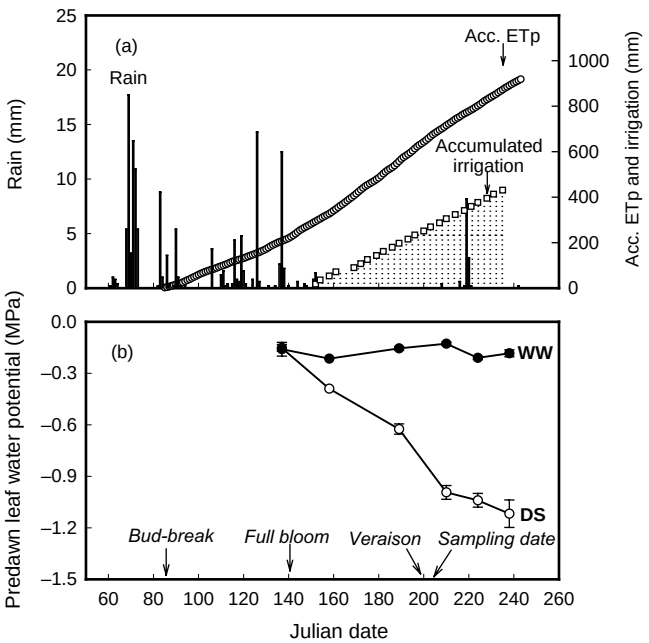

Fig. 1. Seasonal evolution of accumulated potential evapotranspiration (ETp; from bud-break), accumulated irrigation for the well-watered controls and rain (*a*), and seasonal evolution of the predawn leaf water potential (Ψ_{PD}) in drought-stressed (DS) *vs* well-watered (WW) plants (*b*).

stomatal conductance [g_s (Fig. 2*c*; around four times lower)] and quantum yield of PSII reaction centres [Φ_{PSII} (Fig. 2*b*; 35% less, on average, throughout the day)]. The lower Φ_{PSII} of drought-stressed plants was associated with a higher reduction state of the primary acceptors (the Q_A pool) [1-q_P (Fig. 2*f*; 50% more throughout the day)] and lower efficiency of open PSII centres [F_v'/F_m' (Fig. 2*d*; about 20% less, on average, throughout the day)]. A midday depression of photosynthesis was observed in the drought-stressed plants. In well-watered plants, stomatal closure occurred late in the day in response to reduced light levels. This was not the case for drought-stressed plants, where stomatal conductance remained very low and constant throughout the day (Fig. 2*c*). As a result, drought-stressed plants had higher intrinsic water use efficiency [A/g_s (Fig. 2*e*)].

Soluble and insoluble sugars

Sucrose, fructose and glucose, as well as starch, concentrations per unit leaf area were decreased under drought stress (Fig. 3). There was a statistically significant reduction

