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Improving the estimation of mesophyll conductance to CO₂: on the role of electron transport rate correction and respiration

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Abstract

Mesophyll conductance (gₘ) can markedly limit photosynthetic CO₂ assimilation and is required to estimate the parameters of the Farquhar–von Caemmerer–Berry (FvCB) model properly. The variable J (electron transport rate) is the most frequently used method for estimating gₘ, and the correct determination of J is one of its requirements. Recent evidence has shown that calibrating J can lead to some errors in estimating gₘ, but to what extent the parameterization of the FvCB model is affected by calibrations is not well known. In addition to determining the FvCB parameters, variants of the J calibration method were tested to address whether varying CO₂ or light levels, possible alternative electron sinks, or contrasting leaf structural properties might play a role in determining differences in αβ, the product of the leaf absorptance (α) and the photosystem II optical cross-section (β). It was shown that differences in αβ were mainly attributed to the use of A/C₁ or A/PPFD curves to calibrate J. The different αβ values greatly influenced gₘ, leading to a high number of unrealistic values in addition to affecting the estimates of the FvCB model parameters. A new approach was devised to retrieve leaf respiration in the light from combined A/C₁ and A/Cₑ curves and a framework to understand the high variation in observed gₘ values. Overall, a background is provided to decrease the noise in gₘ, facilitating data reporting and allowing better retrieval of the information presented in A/C₁ and A/Cₑ curves.

Key words: A/C₁ curve fitting, chlorophyll fluorescence, Coffea arabica, Limonium gibertii, Nicotiana tabacum, variable J method.

Introduction

Photosynthesis is a major process that affects plant growth and crop productivity. In addition to stomatal and biochemical factors, the photosynthetic capacity of leaves is also determined by the mesophyll conductance (gₘ), which regulates the CO₂ flux from the intercellular airspaces to the sites of carboxylation in the chloroplastic stroma (Flexas et al., 2012). Early gas exchange studies assumed infinite and constant gₘ, implying that the CO₂ concentrations in the substomatal cavities (Cᵢ) and chloroplasts (Cₑ) would be the same (Farquhar et al., 1980). However, the role of finite gₘ limiting photosynthesis in response to several biotic and abiotic stresses (Flexas et al., 2008), as well as its importance in constraining maximum photosynthetic rates, particularly in evergreen sclerophylls (Warren et al., 2004; Niinemets et al., 2011), is now well established. Actually, there is a general consensus that gₘ must be incorporated into the Farquhar–von Caemmerer–Berry (FvCB) model of leaf photosynthesis (Farquhar et al., 1980) because this model underestimates the maximum Rubisco carboxylation rate (Vₑₘₚ) when gₘ is considered to be infinite (Ethier and Livingston, 2004; Niinemets et al., 2009a).

Several methods to estimate gₘ have been reported (Warren, 2006; Pons et al., 2009). Among them, those based...
on gas exchange coupled with the chlorophyll fluorescence technique have been extensively used. In particular, the variable J method (where J stands for electron transport rate, usually measured using chlorophyll fluorescence analysis) is the most widespread method because it allows the point-specific estimation of $g_m$ as well as tracking how $g_m$ supposedly changes in response to varying light or $CO_2$ (Pons et al., 2009). However, the variable J method is highly sensitive to errors in some parameters, especially the $CO_2$ compensation point in the absence of photorespiration ($Γ^*$) and J (Harley et al., 1992). Importantly, J needs to be calibrated due to the uncertainties in both the leaf absorptance ($α$) and photosystem II (PSII) optical cross-section ($β$).

The J calibration is based on the analysis of response curves of photosynthetic rates to light [A/photonsynthetic photon flux density (PPFD)] or $CO_2$ (A/$CO_2$) under non-photorespiratory conditions, typically under low (1–2%) oxygen conditions. Under these circumstances, the relationship between J calculated through gas exchange ($J_A$) and chlorophyll fluorescence ($J_F$) is expected to be linear because electron transport flow is primarily associated with Rubisco carboxylation. Consequently, $J_A$ can be calculated as $J_A=4(A+R_L)$ (Warren, 2006). Other assumptions are that $αβ$ or light respiration ($R_L$) values do not change in response to varying $C_i$ or PPFD.

to date, three different approaches have been used to calibrate J: the relationship between the quantum yield of PSII ($Φ_{PSII}$) and $CO_2$ ($Φ_{CO_2}$) (Valentini et al., 1995), the relationship between $J_A$ and $J_F$ (Pons et al., 2009), and the plot of A versus PPFD $Φ_{PSII}$/4, which is also used to estimate $R_L$ (Yin et al., 2009). Another point deserving attention is the use of $A/PPFD$ or A/$C_i$ curves under low $O_2$ to perform the calibration; ideally, both curves should give the same results. However, as observed by Yin et al. (2009), fundamental differences exist when choosing $A/PPFD$ or A/$C_i$ curves to calibrate J. First, when using the full A/$C_i$ curve, photorespiration can still occur at low $CO_2$ levels. Alternatively, the excess of energy can increase the rate of alternative electron flow. Both cases can compromise the linearity of the relationship between $J_A$ and $J_F$. The same problem occurs in A/$PPFD$ curves, where higher PPFD intensities can induce alternative electron flow (depending on $g_m$, low $C_i$ can occur as well). To overcome this problem, Yin et al. (2009) recommended using the electron transport-limited regions of both curves, namely the combination of low light levels from A/$PPFD$ and high $C_i$ levels from A/$C_i$, which would ultimately minimize the risk of photorespiration and alternative electron flow.

More recently, Gilbert et al. (2012) examined the use of $A/PPFD$ or A/$C_i$ curves to calibrate J and demonstrated dramatic changes in $g_m$ values estimated using either the A/$PPFD$ or A/$C_i$ curves. However, to what extent these calibrations affect estimates of photosynthetic parameters such as $V_{max}$ or $J_{max}$, and whether this effect is dependent on the species studied, remain unresolved. Flexas et al. (2007) found no differences using both calibrations for tobacco, and extrapolated this result to other species where only the A/$PPFD$ calibration was used. Hassiotou et al. (2009), using the calibration based on A/$C_i$ curves performed under two light levels, concluded that calibration is light dependent. Other studies did not use the A/$PPFD$ or A/$C_i$ calibration, but estimated $α$ using integrating spheres and assumed a $β$ value of 0.5 (e.g. Galmés et al., 2007; Tosens et al., 2012), even though $β$ has been reported to vary (Laikis and Loreto, 1996). Collectively, these results reveal no consensus on how to calibrate J, even though $g_m$ estimated by the variable J method is mainly dependent on J. In addition, because the fluorescence signal primarily emanates from the upper mesophyll layers, but gas exchange parameters are volume based (Warren et al., 2006), measured J and estimated $g_m$ are most probably less representative of the whole leaf in species with lower specific leaf area (SLA; leaf area per unit dry mass).

The main questions asked in this study were as follows. (i) How do the J calibrations affect $g_m$? (ii) How might these calibrations be translated into the FvCB parameters? (iii) Would the calibrations be dependent on species? To answer these questions, measurements were carried out using three species with contrasting SLA and photosynthetic capacities. The results highlight how $g_m$, $V_{max}$, and $J_{max}$ may be affected by using A/$C_i$ or A/$PPFD$ curves under low $O_2$ to calibrate J. The results are discussed in the context of current models of $g_m$ estimations.

