Fitting photosynthetic carbon dioxide response curves for C₃ leaves

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ABSTRACT

Photosynthetic responses to carbon dioxide concentration can provide data on a number of important parameters related to leaf physiology. Methods for fitting a model to such data are briefly described. The method will fit the following parameters: V_{cmax} , J, TPU, R_{d} and g_{m} [maximum carboxylation rate allowed by ribulose 1.5-bisphosphate carboxylase/oxygenase (Rubisco), rate of photosynthetic electron transport (based on NADPH requirement), triose phosphate use, day respiration and mesophyll conductance, respectively]. The method requires at least five data pairs of net CO₂ assimilation (A) and [CO₂] in the intercellular airspaces of the leaf (C_i) and requires users to indicate the presumed limiting factor. The output is (1) calculated CO₂ partial pressure at the sites of carboxylation, C_c , (2) values for the five parameters at the measurement temperature and (3) values adjusted to 25 °C to facilitate comparisons. Fitting this model is a way of exploring leaf level photosynthesis. However, interpreting leaf level photosynthesis in terms of underlying biochemistry and biophysics is subject to assumptions that hold to a greater or lesser degree, a major assumption being that all parts of the leaf are behaving in the same way at each instant.

Key-words: A/C_i curves; mesophyll conductance; photosynthesis model.

Accompanying website: http://www.blackwellpublishing. com/plantsci/pcecalculation/

INTRODUCTION

Photosynthesis in plants is composed of interconnected biological processes located in different compartments of photosynthesizing eukaryotic cells. Biophysical processes, which include CO_2 transport through the leaf and stomata, and biochemical processes located in the chloroplast thylakoid membranes, stroma, mitochondria and the cytosol of the cell, determine the net rate of CO_2 assimilation (*A*). These biophysical and biochemical processes, and environmental

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© 2007 The Authors Journal compilation © 2007 Blackwell Publishing Ltd variables such as light intensity and temperature, can have different effects on A. This makes it difficult to predict how A will be affected by genetics, epigenetics and environment. Dissection of the biophysical and biochemical factors, and calculation of photosynthesis parameters, is an important tool for understanding the biology behind changes in A and allows predictions of environmental and genetic influences on plant productivity. This paper describes an approach to determining five of the more important parameters needed to describe A. Using a non-linear curve-fitting routine available in Microsoft Excel, a solution which minimizes the difference between observed and predicted A is found.

MODELLING

The most frequently used method for understanding how C_3 photosynthesis responds to perturbations is the Farquhar *et al.* model of photosynthesis (Farquhar, von Caemmerer & Berry 1980). In this model, the biochemical reactions of photosynthesis are considered to be in one of two distinct steady states. In one state, the rate of photosynthesis can be predicted by the properties of ribulose 1.5-bisphosphate carboxylase/oxygenase (Rubisco) assuming a saturating supply of substrate, RuBP. This state is called Rubisco-limited photosynthesis and normally occurs when the [CO₂] is low. The limitation by Rubisco is associated with the low [CO₂] rather than V_{max} of the enzyme.

In the other state, photosynthetic rates are predicted assuming that the rate of regeneration of RuBP is limiting and so RuBP is used at a constant rate; this is called RuBPregeneration-limited photosynthesis. This condition occurs at higher $[CO_2]$. RuBP-regeneration-limited photosynthesis includes the conditions where light intensity limits the rate of photosynthesis but can also include conditions in which enzymes of the Calvin cycle (other than Rubisco) limit the rate of photosynthesis. RuBP-regeneration-limited photosynthesis increases as $[CO_2]$ increases because increasing $[CO_2]$ causes more RuBP to be carboxylated at the expense of oxygenation. The component processes of photosynthesis can be assigned to one of these two states or a third state explained later (Fig. 1).

Increasing $[CO_2]$ increases A for three different reasons, the most obvious being that (1) CO_2 is a substrate for the



Figure 1. Scheme showing some of the processes that affect photosynthetic rate. For each of the three panels, any process in that panel will cause the photosynthetic rate to vary with [CO₂] in the same way. Rubisco, ribulose 1.5-bisphosphate carboxylase/oxygenase.

reaction. Increasing $[CO_2]$ increases RuBP carboxylation at the expense of oxygenation and this increases A by (2) reducing CO₂ release in photorespiration, and (3) increasing the light use efficiency of photosynthesis. Factors 1 and 2 influence the response of A to CO₂ in Rubisco-limited conditions while factors 2 and 3 influence the response of A to CO₂ in RuBP regeneration-limited conditions. The changes in A with $[CO_2]$ in these two conditions can be used to estimate photosynthesis parameters, provided it is known whether Rubisco or RuBP regeneration is limiting. In practice, different scenarios can be tried and compared, and in some cases it is not possible to unambiguously determine which process is limiting.

