

REVIEW PAPER

# Estimating mesophyll conductance to CO<sub>2</sub>: methodology, potential errors, and recommendations

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## Abstract

The three most commonly used methods for estimating mesophyll conductance ( $g_m$ ) are described. They are based on gas exchange measurements either (i) by themselves; (ii) in combination with chlorophyll fluorescence quenching analysis; or (iii) in combination with discrimination against <sup>13</sup>CO<sub>2</sub>. To obtain reliable estimates of  $g_m$ , the highest possible accuracy of gas exchange is required, particularly when using small leaf chambers. While there may be problems in achieving a high accuracy with leaf chambers that clamp onto a leaf with gaskets, guidelines are provided for making necessary corrections that increase reliability. All methods also rely on models for the calculation of  $g_m$  and are sensitive to variation in the values of the model parameters. The sensitivity to these factors and to measurement error is analysed and ways to obtain the most reliable  $g_m$  values are discussed. Small leaf areas can best be measured using one of the fluorescence methods. When larger leaf areas can be measured in larger chambers, the online isotopic methods are preferred. Using the large CO<sub>2</sub> draw-down provided by big chambers, and the isotopic method, is particularly important when measuring leaves with high  $g_m$  that have a small difference in [CO<sub>2</sub>] between the substomatal cavity and the site of carboxylation in the chloroplast ( $C_i - C_c$  gradient). However, equipment for the fluorescence methods is more easily accessible. Carbon isotope discrimination can also be measured in recently synthesized carbohydrates, which has its advantages under field conditions when large number of samples must be processed. The curve-fitting method that uses gas exchange measurements only is not preferred and should only be used when no alternative is available. Since all methods have their weaknesses, the use of two methods for the estimation of  $g_m$ , which are as independent as possible, is recommended.

**Key words:** Chlorophyll fluorescence, isotope discrimination, mesophyll conductance, methodology, photosynthesis.

## Introduction

The diffusion of CO<sub>2</sub> from the atmosphere to the sites of carboxylation in the chloroplasts of C<sub>3</sub> leaves is restricted by resistances. One is formed by the boundary layer near the leaf surface with impaired air turbulence, another during diffusion through stomatal pores. The last part of the diffusion pathway, from the substomatal cavity to the

sites of carboxylation in the chloroplasts, is complicated and consists of resistances in both the gaseous and liquid phases: through the intercellular airspaces, the cell wall, the plasmalemma and the chloroplast envelope, and the cytosol and chloroplast stroma, which is collectively referred to as the mesophyll resistance. A series of diffusion barriers as

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described above is most conveniently described as a series of resistances. However, when described in conjunction with fluxes, such as the rate of CO<sub>2</sub> uptake, it is more convenient to use the inverse, conductance. The one-dimensional model described above does not take into account the three-dimensional structure of a leaf (Parkhurst, 1994), but a detailed 3D model cannot be routinely solved because of the lack of small-scale data. Therefore, the linear 1D simplification is used for calculating conductances involved in gas exchange, including the mesophyll conductance ( $g_m$ ) discussed here. The  $g_m$  thus represents a value for the bulk of the leaf under consideration and it is expressed per unit leaf area. As mentioned above, the methodologies described here apply for C<sub>3</sub> plants only. In C<sub>4</sub> plants internal diffusion occurs from the intercellular airspace to the mesophyll cytoplasm where phosphoenol pyruvate (PEP) carboxylation takes place. Since the isotope discrimination during C<sub>4</sub> photosynthesis is small and also modulated by bundle sheath leakiness, it cannot be used to quantify this diffusion resistance. The fluorescence method works in C<sub>3</sub> species because of photorespiration (see later). In C<sub>4</sub> species, fluorescence is emitted from mesophyll and bundle sheath chloroplasts and it is difficult to interpret, although sometimes fluorescence is used to quantify bundle sheath leakiness (Evans and von Caemmerer, 1996).

Over the last couple of decades,  $g_m$  has emerged as an important limiting factor for CO<sub>2</sub> diffusion into a leaf. The role of  $g_m$  as a limiting factor is typically similar to or somewhat lower than that of stomatal conductance ( $g_s$ ) (Evans and Loreto, 2000; Warren, 2006). The study of  $g_m$  has increased exponentially in recent years as a result of recognition of its importance and more readily available instrumentation for its measurement (Flexas *et al.*, 2008). Different approaches are used for estimating  $g_m$ . They all rely on measurement of gas exchange. CO<sub>2</sub> and H<sub>2</sub>O share common diffusion pathways across the boundary layer and stomata, which is used to calculate the [CO<sub>2</sub>] in the substomatal cavity ( $C_i$ ). Simultaneously, the [CO<sub>2</sub>] at the sites of carboxylation in the chloroplasts ( $C_c$ ) is estimated (for details, see Long and Bernacchi, 2003). The  $C_i$ – $C_c$  gradient is then used to calculate  $g_m$ . Three types of approaches are used to estimate  $C_c$ , the measurement of the electron transport rate with chlorophyll fluorescence, the discrimination of <sup>13</sup>CO<sub>2</sub> by leaves, and a modelling approach using gas exchange data only. All these methods rely on models that have a number of assumptions, and they have technical limitations and sources of error that need to be considered to obtain reliable estimates of  $g_m$ . Moreover, they all rely on some common assumptions, such as the uniformity of  $C_i$  and  $C_c$  across the leaf, which does not always occur (Terashima *et al.*, 1988).

In the present review, the most commonly used methods are described briefly. While the fundamentals of these methods have already been detailed elsewhere (Harley *et al.*, 1992; Evans and von Caemmerer, 1996; Warren, 2006), here the focus is on technical aspects and on the precautions needed to obtain reliable estimates. The objectives are to provide present and future users of these

techniques guidelines on the most appropriate methodologies to select and warnings about problems to be avoided.

## Gas exchange measurements

All methods for estimating  $g_m$  rely strongly on gas exchange measurements. The accurate measurement of the net photosynthetic rate ( $A_n$ ) and the  $C_i$  is particularly important. Sufficient accuracy of the gas exchange measurements can be obtained relatively easily with large leaf chambers, provided proper calibrations are regularly done and corrections for band broadening by H<sub>2</sub>O and O<sub>2</sub> are made. However, large chambers have their own complications, such as light and temperature gradients across a leaf which affect estimates of  $C_i$ , or on how large the differences in inlet and outlet CO<sub>2</sub> can be, which is also affected by the linearity of infrared gas analysers (IRGAs) or how they are calibrated. Also, very large, custom-built chambers often used for online isotope discrimination have a limit set by the transpiration rate of the enclosed leaf, such that the chosen chamber size often reflects a compromise between maximizing accuracy and avoiding the risk of condensation occurring.

Nevertheless, using a relatively large chamber (i.e. ~10–20 cm<sup>2</sup>) would be the preferred choice when making  $A_n$ – $C_i$  curves for the curve-fitting method and estimations of  $\Gamma^*$  and  $R_L$ . Also when used in combination with online isotope discrimination, larger leaf areas are preferred since small areas result in smaller CO<sub>2</sub> differentials between the air entering and leaving the chamber for a given flow, thereby greatly reducing the accuracy of discrimination measurements. However, such chambers are not very suitable for simultaneous measurement of chlorophyll fluorescence, because most commercial fluorescence instruments cannot sample large areas. Since both measurements should be done as much as possible on the same part of a leaf, chambers enclosing small areas (down to 2 cm<sup>2</sup>) are typically used in commercially available instruments with an integrated optical system for chlorophyll fluorescence measurements. The small chamber is sealed onto a larger leaf by means of gaskets. It has the added advantages that measurements can be done on small leaves as opposed to the isotopic method that requires larger leaf areas, and that there is less likelihood of inhomogeneity in photosynthetic parameters across the measured area. However, these systems have some inherent disadvantages. Increased instrument noise caused by the small leaf area can be reduced, for instance by increased integration times.

More importantly, border effects and leaks related to the gaskets are introduced (Long and Bernacchi, 2003). Respiration ( $R$ ) continues in the part of the leaf under the gasket. Part of the CO<sub>2</sub> produced can escape into the chamber where it increases apparent  $R$  and, hence, decreases apparent  $A_n$  (Pons and Welschen, 2002). A model predicting that the CO<sub>2</sub> produced under the inner half of the gasket escapes to the chamber appeared to be a reasonable estimate and could be used for correcting apparent  $A_n$ .

However, if a large leaf chamber is available where most of the leaf can be kept free from the gaskets, then respiration rates in the dark ( $R_D$ ) can be compared between the two chambers. The large chamber is not necessarily a sophisticated one, since measurements are only done in darkness. When using 2.5–6 cm<sup>2</sup> leaf chambers, the uncorrected  $R_D$  was found to be ~50% higher than the  $R_D$  corrected after considering this effect (Pons and Welschen, 2002). This means that, for instance, in a leaf with an actual  $R_D$  of 2  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and  $A_n$  of 20  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , the measured  $A_n$  would have been 19  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (i.e. a 5% error). However, for a stressed leaf showing  $A_n$  of, say, 2  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , the measured  $A_n$  would have been 1  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (i.e. a 100% error).

Another problem can arise when measuring homobaric leaves. CO<sub>2</sub> can be transported through the leaf under the gasket (Jahnke, 2001). Comparison of homobaric and heterobaric leaves revealed that the magnitude of the process depends on the pressure difference between inside and outside the chamber and on stomatal conductance. With open stomata the error was estimated to be 7  $\mu\text{mol m}^{-2} \text{s}^{-1}$  per each kPa overpressure inside the chamber. This is likely to induce underestimations for commercially available small chambers that have a larger edge to area ratio (Jahnke and Pieruschka, 2006). When measuring homobaric leaves, it is suggested to use minimal overpressure and minimal [CO<sub>2</sub>] difference between inside and outside the chamber, but the phenomenon cannot be avoided altogether when using highly porous leaves such as tobacco. The phenomenon can interfere with the magnitude of the error caused by the CO<sub>2</sub> produced under the gasket mentioned above.

A further complication is the leakage of CO<sub>2</sub> and H<sub>2</sub>O through the gaskets and, more importantly, along the contact zone between gaskets and leaf surfaces. For instance, Flexas *et al.* (2007a) estimated that leakage resulted in apparent respiration and maximum photosynthesis rates of up to -1  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 4  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , respectively, when working with an empty 2 cm<sup>2</sup> chamber. The process is demonstrated when at low [CO<sub>2</sub>] inside an empty chamber an apparent  $R$  is measured and an apparent  $A_n$  at high [CO<sub>2</sub>]. Corrections for this process are suggested by the manufacturers. However, the diffusion rate is likely to be altered by the presence of a leaf. Flexas *et al.* (2007a) observed a decreased leakage of CO<sub>2</sub> in the presence of thermally killed leaves of several species. However, Rodeghiero *et al.* (2007) found an increased leakage for CO<sub>2</sub> when a dried sclerophyllous *Quercus ilex* leaf was clamped between the gaskets. In addition, they also observed significant leakage of H<sub>2</sub>O when the difference in vapour pressure between inside and outside the chamber was large. Errors were found to be substantial (up to 200%), particularly at low photosynthetic rates. The magnitude of the error is apparently difficult to predict. As a first approximation, empty leaf chamber corrections can be applied. Alternatively, corrections can be derived from measurements with a dead leaf, but it is not sure to what extent these are representative for a living leaf. These errors and to some extent also the transport of CO<sub>2</sub> through a homobaric

leaf are minimized by enclosing the chamber and flushing the enclosure with air leaving the chamber. This can be done by a plastic bag or by mounting a second pair of gaskets and flushing the space outside the chamber gaskets with chamber air (Rodeghiero *et al.*, 2007). The bag technique, however, may be difficult to seal totally when using intact plants, and flushing the large volume takes a long time when making  $A_n$ - $C_i$  curves (Flexas *et al.*, 2007a).

