Plant, Cell and Environment (2003) **27**, 41–50

Elevated CO_2 effects on mesophyll conductance and its consequences for interpreting photosynthetic physiology

E. L. SINGSAAS[1], D. R. ORT[1,2] & E. H. DELUCIA[1]

[1]*Department of Plant Biology, University of Illinois Urbana-Champaign, 265 Morrill Hall, 505 S. Goodwin Avenue, Urbana, IL 61801, USA and* [2]*USDA/ARS Photosynthesis Research Unit, 190 ERML, 1201 W. Gregory Drive, Urbana, IL 61801, USA*

ABSTRACT

Mesophyll conductance (g_m) generally correlates with photosynthetic capacity, although the causal relationship between the two is unclear. The response of g_m to various CO_2 regimes was measured to determine its relationship to environmental changes that affect photosynthesis. The overall effect of CO_2 growth environment on g_m was species and experiment dependent. The data did not statistically differ from the previously shown A–g_m relationship and was unaffected by CO_2 treatment. The consequences of the CO_2 effect on g_m for interpreting photosynthesis in individual cases were investigated. Substantial effects of assumed versus calculated g_m on leaf properties estimated from gas-exchange measurements were found. This differential error resulted in an underestimation in ratio of maximum carboxylation to electron transport, especially in plants with high photosynthetic capacity. Including g_m in the calculations also improved the agreement between maximum carboxylation rates and *in vitro* Rubisco measurements. It is concluded that g_m is finite and varies with photosynthetic capacity. Including g_m when calculating photosynthesis parameters from gas-exchange data will avoid systematic errors.

Key-words: acclimation; diffusion; climate change; Rubisco; J_{max}; $V_{c\,max}$.

Abbreviations: A, net photosynthetic CO_2 assimilation; C_c, [CO_2] at the site of carboxylation inside the chloroplast; C_i, [CO_2] inside the leaf airspaces; FACE, Free Air CO_2 Enrichment; g_m, mesophyll conductance; J_{max}, maximum potential rate of RuBP regeneration; LHC, light harvesting complexes; RuBP, Ribulose-1,5-bisphosphate; $V_{c\,max}$, maximum potential rate of RuBP carboxylation.

INTRODUCTION

A better understanding of the mechanism by which elevated atmospheric CO_2 affects photosynthesis is necessary to predict plant responses to future environments (Long 1998). Growth at elevated CO_2 frequently leads to a reduction in photosynthetic capacity (e.g. Curtis 1996; Drake, Gonzàlez-Meler & Long 1997). This reduction is often quantified by monitoring changes in the maximum rates of RuBP carboxylation ($V_{c\,max}$) and regeneration (J_{max}) through leaf gas-exchange measurements (e.g. Sage 1994) and is considered an acclimation response (Gunderson, Norby & Wullschleger 2000). These changes are part of a cascade of acclimation responses to growth at elevated CO_2 affecting the biochemistry, morphology, and phenology of the plant (Curtis & Wang 1998). Measurements of the effect of elevated CO_2 on mesophyll conductance (g_m) are scarce. Mesophyll conductance (Harley *et al*. 1992; Loreto *et al*. 1992) is a measure of the transfer capacity of CO_2 between the leaf internal airspaces and the site of carboxylation in the chloroplast and is a fundamental property of leaves that may influence photosynthetic capacity (Epron *et al*. 1995; Evans & Loreto 2000). Changes in g_m may contribute to the acclimation of photosynthesis to elevated CO_2. The primary goal of this study was to investigate the effects of growth at different ambient CO_2 levels on g_m and its relationship with photosynthetic capacity.

The secondary goal of this study was to investigate the relationship between *in vitro* biochemical and *in vivo* gas-exchange measurements of photosynthetic CO_2 acclimation, and to determine any possible differential effects of a change in g_m on the estimations of photosynthetic acclimation. This potential for error results from the common use of C_i as a basis for calculations of photosynthetic parameters such as $V_{c\,max}$ and J_{max} even though these parameters are defined based on the CO_2 at the site of RuBP carboxylation (C_c; Farquhar, von Caemmerer & Berry 1980). This interchangeable use of C_i and C_c carries with it the implicit assumption that g_m is effectively infinite; any effect of growth CO_2 on g_m may alter the interpretation of CO_2 acclimation studies. For example, if two leaves differed only in mesophyll conductance, the difference between C_i and C_c will be greater for the leaf with the lower g_m. Thus the initial slope of the photosynthesis–C_i relationship would be lower in this plant even when the photosynthesis–C_c relationship is identical between the two. This would make the calculated apparent $V_{c\,max}$ erroneously lower for the plant with lower g_m. Conversely, if a leaf has a lower $V_{c\,max}$ than its counterpart, a compensating decrease in g_m would erro-

Correspondence: Eric L. Singsaas, (present address) University of Wisconsin – Stevens Point, Department of Biology, Room 167 C.N.R. Building, Stevens Point, WI 54481, USA. Fax: +1 715 346 3624; e-mail: Eric.Singsaas@uwsp.edu

neously increase its apparent $V_{c\,max}$ (measured based on C_i), and mask changes in the underlying biochemistry.

These experiments were designed to determine whether growth CO_2 substantially affects g_m, and whether using C_c rather than C_i influences the calculation of photosynthetic parameters and the interpretation of their response to elevated CO_2. We made gas-exchange measurements on trees growing in two different elevated CO_2 experiments. These included a complete set of gas-exchange and chlorophyll fluorescence measurements to quantify g_m using the *constant J* method (Loreto *et al*. 1994). Further measurements were made on herbaceous plants grown in environmental chambers at elevated CO_2. We analysed the CO_2 effects on g_m and on the relationship between photosynthetic capacity (assessed as both net photosynthesis rate at a standard C_i as well as by Rubisco content) and g_m. We used g_m measurements to re-analyse gas-exchange derived measurements of photosynthesis, $V_{c\,max}$ and J_{max}, to compare the estimates based on C_i and C_c. We also investigated the relationship between acclimation to CO_2 treatments as calculated from gas-exchange measurements compared with those measured by biochemical methods.

MATERIALS AND METHODS

Plant growth conditions

Mesophyll conductance was estimated on sweetgum (*Liquidambar styraciflua* L.) and aspen (*Populus tremuloides* L.) trees growing under field conditions but exposed to elevated and ambient levels of CO_2. In the FACTS-1 experiment near Chapel Hill, NC, sweetgum trees had naturally sprouted in the understorey of a 17-year-old Loblolly pine (*Pinus tadaea* L.) plantation. Three 30-m-diameter plots in this plantation are continuously fumigated with CO_2 to raise the ambient CO_2 levels to 200 μmol mol^{-1} above atmospheric levels (to about 560 μmol mol^{-1}). An additional three rings were fully instrumented to serve as controls. The treatment had been applied at the site for 3 years at the time of measurements. Additional details on the site are provided elsewhere (DeLucia *et al*. 1999; Singsaas, Ort & DeLucia 2000). Field measurements on aspen were made at the FACTS-II field site in Rhinelander, WI, which had an array of treatment and control fumigation rings similar to those at FACTS-I. Aspen trees were planted as 6-month-old rooted cuttings propagated from greenhouse stock. All trees were between 2 and 3 m tall and approximately 3-year-old at the time of measurement. Additional details on the FACTS-II experiment can be found in Dickson *et al*. (2000).