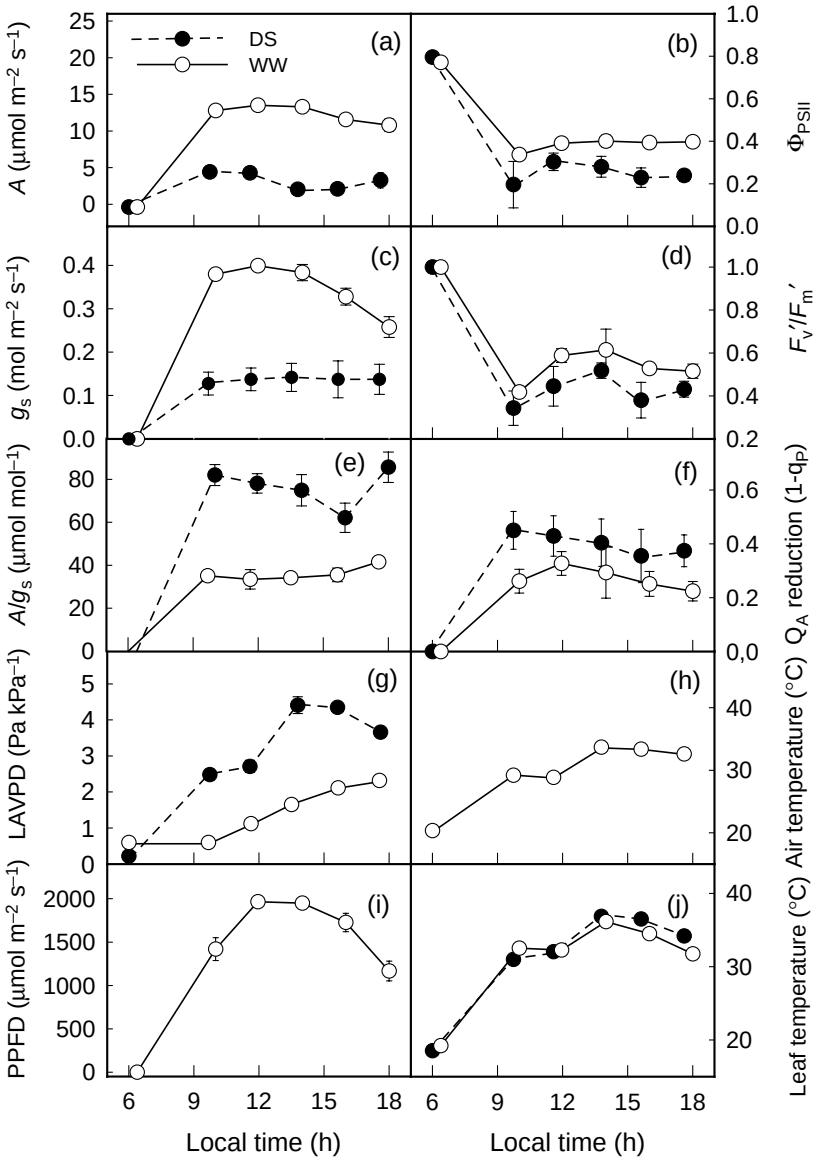

Fig. 2. Daily courses of (*a*) net CO_2 assimilation (*A*), (*b*) quantum yield of PSII centres (Φ_{PSII}), (*c*) stomatal conductance (g_s), (*d*) efficiency of open PSII centres ($F_v'F_m'$), (*e*) intrinsic water use efficiency (A/g_s), (*f*) reduction state of the Q_A pool (1-q_p), (*g*) leaf-to-air vapour pressure deficit (LAVPD), (*h*) air temperature, (*i*) photosynthetic photon flux density (PPFD),(*j*) and leaf temperature in well-watered (○) and drought-stressed (●) plants. Data are shown as mean ± s.e.

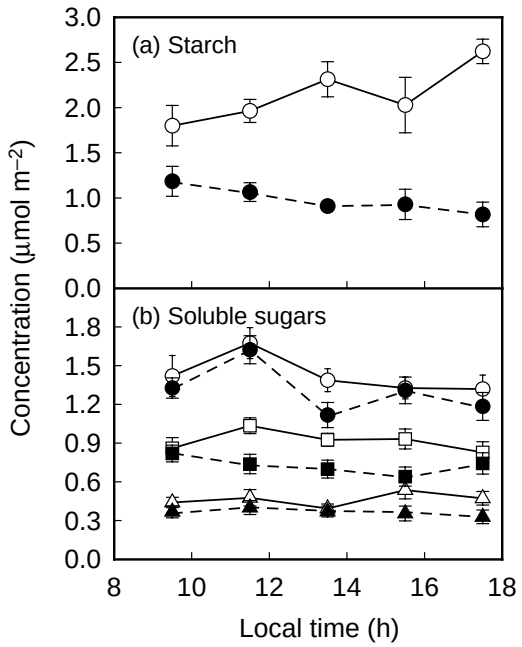

Fig. 3. Daily evolution of starch (*a*) and soluble sugar (*b*) concentrations in leaves of well-watered plants (open symbols) and drought-stressed plants (closed symbols). Data are shown as means ± s.e. ●, ○, glucose; ■, □, fructose; ▲, △, sucrose.

