

RESEARCH PAPER

# Improving the estimation of mesophyll conductance to CO<sub>2</sub>: on the role of electron transport rate correction and respiration

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## Abstract

Mesophyll conductance ( $g_m$ ) can markedly limit photosynthetic CO<sub>2</sub> 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  $g_m$ , 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  $g_m$ , 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 CO<sub>2</sub> or light levels, possible alternative electron sinks, or contrasting leaf structural properties might play a role in determining differences in  $\alpha\beta$ , the product of the leaf absorptance ( $\alpha$ ) and the photosystem II optical cross-section ( $\beta$ ). It was shown that differences in  $\alpha\beta$  were mainly attributed to the use of  $A/C_i$  or  $A/PPFD$  curves to calibrate  $J$ . The different  $\alpha\beta$  values greatly influenced  $g_m$ , 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  $A/C_i$  and  $A/C_c$  curves and a framework to understand the high variation in observed  $g_m$  values. Overall, a background is provided to decrease the noise in  $g_m$ , facilitating data reporting and allowing better retrieval of the information presented in  $A/C_i$  and  $A/C_c$  curves.

**Key words:**  $A/C_i$  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 ( $g_m$ ), which regulates the CO<sub>2</sub> 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  $g_m$ , implying that the CO<sub>2</sub> concentrations in the substomatal cavities ( $C_i$ ) and chloroplasts ( $C_c$ ) would be the same (Farquhar *et al.*, 1980). However, the role of finite  $g_m$  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  $g_m$  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 ( $V_{cmax}$ ) when  $g_m$  is considered to be infinite (Ethier and Livingston, 2004; Niinemets *et al.*, 2009a).

Several methods to estimate  $g_m$  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  $\text{CO}_2$  (Pons *et al.*, 2009). However, the variable  $J$  method is highly sensitive to errors in some parameters, especially the  $\text{CO}_2$  compensation point in the absence of photorespiration ( $\Gamma^*$ ) and  $J$  (Harley *et al.*, 1992). Importantly,  $J$  needs to be calibrated due to the uncertainties in both the leaf absorptance ( $\alpha$ ) and photosystem II (PSII) optical cross-section ( $\beta$ ).

The  $J$  calibration is based on the analysis of response curves of photosynthetic rates to light [ $A$ /photosynthetic photon flux density (PPFD)] or  $\text{CO}_2$  ( $A/C_i$ ) 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  $\alpha\beta$  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 ( $\Phi_{\text{PSII}}$ ) and  $\text{CO}_2$  ( $\Phi_{\text{CO}_2}$ ) (Valentini *et al.*, 1995), the relationship between  $J_A$  and  $J_F$  (Pons *et al.*, 2009), and the plot of  $A$  versus PPFD  $\Phi_{\text{PSII}}/4$ , 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  $\text{O}_2$  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  $\text{CO}_2$  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  $g_s$ , 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  $V_{\text{cmax}}$  or  $J_{\text{max}}$ , 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  $\alpha$  using integrating spheres and assumed a  $\beta$  value of 0.5 (e.g. Galmés *et al.*, 2007; Tosens *et al.*, 2012), even though  $\beta$  has been reported to vary (Laisk 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  $g_m$ ,  $V_{\text{cmax}}$ , and  $J_{\text{max}}$  may be affected by using  $A/C_i$  or  $A$ /PPFD curves under low  $\text{O}_2$  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 gibertii* (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. gibertii*, 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  $\Phi_{\text{PSII}}$  was determined by measuring steady-state fluorescence ( $F_s$ ) and maximum fluorescence during a light-saturating pulse of ~8000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  ( $F_m'$ ), following the procedures of Genty *et al.* (1989):

$$\Phi_{\text{PSII}} = (F_m' - F_s) / F_m' \quad (1)$$

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

$$J_F = \alpha\beta \text{ PPFD } \Phi_{\text{PSII}} \quad (2)$$

where PPFD is the photosynthetically active photon flux density,  $\alpha$  is the leaf absorptance, and  $\beta$  is the PSII optical cross-section. The product  $\alpha\beta$  was determined from the relationship between  $\Phi_{\text{PSII}}$  and  $\Phi_{\text{CO}_2}$  or  $A$  and PPFD  $\Phi_{\text{PSII}}/4$ , obtained by varying either the light

intensity or the CO<sub>2</sub> concentration under non-photorespiratory conditions in an atmosphere containing <1% O<sub>2</sub> (Valentini *et al.*, 1995; Yin *et al.*, 2009).

Four to six  $A/C_i$  and  $A/PPFD$  curves under <1% O<sub>2</sub> ( $A/PPFD$  and  $A/C_i$ ) or 21% O<sub>2</sub> (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 CO<sub>2</sub> concentration ( $C_a$ ) of 400 μmol mol<sup>-1</sup> under a saturating PPFD of 1500 μmol m<sup>-2</sup> s<sup>-1</sup>. Once steady state was reached,  $C_a$  was decreased stepwise down to 50 μmol mol<sup>-1</sup> air. Upon completion of the measurements at low  $C_a$ , it was returned to 400 μmol mol<sup>-1</sup> air to restore the original  $A$ . Next,  $C_a$  was increased stepwise to 2000 μmol mol<sup>-1</sup> air. For the  $A/PPFD$  curves,  $C_a$  was held at 400 μmol mol<sup>-1</sup>, and the curve was initiated at a PPFD of 1500 μmol m<sup>-2</sup> s<sup>-1</sup>; then, PPFD levels were decreased to 0 μmol m<sup>-2</sup> s<sup>-1</sup>. Both the  $A/C_i$  and  $A/PPFD$  curves consisted of 11–13 different  $C_a$  values or PPFD intensities.

$C_c$  was calculated after Harley *et al.* (1992) as:

$$C_c = \{\Gamma^*[J_F + 8(A + R_L)]\} / [J_F - 4(A + R_L)] \quad (3)$$

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

$$\Gamma^* = O/S_{c/o} \quad (4)$$

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

After estimating  $C_c$ ,  $g_m$  was calculated as follows (Harley *et al.*, 1992):

$$g_m = A/(C_i - C_c) \quad (5)$$

From the  $A/C_i$  and  $A/C_c$  curves, the maximum carboxylation capacity ( $V_{cmax}$ ) and maximum capacity for electron transport rate ( $J_{max}$ ) were calculated on a  $C_i$  and  $C_c$  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,  $g_m$ ,  $V_{cmax}$ , and  $J_{max}$  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  $S_{c/o}$  values were used as in the Harley method. Corrections for the leakage of CO<sub>2</sub> 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 CO<sub>2</sub> and water vapour flux rates are shown in Supplementary Table S1 available at JXB online for the different species.

#### Calibration relationships

The relationship between  $J_A$  and  $J_F$  was calibrated using the linear plot of  $\Phi_{PSII}$  and  $\Phi_{CO_2}$ , based on Valentini *et al.* (1995):

$$\Phi_{PSII} = k \Phi_{CO_2} + b \quad (6)$$

$$J_F = 4(\Phi_{PSII} - b) PPFD/k \quad (7)$$

where  $4/k = \alpha\beta$ .  $k$  and  $b$  were obtained through the linear fit of  $\Phi_{PSII}$  versus  $\Phi_{CO_2}$ .

