Journal of
Experimental
Botany

REVIEW ARTICLE: FIELD TECHNIQUES

Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error

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Abstract

The principles, equipment and procedures for measuring leaf and canopy gas exchange have been described previously as has chlorophyll fluorescence. Simultaneous measurement of the responses of leaf gas exchange and modulated chlorophyll fluorescence to light and CO_2 concentration now provide a means to determine a wide range of key biochemical and biophysical limitations on photosynthesis *in vivo*. Here the mathematical frameworks and practical procedures for determining these parameters *in vivo* are consolidated. Leaf CO_2 uptake (A) versus intercellular CO_2 concentration (C_i) curves may now be routinely obtained from commercial gas exchange systems. The potential pitfalls, and means to avoid these, are examined. Calculation of *in vivo* maximum rates of ribulose-1,5-*bis*phosphate (RuBP) carboxylase/oxygenase (Rubisco) carboxylation ($V_{c,max}$), electron transport driving regeneration of RuBP (J_{max}), and triose-phosphate utilization (V_{TPU}) are explained; these three parameters are now widely assumed to represent the major limitations to light-saturated photosynthesis. Precision in determining these in intact leaves is improved by the simultaneous measurement of electron transport via modulated chlorophyll fluorescence. The A/C_i response also provides a simple practical method for quantifying the limitation that stomata impose on CO_2 assimilation. Determining the rate of photorespiratory release of oxygen (R_l) has previously only been possible by isotopic methods, now, by combining gas exchange and fluorescence measurements, R_l may be determined simply and routinely in the field. The physical diffusion of CO_2 from the intercellular air space to the site of Rubisco in C_3 leaves has long been suspected of being a limitation on photosynthesis, but it has commonly been ignored because of the lack of a practical method for its determination. Again combining gas exchange and fluorescence provides a means to determine mesophyll conductance. This method is described and provides insights into the magnitude and basis of this limitation.

Key words: Electron transport, fluorescence, mesophyll conductance, photorespiration, photosynthesis, Rubisco, stomata.

Introduction

Previously, the theory of infrared gas analysis, as used in plant physiology, and its incorporation into portable open gas-exchange systems for the measurement of leaf and canopy photosynthetic and water vapour exchange has been described. In this previous paper, the measurement of the maximum quantum yield of CO_2 uptake and the construction and use of field systems for measuring stand photosynthesis was also described (Long *et al*., 1996). At the time of the earlier paper a series of commercial, portable, open gas exchange systems with options for controlling CO_2, humidity, temperature, and light had entered the market place (e.g. LI-6400, Li-Cor Inc.,

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Lincoln, Nebraska, USA; CIRAS-II, PP Systems, Hitchin, UK; LCA4 ADC-Biosciences, Hoddesdon, UK). These off-the-shelf portable systems provide real-time measurements of CO_2 uptake (A), transpiration (E), leaf conductance (g_1), and the intercellular CO_2 mole fraction (C_i). The precision of measurement possible has meant that custom-built field and laboratory systems have largely been replaced. Whereas 15 years ago many measurements would be made with systems constructed by their operator, today over 95% of measurements of photosynthetic CO_2 uptake in ISI listed journals use commercial off-the-shelf systems (Long and Hällgren, 1993; Long *et al*., 1996). The ease of measurement, which allows the operator to obtain A, E, g_1, and C_i at the push of a few buttons and without any prior experience, does have its pitfalls, as is shown later. In parallel with the development of portable gas exchange systems has been the development of further instrumentation that is greatly extending the ability to interpret the basis of change in CO_2 uptake *in vivo*. In particular, the further development of modulated chlorophyll fluorimetry, differential oxygen analysis and higher resolution infrared gas analysers suited for the measurement of non-steady-state changes in CO_2 fluxes (Bloom *et al*., 2002; Laisk *et al*., 2002; Maxwell and Johnson, 2000). This paper extends upon Long *et al*. (1996), focusing on the measurements now possible through combining gas exchange and fluorescence. It also highlights some of the pitfalls of the off-the-shelf gas exchange systems.

Off-the-shelf gas exchange systems: some pitfalls

By contrast with earlier custom-built gas exchange systems, the modern commercially available systems enclose small areas of leaf, typically less than 10 cm^2 and often as small as 2 cm^2. This has the advantage that, given variability across leaf surfaces, the measurements will be less prone to errors in calculations resulting from spatial heterogeneity of stomatal conductance and photosynthetic capacity (Cheeseman, 1991). It also has the advantage that the exact area is known, with the exception of small or narrow leaves which do not fill even these small chambers. The downside is that a small area, by definition, will have a larger edge-to-area ratio. In the older chambers typically used in custom-built systems, the entire leaf was enclosed and sealed at the petiole. In commercial chambers, a portion of the leaf is sealed into the chamber. A seal is achieved with closed cell foam gaskets appressed to both surfaces of the leaf isolating a small area. Much has been learned from the application of these systems in measuring the response of leaf respiration to elevated $[CO_2]$, but it has also been learnt how misleading results from these systems can be if attention is not given to potential errors. Ten years ago there appeared to be overwhelming evidence, most from commercial portable gas-exchange systems, that

