Plant, Cell & Environment

Plant, Cell and Environment (2016) **39**, 1198–1203

doi: 10.1111/pce.12722

Technical Report

An improved approach for measuring the impact of multiple CO_2 conductances on the apparent photorespiratory CO_2 compensation point through slope–intercept regression

Berkley J. Walker[1,2], Dane C. Skabelund[3], Florian A. Busch[4] & Donald R. Ort[1,2,5]

[1]*Global Change and Photosynthesis Research Unit, USDA/ARS, Urbana, IL 61801, USA,* [2]*Carl R. Woese Institute for Genomic Biology, University of Illinois, Urbana, IL 61801, USA,* [3]*Department of Mathematics, University of Illinois, Urbana, IL 61801, USA,* [4]*Research School of Biology, The Australian National University, Canberra, ACT 2601, Australia and* [5]*Department of Plant Biology, University of Illinois, Urbana, IL 61801, USA*

ABSTRACT

Biochemical models of leaf photosynthesis, which are essential for understanding the impact of photosynthesis to changing environments, depend on accurate parameterizations. One such parameter, the photorespiratory CO_2 compensation point can be measured from the intersection of several CO_2 response curves measured under sub-saturating illumination. However, determining the actual intersection while accounting for experimental noise can be challenging. Additionally, leaf photosynthesis model outcomes are sensitive to the diffusion paths of CO_2 released from the mitochondria. This diffusion path of CO_2 includes both chloroplastic as well as cell wall resistances to CO_2, which are not readily measurable. Both the difficulties of determining the photorespiratory CO_2 compensation point and the impact of multiple intercellular resistances to CO_2 can be addressed through application of slope–intercept regression. This technical report summarizes an improved framework for implementing slope–intercept regression to evaluate measurements of the photorespiratory CO_2 compensation point. This approach extends past work to include the cases of both Rubisco and Ribulose-1,5-bisphosphate (RuBP)-limited photosynthesis. This report further presents two interactive graphical applications and a spreadsheet-based tool to allow users to apply slope–intercept theory to their data.

Key-words: FvCB photosynthesis model; mesophyll conductance; photosynthesis.

INTRODUCTION

Biochemical models of leaf photosynthesis are a critical tool for plant biology given their ability to predict and potentially optimize CO_2 assimilation under present and future climate (Sage and Kubien 2007; Zhu et al. 2008). Rubisco reaction kinetics are the cornerstone of these models (Farquhar *et al.* 1980; von Caemmerer & Farquhar 1981). Rubisco catalyses reactions between Ribulose-1,5-bisphosphate (RuBP) and either CO_2 resulting in carbon gain, or O_2, which results in carbon loss through photorespiration. Rubisco's specificity for reaction with CO_2 as opposed to O_2 and the carbon lost through photorespiration is quantified by measurement of the photorespiratory CO_2 compensation point (Γ^*). Γ^* can be estimated in C3 plants from the *x*-value of the common intersection of several photosynthetic CO_2 response (A-C_i) curves measured at numerous sub-saturating illumination levels (Brooks & Farquhar 1985; Laisk 1977; Fig. 1).

Like any mechanistic model, accuracy depends on correct assumptions of plant physiology. There is active discussion concerning two major assumptions commonly applied to measurements of Γ^* and the standard Farquhar, von Caemmerer and Berry model of leaf photosynthesis generally. The first assumption concerns the accuracy of measurements of Γ^* using the common intersection method and the second concerns how best to account for the diffusion of CO_2 from the intercellular airspace and from the mitochondria to the chloroplast (Tholen & Zhu 2011; Tholen *et al.* 2012; Busch *et al.* 2013; von Caemmerer 2013; Gu & Sun 2014). CO_2 entry into the chloroplast is typically considered as a single mesophyll conductance (g_m) between the intercellular airspace and the chloroplast. This results in a simple relationship between the intercellular CO_2 partial pressure measured at the *x*-value of the common intersection (C_i*) and Γ^* according to

$$\Gamma^* = C_i^* + R_d/g_m, \tag{1}$$

where R_d is the rate of day respiration.