in starch during the course of the day in drought-stressed, relative to well-watered, plants (Fig. 3*a*; 53% reduction, on average, throughout the day). Statistically significant differences were also observed at midday for glucose and fructose, and by the end of the day for sucrose (Fig. 3*b*). Relative to insoluble sugars (starch), the reductions observed in soluble sugars in drought-stressed plants throughout the day were much smaller (only 15%).

Modelled biochemical and stomatal responses

Biochemical modelling of A/C_i response curves suggests that Rubisco maximal activity, RuBP regeneration capacity, and triose-P utilization capacity were decreased in drought-stressed plants relative to well-watered plants (Fig. 4). Model estimates of maximum Rubisco carboxylation capacity (V_{Cmax}) were decreased by 32%, RuBP regeneration capacity (which can be estimated by J_{max}; see, for example, Wullschleger 1993 and Wohlfahrt 1999) was decreased by up to 27%, and triose-P utilization capacity (TPU) was reduced by *ca* 37% in drought-stressed plants relative to well-watered plants (Table 1). There were no significant differences between treatments for estimated dark-respiration rates. Consistent with model estimates, quantum yields of incident PPFD under ambient CO_2 (35 Pa) were reduced by 57% in drought-stressed plants relative to well-watered plants, while maximum rates of photosynthesis under saturating light (2000 µmol photons m^{-2} s^{-1}) and CO_2 (100 Pa) were decreased by 64%. Both maximum CO_2 assimilation and quantum yield increased under very high CO_2 (100 Pa), but the percent reductions observed in drought-stressed plants as compared with well-watered plants were similar to those observed at 35 Pa of CO_2 for A_{max} only (Table 1). Elevated CO_2 did not relieve the inhibition of quantum yield by drought, suggesting that photochemical inhibition did occur, as also indicated by Φ_{PSII} values estimated through chlorophyll fluorescence. Finally, the relative stomatal limitation of photosynthesis increased from 22% in well-watered plants to 31% in drought-stressed plants (Table 1).

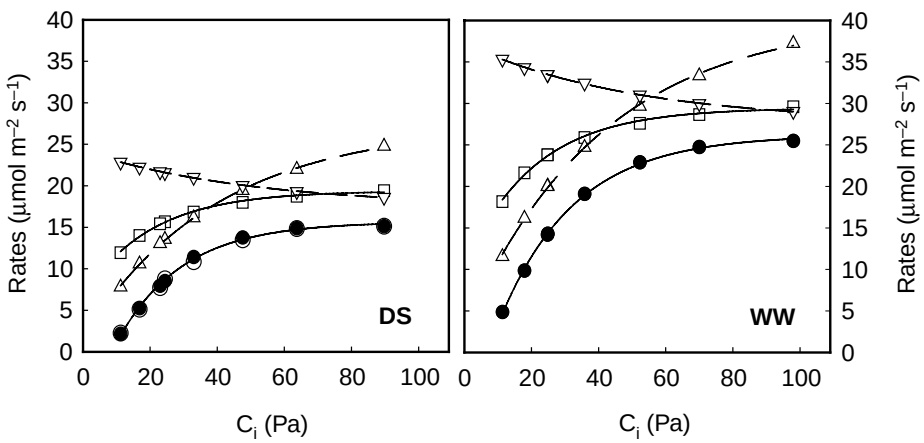

Fig. 4. Measured (○) and modelled (●) net CO_2 assimilation responses to internal CO_2 (C_i) in well-watered (WW) and drought-stressed (DS) plants. Modelled net CO_2 assimilation response to C_i is a minimum function of the rate of carboxylation, limited by either: (i) the amount and kinetic properties of Rubisco (W_c; △); (ii) the rate of RuBP regeneration (W_j; □); or (iii) inorganic phosphate utilization (W_p; ▽) (see 'Materials and methods'). All adjusted models have statistically significant r^2 greater than 0.99.