elevation of $[CO_2]$ would immediately depress leaf respiration (Amthor *et al*., 1992; reviewed in Drake *et al*., 1997). Today it is realized that this conclusion was largely, if not wholly, an artefact of the way these measurements were made (Amthor, 2000; Jahnke, 2001; Jahnke and Krewitt, 2002). The commercial systems were designed for measuring larger fluxes of CO_2 associated with photosynthesis. Errors that might have a small effect with high rates of photosynthesis will have a large impact on measured rates of respiration. These errors will apply equally to photosynthesis at low fluxes, for example, during measurements at low light and low $[CO_2]$, or of stressed plants or plants with inherently low photosynthetic rates. What are some of the problems associated with these commercial leaf chambers?

Leaks

Some CO_2 can escape through the gasket, this may not be a constant and will vary with the leaf. It is worse among leaves with prominent veins where small air channels may form between the gasket and the sides of the vein. This is particularly significant at low fluxes when errors due to artefactual apparent fluxes will have their greatest effect and in the measurement of A/C_i responses, when differences between the air outside and that within the chamber are greatest. A partial solution, recommended commonly by manufacturers is the measurement of flux in the absence of a leaf. Here, when the chamber is closed, a perfect seal should give a zero flux, regardless of the difference in $[CO_2]$ between the inside and outside of the chamber. However, gaskets have some permeability and may release or absorb some CO_2 (Long and Hällgren, 1993). These leaks may be measured and used to correct fluxes. However, when the leaf is placed in the chamber additional leaks may be introduced. There are two partial solutions. (1) Use a dead leaf, formed by rapidly drying a live specimen and establish the rate of leakage at each $[CO_2]$ that will be used in constructing an A/C_i response. (2) Enclose the chamber in a container filled with the gas mixture that is being introduced into the chamber. One means to achieve this is to supply the outer container with the exhaust air from the system.

Edge effects

Because the gasket has a finite thickness it and any other wall structure above the leaf will affect radiation in the chamber, unless the light source is a parallel beam at 90° to the leaf surface. In the field, the lower the sun angle the greater this shading effect may be. The problem is alleviated if an artificial light source is placed above the chamber. The gasket will also cause the photosynthesizing surface to be surrounded by tissue in darkness that is respiring. This respired CO_2 will decrease the measured net flux. This problem may be decreased by maintaining a pressure above atmospheric in the chamber and by

minimizing gasket width (Pons and Welschen, 2002), although this may, in itself, cause other errors (Jahnke and Krewitt, 2002).

Lateral flux through the leaf air space

Another source of error is a flux of air between the chamber and surrounding air via the internal air space of the enclosed leaf. This will only be an issue with homobaric leaves, i.e. those with a continuous internal air space. In this case, the only complete solution will be enclosure of the entire leaf (Jahnke and Krewitt, 2002). Otherwise, minimizing the pressure gradient between the leaf chamber space and the outside air will minimize this error.

Practical interpretation of the *A/C*$_i$ response

The model of Farquhar *et al*. (1980) has provided a tried and tested means quantitatively to partition biochemical and stomatal limitations on photosynthesis, from the response of CO_2 uptake to intercellular mole fraction of CO_2 (A/C_i). Simultaneous measurement of chlorophyll fluorescence now extends this analysis, providing a means to determine the partitioning of energy between photosynthesis and photorespiration, and to determine R_l *in vivo*. Within the mesophyll, biochemical and diffusion limitations may now be separated.

The A/C$_i$ curve

Two of the most commonly reported responses of CO_2 uptake are the responses of *A* to photon flux (*Q*) and to intercellular CO_2 mole fraction (C_i). The measurement and interpretation of the *A/Q* response has been described previously (Long *et al*., 1996; Long and Hällgren, 1993). The *A/C*$_i$ response is determined by measuring *A* and *E* at a series of ambient CO_2 concentrations (C_a) and a defined leaf-air vapour pressure deficit (*D*). By assuming that CO_2 diffuses from the ambient air passing over the leaf to the substomatal cavity, via the pathway by which water vapour escapes the substomatal cavity, the leaf diffusive conductance for carbon dioxide (g_l) can be determined from *E*, *D*, and the diffusivities of CO_2 and water vapour (Long and Hällgren, 1993). From measurements of both the CO_2 concentration surrounding the leaf (C_a) and *A*, C_i can then be calculated.

