Journal of Experimental Botany, Vol. 60, No. 8, pp. 2217–2234, 2009
doi:10.1093/jxb/erp081 Advance Access publication 8 April, 2009

Journal of Experimental Botany
www.jxb.oxfordjournals.org

REVIEW PAPER

Estimating mesophyll conductance to CO_2: methodology, potential errors, and recommendations

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Received 19 December 2008; Revised 22 February 2009; Accepted 25 February 2009

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 $^{13}CO_2$. 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_2 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_2] 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_2 from the atmosphere to the sites of carboxylation in the chloroplasts of C_3 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_2 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_3 plants only. In C_4 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_4 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_3 species because of photorespiration (see later). In C_4 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_2 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_2 and H_2O share common diffusion pathways across the boundary layer and stomata, which is used to calculate the $[CO_2]$ in the substomatal cavity (C_i). Simultaneously, the $[CO_2]$ at the sites of caboxylation 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 $^{13}CO_2$ 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_2O and O_2 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_2 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^2) would be the preferred choice when making $A_n - C_i$ curves for the curve-fitting method and estimations of Γ^* and R_L. Also when used in combination with online isotope discrimination, larger leaf areas are preferred since small areas result in smaller CO_2 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^2) 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_2 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_2 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^2 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 μmol m^{-2} s^{-1} and A_n of 20 μmol m^{-2} s^{-1}, the measured A_n would have been 19 μmol m^{-2} s^{-1} (i.e. a 5% error). However, for a stressed leaf showing A_n of, say, 2 μmol m^{-2} s^{-1}, the measured A_n would have been 1 μmol m^{-2} s^{-1} (i.e. a 100% error).

Another problem can arise when measuring homobaric leaves. CO_2 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 μmol m^{-2} 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_2] 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_2 produced under the gasket mentioned above.

A further complication is the leakage of CO_2 and H_2O through the gaskets and, more importantly, along the contact zone between gaskets and leaf surfaces. For instance, Flexas *et al.* (2007*a*) estimated that leakage resulted in apparent respiration and maximum photosynthesis rates of up to –1 μmol m^{-2} s^{-1} and 4 μmol m^{-2} s^{-1}, respectively, when working with an empty 2 cm^2 chamber. The process is demonstrated when at low [CO_2] inside an empty chamber an apparent R is measured and an apparent A_n at high [CO_2]. 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.* (2007*a*) observed a decreased leakage of CO_2 in the presence of thermally killed leaves of several species. However, Rodeghiero *et al.* (2007) found an increased leakage for CO_2 when a dried sclerophyllous *Quercus ilex* leaf was clamped between the gaskets. In addition, they also observed significant leakage of H_2O 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_2 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.*, 2007*a*).

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.*, 2007*a*; 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_2], 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 CO_2 assimilation (A_n), and the CO_2 concentration at the site of Rubisco (C). The relationship can be modelled according to Farquhar *et al.* (1980):

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

where R_L is the rate of mitochondrial respiration in the light, Γ^* is the 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 H+, 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 $[CO_2]$ (C_i) as the best estimate of the CO_2 concentration in the chloroplast (C_c). When there is a significant decrease in $[CO_2]$ 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 $[O_2]$ 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 $[CO_2]$ (C_c) at atmospheric $[CO_2]$, 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 CO_2 response of A_n at atmospheric $[O_2]$ (Fig. 1). The ranges of C_i (μmol mol^{-1}) and $[O_2]$ 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 μmol m^{-2} s^{-1}, leaf temperature 20 °C, a vapour pressure difference of ~0.8 kPa, and an atmospheric pressure of 100.0 kPa. A_n was 77% of the light-saturated value. A_n and C_i data were corrected for band broadening caused by H_2O and O_2, leakage of respired CO_2 into the chamber, and diffusion of 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**[†]
	C_i range 240–650	**Single C_i=238**	**$[O_2]$ range* C_i=227**	**C_i range 100–180**	**C_i range 40–650**
g_m mmol m^{-2} s^{-1}	155	125	133	143	178
C_c μmol mol^{-1}[‡]	159	141	148	–	168
b_F (J_A/J_F)	1.003	1.078[§]	1.004	0.970	–
Effect on g_m with alternative stoichiometry of J[¶]	+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 $[O_2]$ were included, 1, 10, and 21%.
[†] 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).
[‡] Measurement at atmospheric C_a, where C_i was 238 μmol mol^{-1}.
[§] Estimation of b_F in air without O_2, where J_A and J_F were proportional; b_F for the other methods was solved together with g_m.
[¶] The notation (4 C_c+8 Γ^*) in Equation 3) was changed into (4.5 C_c+10.5 Γ^*) to consider the case that models a limitation of ATP supply instead of NADPH supply (von Caemmerer, 2000).