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:

$$J_A = 4(A + R_L) \quad (8)$$

$$J_F = \alpha\beta PPFD \Phi_{PSII} \quad (9)$$

$J_A$  is rewritten as

$$A = J_A/4 - R_L \quad (10)$$

Assuming  $J_A = J_F$  under non-photorespiratory conditions gives

$$A = \alpha\beta PPFD \Phi_{PSII}/4 - R_L \quad (11)$$

As Equation 11 has the form of  $y = ax + b$ , through the linear fit of  $A$  versus  $PPFD \Phi_{PSII}/4$ ,  $R_L$  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 ( $f_{pseudo}$ ) and cyclic ( $f_{cyc}$ ) electron flow (see further details in Yin *et al.*, 2004):

$$A = \alpha\beta PPFD \Phi_{PSII} \{1 - [f_{pseudo}/(1 - f_{cyc})]\} / 4 - R_L \quad (12)$$

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 low values of  $f_{pseudo}$  and  $f_{cyc}$  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  $\Phi_{PSII}/\Phi_{CO_2}$  relationship, and the third and fourth methods were based on Yin *et al.* (2009) as follows:

- (i)  $\Phi_{PSII}/\Phi_{CO_2}$  ( $A/C_i$ ): this calibration is performed using the entire  $A/C_i$  curve under low O<sub>2</sub>, and the PPFD level is that used in the normal  $A/C_i$  curve (i.e. 1500 μmol m<sup>-2</sup> s<sup>-1</sup>). 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<sub>2</sub> and O<sub>2</sub>). Conversely, this variant most resembles the conditions used in the normal  $A/C_i$  curve.
- (ii)  $\Phi_{PSII}/\Phi_{CO_2}$  (PPFD >400 μmol m<sup>-2</sup> s<sup>-1</sup>): this is the variant more often reported in the literature and consists of using  $A/PPFD$  curves under low O<sub>2</sub> and ambient CO<sub>2</sub>. A disadvantage of this variant is that a loss of linearity occurs at low PPFD, as  $\Phi_{CO_2}$  is affected disproportionately by errors in respiration estimations or by the increase in mitochondrial respiration at low light (the Kok-effect; Brooks and Farquhar, 1985). Thus, it is recommended to exclude  $\Phi_{CO_2}$  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 μmol m<sup>-2</sup> s<sup>-1</sup> had to be excluded, making this method prone to a high reductant supply (high PPFD) and intermediate reductant use (moderate CO<sub>2</sub> and low O<sub>2</sub>).
- (iii) Yin (PPFD <400 μmol m<sup>-2</sup> s<sup>-1</sup>): 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 μmol m<sup>-2</sup> s<sup>-1</sup> 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  $\mu\text{mol m}^{-2} \text{s}^{-1}/C_i$  >500  $\mu\text{mol mol}^{-1}$  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  $\mu\text{mol mol}^{-1}$  air.

#### Retrieving the respiration value from 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  $\text{CO}_2$  compensation point ( $\Gamma$ ), as this parameter is independent of  $g_m$ :

$$\Gamma = [\Gamma^* + K_c(1 + O/K_o)R_L/V_{cmax}]/(1 - R_L/V_{cmax})$$

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  $\Gamma$  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  $R_L$  was set in advance as required to obtain  $C_c$ , 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  $R_L$  used to calculate  $C_c$ , which in turn leads to different  $\Gamma$  values, as obtained from the  $A/C_i$  and  $A/C_c$  curves. Given this fact and that the  $\Gamma^*$  values in this study were reliably estimated from Rubisco kinetics in purified preparations, it is proposed here that  $R_L$  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,  $R_L$  was estimated as described below. First,  $R_L$  was set to be equal to  $R_{dark/2}$ , as set in previous studies (Niinemets *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 ( $C_i < 200 \mu\text{mol mol}^{-1}$  air) were considered. Secondly, the new  $R_L$  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  $R_{A/C_i/A/C_c}$ . 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  $S_{c/o}$  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  $\text{CO}_2$  ( $K_c$ ) and  $\text{O}_2$  ( $K_o$ ) 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 Bicine (pH 8.2), 6% (w/v) polyethylene glycol (PEG) 4000, 2 mM  $\text{MgCl}_2$ , 0.1 mM EDTA, 1 mM benzamidine, 1 mM  $\epsilon$ -aminocaproic acid, 50 mM 2-mercaptoethanol, 10 mM dithiothreitol (DTT), 2  $\mu\text{M}$  pepstain A, 10  $\mu\text{M}$  E64, 10  $\mu\text{M}$  chymostatin, 2 mM phenylmethylsulphonyl fluoride (PMSF), and 2.5% (w/v) polyvinylpyrrolidone (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 Bicine (pH 8.2), 20 mM  $\text{MgCl}_2$ , 10 mM DTT, 1 mM  $\text{KH}_2\text{P}_i$ , 0.5 mM EDTA, 1 mM benzamidine, 1 mM  $\epsilon$ -aminocaproic acid, and 10 mM  $\text{NaHCO}_3$ . The protein peak (in 1 ml) was supplemented with protease inhibitors (4  $\mu\text{M}$  pepstain A, 20  $\mu\text{M}$  E64, and 20  $\mu\text{M}$  chymostatin). Of this extract, 250  $\mu\text{l}$  was supplemented with 2.542  $\mu\text{Ci NaH}^{14}\text{CO}_3$  and activated for 15 min before carboxylase measurements. The remainder was used to assay Rubisco catalytic sites. Measurements of  $K_c$  and  $V_{cmax}$  for the carboxylase activity in nitrogen or air were determined in two sets of eight vials from the amount of  $^{14}\text{C}$  incorporated into PGA, as described elsewhere (Bird *et al.*, 1982). Each set of vials used eight different concentrations of bicarbonate chosen to provide  $\text{CO}_2$  (aq) between 0.7  $\mu\text{M}$  and 75  $\mu\text{M}$ , each with a specific radioactivity of  $3.7 \times 10^{10}$  Bq  $\text{mol}^{-1}$  and containing 375 nmol RuBP.  $K_o$  was calculated from the relationship  $K_c$  (air) =  $K_c$  ( $\text{N}_2$ )  $\times \{1 + [\text{O}_2]/K_o\}$ . The concentration of Rubisco catalytic sites in the extract was measured from the stoichiometric binding of the inhibitor [ $^{14}\text{C}$ ]CABP to  $\text{CO}_2$ - $\text{Mg}^{2+}$ -activated Rubisco active sites (Butz and Sharkey, 1989). Thereafter, the carboxylase catalytic turnover rate  $K_{cat}^c$  was obtained as  $K_{cat}^c = V_{cmax}/[\text{catalytic sites}]$ . The Rubisco kinetic constants are summarized in Table 1.