Materials and methods

Plant material and growth conditions

Limonium giberitii (Senn.) Senn. and Nicotiana tabacum L. seeds were germinated, and plants were grown outdoors under typical Mediterranean climate conditions (Balearic Islands, Spain, 39°38’N, 2°38’E, 85 m a.s.l.) in 4 litre pots with a commercial substrate (horticultural peat) and perlite at a proportion of 4:1. Seedlings of Coffea arabica L. obtained from seeds were grown outdoors under subtropical conditions in Viçosa (20°45’S, 42°15’W, 650 m a.s.l.), southeastern Brazil, using 12 litre pots containing a mixture of soil, sand, and composted manure (4:1:1, v/v/v). Plants were irrigated and fertilized as required. Measurements were performed during the summer (growing season) on ~1-year-old plants in the case of C. arabica and L. giberitii, and 1-month-old plants in the case of N. tabacum, on 4–6 plants per species.

Gas exchange and fluorescence measurements

Leaf gas exchange and chlorophyll a fluorescence were measured simultaneously with an open-flow infrared gas-exchange analyser system equipped with a leaf chamber fluorometer (LI-6400XT, Li-Cor, Lincoln, NE, USA). Environmental conditions in the leaf chamber consisted of a leaf-to-air vapour pressure deficit of 1.2–2.0 kPa and a leaf temperature of 25 °C.

In light-adapted leaves, the actual $Φ_{PSII}$ was determined by measuring steady-state fluorescence ($F_i$) and maximum fluorescence during a light-saturating pulse of ~8000 μmol m$^{-2}$ s$^{-1}$ ($F_m$), following the procedures of Genty et al. (1989):

$$Φ_{PSII}=(F_m−F_i)/F_m$$

(1)

The electron transport rate ($J_e$) was then calculated as:

$$J_e=αβ/PPFDΦ_{PSII}$$

(2)

where PPFD is the photosynthetically active photon flux density, $α$ is the leaf absorptance, and $β$ is the PSII optical cross-section. The product $αβ$ was determined from the relationship between $Φ_{PSII}$ and $Φ_{CO_2}$ or A and $PPFDΦ_{PSII}$/4, obtained by varying either the light
intensity or the CO2 concentration under non-photorespiratory conditions in an atmosphere containing <1% O2 (Valentini et al., 1995; Yin et al., 2009).

Four to six \( A/C_i \) and \( A/PPFD \) curves under <1% O2 (\( A/PPFD \) and \( A/C_i \)) or 21% O2 (only \( A/C_i \)) were obtained from different plants for each species. In light-adapted leaves, \( A/C_i \) curves were initiated at an ambient CO2 concentration (\( C_i \)) of 400 \( \mu \text{mol} \text{ mol}^{-1} \) under a saturating \( PPFD \) of 1500 \( \mu \text{mol} \text{ m}^{-2} \text{ s}^{-1} \). Once steady state was reached, \( C_i \) was decreased stepwise down to 50 \( \mu \text{mol} \text{ mol}^{-1} \) air. Upon completion of the measurements at low \( C_i \), it was returned to 400 \( \mu \text{mol} \text{ mol}^{-1} \) air to restore the original \( A \). Next, \( C_i \) was increased stepwise to 2000 \( \mu \text{mol} \text{ mol}^{-1} \) air. For the \( A/PPFD \) curves, \( C_i \) was held at 400 \( \mu \text{mol} \text{ mol}^{-1} \), and the curve was initiated at a \( PPFD \) of 1500 \( \mu \text{mol} \text{ m}^{-2} \text{ s}^{-1} \); then, \( PPFD \) levels were decreased to 0 \( \mu \text{mol} \text{ m}^{-2} \text{ s}^{-1} \). Both the \( A/C_i \) and \( A/PPFD \) curves consisted of 11–13 different \( C_i \) values or \( PPFD \) intensities. \( C_i \) was calculated after Harley et al. (1992) as:

\[
C_i = \frac{\Gamma^* (J_p - 8 (A_i + R_L))/[J_p - 4 (A_i + R_L)]}{1 - (\Phi_{\text{PSII}}/\Phi_{\text{CO}_2})^4} \tag{3}
\]

where \( \Gamma^* \) was determined from the in vitro Rubisco specificity factor (\( S_{\text{c/o}} \)) (see below) as:

\[
\Gamma^* = \frac{O}{S_{\text{c/o}}} \tag{4}
\]

\( A \) was taken from gas-exchange measurements, and the \( J_p \) values were obtained from chlorophyll \( a \) fluorescence yield. The rate of mitochondrial respiration at darkness (\( R_{\text{dark}} \)) was measured early in the morning in dark-adapted leaves, and it was divided by two (\( R_{\text{dark}}/2 \)) to serve as a proxy for \( R_L \).

After estimating \( C_i \), \( g_m \) was calculated as follows (Harley et al., 1992):

\[
g_m = A (C_i - C) \tag{5}
\]

From the \( A/C_i \) and \( A/C_i \) curves, the maximum carboxylation capacity (\( V_{\text{max}} \)) and maximum capacity for electron transport rate (\( J_{\text{max}} \)) were calculated on a \( C_i \) and \( C_i \) basis using the kinetic parameters of Rubisco described below and, for comparative purposes, those described in Bernacchi et al. (2002). The FvCB model was fitted to the data by applying iterative curve fitting (minimum least square difference) using the Microsoft Excel Solver tool (Microsoft Corporation, Redmond, WA, USA). Additionally, \( g_m \), \( V_{\text{max}} \), and \( J_{\text{max}} \) were estimated using the Ethier and Livingston (2004) method, which is based on fitting \( A/C_i \) curves with a non-rectangular hyperbola version of the FvCB model, relying on the hypothesis that \( g_m \) reduces the curvature of the Rubisco-limited portion of an \( A/C_i \) response curve. For the method based on fitting \( A/C_i \) curves, species-specific \( S_{\text{c/o}} \) values were used as in the Harley method. Corrections for the leakage of CO2 and water vapour into and out of the leaf chamber of the Li-6400–40 have been applied to all gas-exchange data, as described by Rodeghiero et al. (2007). The percentage corrections applied to CO2 and water vapour flux rates are shown in Supplementary Table S1 available at JXB online for the different species.

Calibration relationships

The relationship between \( J_s \) and \( J_p \) was calibrated using the linear plot of \( \Phi_{\text{PSII}} \) and \( \Phi_{\text{CO}_2} \), based on Valentini et al. (1995):

\[
\Phi_{\text{PSII}} = k \Phi_{\text{CO}_2} + b \tag{6}
\]

\[
J_p = 4 (\Phi_{\text{PSII}} - b) PPFD/k \tag{7}
\]

where \( 4/k = \alpha \beta \). \( k \) and \( b \) were obtained through the linear fit of \( \Phi_{\text{PSII}} \) versus \( \Phi_{\text{CO}_2} \).