The model presented in Eqn 1 assumes that all CO_2 release from the mitochondria from photorespiration passes through the chloroplast and is either re-fixed by Rubisco, or diffuses to the intercellular airspace. This assumption can be amended to include multiple diffusive paths of CO_2, but it is currently unclear how important it is to consider these multiple diffusive paths when modelling leaf photosynthesis. Additionally, when multiple diffusive resistances are inserted into Eqn 1, there is no longer a common intersection of A-C_i curves measured at sub-saturating light intensities which invalidates current methods for measuring C_i^* (Tholen *et al.* 2012).

Correspondence: D. R. Ort; e-mail: d-ort@illinois.edu

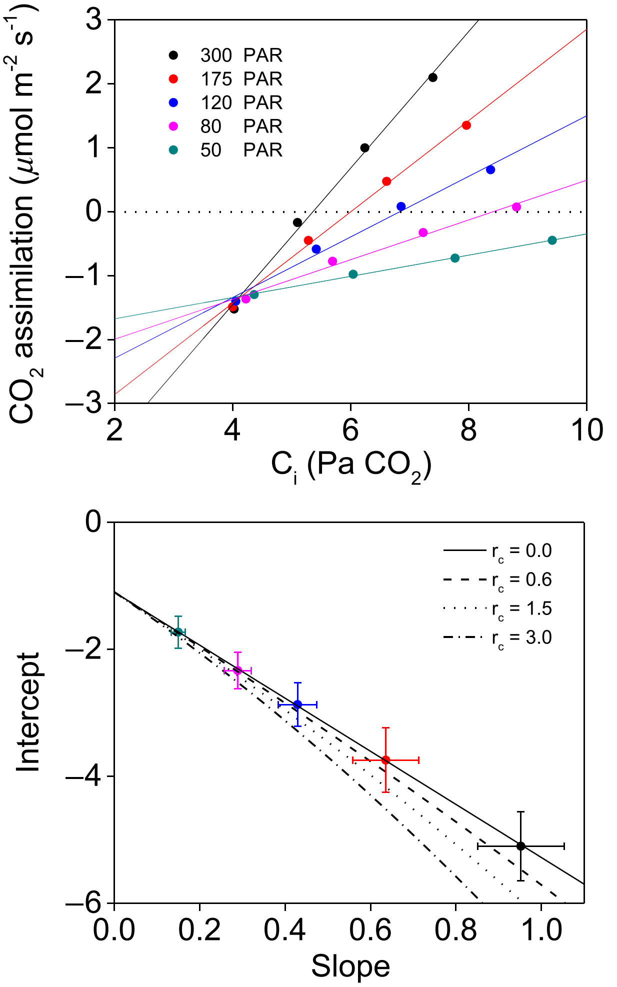

Figure 1. An example intercellular CO_2 photorespiratory CO_2 compensation point (C_i^*) measurement using the common intercept method (a) and slope intercept regression (b). Shown is a single replicate of a measurement of the *Glycine max* CO_2 response of net CO_2 assimilation measured at four sub-saturating light intensities indicated by the PAR values on the plot with linear fits shown (a). Also shown is the relationship between the slope and intercept of a number ($n = 4$) of common intercept measurements (b). The dotted lines represent the expected relationship given different assumptions of chloroplast resistance to CO_2. Data originally from (Walker & Ort 2015).