Minimizing the errors as described above is not sufficient to avoid them completely. Since the highest degree of accuracy is required for the estimation of  $g_m$  by means of gas exchange and fluorometry, corrections should thus be applied where possible for the above-mentioned sources of error, following the procedures described elsewhere (Flexas *et al.*, 2007a; Rodeghiero *et al.*, 2007). They must be applied not only to apparent  $A_n$  but also to  $C_i$ . A larger chamber has a smaller border to area ratio, which reduces the errors (Rodeghiero *et al.*, 2007). A step forward is thus the development of larger chambers with integrated optical systems for fluorometry or fluorescence imaging that are now becoming available.

The above considerations concern reliable measurements of  $A_n$ , and  $C_i$  in so far as  $A_n$  is used for its calculation. However, a correct estimate of  $C_i$  also requires a reliable calculation of stomatal conductance ( $g_s$ ), which is based on measured transpiration rate and leaf temperature. Gaskets of clamp-on chambers can also leak water vapour and, hence, interfere with the measurement of transpiration (Rodeghiero *et al.*, 2007). Leaf temperature is typically measured with thermocouples. However, when leaf to air temperature differences are large, the reading with these devices may not be sufficiently representative for leaf surface temperature. Infrared thermometry is a better choice because it measures temperature remotely over a significant surface of the leaf, and is now also available on commercial systems. At low  $g_s$  [e.g. under severe water stress or abscisic acid (ABA) treatment], the relative importance of the cuticular conductance cannot be ignored (Boyer *et al.*, 1997; Meyer and Genty, 1998). This can be separately determined (e.g. as described by Boyer *et al.*, 1997) and used for the correction of  $g_s$  values. The distribution of  $g_s$  is not always uniform over a leaf. Patchy stomatal closure (Terashima *et al.*, 1988) can occur, for example at low humidity, high [CO<sub>2</sub>], and under water stress. Its occurrence should be evaluated where relevant because it affects the estimation of  $C_i$ . This can be done, for instance, directly using fluorescence imaging (Meyer and Genty, 1998) or indirectly using the method described by Grassi and Magnani (2005), consisting of checking the similarity of  $A_n$ - $C_i$  curves at different vapour pressure deficit (since high vapour pressure deficit drives stomatal closure, if this closure was heterogeneous it would lead to errors in  $C_i$ , therefore modifying the slope of the  $A_n$ - $C_i$  curve). Unfortunately, if evidence for patchy stomatal closure is found, determining  $g_m$  is precluded (although a reliable value of  $C_c$  but not of  $C_i$  can still be obtained), because models of patchy distribution are unclear, variable,

and often complex (Terashima *et al.*, 1988; Buckley *et al.*, 1997) and, hence, not operational for  $g_m$  studies.

## Estimation of $g_m$ with gas exchange and chlorophyll fluorescence

### Theory

Estimating  $g_m$  from gas exchange plus fluorescence relies on the basic relationship between the rate of photosynthetic electron transport ( $J$ ), net  $\text{CO}_2$  assimilation ( $A_n$ ), and the  $\text{CO}_2$  concentration at the site of Rubisco ( $C$ ). The relationship can be modelled according to Farquhar *et al.* (1980):

$$J = (A_n + R_L) \frac{4C + 8\Gamma^*}{C - \Gamma^*} \quad (1)$$

where  $R_L$  is the rate of mitochondrial respiration in the light,  $\Gamma^*$  is the  $\text{CO}_2$  compensation point in the absence of  $R_L$ , and the factor 4 denotes the minimum electron requirement for carboxylation. Equation 1 assumes that linear electron transport only fulfils the demand for ATP by the carbon reduction cycle and photorespiration, and that the NADPH supply is limiting. A more general expression has been proposed recently (Yin *et al.*, 2004, 2009) to include the possible contributions of cyclic electron transport, pseudocyclic electron transport, and variable Q-cycle to balance  $\text{H}^+$ ,  $\text{e}^-$  supply. Equation 1 is thus a special case, which assumes absence of cyclic and pseudocyclic electron transport and that linear electron transport and full operation of the Q-cycle generate a non-limiting ATP supply for carboxylation and oxygenation. When a limitation of ATP supply is considered, alternative derivations have to be used (von Caemmerer, 2000) that are also special cases of the general model of Yin

*et al.* (2004, 2009). The use of alternative assumptions indeed has consequences for the calculation of  $g_m$  (see Table 1).

In the absence of knowledge of  $g_m$ , it was common to use intercellular  $[\text{CO}_2]$  ( $C_i$ ) as the best estimate of the  $\text{CO}_2$  concentration in the chloroplast ( $C_c$ ). When there is a significant decrease in  $[\text{CO}_2]$  from intercellular spaces to the site of carboxylation in the chloroplasts, then  $C_c$  can be related to  $C_i$  as:

$$C_c = C_i - A_n/g_m \quad (2)$$

Equation 1 then becomes

$$J = 4(A_n + R_L) \frac{(C_i - A_n/g_m) + 2\Gamma^*}{(C_i - A_n/g_m) - \Gamma^*} \quad (3)$$

Depending on the method used, Equation 1 can be rearranged for the calculation of  $C_c$ , and  $g_m$  can then be calculated from a rearranged Equation 2, or  $g_m$  can be calculated directly from a rearranged Equation 3. In those cases, values for  $J$  are derived from chlorophyll fluorescence. Alternatively, Equation 3 can be used to solve iteratively for  $g_m$  and  $J$  using least square methods.

When  $C_c$  is lower than  $C_i$  as a result of a finite  $g_m$ , then  $J$  at atmospheric  $[\text{O}_2]$  calculated on the basis of  $C_i$  is lower than calculated from  $C_c$  (Equation 1, Fig. 1). The higher  $J$  results from a higher rate of photorespiration than predicted from  $C_i$ . The magnitude of the difference as estimated by means of gas exchange and chlorophyll fluorescence is indicative of the  $C_i - C_c$  gradient and thus of  $g_m$  under the conditions of the measurements. The difference is typically small, and the accuracy of the calculated  $g_m$  thus depends on the accuracy of the fluorescence and gas exchange parameters. It further depends on model assumptions and on the validity of parameter values.

**Table 1.** Calculations of mesophyll conductance ( $g_m$ ), chloroplastic  $[\text{CO}_2]$  ( $C_c$ ) at atmospheric  $[\text{CO}_2]$ , and the ratio ( $b_F$ ) of the electron transport rate ( $J$ ) calculated from gas exchange ( $J_A$ ) over  $J$  calculated from chlorophyll fluorescence ( $J_F$ )

Different methods were used using gas exchange and chlorophyll fluorescence measurements on a leaf of *Hedera helix*. The calculations use the  $\text{CO}_2$  response of  $A_n$  at atmospheric  $[\text{O}_2]$  (Fig. 1). The ranges of  $C_i$  ( $\mu\text{mol mol}^{-1}$ ) and  $[\text{O}_2]$  used for the different methods are shown in the second row. The change in the estimate of  $g_m$  when using an alternative stoichiometry of  $J$  is shown in the last row. Measurements were done on a detached *H. helix* leaf grown in moderate shady conditions in a garden in October 2008. The leaf was clamped in a Parkinson leaf chamber using a LICOR 6262 IRGA and a PAM-2000 fluorometer (Pons and Welschen 2003). Conditions were: PFD  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ , leaf temperature  $20^\circ\text{C}$ , a vapour pressure difference of  $\sim 0.8 \text{ kPa}$ , and an atmospheric pressure of  $100.0 \text{ kPa}$ .  $A_n$  was 77% of the light-saturated value.  $A_n$  and  $C_i$  data were corrected for band broadening caused by  $\text{H}_2\text{O}$  and  $\text{O}_2$ , leakage of respired  $\text{CO}_2$  into the chamber, and diffusion of  $\text{CO}_2$  across the gaskets in an empty chamber. The  $J_F - J_A$  relationship was linear with a non-significant zero intercept.

	Constant $J$	Variable $J$		Curve-fitting <sup>†</sup>	
	$C_i$ range 240–650	Single $C_i=238$	$[\text{O}_2]$ range* $C_i=227$	$C_i$ range 100–180	$C_i$ range 40–650
$g_m \text{ mmol m}^{-2} \text{ s}^{-1}$	155	125	133	143	178
$C_c \mu\text{mol mol}^{-1}\ddagger$	159	141	148	–	168
$b_F (J_A/J_F)$	1.003	1.078 <sup>§</sup>	1.004	0.970	–
Effect on $g_m$ with alternative stoichiometry of $J$ <sup>¶</sup>	+14%	+14%	+15%	+8%	+15%

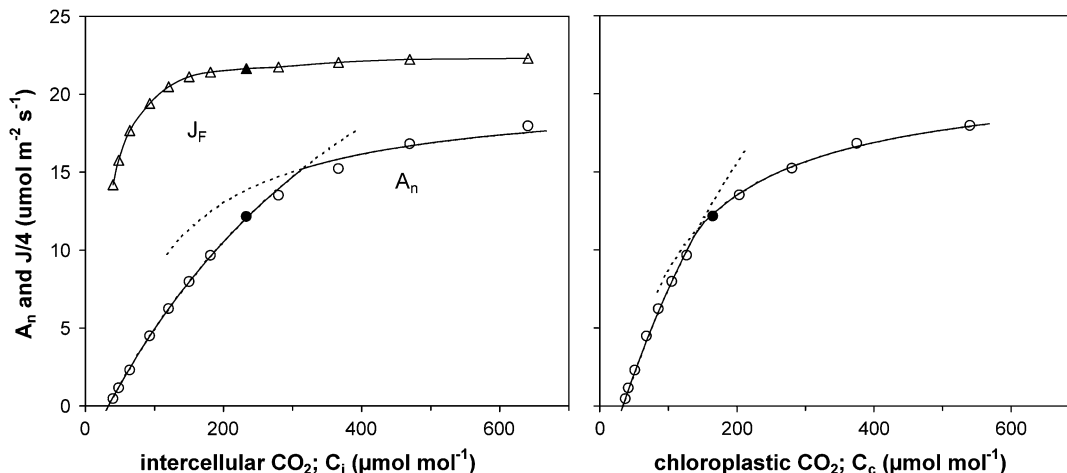
\* Measurements were done on a similar leaf from the same population as the one used for the other measurements. Three  $[\text{O}_2]$  were included, 1, 10, and 21%.

<sup>†</sup> For the Rubisco-limited part of the  $A_n - C_c$  relationship, values for  $K_c$  (23.4 Pa) and  $K_o$  (19.3 kPa) were taken from Bernacchi *et al.* (2001).