Table 1. Estimated model parameters (V_{Cmax}, J_{max}, TPU and R_d), relative stomatal limitation (RSL), maximum net CO_2 assimilation (A_{max}) and quantum yield (QY) measured both at ambient (35 Pa) and elevated CO_2 (100 Pa) at a constant PPFD of 1200 μmol m^{-2} s^{-1} as described in 'Materials and methods' for well-watered (WW) and drought-stressed (DS) plants

Values are mean ± s.e. Different letter superscripts indicate statistically significant differences at α = 0.05

	Model parameters								
	V_{Cmax}	J_{max}	TPU	R_d		A_{max} (μmol m^{-2} s^{-1})		QY (μmol CO_2 μmol photons^{-1})	
Treatment	(μmol m^{-2} s^{-1})	(μmol m^{-2} s^{-1})	(μmol m^{-2} s^{-1})	(μmol m^{-2} s^{-1})	RSL (%)	35 Pa	100 Pa	35 Pa	100 Pa
WW	52.52 ± 0.57^a	129.03 ± 0.57^a	8.79 ± 0.06^a	2.25 ± 0.15^a	21.7 ± 1.7^a	17.33 ± 0.89^a	36.29 ± 0.43^a	0.067 ± 0.01^a	0.094 ± 0.009^a
DS	35.88 ± 1.85^b	94.54 ± 1.01^b	5.57 ± 0.1^b	2.57 ± 0.46^a	31.3 ± 2.9^b	6.19 ± 1.11^b	11.83 ± 1.49^b	0.027 ± 0.02^b	0.031 ± 0.010^b

In vitro *activities of key enzymes of the Calvin cycle*

Total soluble protein and total chlorophyll were reduced by 25 and 20%, respectively, in drought-stressed plants relative to well-watered controls (Table 2). The decrease in total chlorophyll was proportional to the reduction observed in Chlorophyll *a* and *b*, which resulted in non-significant differences in the Chlorophyll *a*/*b* ratio. The *in vitro* activity of Rubisco (a key enzyme in the carboxylation phase of the Calvin cycle), which relates to V_{Cmax} estimated by the biochemical model, was reduced by 37% in drought-stressed plants relative to well-watered controls (Fig. 5). Drought induced a similar reduction (36%) in the activity of G3PDH (a key enzyme in the reductive phase of the Calvin cycle), while the activity of Ru5PK (a key enzyme in the regenerative phase of the Calvin cycle), which is related to J_{max}, was reduced by 40% relative to well-watered plants. Finally, the activity of FruBPase (a key enzyme in the utilization of triose-P for starch and sucrose synthesis), which relates to TPU estimated by the biochemical model, was decreased by 49% in drought-stressed plants compared with well-watered ones (Fig. 5).

Discussion

We observed a significant reduction of net CO_2 assimilation in drought-stressed plants relative to well-watered controls, which was partially associated with stomatal closure and downregulation of PSII activity, but also with biochemical limitations as estimated from maximum *A* rates and quantum yields measured under saturating PPFD and elevated CO_2 (three times ambient) (Fig. 2; Table 1). The reduction of PSII yield observed in light-adapted leaves of drought-stressed plants was associated with a reduction of the quinone A pool and lower efficiency of PSII open centres. These responses may be driven by the lower protein and chlorophyll contents observed in those leaves (Table 2; Fig. 2). However, no permanent damage to PSII centres was observed, as indicated by the quantum yield of PSII of dark-adapted leaves (see data for 6 am in Fig. 2). Drought increased the relative stomatal limitation for CO_2 assimilation, but both mesophyll photosynthesis and stomatal aperture appeared to be tightly coregulated under drought, as has been observed in other studies with grapevines (Correia *et al.* 1990, 1999; Flexas *et al.* 1999*a*, *b*).