To study the CO_2–g_m relationship under a greater range of CO_2 conditions, we performed additional experiments on potted plants grown indoors. Linden bean (*Phaseolus vulgaris* L. var. Linden), cucumber (*Cucumis sativus* L.), and spinach (*Spinacia oleracea* L.) were grown in controlled-environment chambers (Model PGW36; Conviron, Winnipeg, Manitoba, Canada). Light was provided by high-intensity fluorescent lamps and averaged 530 μmol m^{-2} s^{-1} at 1 m above the floor throughout the experiment. Ambient temperature averaged 26.4 °C throughout the experiment. Temperature and illumination in both chambers were monitored weekly with a thermocouple (Type T; Omega Inc., Stamford, CT, USA) and quantum photometer (Model LI-189; Li-Cor Inc., Lincoln, NE, USA).

Ambient CO_2 and dewpoint were monitored in the growth chambers by an automated measurement system. Air was pumped from the chamber to a valve system that allowed airflow from each chamber to be alternately sent through an infrared gas analyser (Model 6262; Li-Cor Inc.). Ambient CO_2 was elevated in the one chamber by injection of 100% CO_2 through a valve controlled by a feedback loop based on the sampled CO_2. A data logger (Model CR10x; Campbell Scientific, Logan, UT, USA) was used for system control. The elevated CO_2 chamber was maintained at 745 μmol mol^{-1} during the first and second experimental blocks and 737 μmol mol^{-1} during the third. Because both cabinets were located in a small room, the lower CO_2 treatment was 478 μmol mol^{-1} in the first and second blocks and 501 μmol mol^{-1} in the third. For simplicity in data reporting, we have labelled the treatments 750 and 500 μmol mol^{-1}, respectively. To further minimize any chamber effects other than CO_2, plants and CO_2 control systems were switched weekly between the two chambers.

Plants were germinated from seed in 3 L pots, watered daily to maintain adequate soil moisture, and fertilized weekly with approximately 500 mL full strength Hoagland's solution. Plants were grown for 4 to 6 weeks before measurements began and then all measurements were made within 1 week. The youngest fully expanded leaf was measured in all cases.

Photosynthesis measurements

Gas-exchange measurements were made at the FACTS-I site on shaded sweetgum leaves <3 m from the ground and on fully sunlight leaves in the upper canopy accessed from canopy towers and hydraulic lifts. At the FACTS-II site, leaves were selected for measurement from the top 1 m of the canopy. On chamber-grown plants, measurements were made on the youngest fully expanded leaf during the measurement period that began 6 weeks after germination.

Measurements were made using an open gas-exchange system (Model LI-6400; Li-Cor Inc.) where the partial pressure of CO_2 in the cuvette (C_a) was controlled using a CO_2 injection system controlled by the instrument. Chlorophyll fluorescence ($\Delta F/F_m'$) was measured simultaneously using a portable pulse-modulated fluorometer (Model OS-500; OptiScience Corporation, Tyngsboro, MA, USA). Light was provided using a 100 W metal halide lamp attenuated to the desired PPFD with neutral-density filters. The PPFD levels were selected to provide saturating light without causing photo-inhibition, by comparison with A versus PPFD measurements made separately before each experiment cycle (data not shown). Saturating PPFD was 2000 μmol m^{-2} s^{-1} for leaves grown in full sunlight, 800 μmol m^{-2} s^{-1} for leaves grown in shade, and

1500 μmol m^{-2} s^{-1} for leaves grown in controlled-environment chambers. Because of the need for an external lamp when making combined gas-exchange and chlorophyll fluorescence measurements, leaves at each of the FACTS sites were excised with the petiole submerged in distilled water and brought to the measurement apparatus inside a portable laboratory trailer. These measurements were used to calculate g_m.

Two input parameters were required to calculate g_m; the rate of mitochondrial respiration (R_d) and the CO_2 compensation point in the absence of mitochondrial respiration (Γ^*). These were calculated from the common intersection points of three A versus C_i response curves (Laisk 1977; Brooks & Farquhar 1985; Villar, Held & Merino 1994, 1995). Briefly, the CO_2 response of photosynthesis was measured at five points below a C_i of 200 μmol mol^{-1}. Three such curves were measured at different PPFD levels (150, 100, and 75 μmol m^{-2} s^{-1}). The parameters are determined from the co-ordinates of the intersection point of the three lines on a graph of A versus C_i. Measurements for these parameters were made *in situ* using attached leaves at both of the FACTS sites and in the environmental chambers. Average Γ^* values (μmol mol^{-1}) determined for each species and used in subsequent calculations were: 42.49 ± 2.9, 46.83 ± 3.0, 42.52 ± 2.1, 41.38 ± 1.2, 43.02 ± 1.7, and 36.8 ± 0.8 for sweetgum sun leaves, sweetgum shade, aspen, bean, cucumber, and spinach, respectively. From the same calculations, daytime respiration (R_d) values (μmol m^{-2} s^{-1}) were –0.92 ± 0.2, –0.32 ± 0.1, –3.73 ± 0.7, –1.74 ± 0.2, –1.16 ± 0.2 and –2.07 ± 0.1 for each species, respectively.

Leaf gas-exchange parameters were calculated using the equations of von Caemmerer & Farquhar (1981). The maximum rate of RuBP carboxylation ($V_{c\,max}$) and RuBP regeneration (J_{max}) were calculated from CO_2 response curves with 11 points measured at ambient CO_2 levels between 1000 and 20 μmol mol^{-1}. These data were fit to the Farquhar *et al*. (1980) model by non-linear least squares regression as described in Harley & Tenhunen (1991). We used *in vitro* model constants from Harley & Baldocchi (1995). We made a further comparison of approaches to determine the correct value of $V_{c\,max}$ by re-fitting the A–C_i data using *in vivo* Rubisco parameters (Bernacchi *et al*. 2001). To compare photosynthesis rates among blocks on an equal basis, we standardized the photosynthesis measurements by choosing light-saturated photosynthesis measured at a C_i of approximately 400 μmol mol^{-1}.

Mesophyll conductance was calculated using the *constant J* method (Loreto *et al*. 1992). Data were selected from CO_2-response measurements in the region where $\Delta F/F_m'$ was constant with increasing CO_2. The gas-exchange data (A, C_i) and constants (Γ^* and R_d; determined separately for each experimental block and species) were used to calculate the rate of electron transport needed to support CO_2 assimilation and photorespiration, J_p, for each point (Loreto *et al*. 1992). Electron transport through PSII was monitored independently from J_p using chlorophyll fluorescence measurements $\Delta F/F_m'$ and is referred to as J_f. The variance in J_p across all the points of known constant J_f was calculated as described in Harley *et al*. (1992), using the Γ^* and R_d values calculated previously for that experimental block. Conductance values were determined by least-squares regressions, minimizing the variance across the selected data points by substituting values of g_m into the J_p calculations. We report g_m in units of mol m^{-2} s^{-1} bar^{-1} to remain consistent with units in most publications (Harley *et al*. 1992; Loreto *et al*. 1992, Evans & Loreto 2000).