<sup>‡</sup> Measurement at atmospheric  $C_a$ , where  $C_i$  was  $238 \mu\text{mol mol}^{-1}$ .

<sup>§</sup> Estimation of  $b_F$  in air without  $\text{O}_2$ , where  $J_A$  and  $J_F$  were proportional;  $b_F$  for the other methods was solved together with  $g_m$ .

<sup>¶</sup> The notation  $(4C_c + 8\Gamma^*)$  in Equation 3) was changed into  $(4.5C_c + 10.5\Gamma^*)$  to consider the case that models a limitation of ATP supply instead of NADPH supply (von Caemmerer, 2000).



**Fig. 1.** The CO<sub>2</sub> response curve of a *Hedera helix* leaf. Net photosynthesis ( $A_n$ ) was plotted against intercellular CO<sub>2</sub> concentration ( $C_i$ ) (left panel), and against the CO<sub>2</sub> concentration in the chloroplast ( $C_c$ ) (right panel). The electron transport rate measured with fluorometry ( $J_F$ ) was also plotted against  $C_i$ . The biochemically based leaf photosynthesis model (Farquhar *et al.*, 1980) was fitted to the data based on  $C_i$  (left panel) and based on  $C_c$  after calculation of  $g_m$  (Sharkey *et al.*, 2007). The data were used for the sensitivity analysis shown in Table 1 and Fig. 2. Filled symbols were measured at atmospheric [CO<sub>2</sub>] (380  $\mu\text{mol mol}^{-1}$ ).

#### Chlorophyll fluorescence in conjunction with gas exchange

For methods involving fluorometry, the electron transport rate ( $J_F$ ) is calculated according to Genty *et al.* (1989):

$$J_F = \alpha\beta\text{PFD}\Phi_{\text{PSII}} \quad (4)$$

where  $\Phi_{\text{PSII}}$  is the photochemical yield of photosystem II estimated from fluorescence, PFD is the photosynthetically active photon flux density incident on the leaf,  $\alpha$  is the leaf absorptance, and  $\beta$  denotes the fraction of photons absorbed by PSII.  $\Phi_{\text{PSII}}$  is calculated as (Genty *et al.*, 1989):

$$\Phi_{\text{PSII}} = (F_m' - F_s) / F_m' \quad (5)$$

where  $F_s$  is the steady state fluorescence in the prevailing light conditions and  $F_m'$  is the maximal fluorescence during a short saturating pulse of light.  $J_F$  requires the measurement of PFD incident on the leaf, and estimates of  $\alpha$  and  $\beta$  (Equation 4). Leaf absorptance ( $\alpha$ ) can either be measured directly or an approximation can be derived from chlorophyll content per unit area using published relationships, for example  $\alpha = [\text{Chl}] / ([\text{Chl}] + 76)$ , where [Chl] is the chlorophyll content per unit leaf area expressed in  $\mu\text{mol m}^{-2}$  (Evans and Poorter, 2001). The partitioning factor  $\beta$  is normally assumed to be 0.5, but may vary (Laisk and Loreto, 1996). A problem related to  $\Phi_{\text{PSII}}$  measurements concerns PSI. While it is assumed that all chlorophyll fluorescence arises from PSII at ambient temperatures, there is evidence that PSI contributes substantially to fluorescence emission at  $F_s$ , but less at  $F_m'$ , thereby leading to serious underestimations of  $\Phi_{\text{PSII}}$  (Genty *et al.*, 1990; Agati *et al.*, 2000; Franck *et al.*, 2002) and consequently  $J_F$ .  $\Phi_{\text{PSII}}$  can be low at high irradiance and in stressed leaves. Therefore, measuring at high light is sometimes problematic, since the signal-to-noise ratio in the determination of  $F_m'$  is decreased, and it exacerbates the problem of ignoring the

contribution of PSI. The resolution can be improved by reducing the PFD.

To overcome the uncertainties linked with the estimation of  $J_F$  and  $J$  calculated from gas exchange ( $J_A$ ) (Equation 1), the relationship between the two can be determined under non-photorespiratory conditions. This is often done at low [O<sub>2</sub>], typically 1% or 2% across a similar range of  $J$  as measured at atmospheric [O<sub>2</sub>]. The small rates of photorespiration ongoing at these low [O<sub>2</sub>] are sometimes ignored. However, they are not negligible at the level of accuracy required for the calculation of  $g_m$ , particularly when  $C_c$  is low as a result of a low  $g_s$  and/or  $g_m$ . The small rate of photorespiration can be included by using Equation 3. Alternatively, the measurement is carried out at a lower [O<sub>2</sub>] (Meyer and Genty, 1998) or in an anoxic atmosphere (Genty *et al.*, 1998). Equation 3 is then reduced to  $J_A = 4(A_n + R_L)$  that does not require any assumption about  $\Gamma^*$  or  $g_m$ . Photosynthesis should be induced in the light at atmospheric [O<sub>2</sub>] before O<sub>2</sub> is removed. However, the approach assumes that  $R_L$  is not affected by the very low [O<sub>2</sub>] generated by photosynthesis. This is difficult to verify, since this O<sub>2</sub> source is not available for the control measurement in darkness.

$J_A$  at low [O<sub>2</sub>] and  $J_F$  are typically similar, but are not necessarily exactly the same.  $J_F$  is thus best considered as a proxy of  $J$ . The  $J_A - J_F$  relationship at low [O<sub>2</sub>] over the range of interest can be expressed as:

$$J_A = b_F(J_F + c) \quad (6)$$

where  $b_F$  is the regression coefficient of a linear relationship with constant  $c$ . This is mostly found with  $b_F$  close to unity and  $c$  normally small or negligible (Genty *et al.*, 1989; Meyer and Genty, 1996), but non-linear relationships have also been reported (Seaton and Walker, 1990).

Apart from the mentioned uncertainty concerning the correct formulation of the relationship between electron

transport, and carboxylation and photorespiration, and possible errors in the estimation of the components of Equation 4, there may be additional reasons for a deviation of  $J_F$  from  $J_A$ . The most important ones are: (i) engagement of alternative electron sinks such as nitrate reduction and the Mehler reaction (pseudocyclic electron transport) (Laisk *et al.*, 2002); and (ii) the chloroplasts in the cross-section of the leaf that are sampled by the fluorometer may not be representative for the gas exchange of the sampled leaf section as a whole (Kingston-Smith *et al.*, 1997). For instance, the measuring light of the fluorometer is often red, which has an absorption profile over the leaf depth different from that of white light, or the combination of red and blue typically used as actinic light. The  $J_A$ – $J_F$  relationship can thus be of a complex nature and may change with measurement condition such as irradiance and light colour. The relationship should be established across the whole range of measurement conditions used for the estimation of  $g_m$ .

#### The model parameter values $\Gamma^*$ and $R_L$

All methods require values for the  $\text{CO}_2$  compensation point ( $\Gamma^*$ ) in the absence of mitochondrial respiration in the light ( $R_L$ ) and  $R_L$  itself.  $\Gamma^*$  is typically taken from a literature source, but the reported values vary (von Caemmerer, 2000; Evans and Loreto, 2000; Pons and Westbeek, 2004). When no values are reported for the species under consideration, the choice is likely to be haphazard. Furthermore, it is most common to report a proxy for the  $\Gamma^*$  value estimated at the level of the intercellular spaces ( $C_i^*$ ), whereas for calculating  $g_m$ , strictly a true value for  $\Gamma^*$  at the chloroplast level is required. The two are related according to (von Caemmerer *et al.*, 1994):

$$\Gamma^* = C_i^* + R_L/g_m \quad (7)$$

which is equivalent to Equation 2. Preferably,  $\Gamma^*$  is measured for the species, and for growth and measurement conditions being used. This is mostly done by measuring  $C_i^*$  using the Laisk method (Brooks and Farquhar, 1985). The method essentially consists of measuring  $A_n$ – $C_i$  relationships at, for instance, three different irradiances around the  $\text{CO}_2$  compensation point. Their linear regressions are predicted to converge at  $C_i^*$  and  $R_L$ . Then the two parameters can be used to calculate  $\Gamma^*$  together with an estimate of  $g_m$  using Equation 7. Alternatively,  $\Gamma^*$  is solved together with  $g_m$  using Equations 3 and 7, but a fixed value is preferred. Evidently, the gas exchange measurements for estimating  $C_i^*$  and  $R_L$  at low  $[\text{CO}_2]$  are preferably done in a large leaf chamber. When performed with a small clamp-on chamber, rigorous corrections are required at low  $[\text{CO}_2]$ . Bernacchi *et al.* (2002) used an alternative method for measuring  $\Gamma^*$  involving labelling with  $^{18}\text{O}$ . The values obtained with this method tend to be lower than those obtained with the Laisk method and have only been measured for a few species. The temperature dependence of  $\Gamma^*$  has been described (von Caemmerer, 2000; Bernacchi *et al.*, 2001, 2002), making conversions to other temper-

atures possible. However, temperature dependencies may be species specific. Hence, the estimation of  $\Gamma^*$  for the conditions of the measurement is preferred, since the value of  $\Gamma^*$  has a substantial effect on the result of  $g_m$  calculations (Harley *et al.*, 1992).

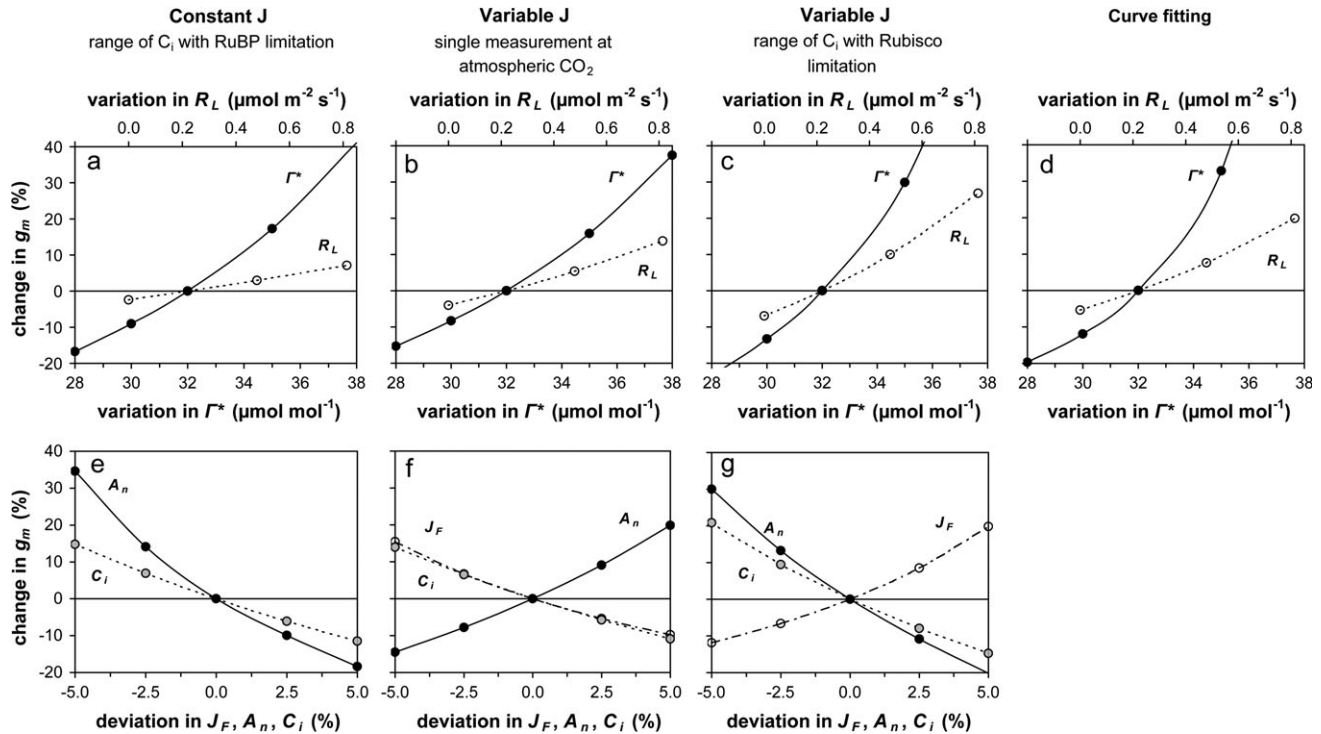
An alternative to measuring  $\Gamma^*$  is to estimate it from published values of the Rubisco specificity factor ( $\tau$ ) for the species under study (Galmés *et al.*, 2007), but in most cases this would be appropriate only for measurements made at leaf temperatures of 25 °C, at which most values of  $\tau$  are reported. The variations of  $\tau$  with temperature are species dependent (Galmés *et al.*, 2005). The search for reliable  $\Gamma^*$  values and its temperature dependence specific for species and growth conditions should continue.