#### Statistical analyses

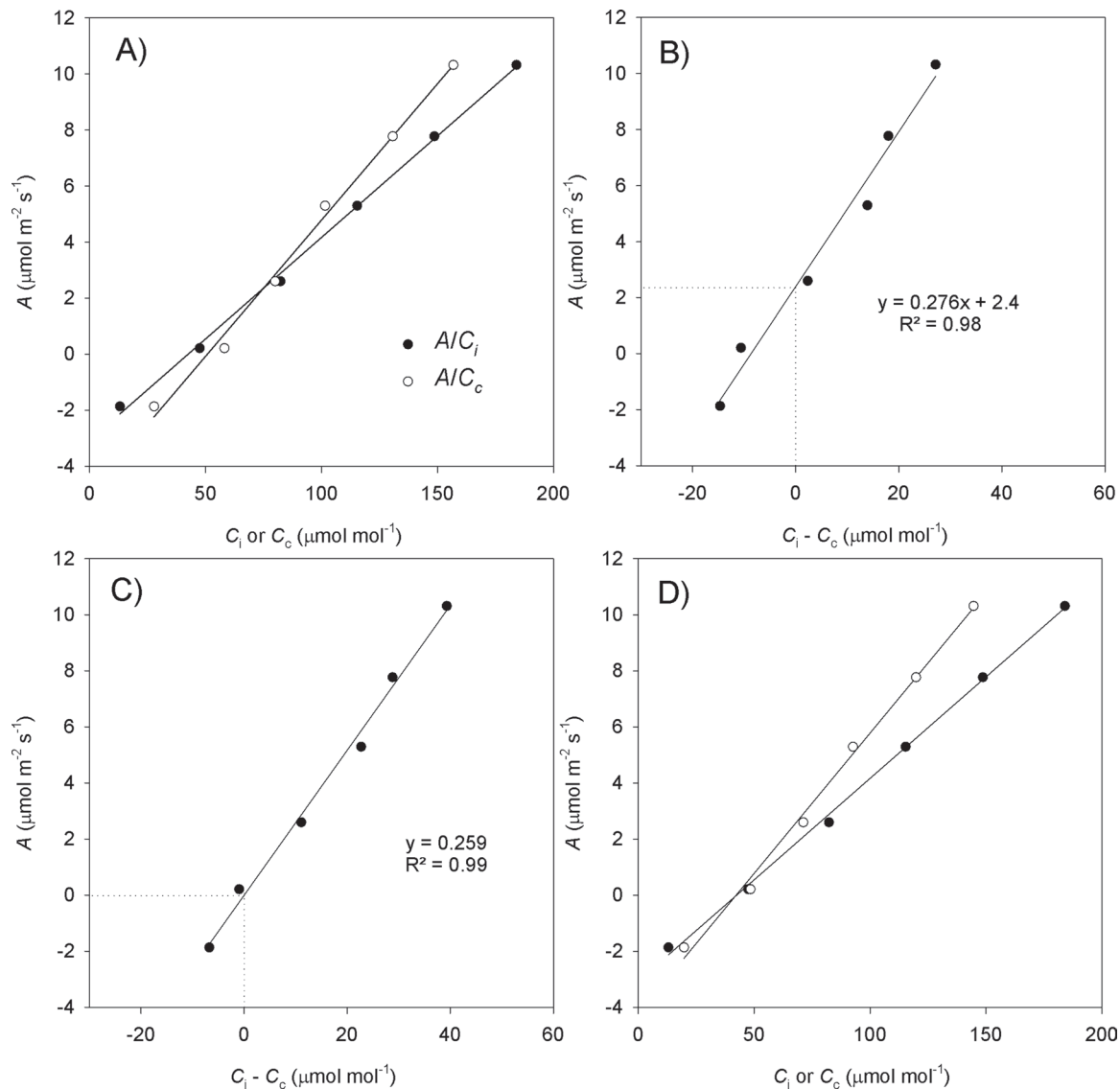
Data are expressed as the means  $\pm$  standard error. Student's *t*-tests were used to compare the photosynthetic parameters calculated with the different sets of Rubisco kinetic constants and to examine whether the intercepts of the regression were significantly different from zero. Linear regression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

## Results

### The effect of the different calibration methods on $\alpha\beta$

The four calibration methods tested here comprised scenarios ranging from a low (Yin PPFD <400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) to a high reductant supply ( $\Phi_{PSII}/\Phi_{\text{CO}_2}$  PPFD >400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), all performed under non-photorespiratory conditions, although potentially affected by the occurrence of an alternative electron flow. Additionally, three species covering a large range in SLA ( $33 \pm 1.4$ ,  $14 \pm 2.7$ , and  $9.0 \pm 1.3$   $\text{m}^2 \text{kg}^{-1}$  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  $\alpha\beta$  product.

High regression coefficients were obtained for all of the relationships based on the four calibration methods ( $r^2 = 0.91$ – $0.98$ ) (Fig. 2). In contrast to expectations, no major differences in the estimation of  $\alpha\beta$  were found between the methods Yin (PPFD <400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and  $\Phi_{PSII}/\Phi_{\text{CO}_2}$  (PPFD >400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) (Fig. 2B, C) and between  $\Phi_{PSII}/\Phi_{\text{CO}_2}$  ( $A/C_i$ ) and Yin (PPFD <400/ $C_i$  >500  $\mu\text{mol mol}^{-1}$  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  $\alpha\beta$ . Instead, the major differences among the calibration methods were dependent on the type of data used to perform the calibration [i.e.  $A/\text{PPFD}$  or  $A/C_i$  curves (Table 2)]. Regardless of the studied species, the calibrations using only  $A/\text{PPFD}$  data gave lower  $\alpha\beta$  values, ranging from 0.37 to 0.50, whereas those using  $A/C_i$  data produced higher  $\alpha\beta$  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



**Fig. 1.** Graphical representation of the method utilized to retrieve the respiration value from the combined  $A/C_i$  and  $A/C_c$  curves ( $R_{AC_i/AC_c}$ ). The example is based on values measured in an *N. tabacum* leaf. (A) The initial part of an  $A/C_i$  (filled circles) and the respective  $A/C_c$  (open circles) curve considering  $R_L = R_{\text{dark}/2}$  ( $1.1 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ). (B)  $C_i - C_c$  from the curves presented in (A) plotted against  $A$ . The  $y$ -axis positive intercept (2.4) when  $x=0$  (see the dotted lines) means negative  $g_m$  for  $A$  lower than the intercept because  $C_i > C_c$ . The slope of the linear regression (0.276) is an estimation of averaged  $g_m$  over the range of  $C_i$  used in the linear fit. (C) By adjusting to zero the intercept in the relationship  $A$  versus  $C_i - C_c$ , a new respiration value,  $R_{AC_i/AC_c}$ , is obtained ( $0.4 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), and the same  $\text{CO}_2$  compensation point is now shared by both curves ( $C_i - C_c = 0$  at  $A=0$ ). The influence of the new respiration on  $g_m$  can be observed as the modified slope (0.259). (D) The modified initial part of the  $A/C_i$  and  $A/C_c$  curves calculated with  $R_{AC_i/AC_c}$ .

similar values for  $\alpha\beta$ , suggesting that leaf structure does not play a major role in determining  $\alpha\beta$ .

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

Given that no major differences were found between the  $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$  relationship and the Yin method,  $g_m$  was estimated using the methods covering the extremes of  $\alpha\beta$  values, namely  $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$  ( $A/C_i$ ) and  $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$  (PPFD  $>400 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ). Because these methods consider only  $A/\text{PPFD}$  ( $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ , PPFD  $>400 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ) or  $A/C_i$  data [ $\Phi_{\text{PSII}}/$

$\Phi_{\text{CO}_2}$  ( $A/C_i$ )], the  $g_m$  estimated using either method is hereafter referred to as the  $g_m$  obtained with the  $A/\text{PPFD}$  or  $A/C_i$   $J$  calibration. Once the two  $J$  calibrations to be used were defined, an  $R_L$  value next had to be chosen to calculate  $C_c$ . Because there was no consistency in the  $R_L$  estimated via the Yin approach using  $A/\text{PPFD}$  (PPFD  $<400 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ) or  $A/C_i$  (PPFD  $<400 \mu\text{mol m}^{-2} \text{ s}^{-1}$  or  $C_i >500 \mu\text{mol mol}^{-1}$  air) (Table 2),  $R_{\text{dark}/2}$  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  $R_L$  ( $R_{AC_i/AC_c}$ ) as the value forcing the  $\text{CO}_2$  compensation point ( $\Gamma$ ) to be equal