Rubisco and chlorophyll

We measured Rubisco activity using a NADH-linked enzyme assay modified from Sharkey, Savitch & Butz (1991). Leaf punches (1.7 cm^2) were excised with a cork borer and immediately ground in extraction buffer in a ground-glass tissue homogenizer at 0 °C. The extraction buffer contained 100 mM bicine–NaOH (pH 7.8), 100 mM $Na_2B_4O_7$, 20 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 4 mM amino-*N*-caprioic acid, 0.8 mM benzamidine, 0.1% (w/w) Triton-X-100, 0.02% (w/v) bovine serum albumin, 150 mM $NaHCO_3$, 5 mM dithiothreitol (DTT), and 30 mg poly(vinylpolypyrrolidone) (insoluble). The crude extract was transferred to a 1.5 mL microcentrifuge tube and spun for 30 s. Initial activity was measured using 10 μL of supernatant assayed immediately in 1 mL of assay buffer (50 mM Bicine–NaOH (pH 8.0), 15 mM $MgCl_2$, 1 mM EDTA, 19 mM NaCl, 9.3 mM $NaHCO_3$, 9.3 mM DTT, 0.2 mM RuBP, 0.1 mM NADH, 4.7 mM photsphocreatine, 4.7 mM ATP, 1.4 U mL^{-1} creatine-P-kinase, 1.4 U mL^{-1} glyceraldehyde-3-P-dehydrogenase, and 2.9 U mL^{-1} phosphoglycerokinase); the reaction was monitored at ΔA_{340} for at least 3 min. A 1 mL aliquot of crude extract was incubated for 10 min with 80 mM $MgCl_2$ and 150 mM HCO_3^- to fully activate Rubisco, and then assayed as described for the crude extract. Five aliquots of activated Rubisco extract were then incubated with CABP (concentrations of 0, 0.58, 1.1 and 1.8 μM) for 10 min to inhibit Rubisco activity, and assayed as above. Rubisco activity was calculated based on the slope of ΔA_{340} versus time. Rubisco content was calculated from the y-intercept of a plot of activity versus $[CABP]^{-1}$.

Chlorophyll was measured spectrophotometrically. Leaf punches (1.7 cm^2) were taken immediately after gas exchange measurements and ground in 96% EtOH using a chilled mortar and pestle. After centrifuging, the optical density of the supernatant was measured at 665, 649 and 654 nm. Chlorophyll concentration was calculated using the specific absorption coefficients in Wintermans & DeMots (1965).

Experimental design and statistical analyses

The FACTS-I experiment consisted of six rings enclosing plants in paired control (ambient CO_2) and treatment (ambient + 200 μmol mol^{-1} CO_2) conditions. The controls were fully instrumented. The FACTS-II experiment consisted of 12 rings in a crossed $CO_2 \times O_3$ experiment. Measurements were only made on plants in the six rings not

receiving ozone treatment, which consisted of three ambient CO_2 and three ambient + 200 μmol mol^{-1} CO_2 rings. Both experiments were designed with three blocks of paired (ambient + elevated) rings, and three replicate measurements were averaged within blocks. Blocked means were calculated across each species and treatment. The CO_2 effects on g_m were analysed for the FACE results using mixed ANOVA (JMP; SAS, Inc., Cary, NC, USA) with g_m as the main effect, treatment as a fixed factor and species and block as random factors. *Post-hoc* comparisons of treatment effects were performed within each species using the Tukey adjustment.

Growth chamber experiments were conducted in a pair of chambers, and blocks were replicated through time. Each block consisted of three to five individual plants of each species and ran for 6 to 8 weeks, after which the chambers were emptied, and new seedlings were started for the next experimental block. Results were analysed using ANOVA with treatment as a fixed factor and species as random. Unequal numbers made the block effects untestable. Tukey-adjusted *post-hoc* comparisons were made within species to investigate treatment effects.

We made several comparisons of relationships common to all experiments. Because Evans & Loreto (2000) found a consistent relationship between net photosynthesis rate and g_m we made the same comparison across our different experiments. We also compared the $V_{c\,max} : J_{max}$ ratio across our experiments. The slopes and intercepts of regression lines were compared using analysis of covariance (ANCOVA) as described by Underwood (1997).

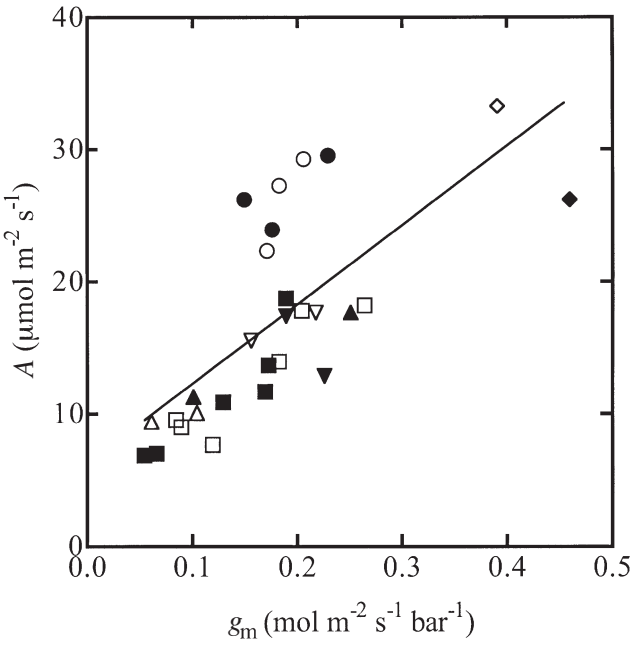

Figure 1. The relationship between net CO_2 assimilation (A) and mesophyll conductance (g_m). Mesophyll conductance was estimated using the *constant J* method. Photosynthesis was measured at C_i between 390 and 450 in all cases. Symbol shapes represent species; sweetgum (■,□) aspen (●,○) cucumber (▲,△) bean (▼,▽) and spinach (◆,◇). Open symbols represent plants grown at elevated CO_2, and closed symbols represent plants grown at ambient CO_2. The solid line represents a linear regression of the combined (all species and treatments) data ($y = A + Bx$; $A = 6.4$, $B = 60$, $r^2 = 0.51$).

RESULTS

Photosynthesis increased with increasing g_m consistently across all species and observations (Fig. 1). We used a standardized measurement (the light saturated rate of net CO_2 assimilation at $C_i \approx 400$ μmol mol^{-1}) as a consistent metric of the leaf capacity for photosynthesis. The linear regressions between g_m and photosynthetic capacity on the elevated and ambient CO_2 data differed neither in slopes (ANCOVA; $F = 0.76$, $P = 0.47$) nor intercepts (ANCOVA; $F = 0.13$, $P = 0.88$), so we show only a single regression for the combined data set. There were similar, apparently linear, relationships between both leaf Rubisco and chlorophyll content and g_m in the growth chamber experiment (Fig. 2). The difference in CO_2 from the intercellular airspaces to the site of carboxylation did not vary in a systematic fashion with net CO_2 assimilation rate (Fig. 3). This relationship also showed no discernable dependence on growth CO_2 or plant species.

The overall effect of the CO_2 treatment on photosynthesis generally co-varied with the change in g_m except in spinach and linden bean (Table 1). The largest CO_2 effects were seen in cucumber and sweetgum (sun leaves) followed by spinach. An analysis of the growth chamber experiment data (top three rows of Table 1) showed significant CO_2 [$F = 5.59$, $P = 0.0248$, degrees of freedom (d.f.) = 1] and species ($F = 39.63$, $P < 0.001$, d.f. = 3) effects. In the FACE experiments, g_m was significantly affected by species ($F = 31.22$, $P < 0.001$, d.f. = 3) and block ($F = 3.87$, $P = 0.03$, d.f. = 3). Mean CO_2 effects were significant only at the 10% level ($F = 1.28$, $P = 0.09$, d.f. = 1). Pair wise comparisons of CO_2 effects within each species were significant at the 5% level in cucumber and at the 10% level in sunlit sweetgum leaves.