As shown above, the Laisk method also provides an independent estimate of  $R_L$ . Values for this  $R_L$  are typically lower than  $R_D$  (Atkin *et al.*, 2006). When measuring  $R_D$  on the same leaf, the  $R_L/R_D$  ratio can be used to calculate  $R_L$  from  $R_D$  measurements on other leaves of the same species under the same conditions. The temperature dependence of  $R_L$  has been described as an exponential function of temperature (Bernacchi *et al.*, 2001). However, this is not invariably so (Pons and Welschen, 2003), and  $R$  typically acclimates to a new temperature and a unique temperature dependence of  $R$  is non-existent (Atkin *et al.*, 2006). Pinelli and Loreto (2003) estimated  $R_L$  using  $^{13}\text{CO}_2$  and did not find evidence for a reduced  $R_L$  relative to  $R_D$ . However, other evidence is in favour of a reduced  $R_L$  (Krömer, 1995; Tcherkez *et al.*, 2005). Nevertheless, it is advisable in many cases to have an independent estimate of  $R_L$  that can be used for the calculations of  $g_m$ . This has the advantage that  $R_L$  can be excluded from the parameter estimation, making the solving for  $g_m$  more robust.

#### Variable J method

One of the approaches for estimating  $g_m$  from gas exchange and chlorophyll fluorescence is the so-called variable  $J$  method. This method was originally described by Di Marco *et al.* (1990) and further elaborated by Harley *et al.* (1992). For the original method,  $A_n$ ,  $C_i$ , and  $J_F$  are measured under a single set of conditions, typically at atmospheric  $[\text{O}_2]$ . The relationship between  $J_A$  and  $J_F$  must be separately established for the same conditions (except  $[\text{O}_2]$ ) as described above. The known  $J$  allows a direct calculation of  $C_c$  and  $g_m$  from Equations 2 and 3.

The method relies on the assumption that the  $b_F$  value, and where applicable other constants describing the  $J_A$ – $J_F$  relationship, measured at zero or low  $[\text{O}_2]$  remains the same at atmospheric  $[\text{O}_2]$ . Harley *et al.* (1992) identified the value of  $J$  as an important source of error, which is also illustrated for the *Hedera* leaf measured here, where a 5% lower  $b_F$  (and thus  $J$ ) caused a 15% higher  $g_m$  (Fig. 2g). The value for  $g_m$  calculated with this method was substantially lower (125  $\text{mmol m}^{-2} \text{s}^{-1}$ ) compared with the constant  $J$  method (155  $\text{mmol m}^{-2} \text{s}^{-1}$ ). This was due to the fact that  $b_F$  measured at 0%  $\text{O}_2$  was higher (1.078) than the value calculated from the constant  $J$  method at atmospheric  $[\text{CO}_2]$



**Fig. 2.** Sensitivity of the estimation of  $g_m$  for variation in parameter values using different methods. Calculations are based on the same data set for a *Hedera helix* leaf as used in Fig. 1 and Table 1, where other data are shown. The methods are the constant  $J$  method (a, e), the variable  $J$  methods with a single measurement at atmospheric CO<sub>2</sub> (b, f), the variable  $J$  method applied to a range of low [CO<sub>2</sub>] where Rubisco limits gas exchange (c, g), and the curve-fitting method (d). In the upper panels, the effects of variation in  $\Gamma^*$  and  $R_L$  are shown. In the lower panels, the effects of a deviation from the measured values of net photosynthesis ( $A_n$ ), electron transport based on fluorescence ( $J_F$ ), and intercellular CO<sub>2</sub> ( $C_i$ ) are shown. In e and g, where a range of measurements is used, a deviation of, for example, +5% in  $A_n$  and  $J_F$  (g only) refers to a 2.5% increase at the highest  $C_i$  and a 2.5% decrease at the lowest  $C_i$ , with the intermediate values in proportion, keeping the mean value constant.

(Table 1). When the latter value (1.003) was used, the result was equal. This method is also sensitive to variation in  $\Gamma^*$  (Harley *et al.*, 1992), but not much for variation in  $R_L$  when the same value is used for the estimation of  $b_F$  (Fig. 2b). When  $R_L$  is only changed at atmospheric [O<sub>2</sub>], the sensitivity is similar to that with the other methods (Fig. 2g). The advantage of this method is that it does not require the assumption that  $g_m$  is independent of [CO<sub>2</sub>]. It is thus suitable to investigate the effect of [CO<sub>2</sub>] on  $g_m$ . However, at high [CO<sub>2</sub>], the rate of photorespiration is low and thus the  $J_A - J_F$  difference becomes small, making the method increasingly sensitive to errors. The variable  $J$  method suggested a decrease of  $g_m$  with increasing [CO<sub>2</sub>] using this method with the *Hedera* data, as reported by Flexas *et al.* (2007b). However, this was not confirmed by the variant of the variable  $J$  method applied to a range of high and low [CO<sub>2</sub>] (see below). Evidence for a CO<sub>2</sub> effect on  $g_m$  obtained with this method should thus be verified using other methods, as done by Flexas *et al.* (2007b) and Vrábl *et al.* (2009).

The measurement of the relationship between  $J_A$  and  $J_F$  is often done at low [O<sub>2</sub>] over a range of [CO<sub>2</sub>] instead of using an anoxic atmosphere. As argued above, the low rate of photorespiration at 1% or 2% [O<sub>2</sub>] and the effect of the  $C_i - C_c$  gradient thereupon cannot be ignored.  $J_A$  should

then be calculated using Equation 3 with  $\Gamma^*$  reduced in proportion to [O<sub>2</sub>]. The calculation of  $b_F$  can then be done iteratively together with  $g_m$  (Pons and Westbeek, 2004). In the example of the *Hedera* leaf presented in Table 1, apart from 1% and 21% also 10% O<sub>2</sub> was included. More concentrations can be used, including higher than atmospheric (Loreto *et al.*, 1992), making the estimation of  $g_m$  more robust. The value calculated for  $g_m$  of 133 mmol m<sup>-2</sup> s<sup>-1</sup> using this method cannot be compared directly with the other values because a different leaf was used. This method also has the advantage that measurements can be made at a single [CO<sub>2</sub>]. However, the assumption remains that  $b_F$  and, where applicable, other constants describing the  $J_A - J_F$  relationship are independent of [O<sub>2</sub>].

A range of [CO<sub>2</sub>] where  $J$  is not constant can also be used. That can be a range of lower [CO<sub>2</sub>] where Rubisco limits  $A_n$  as done with the *Hedera* leaf (Table 1, Fig. 2), but can also be applied to higher [CO<sub>2</sub>] where  $J$  is not exactly constant. The  $J_F$  measurements are used as a relative measure of  $J$ . The calculation is done in a manner similar to that with the [O<sub>2</sub>] range, where  $b_F$  is solved together with  $g_m$ . The method thus assumes that these are constant across the range of [CO<sub>2</sub>]. The method is more sensitive for variation in  $\Gamma^*$  and  $R_L$  compared with the other ones, particularly when low [CO<sub>2</sub>] is included in the range (Fig.

2c). The method yielded a slightly lower value for  $g_m$  than the constant  $J$  method ( $143 \text{ mmol m}^{-2} \text{ s}^{-1}$  and  $155 \text{ mmol m}^{-2} \text{ s}^{-1}$ , respectively), but the two were measured over different ranges of  $[\text{CO}_2]$ , where  $g_m$  is not necessarily the same.

### Constant $J$ method

An alternative approach for estimating  $g_m$  is the constant  $J$  method. Measurements are done across a range of  $[\text{CO}_2]$ , typically higher than atmospheric, where  $A_n$  is limited by RuBP regeneration and  $J$  is constant (Fig. 1). Chlorophyll fluorescence is used to verify this range. Under these conditions,  $A_n$  increases with  $C_i$  because of a decreasing rate of photorespiration. A lower  $C_c$  than  $C_i$  as a result of a finite  $g_m$  increases photorespiration and thus decreases  $A_n$  more at lower compared with higher  $C_i$ . The data are then fitted to Equation 3, solving  $J$  and  $g_m$  iteratively. The deviation of the measured data from the  $A_n$ - $C_i$  curve is illustrated for measurements carried out on a *Hedera helix* leaf. The measured values of  $A_n$  where  $J_F$  is more or less constant increased more steeply than Equation 1 based on  $C_i$  predicts (Fig. 1). Introduction of a  $g_m$  of  $155 \text{ mmol m}^{-2} \text{ s}^{-1}$ , however, generated a perfect fit.

This method was first introduced by Bongí and Loreto (1989) and further elaborated by Harley *et al.* (1992) and Loreto *et al.* (1992). The method assumes that both  $J$  and  $g_m$  are constant across the  $[\text{CO}_2]$  range of the measurements. This is a disadvantage with respect to  $g_m$ , because evidence is emerging that the last condition is not always true (Flexas *et al.*, 2007b; Hassiotou *et al.*, 2009; Vrábl *et al.*, 2009; Yin *et al.*, 2009). The advantage of the method is that no assumption is required about the  $J_A$ - $J_F$  relationship except that the partitioning of electrons remains constant across the  $[\text{CO}_2]$  range of interest. The method is sensitive for the value of  $\Gamma^*$ , since that parameter in combination with  $C_c$  defines the proportion of photorespiration, which is illustrated for measurements done on a *Hedera helix* leaf (Fig. 2). The outcome is also sensitive for the value of  $R_L$ . It is not advisable to solve this parameter together with  $J$  and  $g_m$ . When, as an extreme case, the measured apparent  $\text{CO}_2$  production in darkness was used ( $0.8 \text{ } \mu\text{mol m}^{-1} \text{ s}^{-1}$ ) instead of  $R_L$ ,  $g_m$  increased by 7% (Fig. 2a). That is not too much, but the sensitivity to variation in  $\Gamma^*$  and  $R_L$  increases with increasing  $g_m$  and decreasing  $C_i$ - $C_c$  gradient (Harley *et al.*, 1992).  $J_F$  was not exactly constant in the example shown in Fig. 1; it increased gradually by 3% from  $380 \text{ } \mu\text{mol mol}^{-1} \text{ CO}_2$  to  $1500 \text{ } \mu\text{mol mol}^{-1} \text{ CO}_2$ . When taking the measured variation in  $J_F$  into account and solving  $b_F$  (Equation 6) together with  $g_m$ , then the estimate of  $g_m$  was 18% higher (Fig. 2f). The latter approach is equivalent to a variant of the variable  $J$  method as described above.