The Rubisco-limited state typically occurs at <20 Pa (~200 ppm) CO₂ while the RuBP-regeneration-limited state typically occurs at >30 Pa CO₂. Between 20 and 30 Pa, there is a transition from one limitation to the other. Sometimes this transition can be easily discerned, but often different assumptions can fit the data equally well and the investigator is forced to make a judgement. Data in the transition from one limitation to the next are more likely than other data to represent different limitations in different parts of the leaf (e.g. centre versus leaf margins, adaxial versus abaxial chloroplasts). In some cases, it is useful to exclude potentially 'co-limited' data points at the transition from the analysis. Circumventing the subjective assignment of limitations is an attractive goal but investigators should experiment with different assignments to learn whether the data are robust enough that they can be accurately described using one or another specific assignment of limitations.

A third state occurs when the chloroplast reactions have a higher capacity than the capacity of the leaf to use the products of the chloroplasts; primarily, but not exclusively, triose phosphate. This third state is called triose phosphate use (TPU) limitation. In this condition, photosynthesis does not respond to increasing CO₂, nor is it inhibited by increasing oxygen concentration (Sharkey 1985). This limitation can often set the maximum $A(A_{max})$. Because A_{max} is often set by *TPU*, it gives information about a process that rarely determines A under natural conditions. We do not estimate A_{max} .

Surprisingly, and fortunately for people studying photosynthesis rates, CO_2 assimilation can be modelled using the simple assumption that A is 100% of the lowest rate allowed by these three biochemical conditions. This requires that all parts of the leaf respond the same way to changes in the environment. This is often true for thin leaves but as leaf morphology gets more complex, and especially as leaves get thick, this condition is less likely to be fulfilled. As conditions change, A will change as predicted by the limiting process until one of the other processes becomes limiting.

Because each of these three states causes a distinctive CO_2 response, plotting A against $[CO_2]$ and modelling the response allow researchers to determine the biochemical capacities underlying photosynthesis and to see how internal and external factors affect the components of photosynthesis.

Equations needed to fit the model to data

When A is Rubisco-limited, the response of A to $[CO_2]$ can be described by the following equation:

$$A = V_{\rm cmax} \left[\frac{C_{\rm c} - \Gamma^*}{C_{\rm c} + K_{\rm C} (1 + O/K_{\rm O})} \right] - R_{\rm d}$$
(1)

where V_{cmax} is the maximum velocity of Rubisco for carboxylation, $C_{\rm c}$ is the CO₂ partial pressure at Rubisco, $K_{\rm C}$ is the Michaelis constant of Rubisco for carbon dioxide, O is the partial pressure of oxygen at Rubisco and K_0 is the inhibition constant (usually taken to be the Michaelis constant) of Rubisco for oxygen. This equation lends itself to a linear regression approach to estimating V_{cmax} as the slope and $-R_d$ as the intercept (Long & Bernacchi 2003). The symbol Γ^* is the [CO₂] at which oxygenation proceeds at twice the rate of carboxylation causing photosynthetic uptake of CO₂ to be exactly compensated by photorespiratory CO₂ release [see von Caemmerer 2000 and Ethier & Livingston 2004 for a discussion of the effect of mesophyll conductance (g_m) on this value]. In other words, Γ^* is the photorespiratory compensation point, which is slightly lower than the overall compensation point of the leaf. $R_{\rm d}$ is respiratory CO₂ release other than by photorespiration (day respiration) and is presumed to be primarily mitochondrial respiration. Because of non-photorespiratory CO_2 losses, there is a net release of CO_2 from leaves when air around the leaf has a CO_2 concentration equal to Γ^* . The derivation of this and subsequent equations has been presented many times; readers are referred to von Caemmerer (2000) for a comprehensive review, and to Long & Bernacchi (2003).