To examine the potential error in estimating photosynthetic parameters we calculated $V_{c\,max}$ and J_{max} based on C_i and (using our values of g_m) based on C_c. Calculated values of $V_{c\,max}$ were affected more than J_{max} by the g_m correction, increasing between 20 and 70% when calculated based on C_c rather than C_i (Table 2). The g_m effects often were numerically uneven, affecting the ambient and elevated CO_2 treatments differently, thus revealing that the CO_2 effect on $V_{c\,max}$ could be over- or under-estimated depending on the direction of change in g_m. In sweetgum, aspen, and cucumber this meant that the change in $V_{c\,max}$ was always less than predicted based on C_i. In the case of sweetgum shade leaves, a 5% increase at elevated CO_2 became a 9% decrease when recalculated based on C_c. Spinach and bean behaved differently, increasing the CO_2 effect slightly. Sensitivity of J_{max} to g_m was generally smaller than the sensitivity of $V_{c\,max}$ (Table 3); values of J_{max} varied by approximately 10% with the inclusion of g_m in the calculations, with

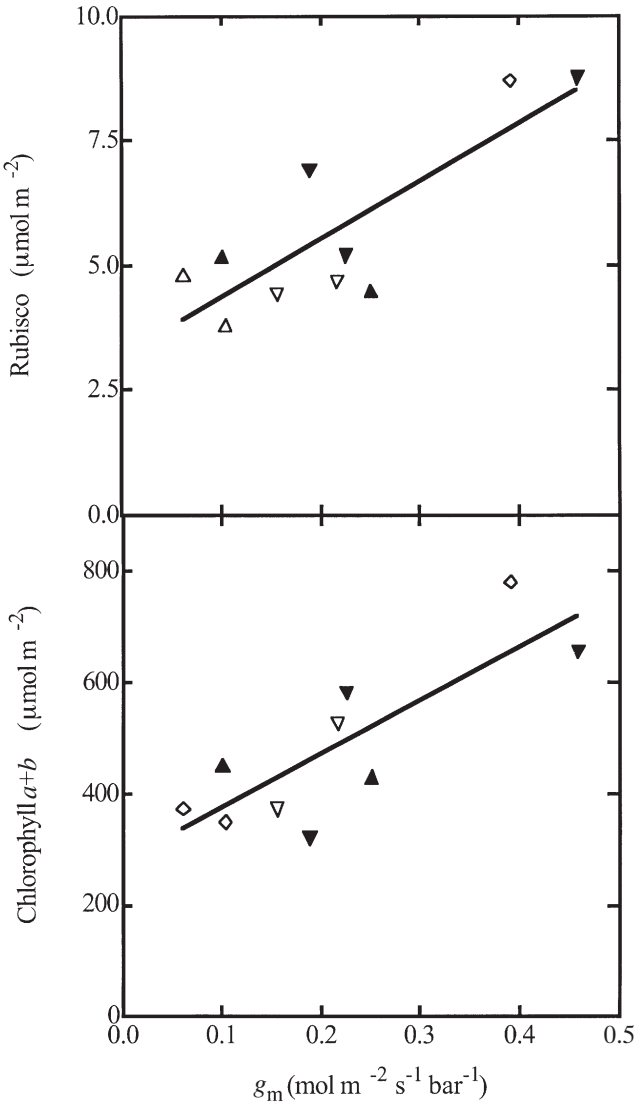

Figure 2. The relationship between mesophyll conductance and leaf Rubisco content (top panel) and chlorophyll (bottom panel). Mesophyll conductance was estimated using the *constant J* method, Rubisco was measured using CABP binding, and chlorophyll was measured spectrophotometrically after extraction in 96% ethanol. Symbols represent species and treatments as in Fig. 1. Linear regressions were calculated with data from all species and treatments ($y = A + Bx$, top panel: $A = 3.2$, $B = 11$, $r^2 = 0.67$; bottom panel $A = 963$, $B = 279$, $r^2 = 0.67$

the exception of shade-grown sweetgum. Using C_i caused a slight underestimate in J_{max} for aspen

Because the choice of using C_i versus C_c in photosynthesis calculations affected $V_{c\,max}$ more than J_{max}, the relationship between the two parameters was sensitive to g_m as seen by the change in the slope of the $J_{max} : V_{c\,max}$ relationship (Fig. 4). The regression lines for the values calculated from C_i and C_c had significantly different slopes (ANCOVA; $F = 27.9$, $P < 0.001$, d.f. = 52). There was no systematic effect of CO_2 treatment on the $J_{max} : V_{c\,max}$ ratio using either calculation, so data from both treatments were grouped for the regressions. We took an alternative approach to 'correcting' C_i-based calculations of $V_{c\,max}$ and J_{max} by calculating $V_{c\,max}$ and J_{max} using Rubisco constants determined from *in vivo* measurements (Bernacchi *et al.* 2001). This approach gave similar results to the C_c-based calculations at low photosynthesis capacity, but deviated from those data at higher values (Fig. 4).

In the growth chamber experiments, we used measurements of Rubisco activity and chlorophyll content as secondary indicators of photosynthetic capacity. Using C_c in calculations of $V_{c\,max}$ somewhat changed the relationship between *in vivo* and *in vitro* carboxylation capacity (Fig. 5; a 1 : 1 line is shown for comparison). RuBP regeneration capacity increased with total leaf chlorophyll content in a seemingly linear fashion (Fig. 6). As the J_{max} calculations were not strongly affected by the g_m, the differences between the two data sets are relatively small.

DISCUSSION

There was a consistent relationship between g_m and photosynthetic capacity across species, growth location, and CO_2. This apparently linear relationship (Fig. 1) is similar to that reported by Evans & Loreto (2000), where they summarized the results of several earlier g_m studies. We calculated a regression line to determine the slope of the A–g_m relationship from those data (not shown) and tested it against the regression of our data. The slopes were not significantly different at the 5% level (ANCOVA: $F = 3.5$, $P = 0.06$, d.f. = 80) although they were different at the 10% level. The aspen data were the main outliers in our data that affected the slope, and without these data the two data-sets were

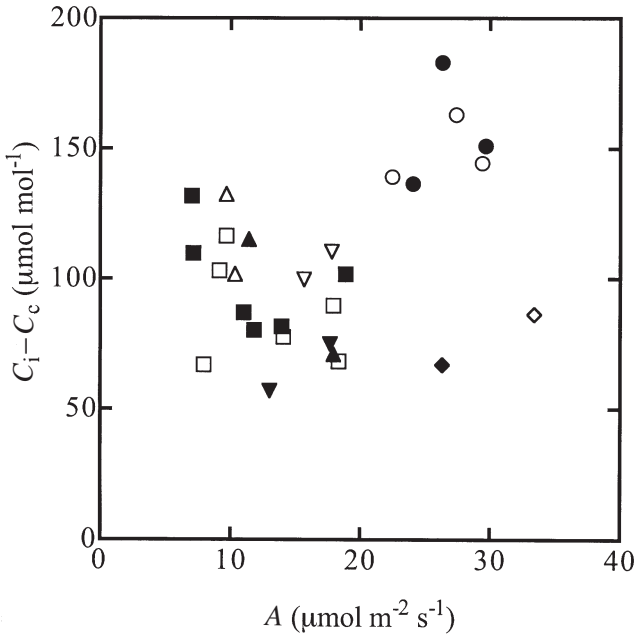

Figure 3. The difference in pCO_2 from the leaf internal airspaces to the sites of carboxylation, C_i–C_c versus net CO_2 assimilation rate, A. Measurements and symbols are as in Fig. 1.