It is concluded that this method is only suitable when there is a truly constant  $J$  across a sufficiently wide range of  $[\text{CO}_2]$ , which should be verified by means of chlorophyll fluorescence. Chances are best for meeting these conditions when measuring slightly below light saturation (although

apparently not for the example shown in Fig. 1). Moreover,  $\Phi_{\text{PSII}}$ , and leaf temperature and thus  $C_i$  can then be measured at a higher precision compared with light saturation, without sacrificing precision of  $A_n$ .

### Estimation of $g_m$ with gas exchange only: the curve-fitting method

An estimation of  $g_m$  can also be obtained from gas exchange measurements only. This curve-fitting method is based on measurements of the  $A_n$  and  $C_i$  over a wide range of  $[\text{CO}_2]$ . The data are then fitted to the biochemically based photosynthesis model of Farquhar *et al.* (1980) with modifications to include  $g_m$  (Ethier and Livingston, 2004; Sharkey *et al.*, 2007). In the model, two  $[\text{CO}_2]$  ranges are distinguished that are limited by different processes. The part limited by the activity of Rubisco is described as:

$$A_n = V_{\text{cmax}} \frac{C_c - \Gamma^*}{C_c + K_c(1 + O/K_o)} \quad (8)$$

where  $V_{\text{cmax}}$  is the carboxylation capacity, and  $K_c$  and  $K_o$  are the catalytic constants for the carboxylation and oxygenation reactions of Rubisco, respectively. The part limited by regeneration of ribulose biphosphate (RuBP) is described by Equation 3 that is solved for  $A_n$ . The method requires values for two additional parameters ( $K_c$  and  $K_o$ ) and their dependency on temperature. As with  $\Gamma^*$ , these parameters have been estimated for only a few species, which can induce bias in the estimations. Measured data often do not completely fit the original model that was based on  $C_i$ , but replacing  $C_c$  for  $C_i$  often improves the fit, as illustrated in Fig. 1. Data points must *a priori* be allocated to the two limitations mentioned above. Sharkey *et al.* (2007) also included a third region at high  $[\text{CO}_2]$  where limitation by triose phosphate utilization (TPU) may occur. This model is available in a spreadsheet at <http://www.blackwellpublishing.com/plantsci/pccalculation/>. When independent estimates for  $R_L$  and  $\Gamma^*$  are available,  $V_{\text{cmax}}$ ,  $J$ , and  $g_m$  can be calculated iteratively. The method evidently assumes a constant  $g_m$  across a wide range of  $[\text{CO}_2]$ , although a change of  $g_m$  with  $[\text{CO}_2]$  can be implemented in the model (Flexas *et al.*, 2007b; TD Sharkey, personal communication).

The model produced a somewhat higher value for  $g_m$  ( $178 \text{ mmol m}^{-2} \text{ s}^{-1}$ ) than the methods that combine with fluorometry. When applied to the RuBP-limited region only, the model assumes a constant  $J$ , which is thus equivalent to the constant  $J$  method, but without an independent check. The RuBP-limited part fitted well with that *Hedera* data set, but the Rubisco-limited part did not result in a sensible solution. This reflects that both lower  $V_{\text{cmax}}$  and lower  $g_m$  will affect modelled data similarly and it can be hard to determine which factor explains variation seen in data sets. However, Tholen *et al.* (2008) found a sound solution for  $g_m$  when their data for *Arabidopsis thaliana* leaves were fitted to the Rubisco-limited part.



Hence, the method asks for good judgement with respect to reliability of the results in addition to the allocation of the data to specific limitations.

## Estimation of $g_m$ with gas exchange and <sup>13</sup>C isotope discrimination

### Theory

These methods are based on carbon isotope fractionation measured simultaneously with gas exchange. Measurements of  $g_m$  using <sup>13</sup>C discrimination were first used by Evans *et al.* (1986) in their landmark paper, which during the following decades stimulated many subsequent studies (e.g. von Caemmerer and Evans, 1991; Lloyd *et al.*, 1992; Evans *et al.*, 1994; Evans and Loreto, 2000) and the development of different approaches to estimate  $g_m$ .

Stable isotopic fractionation occurs during photosynthetic CO<sub>2</sub> fixation. Specifically, the heavier isotope of carbon, <sup>13</sup>C, is discriminated against during diffusion (in the gaseous and the liquid phase) and during biochemical carboxylations (Farquhar *et al.*, 1982). These effects are mainly due to the lower diffusivity of <sup>13</sup>CO<sub>2</sub> in air and liquid phase relative to <sup>12</sup>CO<sub>2</sub> and to discrimination by carboxylating enzymes such as Rubisco, which preferentially bind molecular species containing the lighter isotopes (<sup>12</sup>CO<sub>2</sub>). Hence, the photosynthetic products are generally enriched in the lighter isotope <sup>12</sup>C compared with the substrate atmospheric CO<sub>2</sub>. In C<sub>3</sub> species, the isotopic discrimination is related to the relative contribution of diffusion and carboxylation, which is reflected in the ratio of CO<sub>2</sub> concentration at the sites of carboxylation ( $C_c$ ) to that in the surrounding atmosphere ( $C_a$ ). The model developed by Farquhar *et al.* (1982) predicts that

$$\Delta = a_b \frac{C_a - C_s}{C_a} + a \frac{C_s - C_i}{C_a} + (e_s + a_l) \frac{C_i - C_c}{C_a} + b \frac{C_c}{C_a} - \frac{eR_D + f\Gamma^*}{C_a} \quad (9)$$

where,  $C_a$ ,  $C_s$ ,  $C_i$ , and  $C_c$  are the CO<sub>2</sub> concentrations in the free atmosphere, at the leaf surface within the boundary layer, in the intercellular air spaces before it enters in solution, and at the sites of carboxylation, in that order;  $a_b$  is the discrimination occurring during diffusion in the boundary layer (2.9‰);  $a$  is the fractionation occurring during diffusion in still air (4.4‰);  $e_s$  is the fractionation occurring when CO<sub>2</sub> enters in solution (1.1‰, at 25 °C);  $a_l$  is the fractionation occurring during diffusion in the liquid phase (0.7‰);  $b$  is the net discrimination occurring during carboxylations in C<sub>3</sub> plants;  $e$  and  $f$  are the fractionations occurring during dark respiration ( $R_D$ ) and photorespiration, respectively;  $k$  is the carboxylation efficiency, and  $\Gamma^*$  is the CO<sub>2</sub> compensation point in the absence of dark respiration (Brooks and Farquhar, 1985).

Values of isotopic discrimination can be compared with gas exchange measurements, which, however, normally

provide estimates of the intercellular CO<sub>2</sub> concentration ( $C_i$ ), and not that at the sites of carboxylation. In the case of high conductance to CO<sub>2</sub> diffusion from the substomatal cavities to the chloroplast stroma, concurrent measurements of gas exchange and isotopic discrimination ( $\Delta$ ) by isotope ratio mass spectrometry can provide very good relationships between the  $\Delta$  and the ratio of leaf intercellular CO<sub>2</sub> concentration to that in the surrounding atmosphere ( $C_i/C_a$ ).

If carbon isotopic discrimination and gas exchange are measured in a well-stirred gas exchange cuvette, one can omit the terms related to diffusion in the boundary layer and Equation 9 can be written as

$$\Delta = a \frac{C_a - C_i}{C_a} + (e_s + a_l) \frac{C_i - C_c}{C_a} + b \frac{C_c}{C_a} - \frac{eR_D + f\Gamma^*}{C_a} \quad (10)$$

Values of  $e$  and  $f$  are subjected to uncertainty since different measurements have provided different results. Early indirect measurements indicated  $e$  values close to zero (von Caemmerer and Evans, 1991) and subsequent direct measurements during dark respiration provided no significant difference between the isotopic composition of the respiratory substrate and that of CO<sub>2</sub> respired by isolated protoplasts, indicating no fractionation at all (Lin and Ehleringer, 1997). However, more recent studies in intact leaves (Duranceau *et al.*, 1999; Ghashghaie *et al.*, 2001; Tcherkez *et al.*, 2003; Gessler *et al.*, 2009) indicated a significant enrichment in <sup>13</sup>C in respired CO<sub>2</sub> compared with the putative substrate. The apparent fractionation may be due to non-statistical distribution of carbon isotopes in the substrate molecules and, especially, to the relative contribution of pyruvate dehydrogenase activity and the Krebs cycle to respiratory substrates (Tcherkez *et al.*, 2003; Gessler *et al.*, 2009). Currently, there is no agreement as to the value of  $e$ , although most estimates suggest it should be 0–4‰. If the isotopic composition of the CO<sub>2</sub> used for gas exchange differs from that during the growth of the plant, then it will also contribute to the apparent  $e$  value (Wingate *et al.*, 2007).

Fractionation during photorespiration  $f$  has been estimated by several authors (Gillon and Griffiths, 1997; Gashghaie *et al.*, 2003; Igamberdiev *et al.*, 2004; Lanigan *et al.*, 2008) to be 8–12‰. Other sources of uncertainty in Equation 9 concern the value of  $b$ . This is not simply the discrimination by Rubisco, because in C<sub>3</sub> plants a variable amount of carbon is fixed by PEP carboxylase (Nalborczyk, 1978; Farquhar and Richards, 1984). In C<sub>3</sub> plants, this enzyme operates in parallel with Rubisco, affecting the isotopic composition of C fixed (Brugnoli *et al.*, 1998; Brugnoli and Farquhar, 2000). Obviously, changes in the proportion of  $\beta$ -carboxylations would affect the net fractionation. Also the value of fractionation relative to Rubisco carboxylation ( $b_3$ ) is not universally accepted, and variations have been reported in the literature (Brugnoli and Farquhar, 2000) and confirmed recently (Tcherkez *et al.*, 2006; McNevin *et al.*, 2007). However, in higher

plants, the value of discrimination by Rubisco is thought to be very close to 30‰ with respect to gaseous CO<sub>2</sub> (Brugnoli et al., 1988; Guy et al., 1993; Brugnoli and Farquhar, 2000). Therefore, depending on the proportion of PEP carboxylations in C<sub>3</sub> plants, the value of *b* can be between 27‰ and 30‰. The value assumed for *b* will influence the absolute value calculated for *g<sub>m</sub>*, and is one of the most significant issues present in all <sup>13</sup>C discrimination methods (online slope-based and single point or sugar methods). Sensitivity analysis can provide estimates of the errors introduced in the calculated values of mesophyll conductance associated with different *b* values. One such sensitivity analysis is shown as an example in Table 2 for a real measured leaf of *A. thaliana* displaying an *A<sub>n</sub>* of 12.1 μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup> with a stomatal conductance (*g<sub>s</sub>*) of 0.280 mol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>. As measured in a small gas exchange cuvette (2 cm<sup>2</sup>) at an external CO<sub>2</sub> concentration (*C<sub>e</sub>*, see Equation 12 below) of 400 μmol mol<sup>-1</sup>, the leaf created a CO<sub>2</sub> draw-down in the cuvette of 18.2 μmol mol<sup>-1</sup> (i.e. ξ=22.0, see Equation 12). With a difference in the isotopic composition between the air leaving and that entering the chamber (δ<sub>o</sub>-δ<sub>e</sub>, see below) of 0.709‰, the values of *g<sub>m</sub>* estimated using a range of *b* values from 27‰ to 30‰ differed as much as 20% among them (Table 2). However, the true value of *b* should most probably fall between 28‰ and 29‰, and such a range of variation will have much more limited effects on *g<sub>m</sub>*.