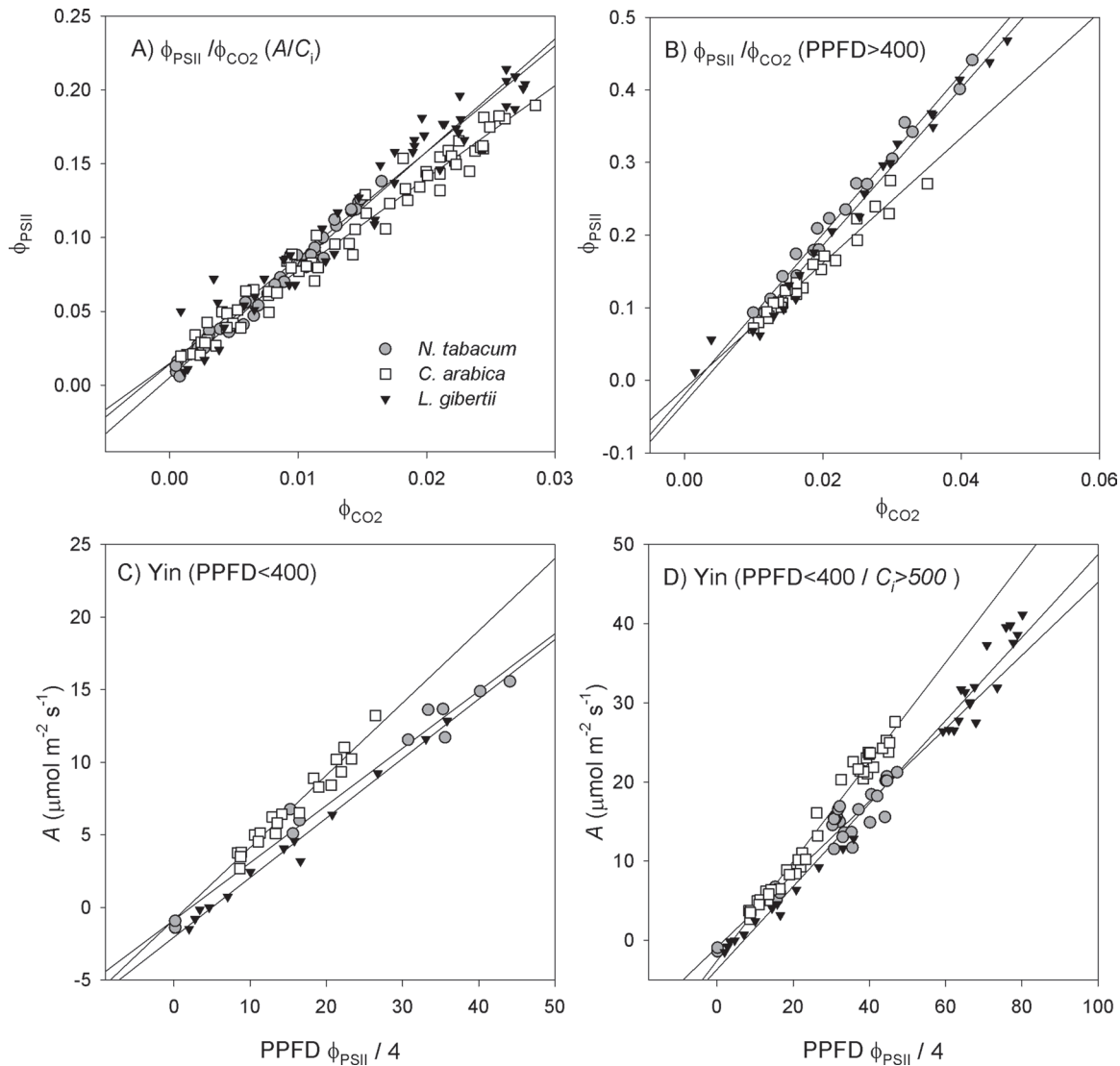
**Table 1.** Rubisco kinetic constants measured for the species studied: specificity factor ( $S_{c/o}$ ),  $CO_2$  compensation point in the absence of respiration ( $\Gamma^*$ ), Michaelis–Menten kinetics for  $CO_2$  ( $K_c$ ) and  $O_2$  ( $K_o$ ), and catalytic turnover rate for the carboxylase reaction ( $K_{cat}$ )

Species	$S_{c/o}$	$\Gamma^*$ ( $\mu\text{bar}$ )	$K_c$ ( $\mu\text{M}$ )	$K_o$ ( $\mu\text{M}$ )	$K_{cat}$ ( $\text{s}^{-1}$ )
<i>N. tabacum</i>	$98.1 \pm 2.6^a$	$39.7 \pm 1.1$	$12.4 \pm 0.7$	$274 \pm 42$	$3.2 \pm 0.3$
<i>C. arabica</i>	$98.4 \pm 4.3$	$39.6 \pm 1.7$	$10.3 \pm 1.3$	$479 \pm 113$	$3.2 \pm 0.1$
<i>L. gibertii</i>	$110.5 \pm 1.6^b$	$35.2 \pm 0.5$	$8.9 \pm 0.5$	$593 \pm 75$	$2.7 \pm 0.8$

Values are the means  $\pm$  standard error of 3–4 replicates per species.

<sup>a</sup> Taken from Galmés et al. (2006)

<sup>b</sup> Taken from Galmés et al. (2005).



**Fig. 2.** Calibration relationships  $\Phi_{PSII}$  versus  $\Phi_{CO_2}$  (A and B) 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  $PPFD > 400 \mu\text{mol m}^{-2} \text{s}^{-1}$  are considered; in (C), only values at  $PPFD < 400 \mu\text{mol m}^{-2} \text{s}^{-1}$  are used; in (D), only values at  $PPFD < 400 \mu\text{mol m}^{-2} \text{s}^{-1}$  plus  $C_i > 500 \mu\text{mol mol}^{-1}$  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  $R_1$  in C and D. The values of the slopes and intercepts are summarized in Table 2. Only  $\Phi_{CO_2}$  values  $< 0.05$  were kept in the plots, as recommended by Seaton and Walker (1990). The scales have different amplitudes according to the calibration. In the plots, data from 4–6  $A/PPFD$  or  $A/C_i$  curves are used for the linear regression.

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  $R_{\text{dark}/2}$  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  $0 < g_m < 1 \text{ mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ . 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

**Table 2.** Slopes ( $\alpha\beta$ ) and light respiration following the method of Yin *et al.* (2011) ( $R_{L,Yin}$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ) or intercept values ( $\Phi_{PSII}/\Phi_{CO_2}$ ) obtained under non-photorespiratory conditions according to different approaches, consisting of higher ( $\Phi_{PSII}/\Phi_{CO_2}$  A/C<sub>i</sub> or PPFD >400  $\mu\text{mol m}^{-2} \text{ s}^{-1}$ ) or lower (Yin PPFD <400 or PPFD <400  $\mu\text{mol m}^{-2} \text{ s}^{-1}$  and C<sub>i</sub> >500  $\mu\text{mol mol}^{-1}$  air) susceptibilities to alternative electron sinks

For comparison, the original slopes ( $k$ ) obtained when using the  $\Phi_{PSII}/\Phi_{CO_2}$  relationship were already converted to  $\alpha\beta$  ( $\alpha\beta=4/k$ ); the intercept refers to the parameter  $b$  in the equation:  $\Phi_{PSII} = k \Phi_{CO_2} + b$ .

		Slope ( $\alpha\beta$ )	Intercept ( $\Phi_{PSII}/\Phi_{CO_2}$ )	$R_{L,Yin}$
<i>N. tabacum</i>	$\Phi_{PSII}/\Phi_{CO_2}$ (A/C <sub>i</sub> )	0.52 ± 0.013	0.005 ± 0.002	
	$\Phi_{PSII}/\Phi_{CO_2}$ (PPFD >400)	0.36 ± 0.011	-0.02 ± 0.008*	
	Yin (PPFD <400)	0.39 ± 0.017		0.87 ± 0.47
	Yin (PPFD <400 C <sub>i</sub> >500)	0.46 ± 0.026		0.87 ± 0.87
<i>C. arabica</i>	$\Phi_{PSII}/\Phi_{CO_2}$ (A/C <sub>i</sub> )	0.64 ± 0.014	0.015 ± 0.002*	
	$\Phi_{PSII}/\Phi_{CO_2}$ (PPFD >400)	0.46 ± 0.018	-0.011 ± 0.007	
	Yin (PPFD <400)	0.50 ± 0.025		0.84 ± 0.41
	Yin (PPFD <400 C <sub>i</sub> >500)	0.63 ± 0.015		2.55 ± 0.45*
<i>L. gibertii</i>	$\Phi_{PSII}/\Phi_{CO_2}$ (A/C <sub>i</sub> )	0.56 ± 0.019	0.014 ± 0.004*	
	$\Phi_{PSII}/\Phi_{CO_2}$ (PPFD >400)	0.37 ± 0.011	-0.03 ± 0.009*	
	Yin (PPFD <400)	0.41 ± 0.013		2.04 ± 0.25*
	Yin (PPFD <400 C <sub>i</sub> >500)	0.50 ± 0.018		3.14 ± 1.02*

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<sub>i</sub> and A/C<sub>c</sub> curves ( $R_{AC_i/AC_c}$ )

All values are in  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ .

Species	$R_{dark}$	$R_{AC_i/AC_c}$ <sup>a</sup>
<i>N. tabacum</i>	2.2 ± 0.1	0.3 ± 0.1
<i>C. arabica</i>	0.9 ± 0.1	0.2 ± 0.1
<i>L. gibertii</i>	4.0 ± 0.3	1.7 ± 0.4

Values are the means ± standard error of four replicates per species.

<sup>a</sup> For  $R_{AC_i/AC_c}$ , the values are averages from the A/C<sub>c</sub> curves

calibrated using the A/PPFD or A/C<sub>i</sub> curves.

highest percentage of data excluded (i.e. unrealistic  $g_m$  values) was found when combining the A/PPFD  $J$  calibration with  $R_{dark/2}$  (88, 56, and 42% for *N. tabacum*, *C. arabica*, and *L. gibertii*, respectively), whereas the lowest percentage of data excluded was obtained with the A/C<sub>i</sub>  $J$  calibration and  $R_{AC_i/AC_c}$  (17% for *N. tabacum* and 0% for the other species) (Table 4).

The different calibrations also affected the magnitude of  $g_m$  in response to C<sub>i</sub>. There was no general trend at C<sub>i</sub> <100  $\mu\text{mol mol}^{-1}$  air, whereas at C<sub>i</sub> >100  $\mu\text{mol mol}^{-1}$  air, lower  $g_m$  values (up to 50%) were observed using the A/C<sub>i</sub>  $J$  calibration (Table 4). In addition, when plotting  $g_m$  versus C<sub>i</sub>, 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<sub>i</sub> of 100–350  $\mu\text{mol mol}^{-1}$  air and the single point  $g_m$  at CO<sub>2</sub> ambient (400  $\mu\text{mol mol}^{-1}$ ) (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  $0 < g_m < 1 \text{ mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ , the Harley *et al.* (1992) criteria were also applied, considering the  $g_m$  data in the range of  $10 < dC_i/dA < 50$  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<sub>i</sub> >350  $\mu\text{mol mol}^{-1}$  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<sub>i</sub> 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<sub>i</sub> curve analysis method suggested by Ethier 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 $\alpha\beta$

In contrast to the  $g_m$  estimates that could be greatly affected by the use of either  $R_{dark/2}$  or  $R_{AC_i/AC_c}$  (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<sub>c</sub> basis were always higher for the A/C<sub>i</sub>  $J$  calibration than for its A/PPFD  $J$

**Table 4.** Mesophyll conductance ( $g_m$ , mol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>) for several intervals of  $C_i$  and percentage of data excluded (DE) after applying a restriction ( $g_m$  restricted to the range of  $0 < g_m < 1$  mol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>)

The  $A/C_i$  or A/PPFD  $J$  calibration refers to the  $\Phi_{PSII}/\Phi_{CO_2}$  ( $A/C_i$ ) or A/PPFD methods, respectively.  $R_{AC_i/AC_c}$  is the light respiration estimate from the combined  $A/C_i$  and  $A/C_c$  curves proposed in this study, and  $R_{dark/2}$  is the dark respiration divided per two to account for the observed reduction in  $R_{dark}$  under light (Niinemets *et al.*, 2005). Note the high DE at low  $C_i$  for  $R_{dark/2}$  and at high  $C_i$  for the A/PPFD  $J$  calibration.

	A/C <sub>i</sub> J calibration				A/PPFD J calibration			
	$R_{AC_i/AC_c}$		$R_{dark/2}$		$R_{AC_i/AC_c}$		$R_{dark/2}$	
	$g_m$	DE (%)	$g_m$	DE (%)	$g_m$	DE (%)	$g_m$	DE (%)
<i>N. tabacum</i>								
$C_i < 100$	0.290 ± 0.065	17	0.243 ± 0.033	58	0.345 ± 0.132	17	0.203 ± 0.031	67
$C_i 100-350$	0.213 ± 0.021	0	0.287 ± 0.037	6	0.468 ± 0.064	50	0.504 ± 0.262	88
$C_i > 350$	0.038 ± 0.005	0	0.045 ± 0.007	0	0.262 ± 0.024	65	0.132 ± 0.130	70
<i>C. arabica</i>								
$C_i < 100$	0.131 ± 0.018	0	0.202 ± 0.042	10	0.164 ± 0.028	0	0.202 ± 0.035	20
$C_i 100-350$	0.117 ± 0.006	0	0.124 ± 0.007	0	0.186 ± 0.029	0	0.191 ± 0.029	6
$C_i > 350$	0.052 ± 0.004	0	0.053 ± 0.004	0	0.219 ± 0.038	41	0.194 ± 0.039	56
<i>L. gibertii</i>								
$C_i < 100$	0.235 ± 0.027	0	0.270 ± 0.050	7	0.310 ± 0.041	7	0.275 ± 0.079	27
$C_i 100-350$	0.214 ± 0.014	0	0.228 ± 0.020	0	0.371 ± 0.044	7	0.406 ± 0.083	21
$C_i > 350$	0.071 ± 0.011	0	0.073 ± 0.012	0	0.226 ± 0.063	32	0.162 ± 0.044	42

Values are the means ± standard error of 4–6  $A/C_i$  curves per species. The SE was calculated according to the points that remained in each  $C_i$  interval after applying the restriction.

counterpart (Table 5). These higher  $V_{cmax}$  and  $J_{max}$  values are in agreement with the lower  $g_m$  values found, in general, with the  $A/C_i$   $J$  calibration (Table 4). The  $V_{cmax}$  obtained using the Ethier and Livingston method was closer to the  $V_{cmax}$  on a  $C_c$  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/PPFD or  $A/C_i$   $J$  calibration was found (Table 5). Apart from the  $J_{max}$  calculated with the  $A/C_i$   $J$  calibration, which had the higher values for all species and reflected the higher  $\alpha\beta$  found (Table 3), there were no major differences among the other calculated values of  $J_{max}$ . 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  $V_{cmax}$  by ~30% 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  $g_m$  (Ethier and Livingston method), which was 28% lower when using the Rubisco kinetics of Bernacchi *et al.* (2002).