Table 1. The effect of growth CO_2 on mesophyll conductance and photosynthesis

Species	CO_2 treatment (μmol mol^{-1})	A (μmol m^{-2} s^{-1})	C_i (μmol mol^{-1})	g_m (mol m^{-2} s^{-1} bar^{-1})	P	n
Cucumber	500	14.6 (3.2)	400 (11)	0.18 (0.08)	0.06	6
	750	9.9 (0.3)	407 (8)	0.08 (0.02)		6
Spinach	500	26.3	403	0.46	0.19	5
	750	33.3	413	0.39		5
Linden bean	500	15.2 (2.3)	421 (11)	0.21 (0.02)	0.47	6
	750	16.7 (1.1)	411 (5)	0.19 (0.03)		8
Aspen	360	26.6 (1.6)	415 (5)	0.18 (0.02)	0.95	9
	560	26.4 (2.0)	412 (3)	0.18 (0.01)		9
Sweetgum[a]	360	14.8 (2.1)	417 (5)	0.17 (0.01)	0.03	9
	560	16.7 (1.4)	415 (6)	0.32 (0.12)		9
Sweetgum[b]	360	8.3 (1.3)	414 (4)	0.08 (0.02)	0.49	8
	560	8.8 (0.5)	415 (5)	0.10 (0.01)		9

To compare measurements on an equal basis, all photosynthesis measurements were compared at a common C_i. Data are means of all observations (SE). Probability (P) values represent the Tukey-adjusted pairwise comparisons between the CO_2 treatments for each species, and n designates the sample size. [a]Sun leaves; [b]shade leaves.

indistinguishable. Excluding these data had a minimal effect on the slope while reducing the y-intercept to 4.3 and increasing the r^2 to 0.84.

The non-zero intercept of our regression differs from Evans & Loreto (2000), which probably results different methods of determining photosynthesis for the y-axis. We defined this as the light-saturated photosynthesis rate at $C_i \approx 400$ μmol mol^{-1} to make consistent comparisons among all treatments. Although the conditions are not specified in Evans & Loreto (2000) most authors use light-saturated photosynthesis rates measured at ambient CO_2 for photosynthetic capacity. Thus all our measurements were made at CO_2 levels between 600 and 1000 μmol CO_2 mol^{-1}, resulting in higher net A. To demonstrate this we re-fit our data using photosynthesis measured at the growth CO_2 in each case, and calculated an intercept of 2.83 μmol m^{-2} s^{-1}(data not shown). This approach also improved the agreement between aspen and all other species. This strongly suggests that the intercept of this relationship depends solely on the conditions under which photosynthetic capacity is defined, and has no physiological interpretation.

By similar comparison with the data in Evans & Loreto (2000), the calculated difference between C_i and C_c determined from g_m and A values averaged 106 ± 6 μmol mol^{-1}, but by excluding the aspen data were indistinguishable from the 78 μmol mol^{-1} value of the data summarized by Evans & Loreto (2000). The relationship between A and g_m, and the consistency of the C_i–C_c draw-down, appear to be general features of leaves that are empirically predictable based on photosynthetic capacity measurements. This is especially notable considering the values from different studies used different methods to measure g_m, seemingly confirming the consistency of g_m measurement regardless of measurement technique (Loreto *et al.* 1992). Thus, this

Table 2. The effect of growth CO_2 on $V_{c\,max}$ from gas-exchange measurements

	$V_{c\,max}$ (μmol m^{-2} s^{-1})			
	Analysis	Ambient CO_2	Elevated CO_2	CO_2 effect (%)
Cucumber	C_i	36.3	26.0	–28
	C_c	48.7	40.1	–18
Spinach	C_i	64.8	85.0	31
	C_c	83.0	112	35
Linden bean	C_i	44.0	39.9	–9
	C_c	57.8	54.2	–6
Aspen	C_i	79.9	78.1	–2
	C_c	102	92.9	–9
Sweetgum[a]	C_i	38.8	46.1	19
	C_c	55.3	62.1	12
Sweetgum[b]	C_i	24.8	26.1	5
	C_c	43.0	39.0	–9

Calculations based on C_i were done with standard gas-exchange equations. C_c was calculated from mesophyll conductance and C_i. All parameters were calculated separately for each leaf. [a]Sun leaves; [b]shade leaves.

Table 3. The effect of growth CO_2 on J_{max} from gas-exchange measurements

	J_{max} (μmol m^{-2} s^{-1})			
	Analysis	Ambient CO_2	Elevated CO_2	CO_2 effect (%)
Cucumber	C_i	91.8	65.1	–29
	C_c	98.4	76.5	–22
Spinach	C_i	163	192	18
	C_c	173	235	36
Aspen	C_i	230	216	–6
	C_c	217	200	–8
Linden bean	C_i	109	88.8	–19
	C_c	120	99.2	–13
Sweetgum[a]	C_i	94.0	106	13
	C_c	109	119	9
Sweetgum[b]	C_i	57.5	64.2	12
	C_c	75.5	86.2	14

Calculations based on C_i were done with standard gas-exchange equations. C_c was calculated from mesophyll conductance and C_i. All parameters were calculated separately for each leaf. [a]Sun leaves; [b]shade leaves.

relationship might in principle be used to improve the estimated CO_2 effect on $V_{c\,max}$ and J_{max} even when g_m was not explicitly measured.

Growth at different CO_2 concentrations caused a small effect on g_m in almost all species studied which was statistically significant at either the 5 or 10% levels (Table 1).

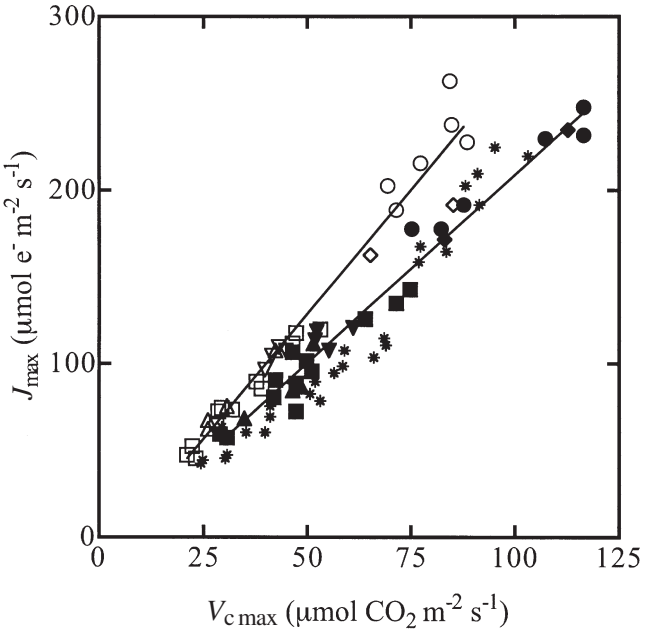

Figure 4. The relationship between maximal carboxylation and electron transport rates as calculated from gas-exchange measurements (A–C_i curves). $V_{c\,max}$ and J_{max} were fitted using non-linear regression using *in vitro* Rubisco constants. Open symbols represent values calculated based on C_i, and closed symbols represent values calculated based on C_c. Symbol shapes represent species as in Fig. 1. The same raw data, re-fitted using *in vivo* Rubisco parameters, are included for comparison (*). The lines were fitted to each data set by linear regression ($y = A + Bx$; C_i: $A = -14.2$, $B = 2.87$, $r^2 = 0.96$; C_c: $A = 5.06$, $B = 2.17$, $r^2 = 0.97$).