$$C_i = C_a - A/g_l \qquad (1)$$

The response of *A* to C_i may then be constructed by measuring these values at a range of CO_2 concentrations. The response of *A* to C_a by itself cannot be interpreted easily since it is affected by boundary layer, stomatal, and mesophyll processes; the response of *A* to C_i, however, eliminates the effect of the boundary layer and stomata, depending solely on mesophyll processes. How the *A/C*$_i$ response is determined depends on the purpose of the
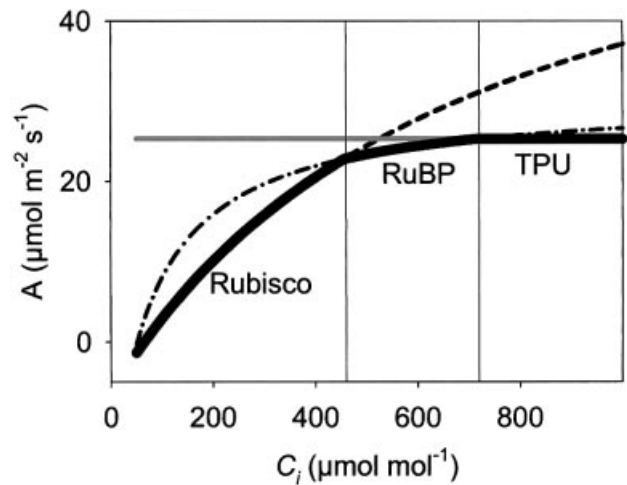

Fig. 1. Idealized A/C_i response. The rates of photosynthesis that would be achieved depending on whether Rubisco, RuBP or TPU are limiting are indicated. The actual photosynthetic rate (solid line) at any given C_i is the minimum of these three potential limitations. Parameters used: $V_{c,max}$=70 μmol m^{-2} s^{-1}, J_{max}=130 μmol m^{-2} s^{-1}, V_{TPU}=9.1 μmol m^{-2} s^{-1}, R_d=2 μmol m^{-2} s^{-1}.

study. The most common purpose is to state *in vivo* apparent Rubisco activity ($V_{c,max}$) and the maximum rate of electron transport used in the regeneration of RuBP (J_{max}), under ambient conditions. A decrease in C_i below ambient will lower *A* and the pools of Calvin cycle intermediates, which can affect the activity of Rubisco and other enzymes. These changes may, in turn, alter *in vivo* Rubisco activity and the capacity for RuBP regeneration. It is therefore important to complete the measurements, particularly at lower [CO_2], as rapidly as possible to avoid significant changes in activation. The response of *A* to C_i describes two, sometimes three, phases (Fig. 1). As C_i is increased from its minimum concentration, dA/dC_i is high and determined by Rubisco activity. With a further increase, there is an inflection to a lower dA/dC_i that approaches zero, where RuBP-regeneration is limiting. In some instances, a further increase in C_i may result in another transition to a plateau or a decrease in *A* with an additional increase in C_i ($dA/dC_i \leqslant 0$) if triose-phosphate utilization (TPU) becomes limiting. These phases are mathematically predicted by the model of Farquhar *et al*. (1980), as modified to account for TPU-limitation (von Caemmerer, 2000). By fitting these phases, as mathematically defined (von Caemmerer, 2000) key biochemical kinetic variables determining photosynthetic rate can be determined *in vivo*, specifically: $V_{c,max}$, J_{max} and V_{TPU}.

The model of Farquhar et al. (1980)

Both CO_2 and O_2 compete for the Rubisco binding site in the processes known as carboxylation and oxygenation, respectively (Farquhar *et al*., 1980). To account for the competitive inhibition between CO_2 and O_2, *A* is mathematically expressed as:

$$A = v_c - 0.5v_o - R_d = v_c\left(1 - \frac{\Gamma*}{C_i}\right) - R_d \qquad (2)$$

The term $(1-\Gamma*/C_i)$ is used to take account of the proportion of recently assimilated carbon that is released in photorespiration. The photosynthetic compensation point ($\Gamma*$) is the CO_2 concentration at which the photorespiratory efflux of CO_2 equals the rate of photosynthetic CO_2 uptake (i.e. when $v_c=2v_o$). It is distinct from Γ which is the CO_2 compensation point, i.e. the CO_2 concentration at which $v_c=2v_o+R_d$. A method for determining $\Gamma*$ from gas-exchange is provided by Brooks and Farquhar (1985). $\Gamma*$ is defined by:

$$\Gamma* = \frac{0.5O}{\tau} \qquad (3)$$

where τ, the Rubisco specificity factor, is derived from Rubisco kinetics as

$$\tau = \frac{V_{c,max}K_o}{V_{o,max}K_c} \qquad (4)$$

and appears a very highly conserved quantity among the Rubisco's of higher plants (Bainbridge *et al.*, 1995; Bernacchi *et al.*, 2001). The actual rate of carboxylation at Rubisco is determined by the minimum of three potential rates (Farquhar *et al.*, 1980; Harley and Sharkey, 1991; von Caemmerer, 2000):