According to previous reports, one of the effects of reduced CO_2 assimilation, in spite of a decrease in assimilate utilization by growth, is the reduction of starch, as well as soluble sugars, in leaves of drought-stressed plants (Sharkey and Seeman 1989; Rodrigues *et al.* 1993; Lawlor 1995). This is consistent with the reduction of FruBPase activity observed in drought-stressed leaves of grapevine (this study) and in sucrose phosphate synthase activity observed by Vassey *et al.* (1991) in *Phaseolus vulgaris*. Our model estimates of C_3 photosynthesis (Fig. 4; Table 1) show that, in addition to increased relative stomatal limitation, drought stress is responsible for the reduction in maximum Rubisco carboxylation activity and electron transport, and therefore RuBP regeneration. The reduction observed in the utilization of triose-P, leading to a diminished supply of inorganic phosphate to the Calvin cycle, is also consistent with the slow-down in growth generally observed in drought-stressed plants (Chaves 1991).

Although genotypic variations may explain differences in the degree of stomatal *vs* non-stomatal limitations on photosynthesis during drought (Quick *et al.* 1992; Wohlfahrt *et al.* 1999), most conclusions have been based on coupled gas-exchange and Chlorophyll *a* fluorescence data. It must

Table 2. Total solubilized protein, chlorophylls (*a*, *b* and total) and chlorophyll *a*/*b* ratio in well-watered (WW) and drought-stressed (DS) plants

Data are mean ± s.e. Different letter superscripts indicate statistically significant differences at α = 0.05

		Chlorophylls (g m^{-2})			
Treatment	Protein (g m^{-2})	*a*	*b*	Total	Ratio *a*/*b*
WW	9.16 ± 1.14^a	0.16 ± 0.01^a	0.089 ± 0.005^a	0.25 ± 0.01^a	1.87 ± 0.05^a
DS	6.86 ± 0.80^a	0.13 ± 0.01^b	0.06 ± 0.003^b	0.20 ± 0.01^b	1.83 ± 0.06^a

be emphasized that effects deduced from gas-exchange data (including our model estimates) may be overestimated, due to erroneous calculations of C_i resulting from stomatal patchiness and cuticular transpiration, especially under drought-stress conditions (Beyschlag *et al.* 1992; Meyer and Genty 1998; Mott and Buckley 2000).

To eliminate ambiguity in model estimates caused by possible errors in C_i calculation, we measured the *in vitro* activities of key enzymes of the Calvin cycle and triose-P utilization, namely Rubisco, G3PDH, Ru5PK and FruBPase, which can be related directly to the model estimates (Farquhar *et al.* 1980; Wullschleger 1993; Wohlfahrt *et al.* 1999). There were striking reductions in maximum Rubisco, G3PDH, Ru5PK and FruBPase activities per unit of leaf area in drought-stressed plants (Fig. 5), which were correlated with a significant reduction in total protein in the same plants. Reductions in soluble protein may result from increased protease activity, which is normally the case under drought (Kanna-Chopra *et al.* 1999). In addition, disequilibrium between source and sink carbohydrates, deficient nitrogen assimilation, and increased carbon:nitrogen ratio, which occur under stress conditions, are known to repress the expression of genes that encode photosynthetic enzymes (Paul and Driscoll 1997; Nielsen *et al.* 1999). This type of response is likely to occur under a slowly-induced water stress. With rapid imposition of water stress, effects on CO_2 assimilation may be caused both by responses involving stomatal closure (Tezara *et al.* 1999; Cornic 2000) and imbalances of key metabolites of the pathway due to the lack of CO_2 in the chloroplast (Gunasekera and Berkowitz 1993; Lawlor 1995; Tezara *et al.* 1999). In this study, we did not address processes associated with the rapid imposition of stress, rather the ones observed during acclimation to water stress developing slowly under field conditions.