In addition, the method based on Yin's approach was also used (Yin et al., 2009), which presents a straightforward way to derive \( \alpha \beta \) and \( R_L \), as follows:

\[
J_s = 4 (A + R_L) \tag{8}
\]

\[
J_s = \alpha \beta PPFD \Phi_{\text{PSII}} \tag{9}
\]

\( J_s \) is rewritten as

\[
A = J_s / (4 - R_L) \tag{10}
\]

Assuming \( J_s = J_s \) under non-photorespiratory conditions gives

\[
A = \alpha \beta PPFD \Phi_{\text{PSII}}(4 - R_L) \tag{11}
\]

As Equation 11 has the form of \( y = ax + b \), through the linear fit of \( A \) versus \( PPFD \Phi_{\text{PSII}} / 4 \), \( R_L \) can be retrieved as the \( y \)-intercept, and \( \alpha \beta \) can be retrieved as the slope of the regression. Notably, this equation presented by Yin et al. (2009) has an extension of the FvCB model to account for alternative electron fluxes in the form of pseudocyclic (\( f_{\text{pseudo}} \)) and cyclic (\( f_{\text{cyc}} \)) electron flow (see further details in Yin et al., 2004):

\[
A = \alpha \beta PPFD \Phi_{\text{PSII}} \left[ 1 - \left( \frac{f_{\text{pseudo}}(1 - f_{\text{cyc}})}{4 - R_L} \right) \right] \tag{12}
\]

According to the updated model, to accomplish Equation 8, not only are non-photorespiratory conditions required but also the down-regulation of alternative electron fluxes so that the \( y \)-values of \( f_{\text{pseudo}} \) and \( f_{\text{cyc}} \) allow Equation 12 to be as close as possible to Equation 11, thus reliably estimating \( \alpha \beta \).

Four types of calibration were devised based on the approaches of Valentini and Yin described above. The first and second calibration methods were based on the \( \Phi_{\text{PSII}} \) relationship, and the third and fourth methods were based on Yin et al. (2009) as follows:

(i) \( \Phi_{\text{PSII}} / \Phi_{\text{CO}_2} \) (\( A/C_i \)): this calibration is performed using the entire \( A/C_i \) curve under low \( O_2 \), and the \( PPFD \) level is that used in the normal \( A/C_i \) curve (i.e. 1500 \( \mu \text{mol} \text{ m}^{-2} \text{ s}^{-1} \)). The \( R_L \) value must be assumed. In this variant, the initial part of the \( A/C_i \) curve is the most susceptible region for the occurrence of alternative electron sinks due to a high reductant (ATP and NADPH) supply associated with high \( PPFD \) and a limitation on reductant use (low \( CO_2 \) and \( O_2 \)). Conversely, this variant most resembles the conditions used in the normal \( A/C_i \) curve.

(ii) \( \Phi_{\text{PSII}} / \Phi_{\text{CO}_2} \) (\( PPFD >400 \mu \text{mol} m^{-2} s^{-1} \)): this is the variant more often reported in the literature and consists of using \( PPFD \) curves under low \( O_2 \) and ambient \( CO_2 \). A disadvantage of this variant is that a loss of linearity occurs at low \( PPFD \), as \( \Phi_{\text{CO}_2} \) is affected disproportionately by errors in respiratory estimations or by the increase in mitochondrial respiration at low light (the Sok-effect; Brooks and Farquhar, 1985). Thus, it is recommended to exclude \( \Phi_{\text{CO}_2} \) values >0.05 to keep the relationship as linear as possible (Seaton and Walker, 1990; Edwards and Baker, 1993). To meet this criterion, \( PPFD \) levels below 400 \( \mu \text{mol} \text{ m}^{-2} \text{ s}^{-1} \) had to be excluded, making this method prone to a high reductant supply (high \( PPFD \)) and intermediate reductant use (moderate \( CO_2 \) and low \( O_2 \)).

(iii) \( PPFD <400 \mu \text{mol} m^{-2} s^{-1} \): this variant was originally described by Yin et al. (2009), and it can also be used to estimate \( R_L \). As this method is not based on quantum efficiency plots, there is not the disadvantage of having to exclude the points at low \( PPFD \) or assume a given \( R_L \) value. Actually, it is recommended to use only the points at the linear phase of the \( A/PPFD \) (corresponding to \( PPFD \) levels <400 \( \mu \text{mol} \text{ m}^{-2} \text{ s}^{-1} \) for the species grown outdoors), which is in the range of a
lower reductant supply and where only the basal components of alternative electron sinks exist.

(iv) Yin (PPFD <400 μmol m⁻² s⁻¹ (C_i >500 μmol mol⁻¹ air); this method has the same advantages and assumptions of method (iii). A particularity of this approach resides in the incorporation of data from the A/C_i curve with C_i above 500 μmol mol⁻¹ air.

Retrieving the respiration combined A/C_i and A/C_c curves

The FvCB model predicts that A/C_i curves and their A/C_c counterparts share the same CO₂ compensation point (G), as this parameter is independent of g_m.

\[
G = [F^* + K_c (1 + O_2/K_c) R_i/V_{cmax}(1 - R_i/V_{cmax})]
\]

Evans and von Caemmerer (1996) proposed that an averaged g_m can be estimated as the slope of the plot of A versus C_i – C_c. Because the plot goes through the origin, both A and C_i – C_c must be zero, which is what occurs when G is shared by both A/C_i and A/C_c curves, as predicted by the FvCB model.

It was realized that for some data sets, when R_i was set in advance as required to obtain C_i, the plot of A versus C_i – C_c in the region which was Rubisco limited showed good linearity, although with an intercept differing from zero. Given the predictions of the FvCB model, this difference should be attributed to a biased R_i used to calculate C_i, which in turn leads to different G values, as obtained from the A/C_i and A/C_c curves. Given this fact and that the F* values in this study were reliably estimated from Rubisco kinetics in purified preparations, it is proposed here that R_i can be obtained as the value that makes the intercept of the plot of A versus C_i – C_c equal zero. The approach was tested using ideal data sets where the respiration values used as inputs were successfully retrieved from the combined A/C_i and A/C_c curves. Consequently, for each curve, R_i was estimated as described below. First, R_i was set to be equal to R_1kcal, as set in previous studies (Ninemets et al., 2005, 2006, 2009b). To maintain the plot A versus C_i – C_c as linear as possible, only points in the C_i range strictly limited by Rubisco (C_i <200 μmol mol⁻¹ air) were considered. Secondly, the new R_i was obtained as the value that forces the intercept of the plot A versus C_i – C_c to be zero using the Goal Seek function available in Microsoft Excel (see Fig. 1 for an example). The new respiration value obtained in this way is hereafter referred to as R_1kcal/A/C_i. An Excel spreadsheet (with modelled and real data) is available (see Supplementary Spreadsheet S1 at JXB online) that shows how this method was performed.