Both of the assumptions mentioned previously were the subject of recent work, which determined optimal measuring conditions for C_i^* and quantified the impact of different assumptions of CO_2 diffusion to the common intersection method using slope–intercept regression analysis (Walker & Ort 2015). Traditionally, C_i^* was determined from the *x*-value of the intersection of several A-C_i curves measured under sub-saturating illumination levels and R_d at the *y*-intersection (Fig. 1a). Determining the precise *x* and *y* values of the intersection of these A-C_i curves is complicated by the fact that intersection between two lines is more sensitive to experimental noise when they have increasingly similar slopes. Slope–intercept regression analysis weights against experimental noise by plotting the slopes and the *y*-intercepts of individual A-C_i curves and determining the linear regression of these lines to produce a slope and *y*-intercept value (Fig. 1, Mathematical Theory and Technique; Walker & Ort 2015). The slope of this linear regression is equal to negative C_i^* and the *y*-intercept equal to negative R_d, but only when CO_2 release from the mitochondria from photorespiration passes exclusively through the chloroplast and Eqn 1 accurately represents CO_2 transfer during photosynthesis (Walker & Ort 2015). Slope–intercept regression results in a more robust measurement of common intersection measurements for two main reasons. The first reason is that when a common intersection is expected, a regression of the slopes and *y*-intercepts determines C_i^* and R_d in a way that is not as sensitive to experimental errors found between A-C_i curves of a similar slope (Walker & Ort 2015). The second reason is that slope–intercept plots of common intercept measurements more clearly show the impact of different assumptions of CO_2 diffusion during photosynthesis, because these result in a curvilinear relationship between the slope and intercept of CO_2 response curves (Fig. 1).

The purpose of this technical report is to present an improved approach for applying slope–intercept analysis to common intersection measurements of Γ^*. This approach is more directly derived from the relevant photosynthetic relationships and additionally considers photosynthesis both under RuBP-saturated and RuBP-limited conditions. This approach can also be more readily used to quantify values of internal cellular resistances through least squares fitting. In addition to providing this framework we present several tools to allow researchers from diverse backgrounds to apply slope–intercept regression analysis to their data. The tools we present provide an example of how complex models can be presented to the research community in a way that allows them to be readily implemented. Finally, we show the capability of slope–intercept analysis by fitting an example data set and discuss the strengths and limitations of this approach.

MODELLING APPROACH

This section introduces the approach behind a novel technique for analyzing and modelling slope–intercept data. The following approach models the slopes and the *y*-intercepts of lines tangent to an A-C_i curve when C_i is equal to C_i^*, which is the common intersection *x*-value when CO_2 is assumed to diffuse through a single path. This framework can be applied to data produced by measuring several A-C_i curves at low intercellular CO_2 partial pressures (C_i) under different illumination levels. These A-C_i curves are individually fitted using linear regression to produce a slope and *y*-intercept for each light intensity (Fig. 1). This approach has the advantage of determining the fundamental relationship between the slope and *y*-intercept of A-C_i curves in a form with fewer assumed or fitted parameters.

The relationship between the slope and intercept of CO_2 response curves measured under sub-saturating illumination was initially determined using a 'brute force' approach (Walker & Ort 2015). In this brute force approach, the slope and *y*-intercept was modelled from lines fitted to modelled CO_2

response curves at several C_i values close to C_i^* assuming RuBP-regeneration limitation. This process was repeated using many values of potential photosynthetic electron transport rates (J) to develop a discontinuous depiction of the relationship between each line's slope and intercept to compare with measured data. This relationship was modelled with different assumptions of chloroplastic (r_c) and cell wall (r_w) resistance to CO_2 diffusion to determine the impact of multiple diffusion paths of photorespired CO_2 to net gas exchange. This approach determines the impact of multiple resistances to C_i^* measurements but is cumbersome to implement and use to determine values of r_c and r_w using least-squared fitting approaches. Additionally, it only considers photosynthesis as RuBP-regeneration limited, which is a good assumption at low light intensities but may not be correct at higher light intensities where photosynthesis may still be Rubisco-limited at the lower values of C_i.