$$v_c = \min \{w_j, w_j, w_p\} \qquad (5)$$

Substituting for v_c into equation (2) yields

$$A = \min\{w_c, w_j, w_p\}\left(1 - \frac{\Gamma*}{C_i}\right) - R_d \qquad (6)$$

where:

$$w_c = \frac{V_{c,max}C_i}{C_i + K_c(1 + O/K_o)} \qquad (7)$$

$$w_j = \frac{JC_i}{4.5C_i + 10.5\Gamma*} \qquad (8)$$

$$w_p = \frac{3V_{tpu}}{\left(1 - \frac{\Gamma*}{C_i}\right)} \qquad (9)$$

w_c, w_j and w_p are the potential rates of CO_2 assimilation that can be supported by Rubisco, RuBP-regeneration and triose-phosphate utilization, respectively. The model assumes that RuBP regeneration is limited by potential whole chain electron transport rate (J) under the given conditions of light and temperature and corrected for partitioning between oxygenation and carboxylation of RuBP. At light saturation, J is equal to J_{max}, but at lower light levels J may be estimated from photon flux (Q) from a non-rectangular hyperbola (von Caemmerer, 2000):

$$J = \frac{Q_2 + J_{max} - \sqrt{(Q_2 + J_{max})^2 - 4\Theta_{PSII}Q_2J_{max}}}{2\Theta_{PSII}} \qquad (10)$$

where Θ_{PSII} represents a curvature factor and Q_2 represents the incident quanta utilized in electron transport through PSII:

$$Q_2 = Q\alpha_l\Phi_{PSII,max}\beta \qquad (11)$$

where α_l is the leaf absorptance, $\Phi_{PSII,max}$ is maximum quantum yield of photosystem II and β is the fraction of absorbed light that reaches photosystem II (von Caemmerer, 2000). Variability in the parameters of equations 10 and 11 with leaf and growth temperature are described by Bernacchi *et al.* (2003).

Estimating $V_{c,max}$, J_{max}, V_{TPU} from the A/C_i response

To deduce the quantities of $V_{c,max}$, J_{max} and V_{TPU}, it is important to obtain measures of A in all three phases, if present, of the response to C_i. Frequently, TPU will not be a limitation at any C_i and so only two phases may be seen. Figure 1 shows a typical A/C_i curve identifying each of the three potential limiting processes. In practice, it has been found that the initial slope of the A/C_i response, representing photosynthesis limited by $V_{c,max}$ (equation 7), will change within 5 min of leaf exposure to a low C_a, while points above the inflexion seem less affected by the duration of exposure to increased C_a. A protocol commonly used in determining this A versus C_i response (Ainsworth *et al.*, 2002) is as follows. (1) Induce photosynthesis at the growth CO_2 concentration and at saturating Q until A is steady-state; where steady-state in practice means that A shows no systematic decrease or increase ($\pm2\%$) over a 5 min period. This is important to ensure a steady-state activation of Rubisco. Values of A and C_i are then recorded and C_a is decreased to 300 µmol mol^{-1}. As soon as C_a is stable (cv <0.7%), but not necessarily steady-state, A and C_i are recorded. This procedure is repeated for C_a values of 250, 200, 150, 100, and finally 50 µmol mol^{-1}. With practice, each measurement in this series can be completed within 2 min. Upon completion of the measurement at a C_a of 50 µmol mol^{-1}, C_a is returned to 370 µmol mol^{-1} to check that the original A can be restored. If this is achieved then C_a is increased stepwise to 450, 550, 650, 800, and 1000 µmol mol^{-1}. Steady-state photosynthesis need not be obtained at each step, and indeed could introduce errors if the purpose is to determine capacities under the ambient growth conditions. It is critical to choose values of C_a that suit your material. First, the use of pilot studies is recommended to determine the C_i at which the transitions between Rubisco- and RuBP-limited photosynthesis and between RuBP- and TPU-limited photosynthesis occur. Then ensure a minimum of five points either side of these. Equation 6 may then be fitted to the whole curve (Wullschleger, 1993). Alternatively, the subcompo-

nents may be fit to different portions of the curve. For example, by substituting equation (7) into (6), A may be fit to C_i with R_d as the y-axis intercept, in which K_c and K_o are assumed constants at any given temperature (Bernacchi *et al.*, 2001):

$$K_c = \exp^{(38.05 - 79.43/(R(T_l + 273.15)))} \qquad (12)$$

$$K_o = \exp^{(20.30 - 36.38/(R(T_l + 273.15)))} \qquad (13)$$

where T_l is the leaf temperature (°C). There are relatively few measurements of the temperature responses of K_o, K_c and Γ^* and the extent to which they vary between species. They are commonly considered to be constant among terrestrial C3 species for the purposes of the Farquhar *et al.* (1980) model (Wullschleger, 1993). Since these parameters depend on the properties of Rubisco, some inter-specific variation should be expected even among terrestrial C3 species. Medlyn *et al.* (2002) review and evaluate different temperature functions for these parameters.