Rubisco kinetic parameters

The Rubisco kinetic parameters used in this study were measured in vitro, except the S_08 values for N. tabacum and L. gibertii, which were taken from Galmés et al. (2006) and Galmés et al. (2005), respectively. All measurements for the determination of Rubisco kinetic parameters were conducted at 25 °C. The Rubisco specificity factor was measured in highly purified extracts and using wheat Rubisco as a reference normalized to 100, following the procedures described in Galmés et al. (2005). The Michaelis–Menten constants for CO₂ (K_c) and O₂ (K_o) and the maximum rate for the carboxylase reaction (V_{cmax}) were measured in rapidly isolated leaf protein extracts (Sharwood et al., 2008). Briefly, ~0.5 g fresh weight of leaves were ground in a mortar with 2 ml of ice-cold extraction buffer containing 100 mM Bicin (pH 8.2), 6% (w/v) polyethylene glycol (PEG) 4000, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM benzamidine, 1 mM ε-aminocaproic acid, 50 mM 2-mercaptoethanol, 10 mM dithiothreitol (DTT), 2 μM pepstain A, 10 μM E64, 10 μM chymostatin, 2 mM phenylmethylsulphonyl fluoride (PMSF), and 2.5% (w/v) polyvinylpolypyrrolidone (PVPP). The liquidized sample was clarified by centrifugation at 12 842 g for 4 min. A 1 ml aliquot of clarified extract was eluted using a Sephadex PD-10 column (GE Healthcare, UK) pre-equilibrated with desalt buffer containing 100 mM Bicin (pH 8.2), 20 mM MgCl₂, 10 mM DTT, 1 mM KH₂PO₄, 0.5 mM EDTA, 1 mM benzamidine, 1 mM ε-aminocaproic acid, and 10 mM NaHCO₃. The protein peak (in 1 ml) was supplemented with protease inhibitors (4 μM pepstain A, 20 μM E64, and 20 μM chymostatin). Of this extract, 250 μl was supplemented with 2.542 μM NaH¹⁴CO₃ and activated for 15 min before carboxylase measurements. The remainder was used to assay Rubisco catalytic sites. Measurements of K_o and V_{cmax} for the carboxylase activity in nitrogen or air were determined in two sets of eight vials from the amount of ¹⁴C incorporated into PGA, as described elsewhere (Bird et al., 1982). Each set of vials used eight different concentrations of bicarbonate chosen to provide CO₂ (aq) between 0.7 μM and 75 μM, each with a specific radioactivity of 3.7 × 10⁶ Bq mol⁻¹ and containing 375 nmol RuBP. K_c was calculated from the relationship K_c (air) = K_c(N₂) × (1 + [O₂]/K_o). The concentration of Rubisco catalytic sites in the extract was measured from the stoichiometric binding of the inhibitor [¹⁴C]CABP to CO₂-Mg²⁺-activated Rubisco active sites (Butz and Sharkey, 1989). Thereafter, the carboxylase catalytic turnover rate K_cat was obtained as K_cat = V_{cmax}/[catalytic sites]. The Rubisco kinetic constants are summarized in Table 1.

Statistical analyses

Data are expressed as the means ± standard error. Student’s t-tests were used to compare the photosynthetic parameters calculated with the different sets of Rubisco kinetic constants and to examine whether the intercepts of the regression were significantly different from zero. Linear regression and statistical analyses were carried out using Microsoft Excel.

Results

The effect of the different calibration methods on αβ

The four calibration methods tested here comprised scenarios ranging from a low (Yin PPFD <400 μmol m⁻² s⁻¹) to a high reductant supply (Φ_psi/Ψ_psi <400 μmol m⁻² s⁻¹), all performed under non-photosynthetic conditions, although potentially affected by the occurrence of an alternative electron flow. Additionally, three species covering a large range in SLA (33 ± 1.4, 14 ± 2.7, and 9.0 ± 1.3 m² kg⁻¹ for N. tabacum, C. arabica, and L. gibertii, respectively) were selected to assess the extent to which structural changes might play a role in determining the αβ product.

High regression coefficients were obtained for all of the relationships based on the four calibration methods (r² =0.91–0.98) (Fig. 2). In contrast to expectations, no major differences in the estimation of αβ were found between the methods Yin (PPFD <400 μmol m⁻² s⁻¹) and Φ_psi/Ψ_psi (Φ_psi >400 μmol m⁻² s⁻¹) (Fig. 2B, C) and between Φ_psi/Ψ_psi (A/C_i) and Yin (PPFD <400/C_i >500 μmol mol⁻¹ air) (Fig. 2A, D). Thus, the inclusion of data at high PPFD or low C_i conditions that favour the prevalence of alternative electron fluxes, had slight effects on the estimated αβ. Instead, the major differences among the calibration methods were dependent on the type of data used to perform the calibration (i.e. A/F_PFFD or A/C_i curves (Table 2)). Regardless of the studied species, the calibrations using only A/F_PFFD data gave lower αβ values, ranging from 0.37 to 0.50, whereas those using A/C_i data produced higher αβ values, ranging from 0.46 to 0.62 (Table 2). Notably, the species displaying the most contrasting SLA values (N. tabacum and L. gibertii) showed
Improving the estimation of mesophyll conductance

similar values for αβ, suggesting that leaf structure does not play a major role in determining αβ.

Mesophyll conductance as affected by the J calibration methods and respiration estimations

Given that no major differences were found between the ΦPSII/ΦCO2 relationship and the Yin method, g_m was estimated using the methods covering the extremes of αβ values, namely ΦPSII/ΦCO2 (A/Ci) and ΦPSII/ΦCO2 (PPFD >400 μmol m^-2 s^-1). Because these methods consider only A/PPFD (ΦPSII/ΦCO2, PPFD >400 μmol m^-2 s^-1) or A/Ci data [ΦPSII/ΦCO2 (A/Ci)], the g_m estimated using either method is hereafter referred to as the g_m obtained with the A/PPFD or A/Ci J calibration. Once the two J calibrations to be used were defined, an R_l value next had to be chosen to calculate Cc. Because there was no consistency in the R_l estimated via the Yin approach using A/PPFD (PPFD <400 μmol m^-2 s^-1) or A/Ci (PPFD <400 μmol m^-2 s^-1 or C_i >500 μmol mol^-1 air) (Table 2), R_dark/2 was preferred because it is the unique respiration value actually measured in planta. To check the consistency of the respiration value used, a new approach was also tested to find an alternative proxy for R_l (R_{AC/ACc}) as the value forcing the CO2 compensation point (Γ) to be equal...
when calculated from $A/C_i$ and $A/C_c$ curves. The estimated $R_{AC_i/AC_c}$ values were always lower than their corresponding $R_{dark/2}$ counterparts (Table 3).

Due to the high amount of negative and extremely high $g_m$ values at low or high $C_i$, respectively (Table 4), a filter was next applied to keep the valid $g_m$ estimates, here defined as those values in the range of $0 < g_m < 1$ mol CO$_2$ m$^{-2}$s$^{-1}$. As expected, the use of $R_{AC_i/AC_c}$ significantly reduced the amount of negative values of $g_m$ at low $C_i$ ($R_{AC_i/AC_c}$ in Table 4), as the rationale for this method works at this $C_i$ range (Fig. 1). Conversely, the use of $A/C_i$ $J$ calibration improved the $g_m$ estimation at high $C_i$ (Table 4) due to a lowering of $C_c$ values (Fig. 3A). Thus, the

Fig. 2. Calibration relationships $\Phi_{PSII}$ versus $\Phi_{CO_2}$ ($A/C_i$) and $A$ versus PPFD $\Phi_{PSII}/4$ ($C$ and $D$) measured under non-photorespiratory conditions (<1% O$_2$) by varying PPFD intensities ($A$/PPFD curves) or substomatal CO$_2$ concentrations ($A/C_i$ curves). For more details on the four calibration methods, see the Materials and methods. In (A), the entire $A/C_i$ curve is utilized; in (B), only values at PPFD >400 $\mu$mol m$^{-2}$s$^{-1}$ are considered; in (C), only values at PPFD <400 $\mu$mol m$^{-2}$s$^{-1}$ are used; in (D), only values at PPFD <400 $\mu$mol m$^{-2}$s$^{-1}$ plus $C_i >$500 $\mu$mol mol$^{-1}$ are considered. The slope of the lines in all graphs refers to the product $\alpha \beta$, whereas the y-intercept should be interpreted as the presence of alternative electron sinks in A and B and as a measure of $R_L$ in C and D. The values of the slopes and intercepts are summarized in Table 2.