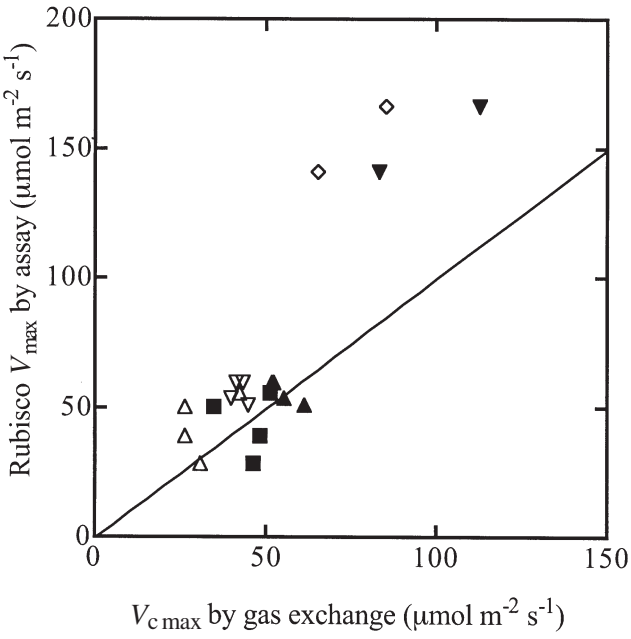

Figure 5. Maximal Rubisco activity (V_{max}) determined by *in vitro* assay versus measured by gas-exchange based on C_i and C_c. Open symbols represent values calculated based on C_i, and closed symbols represent values calculated based on C_c. Symbol shapes represent species as in Fig. 1. The solid line marks a 1 : 1 relationship.

Since the magnitude and direction of photosynthetic acclimation to CO_2 varied considerably across treatments, and g_m was consistently related to photosynthesis, the use of block means is misleading in cases where the direction of acclimation differed between blocks. This was the case in sweetgum (one block showed a decrease in photosynthesis rates whereas the others showed an increase), linden bean (one decrease and one increase) and aspen (two decrease and one increase). Thus the general effect of CO_2 enrichment on g_m is to cause it to move up and down the A versus

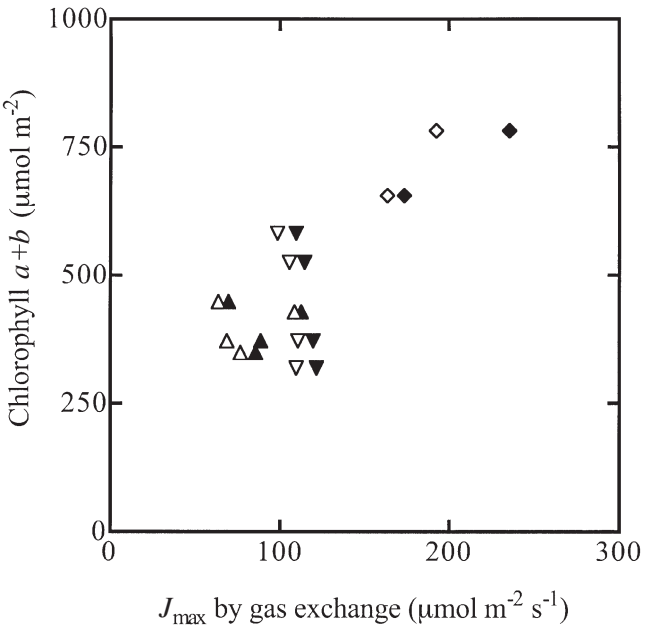

Figure 6. The relationship between leaf chlorophyll content and maximum electron transport activity (J_{max}) estimated based on C_c and C_i. Open symbols represent values calculated based on C_i, and closed symbols represent values calculated based on C_c. Symbol shapes represent species as in Fig. 1.

g_m regression line without substantially changing the relationship between the two.

There remains uncertainty about the mechanism relating g_m to any particular anatomical, biochemical or physical feature of a leaf. Substantial work has gone into relating leaf thickness and cell wall structure to g_m in various species, but the changes in g_m over the growing season and during leaf senescence (Loreto *et al*. 1994; Evans & Vellen 1996), when cell wall structure is relatively fixed, suggest wall structure may only be minimally involved. Gas diffusion within the leaf may contribute to g_m in some instances (Parkhurst & Mott 1990; von Caemmerer & Evans 1991; Syvertsen *et al*. 1995) although this effect has only been found in thick, hypostomatus leaves. A more consistent predictor of g_m is the surface area of chloroplasts appressed to the cell surface exposed to the leaf airspaces (von Caemmerer & Evans 1991; Evans *et al*. 1994). The relationship between chlorophyll content, Rubisco content and g_m (Fig. 2) may be indicative of this relationship, although it is impossible to determine a mechanistic relationship using these data.

Although no single anatomical measurement accurately predicts g_m (Evans *et al*. 1994; Syvertsen *et al*. 1995), empirical models relating several anatomical measurements to g_m have shown promise (Syvertsen *et al*. 1995). Using stable isotope discrimination analysis to partition leaf conductance into its components, Gillon & Yakir (2000) found cell wall conductance was most limiting to overall leaf conductance in oak, whereas resistances in the chloroplasts were more limiting in tobacco and soybeans. Liquid-phase dynamics are likely to be more limiting to g_m, and thus enzymatic processes such as leaf carbonic anhydrase activity and aquaporins (Coleman 2000; Gillon & Yakir 2000, Ono & Terashima 2002) may also play important roles in determining g_m. These conclusions are consistent with the observed temperature response of g_m *in vivo* (Bernacchi *et al*. 2002). Given the substantially different conclusions of the various studies, it seems that growth CO_2 may well affect the various determinants of g_m differently across species. This added complexity might mean that a mechanistic prediction of mesophyll conductance is not possible in the general case, and must be considered on a species-by-species basis.

Given the complex interacting factors contributing to g_m, the consistency of the A–g_m relationship is puzzling. It is likely that this consistency results from covariance of several attributes. For example, the relationship between Rubisco and g_m (Fig. 2) may result from a correlation between Rubisco and carbonic anhydrase (CA; Coleman 2000) rather than through any direct mechanism, given the relationship between CA activity and g_m. Since the relationship between Rubisco and g_m is broken in antisense-Rubisco-transformed plants (Evans *et al*. 1994), we conclude that it is coincidental and not causal.

The most common anatomical changes resulting from growth at elevated CO_2 are associated with stomatal and epidermal cell density (Ferris *et al*. 1996; Masle 2000), neither of which is likely to substantially affect g_m or photosynthetic capacity, at least when measured independently of stomatal conductance. Elevated CO_2 can cause changes in leaf thickness (Kürschner *et al*. 1998), increasing airspace diffusion limitations that in hypostomatous leaves may reduce g_m. Significant CO_2 effects have also been noted on total mesophyll cell cross-sectional area (Ferris *et al*. 1996; Masle 2000), which may correspond to an increase in mesophyll cell surface area and cause an increase in g_m (Evans *et al*. 1994). Studies of growth CO_2 effects on carbonic anhydrase activity have shown either substantial changes in Rubisco and carbonic anhydrase (Majeau & Coleman 1996), or changes in Rubisco with no changes in carbonic anhydrase (Sicher, Kremer & Rodermel 1994). The uncertainty of which leaf properties substantially affect g_m makes the use of these as a proxy for determining CO_2 effects on g_m difficult.