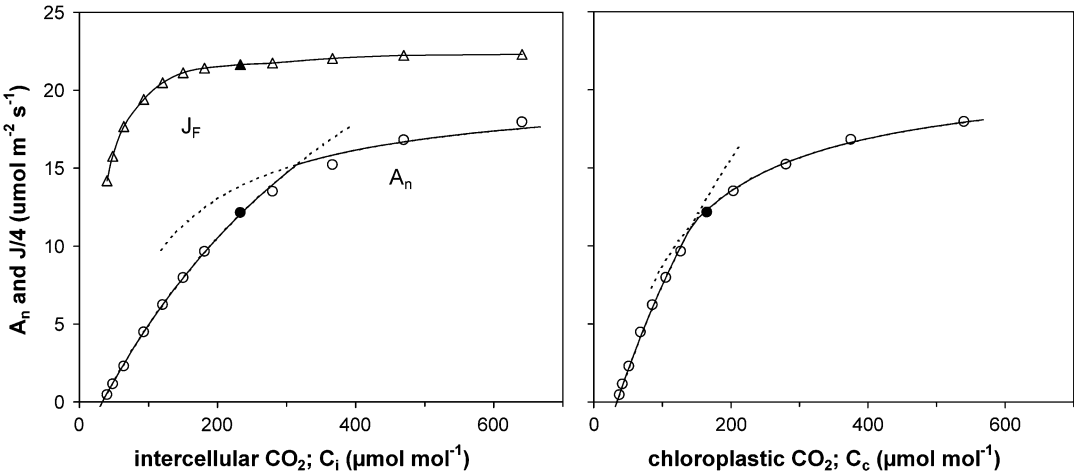

Fig. 1. The CO_2 response curve of a *Hedera helix* leaf. Net photosynthesis (A_n) was plotted against intercellular CO_2 concentration (C_i) (left panel), and against the CO_2 concentration in the chloroplast (C_c) (right panel). The electron transport rate measured with fluorometry (J_F) was also plotted 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_2] (380 µmol 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):

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

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

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

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 α and β (Equation 4). Leaf absorptance (α) can either be measured directly or an approximation can be derived from chlorophyll content per unit area using published relationships, for example α=[Chl]/([Chl]+76), where [Chl] is the chlorophyll content per unit leaf area expressed in µmol m^{-2} (Evans and Poorter, 2001). The partitioning factor β is normally assumed to be 0.5, but may vary (Laisk and Loreto, 1996). A problem related to Φ_{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 Φ_{PSII} (Genty *et al.*, 1990; Agati *et al.*, 2000; Franck *et al.*, 2002) and consequently J_F. Φ_{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_2], typically 1% or 2% across a similar range of J as measured at atmospheric [O_2]. The small rates of photorespiration ongoing at these low [O_2] 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_2] (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 Γ^* or g_m. Photosynthesis should be induced in the light at atmospheric [O_2] before O_2 is removed. However, the approach assumes that R_L is not affected by the very low [O_2] generated by photosynthesis. This is difficult to verify, since this O_2 source is not available for the control measurement in darkness.

J_A at low [O_2] 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_2] 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 Γ* *and* R_L

All methods require values for the CO_2 compensation point (Γ*) in the absence of mitochondrial respiration in the light (R_L) and R_L itself. Γ* 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 Γ* value estimated at the level of the intercellular spaces (C_i*), whereas for calculating g_m, strictly a true value for Γ* 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, Γ* 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 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 Γ* together with an estimate of g_m using Equation 7. Alternatively, Γ* 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 [CO_2] are preferably done in a large leaf chamber. When performed with a small clamp-on chamber, rigorous corrections are required at low [CO_2]. Bernacchi *et al.* (2002) used an alternative method for measuring Γ* involving labelling with ^{18}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 Γ* has been described (von Caemmerer, 2000; Bernacchi et al., 2001, 2002), making conversions to other temperatures possible. However, temperature dependencies may be species specific. Hence, the estimation of Γ* for the conditions of the measurement is preferred, since the value of Γ* has a substantial effect on the result of g_m calculations (Harley *et al.*, 1992).