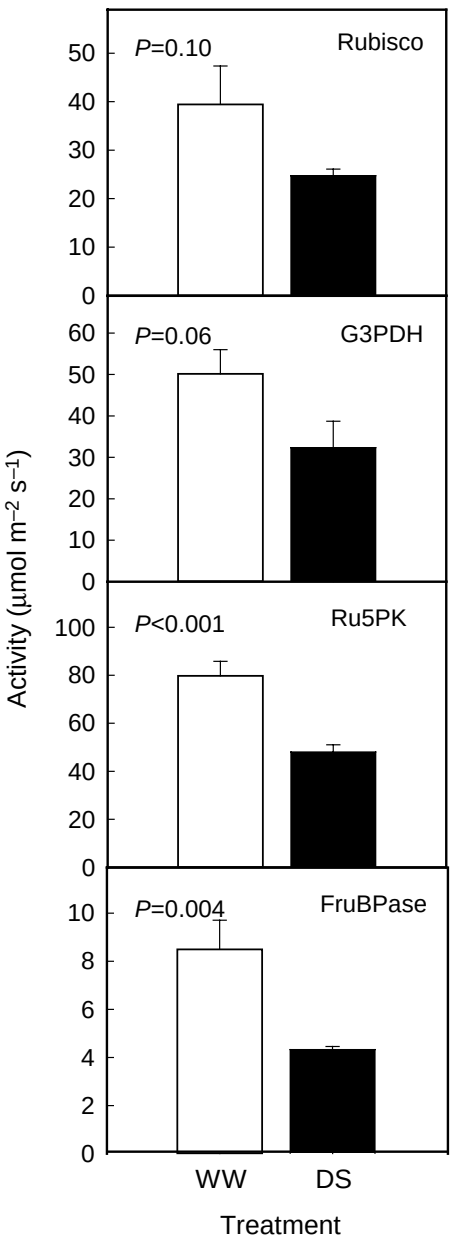

Fig. 5. *In vitro* activities of key enzymes of carbon metabolism (Rubisco, G3PDH, Ru5PK and FruBPase) in well-watered (open bars) and drought-stressed (closed bars) plants. Values are means ± s.e. Statistically significant differences occur for $P<0.05$.

Differences in estimated and measured activities of the enzymes analysed in this study (compare Table 1 and Fig. 5) may be due to the use of model constants and kinetic properties of Rubisco which were determined for spinach (Harley *et al.* 1992). Possible effects of drought over kinetic properties of Rubisco that, again, were estimated for non-stressed spinach plants, may also play a role in the differences observed between modelled and measured activities of the enzymes studied.

The reductions observed in both modelled and measured activities of key enzymes of the carbon assimilation pathway associated with drought stress were of comparable magnitude, suggesting that model estimates are appropriate to evaluate relative (control *vs* stressed) non-stomatal effects under drought. The influence of stomatal patchiness is probably not important under the conditions of the slowly-developed water stress that the plants in our study endured (Wullschleger 1993; Theobald *et al.* 1998).

In conclusion, data gathered in this study suggest that while the limitation of CO_2 assimilation due to stomatal closure in response to slowly-imposed drought is significant; there is also a proportional reduction in Rubisco maximum carboxylation capacity, RuBP regeneration, and triose-P utilization. Reductions in the biochemical capacity for carbon assimilation and utilization under long-term drought is proportional, and may be caused by the shorter-term decrease in CO_2 availability following increased stomatal limitation. However, maximum quantum yield of PSII photochemistry is not greatly affected under these conditions,

while downregulation of PSII activity during the day plays an important role in the tight regulation between photochemical and carbon assimilation reactions under the long-term photosynthetic adaptation to water stress.

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