Although mesophyll conductance defies a complete physical or mathematical description, its consideration when calculating photosynthetic parameters is necessary especially when a treatment is anticipated to affect g_m. Including g_m in photosynthesis calculations changed our interpretation of the effect of growth CO_2 on photosynthesis. In one case, an apparent increase in $V_{c\,max}$ with CO_2 treatment was revealed to actually be a decrease when analysed on a C_c basis (Table 1). In most cases the apparent CO_2 effect was smaller when g_m was considered. The effects of growth CO_2 on J_{max} were quite small in all cases (Table 2). As $V_{c\,max}$ was affected more by the recalculation than J_{max}, the relationship between the two parameters changed and the slope of $J_{max} : V_{c\,max}$ was reduced (Fig. 4).

In both analyses, however, there was no systematic CO_2 effect across species on the ratio of the parameters. A change in the relationship between these parameters was predicted by the mechanistic analysis of CO_2 acclimation and photosynthesis (Medlyn 1996). In the model, lower g_m at elevated CO_2 increased the difference between C_i and C_c, thus reducing the need to reallocate of N from carboxylation (reflected in $V_{c\,max}$) to RuBP regeneration (reflected in J_{max}). This could happen if CO_2 affected g_m independently of any acclimation in photosynthesis, but our data indicate that this is not the case. The C_i–C_c relationship remains unchanged because g_m and photosynthesis change proportionally (Figs 1 & 3). These observations support the work showing that the relationship between carboxylation and RuBP regeneration rates is not affected by growth CO_2 made in other gas-exchange and biochemical analyses (Maxwell, Griffiths & Young 1994; Hymus *et al*. 1999).

Mechanistic photosynthesis models are based on $[CO_2]$ in the chloroplast, yet the majority of studies report values calculated based on C_i. The implicit assumption in such cases is that g_m is infinite. When g_m is not considered, treatment effects such as growth at elevated CO_2 that affect diffusion within the leaf may be falsely attributed to changes in leaf biochemistry (Parkhurst & Mott 1990). This principle is illustrated by Delfine *et al*. (1998) who show apparent treatment differences in the A–C_i response of photosynthesis that are not seen in the A–C_c relationship. The inclusion of g_m in our calculations improved the agreement between gas exchange and biochemical measurements of carboxylation capacity (Fig. 5). This effect was relatively small for the relationship between chlorophyll and RuBP regeneration capacity (Fig. 6). This is more difficult to evaluate, however, because there is no reason to expect a linear relationship between the two values.

Bernacchi *et al*. (2001) avoid the problems associated with the assumption of infinite g_m by measuring Rubisco kinetic parameters from *in vivo* measurements. This approach simplifies gas-exchange analysis because g_m is included in the Rubisco parameters since they were determined from C_i-based measurements. We re-analysed our gas-exchange data from both experiments using these parameters in place of the Baldocchi & Harley (1995) constants, and found the $V_{c\,max}:J_{max}$ relationship matched the ratios we calculated based on C_c at low $V_{c\,max}:J_{max}$, but the two approaches systematically deviate from one another at higher $V_{c\,max}$ (Fig. 4). The deviation between these approaches occurs because the *in vivo* calculations assume g_m is constant (although not infinite), whereas our approach of determining C_c for each set of measurements accounts for the increasing g_m as photosynthetic capacity increases (Fig. 1).

We conclude that there was a potentially important effect of growth CO_2 on g_m that corresponded with photosynthetic acclimation to CO_2 through the consistent linear relationship between photosynthetic capacity and g_m. Many gas-exchange studies calculate $V_{c\,max}$ and J_{max} based on A versus C_i measurements rather than A versus C_c, implicitly assuming that g_m is infinite. We found g_m is neither infinite nor constant in field or growth-chamber experiments. Including g_m in the analysis of photosynthetic responses to the CO_2 environment significantly changed the relationship between parameters estimated by gas-exchange measurements and improved the agreement of Rubisco activity measured with gas-exchange with biochemical assays.

ACKNOWLEDGMENTS

This work was funded by a NSF traineeship in the Integrative Photosynthesis Research Training Program (DBI 96-02240), and by a grant to E.H.D. from the US Department of Energy, Office of Biological and Environmental Research (FG02-95 ER62127). CABP was generously provided by Dr Archie Portis. The authors thank Dr Peter Curtis for helpful comments on the manuscript.

REFERENCES

Baldocchi D.D. & Harley P.C. (1995) Scaling carbon dioxide and water vapour exchange from leaf to canopy in a deciduous forest. 2. Model testing and application. *Plant, Cell and Environment* **18,** 1157–1173.

Bernacchi C.J., Portis A.R., Nakano H., von Caemmerer S. & Long S.P. (2002) Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis *in vivo*. *Plant Physiology* **130,** 1992–1998.

Bernacchi C.J., Singsaas E.L., Pimentel C., Portis A.R. & Long S.P. (2001) Improved temperature response functions for models of Rubisco-limited photosynthesis. *Plant, Cell and Environment* **24,** 253–259.

Brooks A. & Farquhar G. (1985) Effect of temperature on the CO_2/O_2 specificity of ribulose-1,5-biphasphates carboxylase/oxygenase and the rate of respiration in the light. *Planta* **165,** 397–406.

von Caemmerer S. & Evans J.R. (1991) Determination of the CO_2 pressure in chloroplasts from leaves of several C_3 plants. *Australian Journal of Plant Physiology* **18,** 287–305.

von Caemmerer S. & Farquhar G.D. (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* **153,** 376–387.

Coleman J.R. (2000) Carbonic anhydrase and its role in photosynthesis. In *Photosynthesis: Physiology and Metabolism* (eds R.C. Leegood, T.D. Sharkey & S. von Caemmerer), pp. 353–367. Kluwer Academic Publishers, Amsterdam, The Netherlands.

Curtis P.S. (1996) A meta-analysis of leaf gas exchange and nitrogen in trees grown under elevated carbon dioxide. *Plant, Cell and Environment* **19,** 127–137.

Curtis P.S. & Wang X. (1998) A meta-analysis of elevated CO_2 effects on woody plant mass, form, and physiology. *Oecologia* **113,** 299–313.

Delfine S., Alvino A., Zacchini M. & Loreto F. (1998) Consequences of salt stress on conductance to CO_2 diffusion, rubisco characteristics and anatomy of spinach leaves. *Australian Journal of Plant Physiology* **25,** 395–402.

DeLucia E.H., Hamilton J.G., Naidu S.L., *et al.* (1999) Net primary production of a forest ecosystem with experimental CO_2 enrichment. *Science* **284,** 1177–1179.

Dickson R., Lewin K., Isebrands J., *et al.* (2000) *Forest Atmosphere Carbon Transfer and Storage (FACTS-II) The Aspen Free-air CO_2 and O_3 Enrichment (FACE) Project. An Overview*, General

Technical Report No. NC-214. USDA Forest Service, North Central Research Station, St Paul, MN, USA.

Drake B.G., Gonzàlez-Meler M.A. & Long S.P. (1997) More efficient plants: a consequence of rising atmospheric CO_2? *Annual Review of Plant Physiology and Plant Molecular Biology* **48,** 609–639.