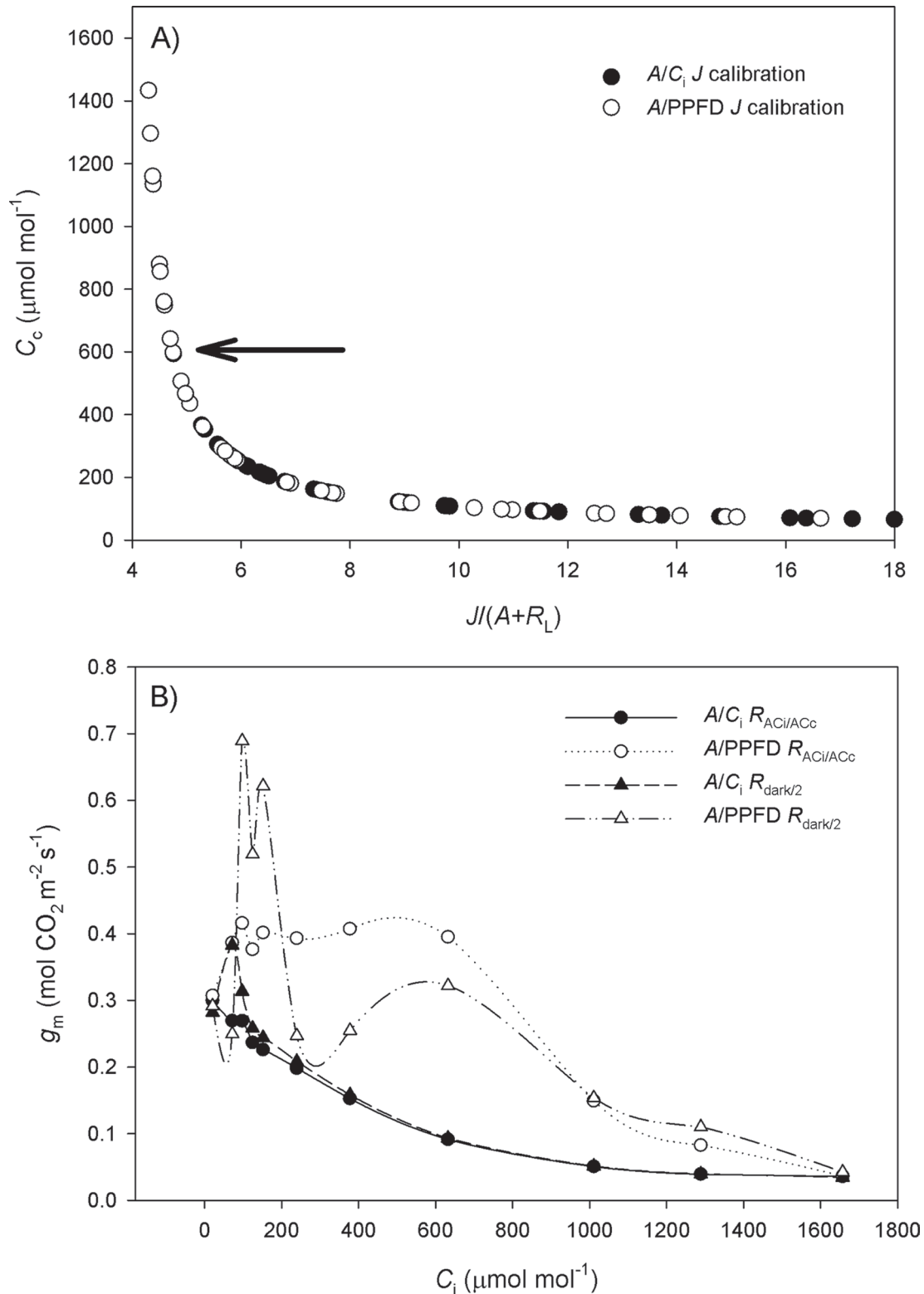
## Discussion

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/PPFD curves under low O<sub>2</sub> to calibrate  $J$  can significantly affect the estimations of  $g_m$ ,  $V_{cmax}$ , and  $J_{max}$ , 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  $g_m$  values at low and high  $C_i$ , and recommendations to improve  $g_m$  estimations accordingly.

### Sensitivity of the variable $J$ method to $\alpha\beta$

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 se*, given the variety of ways in which  $J$  can be calibrated. Despite the  $J$  calibration based on A/PPFD curves being the most common method in the literature, here it is shown that this calibration produced a higher number of unrealistic  $g_m$  estimates than its counterpart based on  $A/C_i$  curves (Table 4). Additionally, it was demonstrated that  $g_m$  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  $R_L$  at low  $C_i$  on the other hand. Notably, the need to develop useful criteria to improve  $g_m$  estimations and to understand the high  $g_m$  variability is crucial considering that the only existing indicator, the Harley *et al.* (1992) criteria which consider that reliable  $g_m$  estimations are those situated in the range of  $dC_c/dA$  (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  $C_c$  and  $J/(A+R_L)$  (Fig. 3A) that  $C_c$  approaches infinity as  $J/(A+R_L)$  approaches four (number of electrons per CO<sub>2</sub> molecule fixed). In contrast,  $C_c$  is negative when the ratio is less than four [the lower  $C_c$  threshold for positive  $J/(A+R_L)$  is defined by the parameter  $\Gamma^*$ ]. Therefore, as  $J/(A+R_L)$  approaches four as photorespiration and other alternative electron sinks tend to decrease (at high  $C_i$ , for example), extremely high  $C_c$  values are to be expected, leading to a greater probability of



**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 ( $\sim 600 \mu\text{mol mol}^{-1}$ ) observed for the  $A/C_i$   $J$  calibration, being much lower than its  $A/PPFD$  counterpart which can reach  $C_c$  values  $>1600 \mu\text{mol mol}^{-1}$ . (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 [ $R_{\text{dark}/2}$  (triangles) or  $R_{ACi/ACc}$  (circles)]. It is remarkable how there are 'spikes' in  $g_m$  at low  $C_i$  when using  $R_{\text{dark}/2}$  and how these are alleviated when using  $R_{ACi/ACc}$ . The curves are from *L. gibertii*, and the points are averages from four plants for  $g_m$  between 0 and  $1 \text{ mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ . 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.

**Table 5.** Maximum carboxylation rate of Rubisco ( $V_{cmax}$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), maximum electron transport rate from gas exchange ( $J_{max}$ ,  $\mu\text{mol e}^- \text{ m}^{-2} \text{ s}^{-1}$ ), mesophyll conductance ( $g_m$ , Ethier,  $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ) according to Ethier and Livingston (2004), and electron transport rate estimated from chlorophyll fluorescence ( $J_{flu}$ ,  $\mu\text{mol e}^- \text{ m}^{-2} \text{ s}^{-1}$ )

All of the photosynthetic parameters were calculated on a  $C_i$  basis from the  $A/C_i$  curves or on a  $C_c$  basis from the  $A/C_c$  curves using the measured Rubisco kinetic constants ( $\Gamma^*$ ,  $K_c$ , and  $K_o$ ) in this study (as reported in Table 1) or the standard Rubisco kinetics for tobacco from Bernacchi et al. (2002).  $A/C_i$   $J$  cal and  $A/PPFD$   $J$  calibration denote the photosynthetic parameters calculated for  $A/C_c$  curves using the  $\alpha\beta$  retrieved from  $\Phi_{PSII}/\Phi_{CO_2}$  ( $A/C_i$ ) or ( $A/PPFD$ )  $J$  calibrations. Note that  $J_{flu}$  is independent of Rubisco kinetics.

	Measured kinetic constants			Bernacchi constants		
	<i>N. tabacum</i>	<i>C. arabica</i>	<i>L. gibertii</i>	<i>N. tabacum</i>	<i>C. arabica</i>	<i>L. gibertii</i>
$V_{cmax}$ $C_i$ basis	71.7 ± 4.6	41.7 ± 2.4*	70.5 ± 1.6*	59.8 ± 3.8	49.8 ± 2.7	104.0 ± 2.4
$V_{cmax}$ $C_c$ basis $A/C_i$ $J$ cal	106.3 ± 8.9	73.9 ± 3.6*	133.4 ± 11.9*	87.0 ± 7.1	89.8 ± 4.4	210.1 ± 20.8
$V_{cmax}$ $C_c$ basis $A/PPFD$ $J$ cal	80.2 ± 5.7	59.8 ± 3.8*	101.7 ± 8.2*	66.7 ± 4.6	70.3 ± 3.1	155.8 ± 14.0
$V_{cmax}$ Ethier	91.8 ± 14.7	80.2 ± 4.0*	124.8 ± 5.0*	81.7 ± 9.2	102.1 ± 5.4	164.6 ± 11.7
$J_{max}$ $C_i$ basis	87.2 ± 7.4	110.7 ± 3.4	175.3 ± 7.3	86.6 ± 7.4	110.7 ± 3.4	176.6 ± 7.4
$J_{max}$ $C_c$ basis $A/C_i$ cal	103.2 ± 10.5	136.6 ± 7.1	228.6 ± 8.7	101.4 ± 10.3	136.6 ± 7.1	233.4 ± 8.9
$J_{max}$ $C_c$ basis $A/PPFD$ cal	84.9 ± 7.3	113.7 ± 5.7	177.0 ± 5.8	84.4 ± 7.3	113.1 ± 5.3	178.2 ± 5.8
$J_{max}$ Ethier	99.5 ± 3.3	115.0 ± 3.2	179.8 ± 8.9	99.5 ± 3.3	114.4 ± 3.2	179.8 ± 8.9
$J_{flu}$ $A/C_i$ $J$ cal	117.5 ± 11.4	139.2 ± 6.7	236.0 ± 9.7		–	
$J_{flu}$ $A/PPFD$ $J$ cal	94.7 ± 7.9	113.1 ± 4.8	181.7 ± 6.4		–	
$g_m$ Ethier	0.412 ± 0.045	0.108 ± 0.006	0.226 ± 0.036	0.296 ± 0.015	0.101 ± 0.005	0.257 ± 0.048