We propose the following approach to determine the impact of changes in r_c and r_w to the relationship between the slope and intercept of common intercept measurements. This improved model has the benefit of representing the relationship between the slope and intercept of A-C_i curves in a more fundamental, but just as robust way. The approach in the succeeding texts also outlines how the relationship between the slope and the intercept of lines tangent to A-C_i curves can be defined independently from J or V_{cmax} when C_i is equal to C_i^*. This simplification serves to decrease the number of parameters used in least-squared fitting of r_c and r_w. Additionally, this expanded approach includes derivations for both RuBP-saturated and Rubisco-limited photosynthesis. The basis of this approach is to determine the slope and y-intercept of a line tangent to the point when C_i is equal to C_i^*. This line is approximated in practice using measured points close to C_i^*. The following is an abbreviated derivation. For a fully detailed step-by-step derivation refer to Text S1.

This improved modelling approach begins with the Fick's law definition of net CO_2 assimilation (A) between the intercellular airspace and the mesophyll

$$A = \frac{C_i - C_m}{r_w}, \tag{2}$$

where C_i and C_m represent the partial pressure of CO_2 (pCO_2) in the intercellular airspace and mesophyll cytoplasm, respectively. Under lower pCO_2, A is limited either by the rate of RuBP-regeneration or the rate of Rubisco catalysis of RuBP (RuBP-saturated). These cases can be modelled assuming RuBP-regeneration limitation as

$$A = \frac{J(C_c - \Gamma^*)}{4C_c + 8\Gamma^*} - R_d \tag{3}$$

or RuBP-saturated as

$$A = \frac{Vcmax(C_c - \Gamma^*)}{C_c + K_c(1 + O/K_o)} - R_d, \tag{4}$$

where C_c, R_d, O, K_c and K_o represent the pCO_2 in the chloroplast, rate of CO_2 release from day respiration, the partial pressure of O_2 and the kinetic affinities of Rubisco for CO_2 and O_2 (von Caemmerer 2000). RuBP-limited and RuBP-saturated photosynthesis can also relate C_c and C_m according to

$$C_c^2 - C_c\left(C_m - \frac{r_c J}{4} - 2\Gamma^*\right) - 2C_m\Gamma^* = 0 \tag{5}$$

for RuBP-limited and

$$C_c^2 - C_c\left(C_m - r_c V_{cmax} - K_c\left(1 + \frac{O}{K_o}\right)\right) - 2C_m K_c\left(1 + \frac{O}{K_o}\right) = 0 \tag{6}$$

for RuBP-saturated as derived previously (von Caemmerer 2013). Both cases of photosynthetic limitation can be derived at once by introducing two constants that are substituted with different values depending on whether RuBP-limited or RuBP-saturated photosynthesis is considered according to

$$f = \begin{cases} 2\Gamma^* & RuBP - limited, \\ K_c\left(1 + \dfrac{O}{K_o}\right) & RuBP - saturated, \end{cases} \text{ and } g = \begin{cases} J/4 & RuBP - limited, \\ V_{cmax} & RuBP - saturated. \end{cases} \tag{7}$$

This allows us to represent Eqns 2, 3, 4 and 5 as simply

$$A = \frac{C_c - \Gamma^*}{C_c + f} g - R_d \tag{8}$$

and

$$C_c^2 - C_c(C_m - r_c g - f) - fC_m = 0. \tag{9}$$

To determine the instantaneous slope of the A-C_i curve when $C_i = C_i^*$ for slope–intercept modelling, Eqns 2, 8 and 9 can be differentiated and re-arranged to produce

$$m = \frac{dA}{dC_i}(C_i) = \left(r_w + r_c\frac{f}{f + \Gamma^*} + \frac{f + \Gamma^*}{g}\right)^{-1}, \tag{10}$$

where m represents the slope of the A-C_i curve when $C_i = C_i^*$.