### Table 1. Rubisco kinetic constants measured for the species studied:
<table>
<thead>
<tr>
<th>Species</th>
<th>$S_{c/o}$</th>
<th>$\Gamma^*$ (µbar)</th>
<th>$K_c$ (µM)</th>
<th>$K_o$ (µM)</th>
<th>$K_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. tabacum</td>
<td>98.1$^{+2.6}$</td>
<td>39.7 ± 1.1</td>
<td>12.4 ± 0.7</td>
<td>274 ± 42</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>C. arabica</td>
<td>98.4$^{+4.3}$</td>
<td>39.6 ± 1.7</td>
<td>10.3 ± 1.3</td>
<td>479 ± 113</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>L. gibertii</td>
<td>110.5$^{+1.6}$</td>
<td>35.2 ± 0.5</td>
<td>8.9 ± 0.5</td>
<td>593 ± 75</td>
<td>2.7 ± 0.8</td>
</tr>
</tbody>
</table>

Values are the means ±standard error of 3–4 replicates per species.

* $^{a}$ Taken from Galmés et al. (2006)

* $^{b}$ Taken from Galmés et al. (2005)
Table 2. Slopes (αβ) and light respiration following the method of Yin et al. (2011) (R_{LYin} μmol CO$_2$ m$^{-2}$ s$^{-1}$) or intercept values (Φ_{Cas}/Φ_{CO2}) obtained under non-photorespiratory conditions according to different approaches, consisting of higher (Φ_{Cas}/Φ_{CO2} A/C, or PPFD >400 μmol m$^{-2}$ s$^{-1}$) or lower (Yin PPFD <400 or PPFD <400 μmol m$^{-2}$ s$^{-1}$ and C$_i$ >500 μmol mol$^{-1}$ air) susceptibilities to alternative electron sinks

For comparison, the original slopes (k) obtained when using the Φ_{Cas}/Φ_{CO2} relationship were already converted to αβ (αβ=4/k); the intercept refers to the parameter b in the equation: Φ_{Cas} = k * Φ_{CO2} + b.

<table>
<thead>
<tr>
<th>Species</th>
<th>Slope (αβ)</th>
<th>Intercept (Φ_{Cas}/Φ_{CO2})</th>
<th>R_{LYin}</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. tabacum</td>
<td>0.52±0.013</td>
<td>0.005±0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.36±0.011</td>
<td>-0.02±0.008*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.39±0.017</td>
<td>0.87±0.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.46±0.026</td>
<td>0.87±0.87</td>
<td></td>
</tr>
<tr>
<td>C. arabica</td>
<td>0.64±0.014</td>
<td>0.015±0.002*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.46±0.018</td>
<td>-0.11±0.007</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.50±0.025</td>
<td>0.84±0.41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.63±0.015</td>
<td>2.55±0.45*</td>
<td></td>
</tr>
<tr>
<td>L. gibertii</td>
<td>0.56±0.019</td>
<td>0.014±0.004*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.37±0.011</td>
<td>-0.03±0.009*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.41±0.013</td>
<td>2.04±0.25*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.50±0.018</td>
<td>3.14±1.02*</td>
<td></td>
</tr>
</tbody>
</table>

Values are the means ±standard error of four replicates per species. An asterisk denotes respiration or intercepts significantly different from zero (P < 0.05).

Table 3. Dark respiration measured at pre-dawn ($R_{dark}$) and light respiration estimated from combined A/C$_i$ and A/C$_c$ curves ($R_{AC/AC_c}$)

All values are in μmol CO$_2$ m$^{-2}$ s$^{-1}$.

<table>
<thead>
<tr>
<th>Species</th>
<th>$R_{dark}$</th>
<th>$R_{AC/AC_c}$ a</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. tabacum</td>
<td>2.2±0.1</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>C. arabica</td>
<td>0.9±0.1</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>L. gibertii</td>
<td>4.0±0.3</td>
<td>1.7±0.4</td>
</tr>
</tbody>
</table>

Values are the means ±standard error of four replicates per species. a For $R_{AC/AC_c}$, the values are averages from the A/C$_i$ curves calibrated using the A/PPFD or A/C$_c$ curves.

The different calibrations also affected the magnitude of $g_m$ in response to $C_i$. There was no general trend at $C_i$ < 100 μmol mol$^{-1}$ air, whereas at $C_i$ > 100 μmol mol$^{-1}$ air, lower $g_m$ values (up to 50%) were observed using the A/C$_i$ J calibration (Table 4). In addition, when plotting $g_m$ versus $C_i$, a high amplitude in $g_m$, depending on the calibration used, was revealed (Fig. 3B). Such amplitude led to a reasonable disagreement between the averaged $g_m$ at $C_i$ of 100–350 μmol mol$^{-1}$ air and the single point $g_m$ at CO$_2$ ambient (400 μmol mol$^{-1}$) (Supplementary Tables S2, S3 at JXB online), which suggests that care must be exercised when reporting single point $g_m$ data. In Supplementary Table S4, in addition to the filter keeping the values in the range of 0 < $g_m$ < 1 mol CO$_2$ m$^{-2}$ s$^{-1}$, the Harley et al. (1992) criteria were also applied, considering the $g_m$ data in the range of 10 < $C_i$/d$A$ < 50 as reliable to see whether they corresponded to the data presented in Table 4. Overall, the $g_m$ values presented in Table 4 and those obtained after applying the Harley criteria varied accordingly, but some exceptions were noticed such as the $g_m$ values estimated at $C_i$ > 350 μmol mol$^{-1}$ air for L. gibertii which were substantially higher than the $g_m$ data shown in Supplementary Table S4. Most importantly and irrespective of this difference, the amount of data excluded using the Harley criteria was always higher (up to 100%) even when the same estimates presented in Table 4 had 0% of data excluded together with small standard errors. Additionally, the Harley criteria worked differently depending on the species, J calibration, and $C_i$ range, given that some species were more affected than others.

To obtain an independent estimate of $g_m$ that was not affected by the need for calibrating $J$, $g_m$ was also estimated using an alternative approach, namely the A – $C_i$ curve analysis method suggested by Ether and Livingston (2004). The $g_m$ values obtained following this approach are given in Table 5.

Maximum Rubisco carboxylation and electron transport rate as affected by αβ

In contrast to the $g_m$ estimates that could be greatly affected by the use of either $R_{dark}$ or $R_{AC/AC_c}$ (Table 4, Fig. 3B), the $V_{cmax}$ and $J_{max}$ estimates were minimally affected by the use of different respiration values (~6% at most; Table 5; Supplementary Table S5 at JXB online).