Equation 10 is often further simplified by assuming that the draw-down of CO<sub>2</sub> between the substomatal cavities and the chloroplast stroma is negligible and that fractionation associated with respiration and photorespiration is also negligible; then

$$\Delta_i = a + (b - a) \frac{C_i}{C_a} \quad (11)$$

This equation is the most used model of discrimination and predicts a linear relationship between Δ<sub>*i*</sub> and C<sub>*i*</sub>/C<sub>*a*</sub>. Hence, one can estimate the value of Δ from the value of C<sub>*i*</sub>/C<sub>*a*</sub> measured by gas exchange on the basis of Equation 11

**Table 2.** Sensitivity analysis on the effects of the selected *b* value (i.e. discrimination due to different proportions of Rubisco versus PEP carboxylations) on the estimated *g<sub>m</sub>* in a measured leaf of *Arabidopsis thaliana* displaying an *A<sub>n</sub>* rate of 12.1 μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup> with a stomatal conductance (*g<sub>s</sub>*) of 0.280 mol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>. As measured in a small gas exchange cuvette (2 cm<sup>2</sup>) at an external CO<sub>2</sub> concentration (*C<sub>e</sub>*) of 400 μmol mol<sup>-1</sup>, the leaf created a CO<sub>2</sub> draw-down in the cuvette of 18.2 μmol mol<sup>-1</sup> (i.e. ξ=2.0). The measured δ<sub>o</sub>-δ<sub>e</sub> was 0.709‰.

<i>b</i> (‰)	Δ <sub><i>i</i></sub> (‰)	<i>g<sub>m</sub></i> (mol CO <sub>2</sub> m <sup>-2</sup> s <sup>-1</sup> )	Deviation from average
27	22.9	0.114	+10%
28	23.7	0.107	+3%
29	24.5	0.100	-4%
30	25.4	0.095	-9%

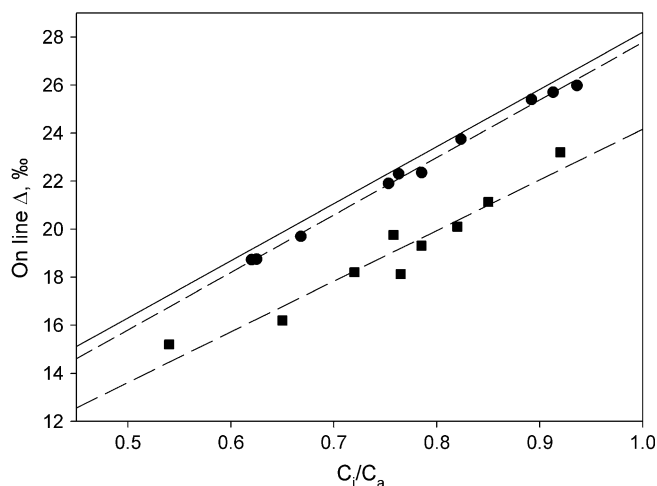
with its underlying assumptions. Comparisons between the expected Δ values and those actually measured (Δ<sub>obs</sub>) provide an insight into the magnitude of mesophyll conductance and of the draw-down of CO<sub>2</sub> between the intercellular air spaces and the sites of carboxylation. Figure 3, for example, shows very different online results for bean and *Fagus* leaves, with the latter showing a much higher deviation between Δ<sub>*i*</sub> and Δ<sub>obs</sub> compared with the former, mainly attributable to lower *g<sub>m</sub>*.

The actual estimate of *g<sub>m</sub>* can be performed using different methods, consisting of the determination of Δ<sub>obs</sub> usually by isotope ratio mass spectrometry and the calculation of the expected Δ<sub>*i*</sub> from C<sub>*i*</sub>/C<sub>*a*</sub> calculated from gas exchange measurements. Irrespective of the method used, high precision in the determination of gas exchange parameters and of isotopic composition is needed.

### Instrument precision

There are several methods to measure the C isotopic composition in CO<sub>2</sub>, with isotope ratio mass spectrometry (IRMS) being the most frequently used under both continuous flow (CF-IRMS) and dual-inlet (DI-IRMS), while tunable-diode laser absorption spectrometry (TDLAS) is increasingly being used for carbon isotope composition analysis (Bowling et al., 2003).

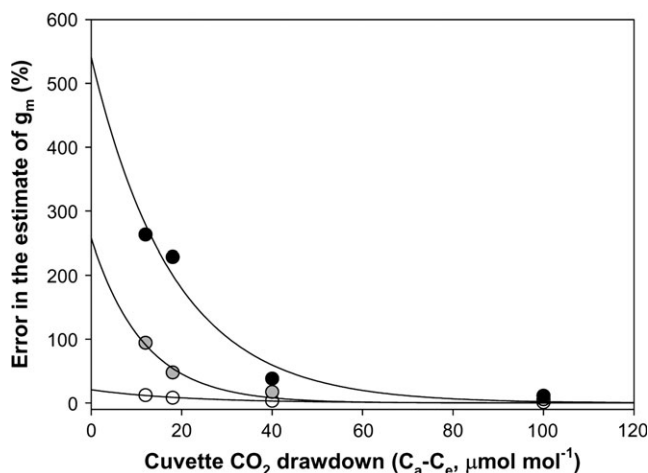
The precision of measurements for δ<sup>13</sup>C depends on the method used. DI-IRMS offers the lowest standard deviation, ranging from 0.01‰ to 0.03‰, while CF-IRMS and TDLAS give an SD not lower than 0.1–0.2‰. The uncertainty in the isotopic composition of <sup>13</sup>CO<sub>2</sub> is translated into precision errors in the measurement of *g<sub>m</sub>*.



**Fig. 3.** Relationships between online isotopic discrimination (Δ) and C<sub>*i*</sub>/C<sub>*a*</sub> in bean (*Phaseolus vulgaris* L., circles) and in beech (*Fagus sylvatica* L., squares) leaves. Differences are due to substantially different *g<sub>m</sub>*. The solid line represents the predicted Δ<sub>*i*</sub> from the equation Δ = a + (b - a) C<sub>*i*</sub>/C<sub>*a*</sub> with a = 4.4‰ and b = 28.2‰. The dashed lines are the regression equations: y = 3.83 + 23.95x, r<sup>2</sup> = 0.99 for bean; y = 3.07 + 21.1x, r<sup>2</sup> = 0.93 (E Brugnoli, unpublished results).

Analysis of  $g_m$  was conducted on the same *A. thaliana* leaf described above, using combined gas exchange measurements performed in a 2 cm<sup>2</sup> cuvette (LI-6400, Li-Cor Inc., NE, USA) with a fluorescence chamber (LI-6400-40) and an offline system where the entering and outgoing gas were collected and further analysed in a DI-IRMS. The precision (standard deviation) of the dual-inlet system was 0.02‰. The value of  $g_m$  obtained from these results was 0.100 mol m<sup>-2</sup> s<sup>-1</sup>, with an uncertainty of 0.005 mol m<sup>-2</sup> s<sup>-1</sup> or a 5% error. Had these measurements been made with a CF-IRMS or a TDALS with an associated error of 0.20‰, the deviation of the calculation of  $g_m$  would have been of 0.075 mol m<sup>-2</sup> s<sup>-1</sup> or a 75% error.

A sensitivity analysis can be performed where the different standard deviations of measurements are converted into deviations of the calculated  $g_m$  as a function of total CO<sub>2</sub> draw-down (Fig. 4). In the well-watered *Arabidopsis* leaf already described, with CO<sub>2</sub> draw-down ranging from 100 μmol mol<sup>-1</sup> to 12 μmol mol<sup>-1</sup>, the errors increased from 1% to 12%, from 6% to 94%, and as much as from 11% to 263% when using a dual-inlet system with a precision of 0.02‰, or a CF-IRMS or TDALS with an associated error of 0.10‰ and 0.20‰, respectively. In a water-stressed plant, showing lower  $A_n$  and  $g_m$ , the errors can be even larger (data not shown). It is worth noticing that even smaller CO<sub>2</sub> draw-downs than those analysed here are often observed in small gas exchange cuvettes, particularly with slowly photosynthesizing leaves. Therefore, although the use of DI-IRMS minimizes the errors associated with instrument precision, it is clear that precautions need to be taken when using either CF-IRMS or TDALS. With such systems, small gas exchange cuvettes cannot be used, since larger chambers are needed to create a sufficient CO<sub>2</sub> draw-down, particularly when photosynthesis rates are low.



**Fig. 4.** A sensitivity analysis showing percentage errors in the calculated  $g_m$  as a function of total CO<sub>2</sub> draw-downs for precisions of 0.02 (white dots), 0.1 (grey dots), or 0.2‰ (black dots) in a leaf of well-watered *Arabidopsis thaliana* displaying an  $A_n$  of 12.1 μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>, stomatal conductance ( $g_s$ ) of 0.28 mol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>, and a  $g_m$  of 0.15 mol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>.

#### The online discrimination: slope method

To measure the instantaneous carbon isotope discrimination simultaneously with leaf gas exchange (called ‘online discrimination’), the air entering and leaving a well-stirred gas exchange chamber has to be sampled so that the C isotopic composition can be measured. This method was developed by Evans *et al.* (1986) who were the first to measure  $g_m$  using online discrimination. Initially, this method involved collecting CO<sub>2</sub> samples simultaneously with gas exchange measurements, using a series of cryogenic traps: a series of alcohol–dry ice traps was used to freeze out water vapour and then the CO<sub>2</sub> was frozen in a second series of traps kept at liquid nitrogen temperature and evacuated under high vacuum while frozen (Evans *et al.*, 1986; von Caemmerer and Evans, 1991). Subsequently, the CO<sub>2</sub> collected was transferred into a mass spectrometer to determine δ<sup>13</sup>C. Because of isotopic discrimination during photosynthesis, the air leaving the chamber will be enriched in <sup>13</sup>C compared with that entering the chamber. Measuring this difference and measuring the CO<sub>2</sub> concentrations in the air entering ( $C_e$ ) and leaving ( $C_o$ ) the chamber by infrared gas analysis makes it possible to estimate the net online discrimination as

$$\Delta = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)} \quad (12)$$

with  $\xi = \frac{C_e}{C_e - C_o}$ , and  $\delta_e$  and  $\delta_o$  being the isotopic compositions of the CO<sub>2</sub> (relative to the standard Pee Dee Belemnite) in the air entering and leaving the leaf chamber, respectively. A more complex expression of online  $\Delta$ , to account for refixation of respired and photorespired CO<sub>2</sub>, has been developed by Gillon and Griffiths (1997).