The y-intercept (b) of the tangent line $Y = mX + b$ to the A-C_i curve at the point when $X = C_i = C_i^*$ and $Y = A = -R_d$ can then be represented in the form

$$b = -C_i^*\frac{dA}{dC_i}(C_i^*) - R_d, \tag{11}$$

which, when combined with Eqn 10, produces

$$b = -R_d - (\Gamma^* - r_w R_d)m - \frac{r_c\Gamma^* m^2}{1 - \left(r_w + r_c\frac{f}{f+\Gamma^*}\right)m}. \tag{12}$$

Although the quantities b and m certainly depend on the value of J (in the RuBP-limited case) or V_{cmax} (in the RuBP-saturated case), the relationship between b and m given in Eqn 12 is independent of these values. This is because the other quantities appearing in the equation (r_c, r_w, R_d, Γ^* and f) have no dependence on J or V_{cmax}. Indeed, obtaining such a relationship was the purpose of the preceding manipulations. As J or V_{cmax} vary, the values of b and m should change, but the relationship will still be related by Eqn 12, which can then be used to model the relationship between m and b from A-C_i curves measured under sub-saturating illumination. The model can be further amended to account for the impact of oxygen partial pressure (O), which is reflected in Γ^* according to

$$\Gamma^* = \frac{0.5O}{S_{c/o}}. \tag{13}$$

Modelling based on this refined approach allows more sensitive insight into how differences in internal CO_2 diffusion paths from the mitochondria during photorespiration (r_c and r_w) can impact rates of net gas exchange.

MATERIAL AND METHODS

Mathematica and SAGE application building

The approach presented within was incorporated into two graphical applications using both closed and open-source computer algebra packages. The first package was Mathematica, a closed-source programme produced by Wolfram Research (Wolfram Research 2015). Mathematica code can only be edited on computers with the Mathematica software installed, but can be exported as a CDF file which can be executed on any computer with the free CDF player installed (www.wolfram.com/cdf-player). The open source package used was Sage (Sage Development Team, 2016). Sage is most easily implemented on non-Linux based computers using the online Math Sage Cloud (www.sagemath.com). The Sage application has all the functionality of the playable CDF file but can be edited with freely available software and allows direct import and export of data for modelling. Details of how to use these two applications may be found in the Supporting Information.

Excel-based tool for least-squared fitting

We developed an Excel-based tool to estimate and model the apparent CO_2 compensation point (C_i*) from A/C_i curves measured at different light intensities. In this tool, the slope and intercept of the tangent at C_i* is approximated by linear regression over the initial parts of the measured A/C_i curves. The obtained slopes and intercepts are then fitted with one of two models: the first model assumes that there is a single mesophyll resistance, which is mathematically equivalent to setting $r_c = 0$ in Eqn 12 and taking r_w as equivalent to r_m. In this case, a linear regression of the slope–intercept data gives an estimate of R_d (equal to the negative of the y-intercept of the regression) and C_i* (equal to the negative of the slope of the regression). Equation 1 can then be used to estimate r_m with a given value of Γ^*, or to estimate Γ^* with an independently determined value of r_m. The second model involves fitting Eqn 12 to the slope–intercept data by varying the parameters r_c, r_w and R_d using the Solver add-on in Excel. The tool gives researchers the opportunity to assess the impact of the assumed Γ^* on the fitted parameters and allows for easy graphing of different values of r_c, r_w and R_d. Please note that given the strong dependence of Γ^* (which may change with species under investigation as well as growth condition) on the fitted values of r_c and r_w, fitting for multiple mesophyll resistances should be used as a tool to explore data rather than to derive absolute values of r_c and r_w. The default parameterization of Γ^* in the Excel spread sheet is taken from Bernacchi *et al.* (2002)

Plant growth and plant measurement setup

Data for slope–intercept fitting were collected previously from *Glycine max* as outlined in Walker & Ort (2015). Briefly, seeds were sown in 3 L pots containing potting soil (Sunshine Mix #1 LC1, SunGro Horticulture, Agawam, MA, USA). Plants were grown in a climate-controlled cabinet (Conviron, Winnipeg, Manitoba, Canada) with day/night cycles of 11/13 h and 25/23 °C under an irradiance of 300 μmol m^{-2} s^{-1}. Plants were watered as needed and fertilized weekly (Peters 20-20-20, J.R. Peters, Allentown, PA, USA).