Values are the means ± standard error of 4–6  $A/C_i$  or  $A/C_c$  curves per species.

$R_{AC_i/AC_c}$  was used when needed.

An asterisk denotes differences in the respective photosynthetic parameter calculated using the different set of Rubisco kinetics.

The graphical representation of the  $A/C_i$  and  $A/C_c$  curves is shown in Supplementray Figure S1 at JXB online.

estimating negative values of  $g_m$ . Thus, for the same ( $A+R_L$ ), 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  $C_c$ , whereas the opposite holds true for a lower  $\alpha\beta$ .

At low  $C_i$  (<100  $\mu\text{mol mol}^{-1}$  air), misestimations of  $g_m$  are unlikely to be affected by  $J/(A+R_L)$ , as this ratio tends to achieve higher values with decreasing  $C_i$ . In 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  $R_L$ , which can ultimately affect  $C_c$ . 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 ( $R_{AC_i/AC_c}$ ) as the value forcing the intercept of the linear relationship of  $A$  versus  $C_i-C_c$  to zero, which is equivalent to forcing the  $\Gamma$  to be the same. It is believed that  $R_{AC_i/AC_c}$  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  $R_{AC_i/AC_c}$  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  $A/C_i$  and  $A/C_c$  sharing the same  $\Gamma$ .

An *a priori* weakness regarding  $R_{AC_i/AC_c}$  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  $C_i-C_c$ , 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  $C_i-C_c$ . 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  $C_c$  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 $A/C_i$ 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  $A/C_i$  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  $A/C_i$ -based  $J$  calibration: it is performed under the same irradiance used in the normal  $A/C_i$  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  $V_{\text{cmax}}$  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  $V_{\text{cmax}}$  value (33%) in relation to the  $V_{\text{cmax}}$  on a  $C_i$  basis. For *C. arabica* and *L. gibertii*, higher  $V_{\text{cmax}}$  values were observed using both  $A/C_i$  and  $A/PPFD$   $J$  calibrations compared with  $V_{\text{cmax}}$  on a  $C_i$  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  $V_{\text{cmax}}$  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  $\alpha\beta$  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 Ethier and Livingston method. Interestingly, the values of  $V_{\text{cmax}}$  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  $V_{\text{cmax}}$  calculated from the Ethier approach presented intermediate values between the  $V_{\text{cmax}}$  from the  $A/PPFD$  or  $A/C_i$  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  $V_{\text{cmax}}$  (~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  $V_{\text{cmax}}$  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  $R_L$  through the determination of  $\Gamma$  (by definition,  $\Gamma$  must be higher than  $\Gamma^*$ ) or by plotting  $A$  versus  $C_i-C_c$  and analysing 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  $R_L$  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  $\text{CO}_2$  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 homobaric leaves) with large gradients of  $\text{CO}_2$  inside and outside the chamber in addition to the lower fluxes of  $\text{CO}_2$  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  $R_{A/C_i/A/C_c}$  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  $\mu\text{mol mol}^{-1}$  air and the single point  $g_m$  at ambient  $CO_2$  concentration (400  $\mu\text{mol mol}^{-1}$  air) using  $R_{\text{dark}/2}$  in the  $g_m$  estimation.

**Table S3.** The same as in **Table S1**, using  $R_{A/C_i/A/C_c}$  rather than  $R_{\text{dark}/2}$  to estimate  $g_m$ .

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

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

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

**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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### References

- Bernacchi CJ, Portis AR, Nakano H, Von Caemmerer S, Long SP.** 2002. Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis *in vivo*. *Plant Physiology* **130**, 1992–1998.
- Bird IF, Cornelius MJ, Keys AJ.** 1982. Affinity of RuBP carboxylases for carbon dioxide and inhibition of the enzymes by oxygen. *Journal of Experimental Botany* **33**, 1004–1013.
- Brooks A, Farquhar GD.** 1985. Effect of temperature on the  $CO_2/O_2$  specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase and the rate of respiration in the light. *Planta* **165**, 397–406.
- Butz ND, Sharkey TD.** 1989. Activity ratios of ribulose-1,5-bisphosphate carboxylase accurately reflect carbamylation ratios. *Plant Physiology* **89**, 735–739.
- Eichelmann H, Laisk A.** 2000. Cooperation of photosystems II and I in leaves as analyzed by simultaneous measurements of chlorophyll fluorescence and transmittance at 800 nm. *Plant and Cell Physiology* **41**, 138–147.
- Edwards GE, Baker NR.** 1993. Can  $CO_2$  assimilation in maize leaves be predicted accurately from chlorophyll fluorescence analysis? *Photosynthesis Research* **37**, 89–102.
- Ethier GJ, Livingston NJ.** 2004. On the need to incorporate sensitivity to  $CO_2$  transfer conductance into the Farquhar–von Caemmerer–Berry leaf photosynthesis model. *Plant, Cell and Environment* **27**, 137–153.
- Evans JR.** 2009. Potential errors in electron transport rates calculated from chlorophyll fluorescence as revealed by a multilayer leaf model. *Plant and Cell Physiology* **50**, 698–706.
- Evans JR, von Caemmerer S.** 1996. Carbon dioxide diffusion inside leaves. *Plant Physiology* **110**, 339–346.
- Farquhar GD, von Caemmerer S, Berry JA.** 1980. A biochemical model of photosynthetic  $CO_2$  assimilation in leaves of C3 species. *Planta* **149**, 78–90.
- Flexas J, Barbour MM, Brendel O, *et al.*** 2012. Mesophyll diffusion conductance to  $CO_2$ : an appreciated central player in photosynthesis. *Plant Science* **193–194**, 70–84.