Irrespective of species, $V_{cmax}$ and $J_{max}$ on a $C_c$ basis were always higher for the A/C$_i$ J calibration than for its A/PPFD J
counterpart (Table 5). These higher \( V_{\text{max}} \) and \( J_{\text{max}} \) values are in agreement with the lower \( g_{\text{m}} \) values found, in general, with the \( A/C_i \) \( J \) calibration (Table 4). The \( V_{\text{max}} \) obtained using the Ethier and Livingston method was closer to the \( V_{\text{max}} \) on a \( C_i \) basis calculated using the \( A/C_i \) \( J \) calibration for \( L. \ gibertii \) and \( C. \ arabica \), whereas for \( N. \ tabacum \) an intermediate value between those obtained with the \( A/PFJD \) or \( A/C_i \) \( J \) calibration was found (Table 5). Apart from the \( J_{\text{max}} \) calculated with the \( A/C_i \) \( J \) calibration, which had the higher values for all species and reflected the higher \( q_{\text{f}} \) found (Table 3), there were no major differences among the other calculated values of \( J_{\text{max}} \).

The use of the standard Rubisco kinetics (\( K_c \), \( K_o \), and \( \Gamma^* \)) originally obtained for \( N. \ tabacum \) by Bernacchi et al. (2002) would lead to an overestimation of \( V_{\text{max}} \) by \( \sim30\% \) and \( 20\% \) for \( L. \ gibertii \) and \( C. \ arabica \), respectively. The other photosynthetic parameters for these two species were unaffected by the use of different Rubisco kinetic constants (Table 5). No significant differences were observed for \( N. \ tabacum \) when using the different set of Rubisco kinetics, with the exception of \( g_{\text{m}} \) (Ethier and Livingston method), which was \( 28\% \) lower when using the Rubisco kinetics of Bernacchi et al. (2002).

### Discussion

To the best of the authors’ knowledge, this study is the first to address the idea that the use of \( A/C_i \) or \( A/PFJD \) curves under low \( O_2 \) to calibrate \( J \) can significantly affect the estimations of \( g_{\text{m}}, V_{\text{max}} \), and \( J_{\text{max}} \) as demonstrated in species with contrasting leaf structural properties and photosynthetic capacities. A background is also provided to understand potential factors that could be translated into unrealistic \( g_{\text{m}} \) values at low and high \( C_i \), and recommendations to improve \( g_{\text{m}} \) estimations accordingly.

### Sensitivity of the variable \( J \) method to \( q_{\beta} \)

The high sensitivity of the variable \( J \) method has been known since its introduction by Harley et al. (1992). However, among the several sources of error, more attention has been given to an accurate determination of \( \Gamma^* \) (Warren et al., 2006; Pons et al., 2009) than to \( J \) per \( C_i \), given the variety of ways in which \( J \) can be calibrated. Despite the \( J \) calibration based on \( A/PFJD \) curves being the most common method in the literature, here it is shown that this calibration produces a higher number of unrealistic \( g_{\text{m}} \) estimates than its counterpart based on \( A/C_i \) curves (Table 4). Additionally, it was demonstrated that \( g_{\text{m}} \) misestimations could be directly associated with the \( J \) calibration method at high \( C_i \) on the one hand, and the proper choice of \( \Gamma^* \) and \( R_e \) at low \( C_i \) on the other hand. Notably, the need to develop useful criteria to improve \( g_{\text{m}} \) estimations and to understand the high \( g_{\text{m}} \) variability is crucial considering that the only existing indicator, the Harley et al. (1992) criteria which consider that reliable \( g_{\text{m}} \) estimations are those situated in the range of \( dC_i/dA \) (10–50), performed poorly in retrieving acceptable data in addition to being dependent on the species, applied \( C_i \) range, and \( J \) calibration (Supplementary Table S4 at JXB online).

It can be deduced from the relationship between \( C_i \) and \( J(A+R_e) \) (Fig. 3A) that \( C_i \) approaches infinity as \( J(A+R_e) \) approaches four (number of electrons per \( CO_2 \) molecule fixed). In contrast, \( C_i \) is negative when the ratio is less than four [the lower \( C_i \) threshold for positive \( J(A+R_e) \) is defined by the parameter \( \Gamma^* \)]. Therefore, as \( J(A+R_e) \) approaches four as photosynthesis and other alternative electron sinks tend to decrease (at high \( C_i \), for example), extremely high \( C_i \) values are to be expected, leading to a greater probability of

### Table 4. Mesophyll conductance (\( g_{\text{m}}, \text{mol CO}_2 \text{m}^{-2} \text{s}^{-1} \)) for several intervals of \( C_i \) and percentage of data excluded (DE) after applying a restriction (\( g_{\text{m}} \) restricted to the range of \( 0 \leq g_{\text{m}} < 1 \text{mol CO}_2 \text{m}^{-2} \text{s}^{-1} \))

<table>
<thead>
<tr>
<th>( A/C_i ) ( J ) calibration</th>
<th>( A/PFJD ) ( J ) calibration</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R_{ACi/AC} ) ( R_{ACi/AC} )</td>
<td>( R_{ACi/AC} ) ( R_{ACi/AC} )</td>
</tr>
<tr>
<td>( g_{\text{m}} ) (mol CO ( _2 ) m ( ^{-2} ) s ( ^{-1} ))</td>
<td>( g_{\text{m}} ) (mol CO ( _2 ) m ( ^{-2} ) s ( ^{-1} ))</td>
</tr>
<tr>
<td>( N. \ tabacum ) ( C_i \leq 100 )</td>
<td>0.290 ± 0.065</td>
</tr>
<tr>
<td>( N. \ tabacum ) ( C_i &gt; 100 ) and ( \leq 350 )</td>
<td>0.213 ± 0.021</td>
</tr>
<tr>
<td>( N. \ tabacum ) ( C_i &gt; 350 )</td>
<td>0.038 ± 0.005</td>
</tr>
<tr>
<td>( C. \ arabica ) ( C_i &lt; 100 )</td>
<td>0.131 ± 0.018</td>
</tr>
<tr>
<td>( C. \ arabica ) ( C_i &gt; 100 ) and ( \leq 350 )</td>
<td>0.117 ± 0.006</td>
</tr>
<tr>
<td>( C. \ arabica ) ( C_i &gt; 350 )</td>
<td>0.052 ± 0.004</td>
</tr>
<tr>
<td>( L. \ gibertii ) ( C_i &lt; 100 )</td>
<td>0.235 ± 0.027</td>
</tr>
<tr>
<td>( L. \ gibertii ) ( C_i &gt; 100 ) and ( \leq 350 )</td>
<td>0.241 ± 0.014</td>
</tr>
<tr>
<td>( L. \ gibertii ) ( C_i &gt; 350 )</td>
<td>0.071 ± 0.011</td>
</tr>
</tbody>
</table>