The two unknowns $V_{c,max}$ and R_d can be solved using a non-linear maximum likelihood best-fit (Sigmaplot 2000, SPSS Inc.). Alternatively, A may be plotted as a linear function of C_i:

$$A = f'V_{c,max} - R_d \qquad (14)$$

where $V_{c,max}$ is the slope and R_d the intercept. f' is obtained from equations 6 and 7 and expressed as

$$f' = \frac{C_i - \Gamma^*}{C_i + K_c(1 + O/K_o)} \qquad (15)$$

Because of the highly temperature-dependent nature of $V_{c,max}$ it is commonly expressed at a common temperature, 25 °C. The A that would have been obtained at 25 °C may be determined from the functions of Bernacchi *et al.* (2001). $V_{c,max}$ reports the apparent activity of Rubisco *in vivo*, which will vary both with the amount of Rubisco and its activation state. It is important to note that even in healthy well-illuminated leaves under optimal conditions Rubisco is rarely 100% activated. Above 35 °C a further loss of activation may occur due to high-temperature effects on Rubisco activase (Spreitzer and Salvucci, 2002).

Similarly, J_{max} may be obtained by fitting A to C_i with equations (6) and (8), assuming that w_j is limiting. This can also be made amenable to linear regression with

$$g' = \frac{C_i - \Gamma^*}{4.5C_i + 10.5\Gamma^*} \qquad (16)$$

This may be written as:

$$A = g'J - R_d \qquad (17)$$

An important consideration when calculating J_{max}, as the slope in equation (17), is that the small errors in the higher rates of A associated with RuBP-limited photosyn-
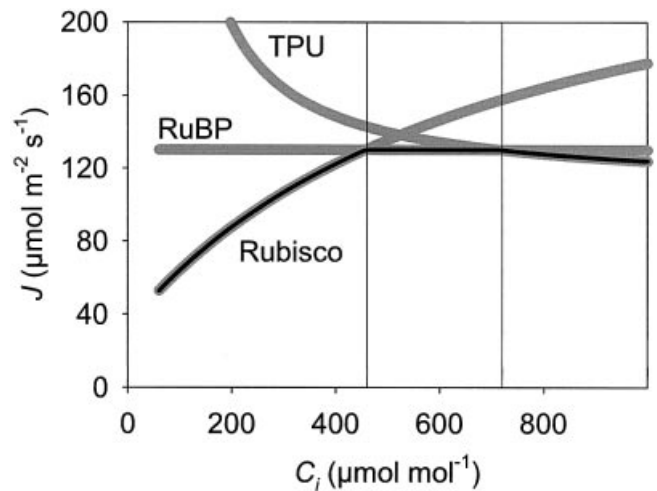

Fig. 2. Using the same parameters as in Fig. 1, electron transport (J) is predicted for photosynthesis and photorespiration assuming Rubisco, RuBP or TPU limitation. The actual electron transport rate at any given C_i is the minimum of these three potential limiting processes.

thesis may introduce large errors in estimates of the intercept, R_d. Therefore, a common practice is to solve for $V_{c,max}$ and R_d first and to use the value of R_d from equation (14) as a fixed parameter when estimating J in equation (17). This approach assumes R_d is constant in the light over all CO_2 concentrations. Given the small magnitude of R_d this would have little effect on estimates of $V_{c,max}$. Alternatively, Loreto *et al.* (1999) provide a combined gas-exchange and mass isotope approach to determine R_d, which can be used at numerous light fluxes. For all curve fits, Γ^* is assumed a constant at any given temperature—an estimate may be obtained from Bernacchi *et al.* (2001):

$$\Gamma^* = \exp^{(19.02 - 38.83/(R(T_l + 273.15)))} \qquad (18)$$

Brooks and Farquhar (1985) present a gas-exchange method for the direct measurement of Γ^*. While w_j is limiting, A should continue to increase slightly with further increases in C_i. A lack of increase in A, indicates TPU is limiting. V_{TPU} in its most simple form is represented by:

$$V_{TPU} = (A + R_d)/3 \qquad (19)$$

Often, however, a decrease in A is observed at higher [CO_2]. In these cases, a more complex representation of V_{TPU} is given as:

$$V_{TPU} = \frac{(A + R_d)(C_i - (1 + 3\alpha_g/2)\Gamma^*)}{3(C_i - \Gamma^*)} \qquad (19a)$$

where α_g represents glycolate carbon not returned to the chloroplast and is linked with the release of phosphate. This more complex variation of TPU-limited photosynthesis is discussed in detail elsewhere (Harley and Sharkey, 1991; von Caemmerer, 2000). Because the transition to TPU-limited photosynthesis, if present, may occur at high C_i it is difficult to separate graphically RuBP-
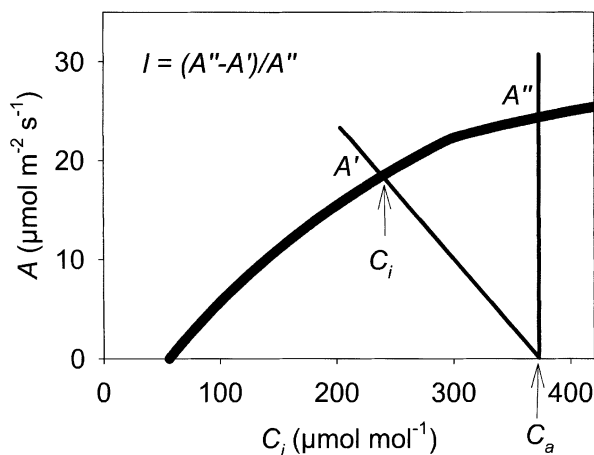
Fig. 3. Graphical method of estimating the limitation (*l*) placed on *A* by the stomata and leaf boundary layer. The line originating at C_i=370 µmol mol^{-1} and intercepting the A/C_i curve at *A'* is the supply function, i.e. the decrease in C_i that will occur as *A* increases above 0. The slope of the supply function is $-1/g_l$. In the absence of any limitation to diffusion of CO_2 from the atmosphere to the site of carboxylation, this line would intercept the A/C_i response at *A''*.

from TPU-limited photosynthesis. Here, parallel measurements of modulated fluorescence to determine J_{PSII} provides important confirmatory information. As C_i increases it follows from equation (6) that J_{PSII} will increase if Rubisco is limiting, stay constant if RuBP-regeneration is limiting, and decrease if TPU is limiting (Fig. 2).

Assessing stomatal limitation

It is frequently seen that leaf conductance decreases with any factor that decreases photosynthesis. However, this does not necessarily mean that stomata are any more limiting to photosynthesis. But do stomata contribute to the decrease in photosynthesis? Several methods have been suggested to quantify the limitation that the combined stomatal and boundary layer conductances (g_l) impose on leaf CO_2 uptake (*A*) (see Jones, 1998, for a detailed consideration of alternative approaches and their limitations). Farquhar and Sharkey (1982) provided a very simple graphical method that makes further use of the A/C_i response. If a leaf is considered with photosynthetic rate *A'* and g_l at the ambient atmospheric CO_2 concentration (Fig. 1), a prediction can be made of the the hypothetical *A* (*A''* in Fig. 3) that would be obtained if the mesophyll had free access to the CO_2 in the ambient air (i.e. g_l=∞) then C_i=C_a. The limitation (*l*) imposed by g_l is given by:

$$l = (A''-A')/A'' \qquad (20)$$

Assessing photorespiration *in vivo*

A measured by gas exchange in C_3 plants is the rate of carboxylation less the release of CO_2 from photorespiration and mitochondrial respiration (equation 6). The rate of electron transport through PSII (J_{PSII}), less any flux to alternative sinks, is the sum of flux both to photosynthesis and photorespiration. Given the electron requirements to support photorespiration and photosynthesis, the rate of photorespiration may be calculated via simultaneous measurement of *A* and Φ_{PSII}.

To make these measurements it is first necessary to determine alternative electron sinks. This may be achieved by determining the responses of *A* and Φ_{PSII} to *Q* under non-photorespiratory conditions (i.e. 10 mmol mol^{-1} O_2). Note, though, that the sensitivity of infrared gas analysers to water vapour and to CO_2 are affected by absorption line broadening when the composition of air is changed, this includes altering the O_2 content (Burch *et al.*, 1962). It is therefore essential to recalibrate the analyser for 1% O_2. Leaf absorptance (α) must also be determined, using an integrating sphere and in the same light source that is used for measuring *A* and J_{PSII} (Bernacchi *et al.*, 2003). From these measurements, the quantum efficiency of CO_2 uptake (Φ_{CO_2}) is calculated as:

$$\Phi_{CO_2} = (A-R_d)/(\alpha Q) \qquad (21)$$

When measured in 1% O_2, the relationship of Φ_{PSII} to Φ_{CO_2} is linear (Genty *et al.*, 1989):