The youngest fully expanded leaves of 30–40 day old plants were used for gas exchange measurements using a LI-COR 6400 XT modified to reach low CO_2 partial pressures using a 6 cm^2 chamber with a red/blue light source (LI-COR Biosciences, Lincoln, NE, USA, LI-COR Biosciences 2010). During the measurements, leaf temperature was kept constant at 25 °C. Assimilation measurements were corrected for CO_2 leakage between the chamber and surrounding atmosphere according standard methods as prescribed by the manufacturer (LI-COR Biosciences 2011).

RESULTS AND DISCUSSION

The approach presented here provides a new tool for understanding the impact of multiple CO_2 conductances to net gas exchange and for improving measurements of Γ^*, a vital component of leaf models of photosynthesis. One major advantage of this improved approach is that it removes the need to assume values of additional modelling terms J or V_{cmax}, when comparing the model to measured slope–intercept data by isolating the relationship between the slope and intercept (Eqn 12). With J or V_{cmax} thus removed from the model, r_c and r_w can be more robustly fitted to slope and intercept data with fewer assumed (or fitted) variables.

The approach taken here also revealed that the curvature of the slope–intercept regression is not sensitive to assumptions of photosynthetic limitation when r_c is included as a non-zero value. This can be seen by the similarities in the modelled lines of the slope–intercept relationship assuming either RuBP-saturated or RuBP-limited photosynthesis (Fig. 2). Minor differences between the assumptions of photosynthetic limitation become apparent at higher values of slopes and attendant lower values of intercepts (Fig. 2a). However, these differences are not seen when the modelling is scaled to within physiologically relevant ranges (Fig. 2b). Insensitivity to assumptions of limitations to photosynthesis makes slope–intercept regression modelling an especially robust tool to understand the impact of multiple intercellular CO_2 diffusion paths because one does not need to assume that photosynthesis is RuBP-saturated or RuBP-limited to observe a change in curvature.

While this slope–intercept model eliminates the need to assume or fit J values in order to fit the CO_2 response of photosynthesis measured at different light intensities, it does require