Values are the means ±standard error of 4–6 A/Ci curves per species. The SE was calculated according to the points that remained in each Ci interval after applying the restriction.
Fig. 3. (A) Relationship between $C_c$ and $J/(A+R_L)$, as affected by the $J$ calibration using the $A/PPFD$ (open circles) or $A/C_i$ curves (filled circles). As $J/(A+R_L)$ approaches four (likely to occur at high $C_i$), $C_c$ tends to infinity, thus increasing the probability of being higher than $C_i$ and resulting in negative $g_m$ values. The arrow indicates the $C_c$ upper limit (~600 μmol mol$^{-1}$) observed for the $A/C_i$ calibration, being much lower than its $A/PPFD$ counterpart which can reach $C_c$ values >1600 μmol mol$^{-1}$. (B) $g_m$ response to $C_i$ as affected by the $A/C_i$ (filled symbols) or $A/PPFD$ (open symbols) $J$ calibration method and the light respiration estimations [$R_{dark/2}$ (triangles) or $R_{AC/AC_i}$ (circles)]. It is remarkable how there are ‘spikes’ in $g_m$ at low $C_i$ when using $R_{dark/2}$ and how these are alleviated when using $R_{AC/AC_i}$. The curves are from $L. giberti$, and the points are averages from four plants for $g_m$ between 0 and 1 mol CO$_2$ m$^{-2}$ s$^{-1}$. The $A/C_i$ or $A/PPFD$ $J$ calibration refers to the $\alpha\beta$ obtained from the curves shown in Fig. 2A and B, respectively.
estimating negative values of $g_m$. Thus, for the same $(A+R_i)$, the higher the $\alpha\beta$, the higher the $J$, ultimately resulting in a greater probability of obtaining positive $g_m$ values due to a lowering of $C_i$, whereas the opposite holds true for a lower $\alpha\beta$.

At low $C_i$ (<100 $\mu$mol mol$^{-1}$), misestimations of $g_m$ are unlikely to be affected by $J(A+R_i)$, as this ratio tends to achieve higher values with decreasing $C_i$, as any case, reliable estimations of $g_m$ at low $C_i$ are extremely challenging given that the estimations are highly dependent on the proper choice of values of $\Gamma^*$ or $R_i$, which can ultimately affect $C_i$.

In the present study, the authors are quite confident about their $\Gamma^*$ values because they were estimated from Rubisco kinetics in purified preparations. Given this, it was possible to retrieve a respiration estimate (R$_{AC/IC}$) as the value forcing the intercept of the linear relationship of $A$ versus $C_i$ to zero, which is equivalent to forcing the $\Gamma$ to be the same. It is believed that R$_{AC/IC}$ obtained in this way is a valid estimate, as its magnitude was lower than that of $R_{dark}$ (Table 3), in agreement with the general consensus that respiration is inhibited in the light (Tcherkez et al., 2005). In addition, the use of R$_{AC/IC}$ significantly improved the number of valid $g_m$ estimates (Table 4), highlighting the importance of respiration in estimating $g_m$ at low $C_i$ with the advantage of better matching a biochemical prediction of the FvCB model, namely A/IC and A/IC sharing the same $\Gamma$.

An a priori weakness regarding R$_{AC/IC}$ resides in whether $g_m$ is kept constant at a low $C_i$ range. However, the authors argue in favour of a constant $g_m$ where a high linearity can be observed when plotting $A$ versus $C_i$ in the region strictly limited by Rubisco (Fig. 1C). Furthermore, it can also be noted that $g_m$ estimated using the variable $J$ method is closer to the constant $g_m$ as the individual points are close to the regression line in the plot of $A$ versus $C_i$. The apparent variability of $g_m$ that might be identified using the variable $J$ method could simply be a result of the high sensitivity of $A$, $C_i$, or $C_r$ to random errors at this low $C_i$ range. This conclusion is supported when comparing the data of Flexas et al. (2007), who showed a high variability of $g_m$ at low $C_i$ using the variable $J$ method, with those recorded by Tazoe et al. (2011), who found almost constant values of $g_m$ using the isotopic method.

### Why do A/IC and A/PPFD curves result in different $\alpha\beta$?

Given that $\alpha$ can be measured and that large changes in this parameter during the execution of A/PPFD or A/IC curves are unlikely to occur, the uncertainty in $J$ is particularly related to $\beta$. Eichelmann and Laisk (2000) found $\beta$ varying from 0.38 to 0.51 in N. tabacum under varying light and temperature conditions. Similar results were reported by Loreto et al. (2009) while studying the effect of blue light on $g_m$. Hassiotou et al. (2009) also concluded that $J$ calibration is light dependent. Collectively, all of this information highlights the importance of irradiance in determining the $\beta$ value and helps explain the apparently better suitability of the A/IC-based $J$ calibration: it is performed under the same irradiance used in the normal A/IC curves. In fact, another advantage of keeping a fixed irradiance during the calibration is to avoid changes in the profiles of light absorption through the leaf that may occur under changing irradiance (Evans, 2009; Oguchi et al., 2011); this can be especially important for
the Li-Cor LED-based fluorescence/light source which consists of blue and red light with narrow bandwidths. However, to what extent changes in spectral light could be responsible for differences in $\beta$ is unclear (Evans, 2009). Further explanations for this statement might be linked to the engagement of alternative electron sinks and the fluorescence signals that might not be representative of the whole leaf, in contrast to the gas-exchange signals (Hassiotou et al., 2009). Even if the uncertainty about the fluorescence signal could be resolved using calibration curves, as in this study, conflicting information on the possible effects of alternative electron sinks on $g_m$ measurements has been reported. Whereas several authors (e.g. Loreto et al., 1994; Ruuska et al., 2000; Flexas and Medrano, 2002) showed circumstantial evidence suggesting that alternative electron sinks play no major role in $g_m$ estimations, other investigators reported that up to 24% of the total electron flux can be associated with alternative sinks (see Gilbert et al., 2012, and references therein). In any case, the present results argue against the relevance of alternative electron sinks as potential bias for proper $g_m$ estimations, given that the calibration methods most likely to be affected by these sinks (calibrations performed at high PPFD and low $C_i$) did not differ from others which used a range of data under strictly limited electron transport (low PPFD and high $C_i$).

**Mesophyll conductance, maximum velocity of Rubisco carboxylation, and electron transport rate**

The choice of the method to calibrate $J$ can significantly affect the estimation of photosynthetic parameters and even alter their interpretation in the context of the diffusive versus biochemical limitations to photosynthesis. In N. tabacum, the use of $A/PPFD$ calibration would lead to the conclusion that the assumption of infinite $g_m$ is plausible because no difference in $V_{\text{max}}$ on a $C_i$ or $C_c$ basis was observed (Table 5). In sharp contrast, the use of $A/C_i$ calibration points to a finite $g_m$ and a higher $V_{\text{max}}$ value (33%) in relation to the $V_{\text{max}}$ on a $C_c$ basis. For C. arabica and L. gibertii, higher $V_{\text{max}}$ values were observed using both $A/C_i$ and $A/PPFD$ $J$ calibrations compared with $V_{\text{max}}$ on a $C_c$ basis, but the degree of underestimation varied considerably (Table 5). It is important to bear in mind that such alterations also imply changes in biochemical aspects of the leaf because $V_{\text{max}}$ is related to the amount of activated Rubisco, whereas $J_{\text{max}}$ is associated with components of the electron transport chain, and both parameters can affect the leaf nitrogen economy (Niinemets and Tenhunen, 1997). Thus, it is highly unlikely that both calibrations can hold true, as they can considerably change the leaf biochemical signature and hence ultimately pose uncertainties on how to decide which calibration to use. To address this issue, a first cut-off point is to check if the minimum requirement of four electrons per carboxylation is attained, which can be achieved by dividing $J$ by $A$. If this ratio is less than four, the calibration is, on a theoretical basis, inadequate. This criterion allowed the exclusion of the standard $q$ for C. arabica (Supplementary Table S2 at JXB online) and the $A/PPFD$ calibration for N. tabacum. In addition, $g_m$ and the FvCB photosynthetic parameters were estimated using the $J$-independent Ether and Livingston method. Interestingly, the values of $V_{\text{max}}$ and $g_m$ obtained with this method reasonably matched those obtained with the $A/C_i$ $J$ calibration for L. gibertii and C. arabica. In N. tabacum, the $V_{\text{max}}$ calculated from the Ether approach presented intermediate values between the $V_{\text{max}}$ from the $A/PPFD$ or $A/C_i$ $J$ calibration, and, given the high standard errors in its $g_m$ estimate, it is believed that, even in this species, the $A/C_i$ $J$ calibration might be a better option to recommend, as it allows the retrieval of a greater number of realistic $g_m$ estimations regardless of the respiration value (Tables 4, 5). Therefore, it is proposed that the $A/C_i$ $J$ calibration seems to be more reliable than its $A/PPFD$ counterpart in terms of producing acceptable data, in accordance with the results of Gilbert et al. (2012).