- Flexas J, Diaz-Espejo A, Galmés J, Kaldenhoff R, Medrano H, Ribas-Carbo M.** 2007. Rapid variations of mesophyll conductance in response to changes in CO<sub>2</sub> concentration around leaves. *Plant, Cell and Environment* **30**, 1284–1298.
- Flexas J, Medrano H.** 2002. Energy dissipation in C<sub>3</sub> plants under drought. *Functional Plant Biology* **29**, 1209–1215.
- Flexas J, Ribas-Carbo M, Diaz-Espejo A, Galmés J, Medrano H.** 2008. Mesophyll conductance to CO<sub>2</sub>: current knowledge and future prospects. *Plant, Cell and Environment* **31**, 602–621.
- Harley PC, Loreto F, Di Marco G, Sharkey TD.** 1992. Theoretical considerations when estimating the mesophyll conductance to CO<sub>2</sub> flux by analysis of the response of photosynthesis to CO<sub>2</sub>. *Plant Physiology* **98**, 1429–1436.
- Hassiotou F, Ludwig M, Renton M, Veneklaas EJ, Evans JR.** 2009. Influence of leaf dry mass per area, CO<sub>2</sub>, and irradiance on mesophyll conductance in sclerophylls. *Journal of Experimental Botany* **60**, 2303–2314.
- Galmés J, Flexas J, Keys AJ, Cifre J, Mitchell RAC, Madgwick PJ, Haslam RP, Medrano H, Parry MAJ.** 2005. Rubisco specificity factor tends to be larger in plant species from drier habitats and in species with persistent leaves. *Plant, Cell and Environment* **28**, 571–579.
- Galmés J, Medrano H, Flexas J.** 2006. Acclimation of Rubisco specificity factor to drought in tobacco: discrepancies between *in vitro* and *in vivo* estimations. *Journal of Experimental Botany* **57**, 3659–3667.
- Galmés J, Medrano H, Flexas J.** 2007. Photosynthetic limitations in response to water stress and recovery in Mediterranean plants with different growth forms. *New Phytologist* **175**, 81–93.
- Genty B, Briantais JM, Baker NR.** 1989. The relationship between the quantum yield of photosynthetic electron-transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* **990**, 87–92.
- Gilbert ME, Pou A, Zwieniecki MA, Holbrook NM.** 2012. On measuring the response of mesophyll conductance to carbon dioxide with the variable *J* method. *Journal of Experimental Botany* **63**, 413–425.
- Laik A, Loreto F.** 1996. Determining photosynthetic parameters from leaf CO<sub>2</sub> exchange and chlorophyll fluorescence. *Plant Physiology* **110**, 903–912.
- Loreto F, Marco G, Tricoli D, Sharkey T.** 1994. Measurements of mesophyll conductance, photosynthetic electron transport and alternative electron sinks of field grown wheat leaves. *Photosynthesis Research* **41**, 397–403.
- Loreto F, Tsonev T, Centritto M.** 2009. The impact of blue light on leaf mesophyll conductance. *Journal of Experimental Botany* **60**, 2283–2290.
- Morison JIL, Lawson T.** 2007. Does lateral gas diffusion in leaves matter? *Plant, Cell and Environment* **30**, 1072–1085.
- Niinemets U, Cescatti A, Rodeghiero M, Tosens T.** 2005. Leaf internal diffusion conductance limits photosynthesis more strongly in older leaves of Mediterranean evergreen broad-leaved species. *Plant, Cell and Environment* **28**, 1552–1566.
- Niinemets U, Cescatti A, Rodeghiero M, Tosens T.** 2006. Complex adjustments of photosynthetic potentials and internal diffusion conductance to current and previous light availabilities and leaf age in Mediterranean evergreen species *Quercus ilex*. *Plant, Cell and Environment* **29**, 1159–1178.
- Niinemets U, Díaz-Espejo A, Flexas J, Galmés J, Warren CR.** 2009a. Role of mesophyll diffusion conductance in constraining potential photosynthetic productivity in the field. *Journal of Experimental Botany* **60**, 2249–2270.
- Niinemets U, Flexas J, Peñuelas J.** 2011. Evergreens favored by higher responsiveness to increased CO<sub>2</sub>. *Trends in Ecology and Evolution* **26**, 136–142.
- Niinemets U, Tenhunen JD.** 1997. A model separating leaf structural and physiological effects on carbon gain along light gradients for the shade-tolerant species *Acer saccharum*. *Plant, Cell and Environment* **20**, 845–866.
- Niinemets U, Wright IJ, Evans JR.** 2009b. Leaf mesophyll diffusion conductance in 35 Australian sclerophylls covering a broad range of foliage structural and physiological variation. *Journal of Experimental Botany* **60**, 2433–2449.
- Oguchi R, Douwstra P, Fujita T, Chow WS, Terashima I.** 2011. Intra-leaf gradients of photoinhibition induced by different color lights: implications for the dual mechanisms of photoinhibition and for the application of conventional chlorophyll fluorometers. *New Phytologist* **191**, 146–159.
- Pons TL, Flexas J, Von Caemmerer S, Evans JR, Genty B, Ribas-Carbo M, Brugnoli E.** 2009. Estimating mesophyll conductance to CO<sub>2</sub>: methodology, potential errors, and recommendations. *Journal of Experimental Botany* **60**, 2217–2234.
- Rodeghiero M, Niinemets U, Cescatti A.** 2007. Major diffusion leaks of clamp-on leaf cuvettes still unaccounted: how erroneous are the estimates of Farquhar *et al.* model parameters? *Plant, Cell and Environment* **30**, 1006–1022.
- Ruuska SA, Badger MR, Andrews TJ, von Caemmerer S.** 2000. Photosynthetic electron sinks in transgenic tobacco with reduced amounts of Rubisco: little evidence for significant Mehler reaction. *Journal of Experimental Botany* **51**, 357–368.
- Seaton GGR, Walker DA.** 1990. Chlorophyll fluorescence as a measure of photosynthetic carbon assimilation. *Proceedings of the Royal Society B: Biological Sciences* **242**, 29–35.
- Sharwood RE, von Caemmerer S, Maliga P, Whitney SM.** 2008. The catalytic properties of hybrid Rubisco comprising tobacco small and sunflower large subunits mirror the kinetically equivalent source Rubiscos and can support tobacco growth. *Plant Physiology* **146**, 83–96.
- Tazoe Y, von Caemmerer S, Estavillo GM, Evans JR.** 2011. Using tunable diode laser spectroscopy to measure carbon isotope discrimination and mesophyll conductance to CO<sub>2</sub> diffusion dynamically at different CO<sub>2</sub> concentrations. *Plant, Cell and Environment* **34**, 580–591.
- Tcherkez G, Cornic G, Bligny R, Gout E, Ghashghaie J.** 2005. *In vivo* respiratory metabolism of illuminated leaves. *Plant Physiology* **138**, 1596–1606.
- Tosens T, Niinemets Ü, Westoby M, Wright IJ.** 2012. Anatomical basis of variation in mesophyll resistance in eastern Australian sclerophylls: news of a long and winding path. *Journal of Experimental Botany* **63**, 5105–5119.

**Valentini R, Epron D, Angelis P, Matteucci G, Dreyer E.** 1995. *In situ* estimation of net CO<sub>2</sub> assimilation, photosynthetic electron flow and photorespiration in Turkey oak (*Q. cerris* L.) leaves: diurnal cycles under different levels of water supply. *Plant, Cell and Environment* **18**, 631–640.

**Warren CR, Adams MA.** 2004. Evergreen trees do not maximize instantaneous photosynthesis. *Trends in Plant Science* **9**, 270–274.

**Warren CR.** 2006. Estimating the internal conductance to CO<sub>2</sub> movement. *Functional Plant Biology* **33**, 431–442.

**Xu C, Fisher R, Wullschlegel SD, Wilson CJ, Cai M, McDowell NG.** 2012. Toward a mechanistic modeling of nitrogen limitation on vegetation dynamics. *PLoS One* **7**, e37914

**Yin X, Struik PC, Romero P, Harbinson J, Evers JB, van der Putten PEL, Vos J.** 2009. Using combined measurements of gas exchange and chlorophyll fluorescence to estimate parameters of a biochemical C<sub>3</sub> photosynthesis model: a critical appraisal and a new integrated approach applied to leaves in a wheat (*Triticum aestivum*) canopy. *Plant, Cell and Environment* **32**, 448–464.

**Yin X, Sun Z, Struik PC, Gu J.** 2011. Evaluating a new method to estimate the rate of leaf respiration in the light by analysis of combined gas exchange and chlorophyll fluorescence measurements. *Journal of Experimental Botany* **62**, 3489–3499.

**Yin X, van Oijen M, Schapendonk AHCM.** 2004. Extension of a biochemical model for the generalized stoichiometry of electron transport limited C<sub>3</sub> photosynthesis. *Plant, Cell and Environment* **27**, 1211–1222.