Epron D., Godard D., Cornic G. & Genty B. (1995) Limitation of net CO_2 assimilation rate by internal resistances to CO_2 transfer in the leaves of two tree species (*Fagus sylvatica* L. & *Castanea sativa* Mill.). *Plant, Cell and Environment* **18,** 43–51.

Evans J.R. & Loreto F. (2000) Aquisition and diffusion of CO_2 in higher plant leaves. In *Photosynthesis: Physiology and Metabolism* (eds R.C. Leegood, T.D. Sharkey & S. von Caemmerer), pp. 321–351. Kluwer Academic Publishers, Amsterdam, The Netherlands.

Evans J.R. & Vellen L. (1996) Wheat cultivars differ in transpiration efficiency and CO_2 diffusion inside their leaves. In *Crop Research in Asia: Achievements and Perspective* (eds R. Ishii & T. Horie), pp. 326–329. Asian Crop Science Association, Tokyo, Japan.

Evans J.R., von Caemmerer S., Setchell B.A. & Hudson G.S. (1994) The relationship between CO_2 transfer conductance and leaf anatomy in transgenic tobacco with a reduced content of Rubisco. *Australian Journal of Plant Physiology* **21,** 475–495.

Farquhar G.D., von Caemmerer S. & Berry J.A. (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta* **149,** 78–90.

Ferris R., Nijs I., Behaeghe T. & Impens I. (1996) Elevated CO_2 and temperature have different effects on leaf anatomy of perrennial ryegrass in spring and summer. *Annals of Botany* **78,** 489–497.

Gillon J.S. & Yakir D. (2000) Internal conductance to CO_2 diffusion and $C^{18}OO$ discrimination in C_3 leaves. *Plant Physiology* **123,** 201–213.

Gunderson C., Norby R. & Wullschlegger S. (2000) Acelimation of photosynthesis and respiration to simulated climactic warming in northern and southern populations of *Acer saccharum*: Laboratory and field evidence. *Tree Physiology* **20,** 87–95.

Harley P.C. & Baldocchi D.D. (1995) Scaling carbon dioxide and water vapour exchange from leaf to canopy in a deciduous forest. 1. Leaf model parametrization. *Plant, Cell and Environment* **18,** 1146–1156.

Harley P.C. & Tenhunen J.D. (1991) Modeling the photosynthetic response of C_3 leaves to environmental factors. In *Modeling Crop Photosynthesis – from Biochemistry to Canopy* (eds K.J. Boote & R.S. Loomis), pp. 17–39. Crop Science Society of America, Madison, WI, USA.

Harley P.C., Loreto F., Di Marco G. & Sharkey T.D. (1992) Theoretical considerations when estimating the mesophyll conductance to CO_2 flux by analysis of the response of photosynthesis to CO_2. *Plant Physiology* **98,** 1429–1436.

Hymus G.J., Ellsworth D.S., Baker N.R. & Long S.P. (1999) Does free-air carbon dioxide enrichment affect photochemical energy use by evergreen trees in different seasons? A chlorophyll fluorescence study of mature loblolly pine. *Plant Physiology* **120,** 1183–1191.

Kürschner W., Stulen I., Wagner F. & Kuiper P. (1998) Comparision of palaeobotanical observations with experimental data on the leaf anatomy of oak [*Quercus petraea* (Fagaceae)] in response to environmental change. *Annals of Botany* **81,** 657–664.

Laisk A. (1977) *Kinetics of Photosynthesis and Photorespiration of C_3 Plants.* Nauka, Moscow, Russia (in Russian).

Long S. (1998) Understanding the impacts of rising CO_2: the contribution of environmental physiology. Paper presented at *Physiological Plant Ecology, the 39th Symposium of the British Ecological Society*. Oxford University, Oxford, UK.

Loreto F., Di Marco G., Tricoli D. & Sharkey T.D. (1994) Measurements of mesophyll conductance, photosynthetic electron transport and alternative electron sinks of field grown wheat leaves. *Photosynthesis Research* **41,** 397–403.

Loreto F., Harley P.C., Di Marco G. & Sharkey T.D. (1992) Estimation of mesophyll conductance to CO_2 flux by three different methods. *Plant Physiology* **98,** 1437–1443.

Majeau N. & Coleman J.R. (1996) Effect of CO2 concentration on carbonic anhydrase and ribulose-1,5-bisphosphate carboxylase/oxygenase expression in pea. *Plant Physiology* **112,** 569–574.

Masle J. (2000) The effects of elevated CO_2 concentrations on cell division rates, growth patterns, and blade anatomy in young wheat plants are modulated by factors related to leaf position, vernalization, and genotype. *Plant Physiology* **122,** 1399–1415.

Maxwell C., Griffiths H. & Young A.J. (1994) Photosynthetic acclimation to light regime and water stress by the C_3-CAM epiphyte *Guzmania monostachia*: gas-exchange characteristics, photochemical efficiency and the xanthophyll cycle. *Functional Ecology* **8,** 746–754.

Medlyn B. (1996) The optimal allocation of nitrogen within the C_3 photosynthetic system at elevated CO_2. *Australian Journal of Plant Physiology* **23,** 593–603.

Ono K. & Terashima I. (2002) Effects of $HgCl_2$ on CO_2 dependence of leaf photosynthesis: evidence indicating involvement of aquaporins in CO_2 diffusion across the plasma membrane. *Plant and Cell Physiology* **43,** 70–78.

Parkhurst D.F. & Mott K.A. (1990) Intercellular diffusion limits to CO_2 uptake in leaves. Studies in air and helox. *Plant Physiology* **94,** 1024–1032.

Sage R.F. (1994) Acclimation of photosynthesis to increasing atmospheric CO_2: the gas exchange perspective. *Photosynthesis Research* **39,** 351–368.

Sharkey T.D., Savitch L.V. & Butz N.D. (1991) Photometric method for routine determination of k_{cat} and carbamylation of Rubisco. *Photosynthesis Research* **28,** 41–48.

Sicher R.C., Kremer D.F. & Rodermel S.R. (1994) Photosynthetic acclimation to elevated CO_2 occurs in transformed tobacco with decreased ribulose-1,5-bisphosphate carboxylase/oxygenase content. *Plant Physiology* **104,** 409–415.

Singsaas E.L., Ort D.R. & DeLucia E.H. (2000) Diurnal regulation of photosynthesis in understory saplings. *New Phytologist* **145,** 39–49.

Syvertsen J.P., Lloyd J., McConchie C., Kriedemann P.E. & Farquhar G.D. (1995) On the relationship between leaf anatomy and CO_2 diffusion through the mesophyll of hypostomatous leaves. *Plant, Cell and Environment* **18,** 149–157.

Underwood A.J. (1997) *Experiments in Ecology*. Cambridge University Press, Cambridge, UK.

Villar R., Held A.A. & Merino J. (1994) Comparison of methods to estimate dark respiration in the light in leaves of two woody species. *Plant Physiology* **105,** 167–172.

Villar R., Held A. & Merino J. (1995) Dark leaf respiration in light and darkness of an evergreen and a deciduous plant species. *Plant Physiology* **107,** 421–427.

Wintermans J.G.F.M. & DeMots A. (1965) Spectrophotometric characteristics of chlorophylls *a* and *b* and their pheophytins in ethanol. *Biochimica et Biophysica Acta* **109,** 448–453.

Received 28 May 2003; accepted for publication 11 September 2003