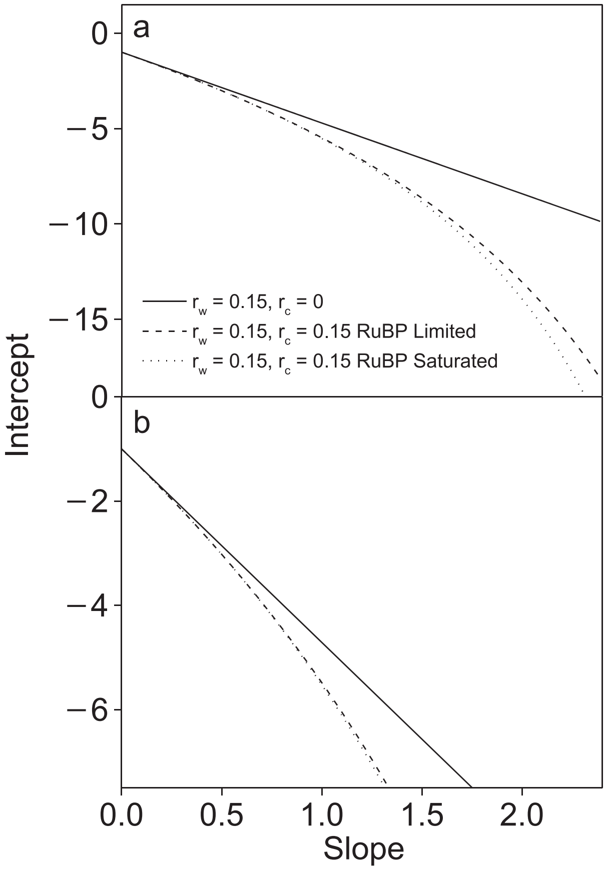

Figure 2. Comparison of modelled RuBP-limited and RuBP-saturated slope–intercept regressions of common intercept measurements. Relationships are shown with an expanded *y*-axis (a) and an axis more in line with what is physiologically relevant (b). Modelled lines represent the relationship of the slope and intercept assuming no chloroplastic resistance to CO_2 ($r_w = 0.15$ and $r_c = 0.00\ m^2\ s\ MPa\ mol^{-1}$, solid), chloroplastic resistance to CO_2 under RuBP-limited photosynthesis ($r_w = 0.15$ and $r_c = 0.15\ m^2\ s\ MPa\ mol^{-1}$, dashed) and RuBP-saturated photosynthesis ($r_w = 0.15$ and $r_c = 0.15\ m^2\ s\ MPa\ mol^{-1}$, dotted). Lines were modelled assuming $R_d = 1$ and $\Gamma^* = 3.86$ Pa CO_2.

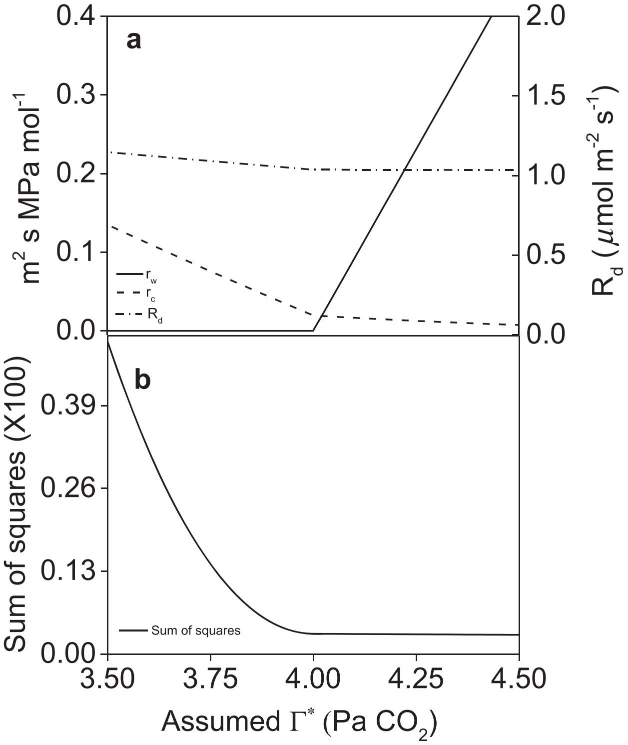

Figure 3. (a) The impact of assumptions of the CO_2 photorespiratory CO_2 compensation point (Γ^*) on fitted values of chloroplastic (r_c, dashed line) and cell wall resistance to CO_2 (r_w, solid line) determined from slope–intercept regressions of common intercept measurements. (b) The sum of squares values (dotted line) is also presented.

assumed values of R_d and Γ^* (Eqn 12). While R_d can be determined from the *y*-intersection of the slope–intercept plot regardless of assumptions of r_c or r_w, values of Γ^* are more difficult to determine because they are often obtained from measurements of C_i^* assuming a simple g_m. The value of Γ^* can theoretically be determined from *in vitro* measurements of $S_{c/o}$ (Eqn 13), but often Γ^* determined this way is lower than would be expected based on common intercept measurements. The value assumed for Γ^* has a significant impact on fitted values of r_c and r_w, as can be seen from Fig. 3 and can be explored with the tools provided in the Supporting Information. At assumed values of Γ^* below the C_i^* value (4.08 Pa CO_2), r_c is larger than r_w. As assumed values of Γ^* increase above C_i^*, r_w rapidly increases and r_c gradually declines.

It is interesting that even at relatively high values assumed for Γ^*, r_c does not decrease to zero. This suggests that the chloroplast may impose a non-trivial resistance to CO_2 diffusion, although the significance of this resistance to net gas exchange is not large enough to produce a visual curvature to the slope–intercept regression. Stronger conclusions concerning the absolute values and impact of r_c and r_w are limited by the availability of accurate and reliable Γ^* values.