Recently, the use of CF-IRMS coupled with gas chromatographs (GC-IRMS) or membrane inlet mass spectrometry coupled directly to the air from gas exchange systems allows real-time simultaneous measurements of online discrimination and gas exchange (Cousins *et al.*, 2006). The direct injection of CO<sub>2</sub> into the GC-IRMS system is faster and can provide real-time measurements, but it is not easily usable outside the laboratory. On the other hand, while the use of cryogenic trapping is slower and more time-consuming, it can be applied in the field. Nowadays, TDLAS systems can be used to perform continuous measurements of online  $\Delta$  (Bowling *et al.*, 2003; Flexas *et al.*, 2006; Barbour *et al.*, 2007; Schaeffer *et al.*, 2008), providing an alternative to mass spectrometers and opening up new possibilities for extensive measurements in the field. The cost of a TDLAS is less than that of an IRMS, but it does require liquid nitrogen and frequent calibration. The high frequency of measurement (250 Hz) offers the potential for good precision despite short sampling times. For example, a cycle of two calibration gases, followed by inlet and sample gases, might take 80 s and yield a precision of 0.5‰ when sampled at 10 Hz, which equates to ~0.05‰ per cycle.

The  $\Delta$  value measured using either IRMS or TDLAS should depend on the CO<sub>2</sub> concentration in the chloroplast stroma ( $C_c$ ), according to Equation 10, while the simplified

model of Equation 11 allows the calculation of  $\Delta$  expected when mesophyll conductance is infinite and  $e$  and  $f$  are negligible.

Subtracting Equation 10 from Equation 11 we obtain

$$\Delta_i - \Delta_{obs} = (b - e_s - a_l) \frac{C_i - C_c}{C_a} + \frac{eR_D + f\Gamma^*}{C_a} \quad (13)$$

and since from the first Fick's law the net assimilation rate ( $A_n$ ) is given by

$$A_n = g_m(C_i - C_c) \quad (14)$$

so we can substitute Equation 14 into Equation 13 to obtain

$$\Delta_i - \Delta_{obs} = (b - e_s - a_l) \frac{A_n}{g_m C_a} + \frac{eR_D + f\Gamma^*}{C_a} \quad (15)$$

Equation 15 is the basis of the 'slope method' to assess  $g_m$ . It shows that the deviation between the observed  $\Delta$  value and that predicted assuming that  $g_m$  is infinite and  $C_i = C_c$  is linearly related to  $A_n/C_a$ , with the slope proportional to  $1/g_m$  (i.e. the mesophyll resistance,  $r_m$ ) and the intercept reflecting the respiratory and photorespiratory term. This method consists of enclosing a leaf in a gas exchange chamber and taking several measurements under varying environmental conditions (e.g. different irradiances,  $\text{CO}_2$  concentrations, or air humidity) to obtain a range of  $A_n/C_a$  values.

As mentioned above, a large draw-down of  $\text{CO}_2$  is needed to obtain the required precision and accuracy. This can be achieved by enclosing a large leaf area in a custom-built chamber, or, alternatively, by reducing the air flow rate through a small leaf chamber. However, the use of small leaf chambers, especially those clamped to leaves, has several disadvantages. They are more prone to problems such as border effects, gas leaks, and transport of  $\text{CO}_2$  through homobaric leaves, as discussed earlier. In addition, reduced flow rates can increase the magnitude of leaks and related errors. Hence, it may be preferable to use large leaf chambers capable of entirely enclosing relatively big leaves, although then other problems appear (see above).

To obtain the range in  $A_n/C_a$  values required, normally irradiance,  $\text{CO}_2$  concentration, or both are varied. This assumes that mesophyll conductance does not vary with changes in irradiance or  $[\text{CO}_2]$ . However, it has been shown (Centritto *et al.*, 2003; Flexas *et al.* 2007b; Hassiotou *et al.*, 2009) that  $g_m$  can strongly respond to changes in  $[\text{CO}_2]$ , which would impair this assumption. Nevertheless, recent tests using the  $^{13}\text{C}$  discrimination method (Tazoe *et al.*, 2009) have shown for wheat leaves that  $g_m$  was independent of changes in PFD between  $200 \mu\text{mol m}^{-2} \text{s}^{-1}$  and  $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$  and independent of  $C_i$  between  $80 \mu\text{mol mol}^{-1}$  and  $500 \mu\text{mol mol}^{-1}$ . Tazoe *et al.* (2009) also found that the isotopic composition of the source  $\text{CO}_2$  was important because compressed  $\text{CO}_2$  cylinders typically differ considerably from atmospheric  $\text{CO}_2$ . This affects the apparent fractionation factor associated with respiratory

fractionation if respiratory  $\text{CO}_2$  release is derived from previously fixed carbon (Wingate *et al.*, 2007). The fractionation associated with respiration is generally small relative to carboxylation (see above) but, if the isotopic composition of the source  $\text{CO}_2$  during measurement differs from that during growth, then the isotopic contribution associated with respiration can become significant. Then, since the ratio of respiration to carboxylation varies with PFD, the effect needs to be considered. At present, all respiratory substrate is treated as a single pool because finer detail could not be resolved. Future refinements to the methodology may justify a more complex analysis.

The theory of carbon isotope discrimination underlying this method has proven to be very robust and universally valid in  $\text{C}_3$  species. In addition, measurements of gas exchange and online discrimination both utilize the same  $\text{CO}_2$  signal from the entire leaf enclosed in the chamber, whereas fluorescence methods compare a  $\text{CO}_2$  signal with an optical signal that varies with the depth through the mesophyll. An advantage of repeated measurements on the same leaf is that it provides a good average estimate, which reduces the influence of outliers associated with error from any signal.

#### Online discrimination: 'single point method'

This method first introduced by Lloyd *et al.* (1992) is essentially the same as the 'slope method' in all experimental procedures, but it can provide an assessment of  $g_m$  from a single  $\Delta$  measurement. Hence, gas exchange and online  $\Delta$  measurements have to be taken as described above.

By rearranging Equation 15,  $g_m$  is derived as:

$$g_m = \frac{(b - e_s - a_l) \frac{A_n}{C_a}}{(\Delta_i - \Delta_{obs}) - \frac{eR_D/k + f\Gamma^*}{C_a}} \quad (16)$$

Then  $g_m$  can be calculated either by ignoring the respiratory and photorespiratory term (i.e. assuming that either are zero or that they cancel out) or by attributing specific constant values to  $e$  and  $f$ . This approach, being based on a single measurement, is faster and does not require changes in irradiance and/or  $[\text{CO}_2]$ , with related uncertainties. On the other hand, ignoring the terms  $e$  and  $f$  can lead to significant errors in the estimation of  $g_m$  (Gillon and Griffiths, 1997). It is advisable to use constant estimated values of  $e$  and  $f$  across different measurements, although variations of these fractionations might occur among different conditions such as environmental stress. A recent study by Flexas *et al.* (2007b) has shown similar  $g_m$  values obtained under normal air or when  $<1\%$   $\text{O}_2$  was used, to suppress respiratory and photorespiratory components. While this was interpreted as indicating that  $e$  and  $f$  could sometimes be safely ignored, it would depend on the isotopic composition of the  $\text{CO}_2$  in use during gas exchange measurements. Another disadvantage associated with using a single measurement is that it will accumulate all potential errors in the estimate of  $g_m$ . Comparisons between the slope

and the single point methods so far available indicate that they yield similar values for  $g_m$ , but it would certainly be useful to have more studies comparing these two variants.

#### Discrimination in recently synthesized carbohydrates

Another variant to the discrimination methods described above is that introduced by Brugnoli *et al.* (1994). This method uses the value of  $\Delta$  measured by mass spectrometers in recently fixed carbohydrates, namely leaf soluble sugars, instead of that measured online. Leaf carbohydrates accumulate in leaves during the day and are then exported later via the phloem to all plant compartments. It has been shown (Brugnoli *et al.*, 1988) that  $\Delta$  in leaf soluble sugars is correlated with an assimilation-weighted average of  $C_i/C_a$  (and  $C_i/C_a$ ) integrated over a period ranging from a few hours to 1–2 d. Therefore, this signal is intermediate between that instantaneous of online  $\Delta$  and that of bulk-biomass  $\Delta$  integrating the entire lifespan of the plant/organ analysed.

This method uses Equation 16 to calculate  $g_m$  as described above, except that  $\Delta_{\text{obs}}$  is represented by the isotopic discrimination measured in leaf soluble sugars. The earliest method to analyse  $\Delta$  in leaf soluble sugars was introduced by Brugnoli *et al.* (1988). This consisted essentially of the extraction of the water-soluble fraction from leaves. Subsequently, the extract was purified by ion-exchange chromatography, to remove the ionic fraction including amino acids and organic acids. This method has been modified by several authors to adapt it to different species (Scartazza *et al.*, 1998; Brugnoli *et al.*, 1998; Wanek *et al.*, 2001; Richter *et al.*, 2009). Other approaches use high-performance liquid chromatography (HPLC) to purify and analyse sugars. Initially the sugar purified by HPLC were combusted and analysed offline (Duranceau *et al.*, 1999), while, subsequently, online compound-specific LC-IRMS has become commercially available (Krummen *et al.*, 2004) offering promising possibilities for extensive applications. Soluble sugars (sucrose, glucose, and fructose) can also be purified and analysed by gas chromatography and mass spectrometry (GC-IRMS). However, GC-IRMS requires derivatization of individual carbohydrates, introducing external carbon into molecules with the consequent need to correct the  $\delta^{13}\text{C}$  measured.

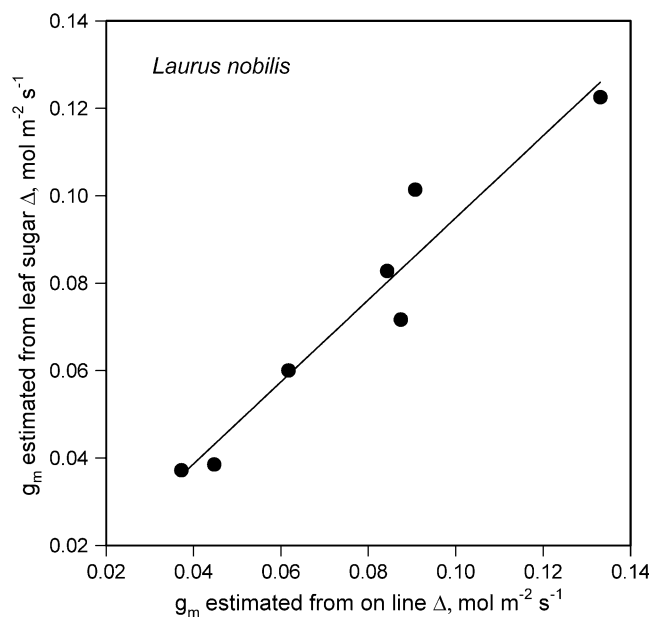
The soluble sugar method offers the advantage of being fast and easy to apply in ecophysiological applications in the field where it is relatively easy to collect many leaves, allowing comparisons of different species or genotypes and treatments, after taking gas exchange measurements (Lauteri *et al.*, 1997; Monti *et al.*, 2006). It does not require complex equipment or electricity, but only dry ice to refrigerate samples. Leaves can be subsequently extracted and sugars analysed in the laboratory. Another advantage is that the soluble sugar method allows estimation of  $g_m$  in nature and integrates the isotopic signal of plant biomass.