Regarding the use of Rubisco kinetic constants, a considerable overestimation of $V_{\text{max}}$ (~30%) would occur in L. gibertii if standard values of those constants (Bernacchi et al., 2002) were used (Table 5). Importantly, even if good fits could be obtained irrespective of the kinetic constants, the relationship between $V_{\text{max}}$ and leaf nitrogen might be compromised. Thus, if the main goal is to use the FvCB model to characterize photosynthetic capacities and relate them to nitrogen partitioning (e.g. Xu et al., 2012), species-specific kinetic constants for Rubisco should be implicitly used.

**Further recommendations to improve $g_m$ estimation**

Given the uncertainties in $g_m$ estimations, criteria were provided to assess the consistency of $\Gamma^*$ and $R_i$ through the determination of $\Gamma$ (by definition, $\Gamma$ must be higher than $\Gamma^*$) or by plotting $A$ versus $C_i-C_c$ and analyzing the intercept of the linear relationship. In addition, provided that the estimates of $\Gamma$ and $\Gamma^*$ are reliable, it is possible to retrieve a respiration estimate from $A/C_i$ and $A/C_c$ curves. The respiration value was the focus here because of the availability of the current methods to estimate $R_i$ using gas exchange and/or chlorophyll fluorescence (see Yin et al., 2011), all of which were performed under low irradiance conditions that do not match those used in $A/C_i$ curves, which ultimately makes the choice of a proper respiration value a very complicated task.

If an inconsistency between $\Gamma$ for the $A/C_i$ curve and $\Gamma^*$ ($\Gamma$ lower than $\Gamma^*$) is found, or if the value of respiration retrieved by forcing the intercept of $A$ versus $C_i-C_c$ to zero is negative, four potential sources of error need to be checked: (i) correction for $CO_2$ and water vapour leakage through the chamber gaskets is crucial to the determination of $\Gamma$ and very important for species with low photosynthetic potential (see Rodeghiero et al., 2007); (ii) the influence of lateral diffusion which can become significant (especially for homoboric leaves) with large gradients of $CO_2$ inside and outside the chamber in addition to the lower fluxes of $CO_2$ near the compensation point (Morison and Lawson, 2007); (iii) $\Gamma^*$ determination; and (iv) Rubisco deactivation. The first two sources of error are particularly dependent on the leaf chamber size and, given the magnitude of the corrections in $A$ at low $C_i$ (Supplementary Table S1 at JXB online), the need to use larger leaf chambers to obtain more reliable $g_m$, $\Gamma$, and $R_i$/AC curves estimates is emphasized. For species displaying low
respiration rates, it is possible to obtain an estimate of $\Gamma^*$ by fixing $R_L$, inputting a test $\Gamma^*$, and changing this parameter to obtain the intercept of $A$ versus $C_i-C_c$ closest to zero. All of these hints provide a good framework to extract as much information as possible from the $A/C_i$ and $A/C_c$ curves. Nevertheless, because all of these adjustments rely on fitting processes, proper judgement about the biological meaning of the obtained estimates is obviously required.

Regarding the $J$ calibration, independent of it being based on $A/C_i$, $A/PPFD$, or both, one should first check whether all of the positive $J/A$ values are higher than four. If not, there is an inconsistency with the calibration because this implies that fewer than four electrons would be used per carboxylation event. If yes, the next step is to verify the suitability of the respiration value by checking if all of the positive $J(A+R_L)$ values are still higher than four. If not, there is an inconsistency for the same reason stated previously. Another checkpoint is to verify that the maximum observed $C_c$ is at least equal to the maximum $C_i$ that would implicate infinite $g_m$. If $C_c$ values higher than the maximum $C_i$ are present for positive $A$, there is again an inconsistency with the calibration, as Fick’s first law of diffusion predicts a drawdown of $CO_2$ between $C_i$ and $C_c$. In fact, it is theoretically possible to define an upper limit for $\alpha\beta$ which would be the value making the $A/C_i$ and $A/C_c$ curves identical, implicating infinite $g_m$.

Conclusion

This work brings new information to the growing amount of published papers trying to improve $g_m$ estimation. Criteria are provided to examine the amount of unrealistic $g_m$ data and identify inconsistencies with the biochemical model utilized, leading to a higher amount of acceptable data. In addition, the need for additional measurements to validate the $J$ calibration to improve the estimation of photosynthetic parameters is emphasized. Moreover, given the observed high variability in $g_m$, it is important that comparisons among studies reporting data consider the variability due to the $J$ calibration method, and, if necessary, normalize data according to the reported $\alpha\beta$ values.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Graphical representation of the $A/C_i$ and their respective $A/C_c$ curves calibrated with the $A/C_i$ or $A/PPFD$ $J$ calibration.

Table S1. Leakage correction for $CO_2$ and water vapour.

Table S2. Averaged mesophyll conductance ($g_m$) for the interval of $C_i$ ranging from 100 to 350 $\mu$mol mol$^{-1}$ air and the single point $g_m$ at ambient $CO_2$ concentration (400 $\mu$mol mol$^{-1}$ air) using $R_{dark}^{1/2}$ in the $g_m$ estimation.

Table S3. The same as in Table S1, using $R_{dark}^{1/4}$ rather than $R_{dark}^{1/2}$ to estimate $g_m$.

Table S4. Mesophyll conductance ($g_m$, mol $CO_2$ m$^{-2}$ s$^{-1}$) for several intervals of $C_i$ and percentage of data excluded (DE) after applying two restrictions ($g_m$ restricted to the range of

0 < $g_m$$<$1 mol $CO_2$ m$^{-2}$ s$^{-1}$ and $dC_i/dA$ of 10–50 [the Harley et al. (1992) criteria].

Table S5. Maximum carboxylation rate of Rubisco ($V_{cmax}$) and maximum electron transport rate from gas exchange ($J_{max}$), as affected by the different $J$ calibrations and calculated with $R_{dark}^{1/2}$.

Spreadsheet S1. An Excel spreadsheet is provided that allows the user to retrieve the leaf respiration from combined $A/C_i$ and $A/C_c$ curves.

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