The common intersection method was initially developed in a time when personal computing power was limited (Laisk 1977; Brooks & Farquhar 1985). Now that computing power is readily available, is it still necessary to use these older methods and simplifications to determine values for Γ^* or C_i^*? For example, one approach would be to fit for Γ^* in common intersection data to the entire RuBP-limited model; however, the model is already over-parameterized when estimating $J_{\max}$, V_{cmax} and R_d (Gu *et al.* 2010). This fitting would additionally require further fitting for either a single or multiple CO_2 diffusive resistances to CO_2. It is unlikely that fitting to the model would result in meaningful results with the addition of two to three terms, but future progress in fitting using statistical-based methods or with measurements under different measuring conditions may hold promise.

Another weakness in slope–intercept regression analysis is the assumption that an A-C_i curve at lower values of C_i is linear. In reality, the slope of an A-C_i curve is more sensitive to pCO_2 at lower values because C_c is in the denominator of the derivative (S1, Eqn A10). This assumption of linearity results in underestimations of C_i^* and R_d using the common intersection method (Gu & Sun 2014). Fortunately, underestimations of C_i* and R_d resulting from assumptions of linearity can be minimized if the range of C_i values is restricted. For example, this approach was tested using measured and simulated datasets and revealed that C_i* was only underestimated by 1% and R_d by 10% when A- C_i curves used points under 10 Pa (Walker & Ort 2015). Selecting these measurement points is complicated by the fact that C_i^* is at the low end of this range (usually ~4 Pa CO_2). Ideally, an equal number of points would be selected above and below C_i^* to minimize the fitting bias to the upper part of the A-C_i curve; however, we observe that measurements made below ~3 Pa CO_2 often result in higher-than-expected photosynthetic rates. This observation suggests that there are changes in CO_2 exchange at low pCO_2 not currently described in the standard biochemical model of photosynthesis. Linear fits may also therefore be valuable in situations where the standard biochemical model of leaf photosynthesis does not fully describe an A-C_i curve and data need to be fitted independently from modelling assumptions.

This improved approach has been incorporated into three easy-to-use tools for researchers who have common intercept data and would like to re-examine it in the context of slope–intercept regression (Files S3, S4, and S5). The first application consists of a Mathematica file formatted to be used with the free-to-download Wolfram CDF Player (www.wolfram.com/cdf-player/). This tool allows non-specialists to freely explore the parametrizations of the slope–intercept model without having to build their own models or have access to the full version of Mathematica. Similar CDF-based tools have been built for leaf and canopy-level models of leaf photosynthesis and allow easy manipulation of key parameters of photosynthesis and environment (Bernacchi *et al.* 2013). The second tool was developed on the open-source computer algebra system Sage (http://www.sagemath.com/). This second tool allows researchers to import their own common intercept data for analysis and export modelled results. The third tool, which uses the Excel solver add-on, allows for a simple analysis of A/C_i curves measured at sub-saturating illumination, estimating C_i* and R_d assuming a single mesophyll resistance, and r_c, r_w and R_d assuming multiple mesophyll resistances. Together these tools also provide a unique example of how biological models in general can be made more accessible to the research community.

CONCLUSION

In this technical report we presented an improved approach for analyzing common intercept measurements of C_i* using slope–intercept regression. This approach further shows insensitivity to key assumptions of photosynthetic rate limitation and has been packaged into three easy-to-use applications that allow it to be used more broadly by the non-specialist. Slope–intercept regression analysis of *Glycine max* common intercept data indicated that fitted values of r_c and r_w are limited by the availability of independently determined Γ* values.

ACKNOWLEDGMENTS

This research was supported via subcontract by the Bill and Melinda Gates Foundation (OPP1060461) titled 'RIPE-Realizing Increased Photosynthetic Efficiency for Sustainable Increases in Crop Yield'. F.A.B. is supported by the ARC Centre of Excellence for Translational Photosynthesis.

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Received 15 October 2015; accepted for publication 22 January 2016

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