One inherent problem of this method is represented by the need for integrating gas exchange measurements over a longer time period (hours to the entire day) or, alternatively, taking several measurements during the day

and averaging them over the assimilation rate. Otherwise, differences in integration times between  $\Delta$  in soluble sugars and gas exchange can lead to significant errors in the estimate of  $g_m$ , especially when photosynthesis and  $C_i/C_a$  vary significantly during the diurnal course.

Another intrinsic disadvantage is that, being destructive, this method does not allow multiple measurements over the same sample as do the others described above. Furthermore, the sugar method shares the same problems with the online discrimination methods, such as uncertainties about the exact values for  $b$ ,  $e$ , and  $f$ . In particular, after purification and removal of amino acids and organic acids, one might expect that the  $\Delta$  in sucrose may be related to fractionation associated with Rubisco carboxylations ( $b_3$ ) only, with no contribution of PEP carboxylase (Brugnoli *et al.*, 1998). In this case, the value of  $b$  should be close to 30‰ (Brugnoli *et al.*, 1998). However, based on results so far reported, it is likely that some carbon skeletons partly derived from PEP carboxylation may contribute to sucrose formation, leading to  $b$  values ranging again between 27‰ and 30‰.

Notwithstanding these problems and uncertainties,  $\Delta$  in leaf sugars provides  $g_m$  values very similar to those obtained using all the other methods (online discrimination and combined fluorescence/gas exchange), indicating the reliability of this approach (Fig. 5). Certainly, this cannot be regarded as a method to measure  $g_m$  precisely in the short



**Fig. 5.** Relationship between mesophyll conductance ( $g_m$ ) estimated from isotopic discrimination ( $\Delta$ ) in leaf sugars and that estimated from online  $\Delta$ , in bay-laurel plants (*Laurus nobilis* L.). Data from E Brugnoli (unpublished results). Plants were subjected to different water availability in order to obtain a wide range of variation in  $g_m$ . Gas exchange and online  $\Delta$  were measured in a laboratory gas exchange system. At the end of the experiment, leaves were frozen and soluble sugars extracted and analysed as described in Scartazza *et al.* (1998).

term but rather an estimation of the assimilation-weighted average value of  $g_m$  integrated over the photoperiod, useful in ecophysiological studies, in the comparisons of different genotypes, and in breeding programmes for increased tolerance to environmental stresses.

## Conclusions

In the sections above, the currently most commonly used methods for the estimation of  $g_m$ , have been described, and their underlying assumptions, their technical aspects, and the precautions needed to obtain reliable estimates have been highlighted. The recommended steps to be followed when planning measuring  $g_m$  with the techniques described are summarized here (Table 3).

All methods rely on gas exchange for the measurement of  $A_n$  and  $C_i$ . For highest accuracy, it is preferable to use large leaf chambers when possible. This minimizes leaks and border effects, and, especially in the case of the isotopic methods, it maximizes the  $CO_2$  draw-down. However, this is not always an option with the chlorophyll fluorescence-based methods, since they rely on measuring gas exchange and chlorophyll fluorescence over the same leaf area, which limits the maximum area measurable for chlorophyll fluorescence. It is necessary to check for leaks

and border effects and correct the values accordingly, particularly when working with clamp-on chambers at  $[CO_2]$  other than in the surrounding air, and when  $A_n$  is low. The  $[CO_2]$  gradient over the gaskets can be minimized by flushing the outside space with chamber air.  $C_i$  is the other critical gas exchange parameter that requires attention. It affects the estimation of  $g_m$ , but not necessarily  $C_c$ . The measurement of  $C_i$  can be affected by patchy stomatal closure, gaskets that are leaky for water vapour, and errors with measuring leaf temperature. Conditions of very low stomatal conductance (e.g. drought stress) can also cause significant errors in  $C_i$  because of relatively high cuticular conductance to water vapour. It is recommended to select measurement conditions that minimize such errors, estimate the magnitude of the error, and apply corrections where possible.

When using the variable  $J$  approach of the chlorophyll fluorescence-based methods, an important issue is the establishment of the relationship between  $J_A$  and  $J_F$  under non-photorespiratory conditions across the range used for the  $g_m$  measurement. An atmosphere near oxygen depletion is preferred but, when low  $[O_2]$  is used, corrections should be applied for the low rates of photorespiration going on under these conditions. This is most critical when using the variant with single measurements, but the accuracy of the variants using ranges of  $[CO_2]$  or  $[O_2]$  is also improved

**Table 3.** Points of attention and recommendations for obtaining best results with estimating mesophyll conductance ( $g_m$ ) using different techniques

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### General

When possible, use two independent methods to estimate  $g_m$ .

Apply a sensitivity analysis to estimate the reliability of the  $g_m$  calculations.

### Gas exchange measurements

Use large leaf exchange chambers, and where possible without gaskets that clap on the leaf.

When working with clamp-on chambers, check for leaks and correct the values accordingly, particularly at  $[CO_2]$  different from the surrounding atmosphere. Alternatively, flush the surrounding space with chamber air.

Check for border effects and correct values accordingly, particularly when measuring low photosynthesis rates.

Verify the validity of leaf temperature readings.

Check for cuticular conductance and patchy stomatal closure, particularly under stress conditions.

### Chlorophyll fluorescence methods

For the variable  $J$  method, establish the  $J_A$ – $J_F$  relationship in non-photorespiratory conditions.

An estimate of leaf absorptance is useful for the calculation of  $J_F$ .

When not available for species and measurement temperature, make estimates for  $\Gamma^*$ .

$R_L$  can be estimated or derived from measured  $R_D$  and a separate estimate of the  $R_L/R_D$  ratio.

Be aware of a possible effect of  $[CO_2]$  on  $g_m$  when using ranges of  $[CO_2]$  with the constant  $J$  and variable  $J$  methods.

### Curve-fitting method

Independent estimates of  $\Gamma^*$  and  $R_L$  are preferred to reduce the degrees of freedom.

Compare the  $g_m$  values estimated by fitting separately the Rubisco- and the RuBP-limited regions for possible  $CO_2$  effect on  $g_m$ .

Experience is required for allocating data points to limitations and judging the reliability of the result.

### Online isotopic methods

Check for the precision of the measurements, and perform a sensitivity analysis of the error in estimating  $g_m$  as a function of  $CO_2$  draw-down.

According to the results of the above checking, choose an appropriate chamber size to set the proper  $\xi$  value depending on photosynthesis and transpiration rates, and decide what the range of validity of the estimates is.

Perform a sensitivity analysis to show how different Rubisco and PEPC discrimination ( $b$  value) would affect the estimates of  $g_m$ .

If using the 'slope method', check with an independent method for the possible incidence of light- and/or  $CO_2$ -induced variations of  $g_m$ .

Using the single point methods, an assessment of fractionations associated with respiration and photorespiration is needed.

### Carbon isotopes in carbohydrates

With the soluble carbohydrate discrimination method one should check that gas exchange parameters are averaged (assimilation weighed) over the same time frame (few hours to a full day).

Check that there is no metabolic fractionation between the initial  $C_3$  products and glucose, fructose, and sucrose.

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when  $J_A$  and  $J_F$  are not proportional. Other critical parameters to estimate  $g_m$  using these methods are  $\Gamma^*$  and  $R_L$ . These can be estimated using the so-called ‘Laik method’. Alternatively,  $\Gamma^*$  is derived from Rubisco kinetic parameters. Where possible, species-specific values should be used, including their temperature dependence where relevant.  $R_L$  can be estimated from measured  $R_D$  and a separately measured  $R_L/R_D$  ratio.

The curve-fitting method requires that limitations are allocated to data points (Rubisco, RuBP regeneration, possibly TPU). When the method is applied to the Rubisco-limited part of the  $A_n$ - $C_i$  curve, additional kinetic constants for Rubisco are required. However, these have been measured for a limited number of species and, as for  $\Gamma^*$ , there is increasing evidence for species-specific variation. As with a value for  $\Gamma^*$ , it is recommended to use an independent estimate of  $R_L$  to reduce the degrees of freedom. The method is not the preferred choice and should only be used when the other methods are not available.

Using online isotopic methods, the most important issue is to determine *a priori* the precision of the instrument used, and to design a gas exchange chamber with the appropriate size. It is important to stress that, despite general agreement that the isotopic methods are the most robust, the precision of current instruments is not always sufficient to allow an accurate estimate of  $g_m$  when CO<sub>2</sub> differentials are small, such as with low photosynthesis rates, small gas exchange chambers, small leaves, etc. In such cases, using a dual-inlet system is the only valid solution for a proper estimate of  $g_m$ , and, since dual-inlet systems are not available in many labs, chlorophyll fluorescence methods may be preferred. In addition, a sensitivity analysis must be performed to assess the effects of different Rubisco and PEPC discrimination, and different fractionation during respiration and photorespiration on the estimates of  $g_m$ . The latter may not be necessary when using the ‘slope method’. Alternatively,  $g_m$  is measured under non-photorespiratory conditions.

When many measurements in the field are needed to obtain an average value of  $g_m$  to be compared in various species, genotypes, and treatments, measuring carbon isotope discrimination in leaf carbohydrates is a valid option. It is easy to apply and does not require complex equipment in the field. A requirement is that  $A_n$ -weighted averages for gas exchange parameters ( $A_n$  and  $C_i$ ) are measured.

Reliability of  $g_m$  calculations with all methods depends on accuracy of the data, model assumptions, and estimates of parameter values. To evaluate the reliability of the result, a sensitivity analysis should be carried out where the effect of variation in the above factors is calculated. Examples of sensitivity analysis are given in Tables 1 and 2, and in Figs 2 and 4. Both types of techniques for estimating  $g_m$ , fluorescence and isotope discrimination based, have their limitations. The isotopic method is generally considered as less sensitive to errors and more reliable. Particularly at high conductances when the  $C_i$ - $C_c$  gradient is small, the fluorescence methods are less reliable. The isotopic methods are more suitable for such leaves. However, the required instrumentation is not always available. Alternatively, the

isotopic method has its limitations when measuring small leaves and at low  $A_n$  because the required CO<sub>2</sub> draw-down cannot be achieved. In that case the fluorescence method may be the preferred choice. Ideally, both methods should be used when possible for increased confidence in the results. Notwithstanding the many sources of potential error mentioned above, the different methods often agree remarkably well, adding to their confidence.

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