When A is limited by RuBP regeneration,

$$A = J \frac{C_{\rm c} - \Gamma^*}{4C_{\rm c} + 8\Gamma^*} - R_{\rm d}$$
(2)

where J is the rate of electron transport. This equation assumes four electrons per carboxylation and oxygenation. There are significant uncertainties in the relationship between electron transport and ATP synthesis (Baker, Harbinson & Kramer 2007). Common fluorescence techniques estimate the rate of electron transport through photosystem II and this is most closely associated with NADPH production. Based on the number of electrons required for NADP+ reduction, the conservative values of 4 and 8 are used here, but 4.5 and 10.5 have also been used. The parameter J is sometimes used to estimate a maximum rate that could be obtained at saturating light, and this is called J_{max} . The J provided here is that rate of electron transport going to support NADP+ reduction for RuBP regeneration at the measurement light intensity.

When A is limited by TPU, it is simply

$$A = 3TPU - R_{\rm d} \tag{3}$$

where TPU is the rate of use of triose phosphates but can also be any export of carbon from the Calvin cycle including direct use of photorespiratory glycine or serine. When significant glycine or serine use occurs, TPU-limited photosynthesis can *decrease* with a decrease in $[O_2]$ or increase in $[CO_2]$ (Harley & Sharkey 1991). The equation that models this effect is given by von Caemmerer (2000). The reverse sensitivity to $[CO_2]$ and $[O_2]$ can also occur because during TPU-limited photosynthesis, high phosphoglyceric acid (PGA) levels can suppress starch synthesis by inhibiting phosphoglucoisomerase (Sharkey & Vassey 1989). Therefore, TPU-limited photosynthesis is seen as no increase in A with increasing $[CO_2]$ or a decrease with increasing $[CO_2]$, but the reverse sensitivity is not reliable enough to model.

The accuracy of the photosynthesis model depends on proper representation of the kinetic properties of Rubisco. Fortunately, the kinetic properties of Rubisco among C_3 plants have been shown to be relatively conserved and thus we use a general set of kinetic parameters (Table 1; see also von Caemmerer 2000) but with caution (Tcherkez, Farquhar & Andrews 2006).

Equations 1, 2 and 3 can be put into a spread sheet and V_{cmax} , J and TPU can be adjusted manually until each modelled line meets or exceeds all of the data points. This simple approach requires prior information about R_d and g_m . Long & Bernacchi (2003) used a linear modelling method to estimate V_{cmax} , J and R_d , but g_m had to be known or estimated by iteration. Ethier and Livingston developed quadratic equations so that g_m could be estimated from the Rubisco-limited data (Ethier & Livingston 2004; Ethier *et al.* 2006). It is also possible to estimate g_m from the RuBP-regeneration-limited data using this method. The approach used here relies on a non-linear curve-fitting program to provide an estimate of the parameters. In this approach,

Table 1. The scaling constant (*c*) and enthalpies of activation (ΔH_a) , deactivation (ΔH_d) and entropy (ΔS) describing the temperature responses for ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) enzyme kinetic parameters and mesophyll conductance that are necessary for $A-C_i$ analysis over a range of temperature

	25 °C	с	$\Delta H_{\rm a}$	$\Delta H_{\rm d}$	ΔS
Parameters used for fittin	ıg				
$K_{\rm C}$ (Pa)	27. 238	35. 9774	80. 99		
$K_{\rm O}$ (kPa)	16. 582	12. 3772	23. 72		
Г* (Pa)	3. 743	11. 187	24. 46		
Used for normalizing					
V _{cmax}	1	26. 355	65. 33		
J	1	17. 71	43.9		
TPU	1	21. 46	53.1	201. 8	0.65
$R_{\rm d} \; (\mu { m mol} \; { m m}^{-2} \; { m s}^{-1})$	1	18. 7145	46. 39		
$g_{\rm m} \; (\mu { m mol} \; { m m}^{-2} \; { m s}^{-1} \; { m Pa}^{-1})$	1	20. 01	49. 6	437. 4	1.4

Estimates of each parameter at 25 °C are also provided. Values are taken from Bernacchi, *et al.* (2001, 2002) and Bernacchi, Pimentel & Long (2003) except for the *TPU* data, which are from Harley *et al.* (1992). Numbers not in bold may not be significant but help prevent rounding discrepancies.

 $K_{\rm C}$, Michaelis constant of Rubisco for carbon dioxide; $K_{\rm O}$, inhibition constant; Γ^* , photorespiratory compensation point; $V_{\rm cmax}$, maximum carboxylation rate allowed by Rubisco; J, rate of photosynthetic electron transport (based on NADPH requirement); TPU, triose phosphate use; $R_{\rm d}$, day respiration; $g_{\rm m}$, mesophyll conductance.

information in both the Rubisco-limited and RuBPregeneration-limited portions of the curve affects the estimates of g_m and R_d .