$$\Phi_{PSII} = k\Phi_{CO_2}+b \qquad (22)$$

where *k* is the slope of the line and represents the apparent number of electrons needed to fix one CO_2. The intercept, *b*, represents the fraction of electrons going to alternative electron sinks at infinite *Q*. In theory, *k* should be 4, which is the number of electron equivalents needed to reduce one molecule of CO_2. In reality, combining measurements of gas exchange and chlorophyll fluorescence does not yield such a low number. As such, it is necessary to 'calibrate' the value of Φ_{PSII} (Valentini *et al.*, 1995). With the above measurements of Φ_{PSII} in 10 mmol O_2 mol^{-1} the calibrated quantum efficiency of PSII, Φ_e, is calculated as

$$\Phi_e = 4\Phi_{CO_2} \qquad (23)$$

Equations (22) and (23) can be combined and rearranged as:

$$\Phi_e = 4(\Phi_{PSII}-b)/k \qquad (24)$$

Equation (24) will hold true regardless of whether photorespiration is present. Because the term *b* is included in this equation, Φ_e represents only electrons being used by photosynthesis and photorespiration. If there is perfect agreement between chlorophyll fluorescence and gas exchange, then the 4 and the *k* in equation (24) would cancel.

The total rate of electron transport though photosystem II to photosynthesis and photorespiration, J_t, can then be calculated as

$$J_t = \Phi_e Q \qquad (25)$$

Using the equations of Valentini *et al.* (1995), the partitioning of electrons to photosynthesis, J_c, and to photorespiration, J_o can be determined as:

$$J_t = J_c + J_o \tag{26}$$

Again assuming that four electrons are needed to fix one CO_2, then:

$$J_c = 4(A + R_d + R_l) \tag{27}$$

where R_d and R_l represent the CO_2 being released through mitochondrial respiration and through photorespiration, respectively. Assuming eight electrons for the release of one CO_2 via photorespiration:

$$J_o = 8R_l \tag{28}$$

Combining equations (26), (27), and (28) and rearranging to solve for R_l yields the equation:

$$R_l = \frac{[J_t - 4(A + R_d)]}{12} \tag{29}$$

Partitioning of electrons between photosynthesis and photorespiration is then given by incorporating equation (29) into equation (27):

$$J_o = 1/3[J_t + 8(A + R_d)] \tag{30}$$

and incorporating equation (29) into equation (28):

$$J_o = 2/3[J_t - 4(A + R_d)] \tag{31}$$

Assessing mesophyll conductance (g_m)

An assumption of much gas exchange work, including the original model of Farquhar *et al.* (1980) has been that C_i approximates to the $[CO_2]$ at Rubisco (C_c), i.e. $C_i = C_c$. However, CO_2 has to diffuse from the intercellular air space through the mesophyll cell wall, plasma membrane and chloroplast membrane. It has become apparent that this pathway can result in a significant difference between C_i and C_c (Harley *et al.*, 1992; Loreto *et al.*, 1992; von Caemmerer, 2000). However, g_m could not easily be measured until recently. Previously g_m was estimated from pathway dimensions and conductances across membranes, requiring many assumptions (Nobel, 1999). By combining gas exchange and fluorescence it is now possible to measure mesophyll conductance directly. This is based on the assumption that the difference between J estimated from gas exchange and J measured from chlorophyll fluorescence is a function of g_m. Two methods for estimating g_m have been used (Loreto *et al.*, 1992; Bernacchi *et al.*, 2002).

The constant J *method*

This method requires measurements of A, R_d and J_{PSII} when photosynthesis is limited by the regeneration of

RuBP and therefore J_{PSII} remains constant as C_i is increased (Bongi and Loreto, 1989). Electron transport (J) estimated from chlorophyll fluorescence is a function of A, C_i, Γ^*, and g_m (Harley *et al.*, 1992). Using Γ^* for a given temperature from Bernacchi *et al.* (2001) and the measured response of A to a range of C_i at which J is observed to be constant:

$$J = (A + R_d)\frac{4((C_i - A/g_m) + 2\Gamma^*)}{(C_i - A/g_m) - \Gamma^*} \tag{32}$$

Equation (32) may then be solved for g_m for the range of C_i. Harley *et al.* (1992) provide a statistical method for this solution.

The variable J *method*

This uses A and R_d measured from gas exchange and J estimated from fluorescence to solve for g_m (Bongi and Loreto, 1989; Harley *et al.*, 1992). This equation requires that Rubisco is limiting photosynthesis and that J will increase with C_i:

$$g_m = \frac{A}{C_i - \frac{\Gamma^*(J + 8(A + R_d))}{J - 4(A + R_d)}} \tag{33}$$

The value of Γ^* at a given temperature from Bernacchi *et al.* (2002) can be used for both the constant and variable J methods of calculating g_m. Other estimates of Γ^* are also available (Brooks and Farquhar, 1985; von Caemmerer *et al.*, 1994; Bernacchi *et al.*, 2001), however, estimates based on C_i, rather than on C_c may introduce error to the calculations.