An alternative to measuring Γ* is to estimate it from published values of the Rubisco specificity factor (τ) 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 τ are reported. The variations of τ with temperature are species dependent (Galmés *et al.*, 2005). The search for reliable Γ* 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 $^{13}CO_2$ 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 [O_2]. The relationship between J_A and J_F must be separately established for the same conditions (except [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 [O_2] remains the same at atmospheric [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 mmol m^{-2} s^{-1}) compared with the constant J method (155 mmol m^{-2} s^{-1}). This was due to the fact that b_F measured at 0% O_2 was higher (1.078) than the value calculated from the constant J method at atmospheric [CO_2]

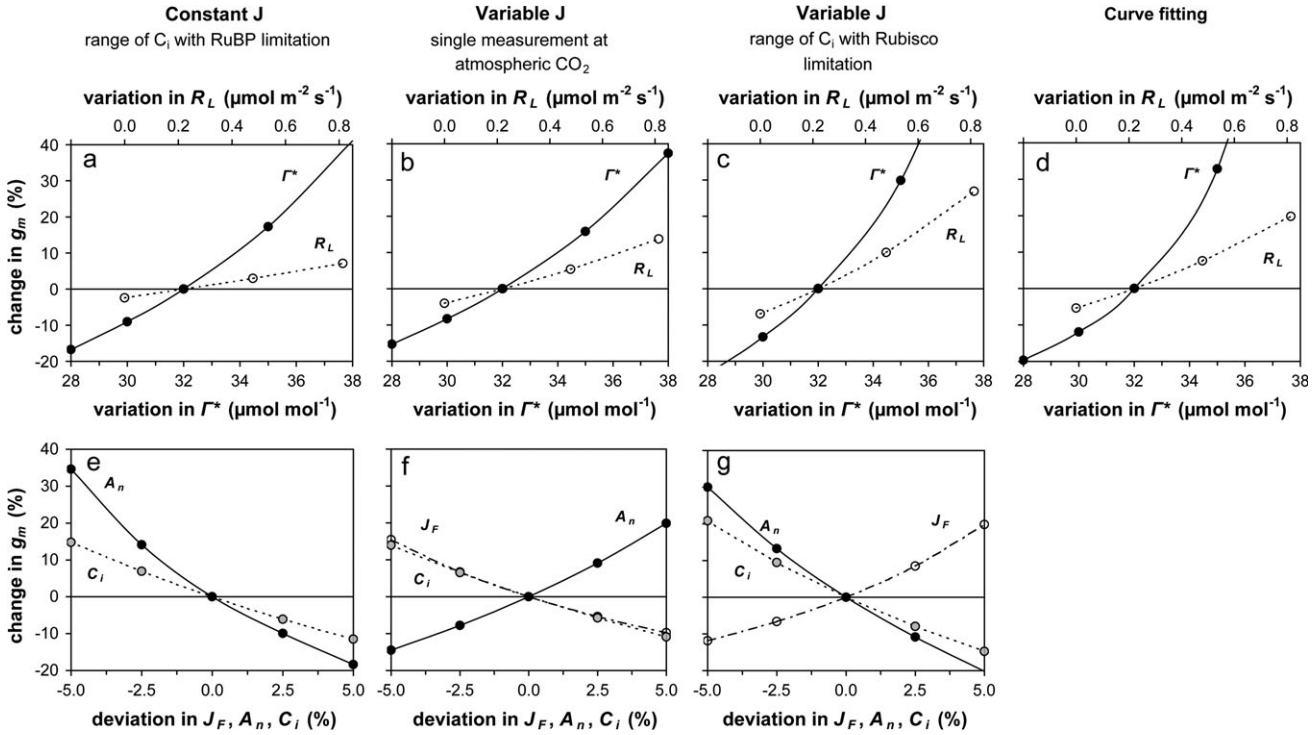

Fig. 2. Sensitivity of the estimation of g_m for variation in parameter values using different methods. Calculations are based on the same data set for a *Hedera helix* leaf as used in Fig. 1 and Table 1, where other data are shown. The methods are the constant *J* method (a, e), the variable *J* methods with a single measurement at atmospheric CO_2 (b, f), the variable *J* method applied to a range of low [CO_2] where Rubisco limits gas exchange (c, g), and the curve-fitting method (d). In the upper panels, the effects of variation in Γ^* 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_2 (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 Γ* (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_2], 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_2]. It is thus suitable to investigate the effect of [CO_2] on g_m. However, at high [CO_2], 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_2] using this method with the *Hedera* data, as reported by Flexas *et al.* (2007*b*). However, this was not confirmed by the variant of the variable *J* method applied to a range of high and low [CO_2] (see below). Evidence for a CO_2 effect on g_m obtained with this method should thus be verified using other methods, as done by Flexas *et al.* (2007*b*) and Vrábl *et al.* (2009).