The best practice for expressing carbon dioxide levels

The mole fraction of carbon dioxide is the most commonly used measure of carbon dioxide. This is the very familiar ppm (by volume) used with gases and can be expressed as μ mol mol⁻¹, μ L L⁻¹ or μ Pa Pa⁻¹. This way of describing CO₂ is convenient because it is independent of pressure. The mole fraction of CO_2 at the top of a mountain is the same as that at the bottom of the mountain. The mole fraction of CO₂ at the beginning part of a gas-exchange system (high pressure) is the same as that at the end (low pressure). However, photosynthesis depends on the chemical activity of CO₂ at Rubisco. Chemical activity of a gas dissolved in a liquid is normally described by fugacity. When CO₂ behaves as an ideal gas, fugacity is proportional to the partial pressure of the gas in equilibrium with the air above the liquid, so partial pressure is the better measure of carbon dioxide when comparing photosynthetic rates.

The carbon dioxide is fixed (attached to an acceptor) in the stroma of the chloroplast and so A should be plotted against the CO₂ partial pressure inside the chloroplast (C_c). When CO₂ is being taken up, C_c is lower than the partial pressure in ambient air (C_a) because of the partial pressure drop as CO₂ diffuses from the air to the intercellular spaces of the leaf (C_i) and then to the inside of the chloroplast (C_c). Methods for estimating C_i from gas exchange are now routine but it was difficult in the past to estimate C_c , and because, in some species, the difference between C_i and C_c can be small, it was common to use C_i in place of $C_{\rm c}$. Thus, the analysis is normally called fitting an A/C_i curve. However, the original model was developed on the basis of chloroplast metabolism and relating all biochemistry to the conditions in the chloroplast allows direct comparisons between leaf gas exchange and the biochemistry of Rubisco and stoichiometry of electron transport. If A and C_i are known, C_c can be estimated using a mesophyll conductance (g_m) . Since g_m is, in effect, that part of the CO₂ diffusion pathway beyond the diffusion pathway of water vapour, it is often assumed to be dominated by liquid phase diffusion resistances and has the units of μ mol m⁻² s⁻¹ Pa⁻¹.

$$C_{\rm c} = C_{\rm i} - A/g_{\rm m} \tag{4}$$

Assessing mesophyll conductance

Mesophyll conductance is the inverse of the biophysical diffusion resistance encountered by CO₂ as it diffuses from the intercellular air spaces to the sites of carboxylation. It has been measured independently of any assumptions used in A/C_i curve fitting using stable carbon isotope discrimination (Evans et al. 1986). This technique is equipmentintensive and laborious. Fortunately, a reasonable estimate of g_m can be made directly from A/C_i data. Mesophyll conductance affects the effective partial pressure of CO₂ inside the chloroplast. A low mesophyll conductance has the effect of reducing the curvature of the A/C_i curve. It is possible to estimate g_m using Eqns 1 and 2 but with C_c replaced by $(C_i - A/g_m)$. By non-linear curve fitting minimizing the sum of squared model deviations from the data, g_m can be estimated from observed data. The estimate of $V_{\rm cmax}$ is especially sensitive to the estimate of $g_{\rm m}$.

Estimating limiting factors

The routine described here requires identifying whether a data point is limited by Rubisco, RuBP regeneration or *TPU*. A good starting point is to assign points above 30 Pa as RuBP-regeneration-limited and points below 20 Pa as Rubisco-limited; points between 20 and 30 Pa might be either. Data right at the transition from Rubisco to RuBP-regeneration limitations may represent a condition where some parts of the leaf are limited by one process and other parts are limited by the other process. If there are sufficient data points, it can be helpful to exclude the data point closest to the transition from the analysis. Data at very low $[CO_2]$ can be limited by Rubisco deactivation and it may be useful to exclude them from the analysis. It is possible to vary the limitation assignment to minimize the differences or to use some other algorithm to make

the assignment, but we feel it is important to examine the assignments to make the best judgement for how to fit the data.

It is common to find data sets for which one or another limitation is not apparent. Most often, TPU is the limitation that is not apparent but any limitation can be missing from a given data set. At low light intensity, there is a good chance that RuBP regeneration may limit at all C_i values. If the model output suggests that the initial estimation of limiting factors was incorrect, these should be changed and the analysis reran.