Bernacchi *et al.* (2002) used both methods at a range of temperatures for tobacco; they gave very close agreement. This study also revealed that g_m has a high Q_{10} suggesting that the dominant process(es) determining g_m was not physical diffusion, but probably protein mediated; possibly involving a carbonic anhydrase or aquaporins. This is consistent with Centritto *et al.* (2003) who showed that g_m could change as rapidly as g_s, indicating that g_m is not determined by intrinsic anatomical features. Bernacchi *et al.* (2002) also showed that transfer from the intercellular space to Rubisco represented a limitation of 0.1–0.2, i.e. if g_m was infinite photosynthetic rates would be 10–20% higher. Clearly, there is more to learn about mesophyll conductance, its basis and significance, but it is one more example of how simultaneous measurement of gas exchange and fluorescence are extending the range of measurements of key processes that may now be achieved with modern off-the-shelf gas exchange and fluorescence systems in the most meaningful situation possible, *in vivo*. These are only a fraction of the possible *in vivo* measurements that may potentially be made on a single leaf (Laisk *et al.* 2002).

Appendix

Abbreviations

A Net rate of CO_2 uptake per unit of projected leaf area (μmol m^{-2} s^{-1})

C_i Intercellular CO_2 concentration (μmol mol^{-1})

D Vapour pressure deficit (kPa)

E Transpiration (mmol m^{-2} s^{-1})

g_l Leaf conductance, the combined gas phase conductances of the boundary layer and stomata (mmol m^{-2} s^{-1})

J Rate of electron transport (μmol m^{-2} s^{-1})

J_{max} Maximum rate of electron transport (μmol m^{-2} s^{-1})

J_c J resulting from carboxylation of RuBP (μmol m^{-2} s^{-1})

J_o J resulting from oxygenation of RuBP (μmol m^{-2} s^{-1})

J_{PSII} Total rate of whole chain electron transport measured via fluorescence (μmol m^{-2} s^{-1})

J_t J_{PSII} corrected for any flux to alternative sinks, i.e. other than photosynthetic carbon metabolism, but including photorespiratory metabolism (μmol m^{-2} s^{-1}).

K_c Rubisco Michaelis constant for CO_2 (μmol mol^{-1})

K_o Rubisco Michaelis constant for O_2 (mmol mol^{-1})

O Concentration of oxygen in air (mmol mol^{-1})

Q Photosynthetic photon flux density (μmol m^{-2} s^{-1}).

Q_2 Quanta utilized in electron transport through PSII (μmol m^{-2} s^{-1})

R Molar gas constant (kJ K^{-1} mol^{-1})

R_d Mitochondrial respiration rate in the light (μmol m^{-2} s^{-1})

R_l Rate of CO_2 release in photorespiration (μmol m^{-2} s^{-1})

T_l Leaf absolute temperature (°C)

TPU   Triose-phosphate utilization

$V_{c,max}$ Maximum RuBP saturated rate of carboxylation (μmol m^{-2} s^{-1})

v_c Rate of carboxylation of RuBP via Rubisco (μmol m^{-2} s^{-1})

v_o Rate of oxygenation of RuBP via Rubisco (μmol m^{-2} s^{-1})

v_{TPU} Rate of triose-phosphate utilization (μmol m^{-2} s^{-1})

w_c Rubisco-limited rate (= RuBP saturated rate) of carboxylation (μmol m^{-2} s^{-1})

w_j RuBP-limited rate of carboxylation (μmol m^{-2} s^{-1})

w_p TPU-limited rate of carboxylation (μmol m^{-2} s^{-1})

α Leaf absorbance (0–1, dimensionless)

β Fraction of absorbed quanta reaching PSII (0–1, dimensionless)

Γ^* CO_2 compensation point in the absence of dark respiration (μmol mol^{-1})

Θ Convexity of the non-rectangular curve describing the dependence of A on Q (0–1 dimensionless)

Θ_{PSII} Convexity of the non-rectangular curve describing the dependence of J_t on Q (0–1 dimensionless)

Φ_a Apparent maximum quantum yield of CO_2 uptake; initial slope of the light response curve; A versus Q (0–1, dimensionless)

Φ_{CO_2} Quantum yield of CO_2 uptake, for any given Q (0–1, dimensionless)

Φ_e Maximum quantum yield of photosynthetic linear electron flow through PSII; initial slope of the light response curve; J versus Q (0–1, dimensionless)

Φ_{PSII} Quantum yield of PSII, for any given Q (0–1, dimensionless)

τ Rubisco specificity factor

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