Using chlorophyll fluorescence to predict limiting factors and g_m

Chlorophyll fluorescence analysis (Baker et al. 2007) is a very powerful tool for identifying the limiting process for any given data point. If chlorophyll fluorescence indicates that photosynthetic electron transport was increasing with increasing [CO₂], then the data are Rubisco-limited. For those data points where fluorescence (and hence electron transport) did not change with [CO₂], the data belong to the RuBP-regeneration limitation. If fluorescence indicates that electron transport fell with increasing $[CO_2]$, then the points are TPU-limited. If fluorescence data are available, it may be possible to estimate g_m from those data and use the fluorescence-based g_m as an input, reducing the number of parameters that are varied in fitting the data. Using additional data from fluorescence can improve the reliability of the estimation of the rest of the parameters. Fluorescence-based estimates of g_m would also be very helpful if g_m varies significantly with $[CO_2]$ as reported by Flexas et al. (2007).

Adjusting for temperature

There are numerous data sets representing the temperature response functions for the kinetic parameters; however, the majority of these are based on C_i . As mentioned earlier, CO_2 concentrations at the enzyme are necessary to remove the impact of the diffusion resistances on A/C_i analysis; thus, the biochemically based parameters determined after accounting for diffusion resistances (e.g. K_m) are preferable over parameters that do not (e.g. 'effective K_m '; Bernacchi *et al.* 2002).

In vitro-derived temperature responses of Rubisco kinetic parameters require assumptions of the *in vivo* chloroplast conditions, for example, pH, and thus it is preferable to use *in vivo*-derived parameters (Bernacchi *et al.* 2001). One data set providing the *in vivo* temperature response functions of these parameters based on the chloroplast CO_2 concentrations is given in Table 1. Analysis of an A/C_i curve should incorporate the values for the parameters corresponding to the measurement temperature to obtain a proper estimate of each parameter.

The parameters estimated from the analysis of an A/C_i curve respond to measurement temperature, thus

comparisons between two treatments are often made at a single temperature. Representative temperature responses of the fitted parameters are used to adjust these values to a single temperature, in this case, $25 \,^{\circ}$ C.

The dependence of reaction rates on temperature is exponential. The equations used here can be found in Harley *et al.* (1992):

Parameter =
$$e^{\left(c - \frac{\Delta H_a}{R \cdot T}\right)}$$
 (5)

or

Parameter =
$$\frac{e^{\left(c - \frac{\Delta H}{R \cdot T}\right)}}{1 + e^{\left(\Delta S \cdot T - \frac{\Delta H_{d}}{R \cdot T}\right)}}$$
(6)

where c is a scaling constant, ΔH_a is an enthalpy of activation, ΔH_d is enthalpy of deactivation and ΔS is entropy. The equation for the high temperature decline in *TPU* and g_m is provided as an example but the decline in photosynthetic parameters at high temperature may result from (1) inherent sensitivities to temperature which could vary from species to species and could be affected by growth conditions, or (2) compensatory mechanisms designed to reduce deleterious processes such as photorespiration. Other deactivation equations are not given here but this does not mean that these processes are expected to continuously increase with temperature.

The scaling constant for the equations used to adjust the parameters is chosen to cause the result to be 1 at 25 °C and the calculated value at other temperatures can be used to scale the parameter to 25 °C. The values here assume $R = 8.314 \text{ J} \text{ mol}^{-1} \text{ K}^{-1}$ and 0 °C = 273.15 K. If different degrees of rounding are used, the values at 25 °C do not perfectly equal those shown in Table 1.

The parameters to be estimated

In summary, there are five parameters that need to be estimated to analyse A/C_i curves. These are V_{cmax} , J, TPU, R_d and g_m . With five variables, it is clear that small or noisy data sets will be subject to significant estimation problems.

The principles described here have been used to make an estimator utility that can be found at http:// www.blackwellpublishing.com/plantsci/pcecalculation/. The sum of squares of the deviations between the observed and modelled points can be 1 or less as a result of the fitting routine, likely much less than the noise present in the data. With five parameters that can be varied, it is relatively easy to get very good fits, especially for small data sets. For this reason, users must make judgements about limiting factors, and about which points to include in the fitting. If one or another parameter can be constrained using other data (e.g. independent assessments of g_m), fewer degrees of freedom are present and the analysis may be improved. It is important when interpreting the data from this or any other curve-fitting exercise to keep in mind that precision can far outstrip accuracy.

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