The measurement of the relationship between J_A and J_F is often done at low [O_2] over a range of [CO_2] instead of using an anoxic atmosphere. As argued above, the low rate of photorespiration at 1% or 2% [O_2] and the effect of the C_i-C_c gradient thereupon cannot be ignored. J_A should then be calculated using Equation 3 with Γ* reduced in proportion to [O_2]. 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_2 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^{-2} s^{-1} 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_2]. However, the assumption remains that b_F and, where applicable, other constants describing the J_A-J_F relationship are independent of [O_2].

A range of [CO_2] where *J* is not constant can also be used. That can be a range of lower [CO_2] where Rubisco limits A_n as done with the *Hedera* leaf (Table 1, Fig. 2), but can also be applied to higher [CO_2] 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_2] range, where b_F is solved together with g_m. The method thus assumes that these are constant across the range of [CO_2]. The method is more sensitive for variation in Γ* and R_L compared with the other ones, particularly when low [CO_2] is included in the range (Fig.

2c). The method yielded a slightly lower value for g_m than the constant J method (143 mmol m^{-2} s^{-1} and 155 mmol m^{-2} s^{-1}, respectively), but the two were measured over different ranges of $[CO_2]$, 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 $[CO_2]$, 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 mmol m^{-2} s^{-1}, however, generated a perfect fit.

This method was first introduced by Bongi 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 $[CO_2]$ 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.*, 2007*b*; 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 $[CO_2]$ range of interest. The method is sensitive for the value of Γ*, 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 CO_2 production in darkness was used (0.8 μmol m^{-1} s^{-1}) instead of R_L, g_m increased by 7% (Fig. 2a). That is not too much, but the sensitivity to variation in Γ* 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 μmol mol^{-1} CO_2 to 1500 μmol mol^{-1} 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 $[CO_2]$, 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, Φ_{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 $[CO_2]$. 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 $[CO_2]$ ranges are distinguished that are limited by different processes. The part limited by the activity of Rubisco is described as:

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

where V_{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 bisphosphate (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 Γ*, 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 $[CO_2]$ where limitation by triose phosphate utilization (TPU) may occur. This model is available in a spreadsheet at http://www.blackwellpublishing.com/plantsci/pcecalculation/. When independent estimates for R_L and Γ* are available, V_{cmax}, J, and g_m can be calculated iteratively. The method evidently assumes a constant g_m across a wide range of $[CO_2]$, although a change of g_m with $[CO_2]$ can be implemented in the model (Flexas *et al.*, 2007*b*; TD Sharkey, personal communication).

The model produced a somewhat higher value for g_m (178 mmol m^{-2} 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_{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 ^{13}C isotope discrimination

Theory

These methods are based on carbon isotope fractionation measured simultaneously with gas exchange. Measurements of g_m using ^{13}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_2 fixation. Specifically, the heavier isotope of carbon, ^{13}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 $^{13}CO_2$ in air and liquid phase relative to $^{12}CO_2$ and to discrimination by carboxylating enzymes such as Rubisco, which preferentially bind molecular species containing the lighter isotopes ($^{12}CO_2$). Hence, the photosynthetic products are generally enriched in the lighter isotope ^{12}C compared with the substrate atmospheric CO_2. In C_3 species, the isotopic discrimination is related to the relative contribution of diffusion and carboxylation, which is reflected in the ratio of CO_2 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

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

where, C_a, C_s, C_i, and C_c are the CO_2 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_2 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_3 plants; e and f are the fractionations occurring during dark respiration (R_D) and photorespiration, respectively; k is the carboxylation efficiency, and Γ^* is the CO_2 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_2 concentration (C_i), and not that at the sites of carboxylation. In the case of high conductance to CO_2 diffusion from the substomatal cavities to the chloroplast stroma, concurrent measurements of gas exchange and isotopic discrimination (Δ) by isotope ratio mass spectrometry can provide very good relationships between the Δ and the ratio of leaf intercellular CO_2 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

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

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_2 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 ^{13}C in respired CO_2 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_2 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_3 plants a variable amount of carbon is fixed by PEP carboxylase (Nalborczyk, 1978; Farquhar and Richards, 1984). In C_3 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 β-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_2 (Brugnoli *et al.*, 1988; Guy *et al.*, 1993; Brugnoli and Farquhar, 2000). Therefore, depending on the proportion of PEP carboxylations in C_3 plants, the value of b can be between 27‰ and 30‰. The value assumed for b will influence the absolute value calculated for g_m, and is one the most significant issues present in all ^{13}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_n of 12.1 μmol CO_2 m^{-2} s^{-1} with a stomatal conductance (g_s) of 0.280 mol H_2O m^{-2} s^{-1}. As measured in a small gas exchange cuvette (2 cm^2) at an external CO_2 concentration (C_e, see Equation 12 below) of 400 μmol mol^{-1}, the leaf created a CO_2 draw-down in the cuvette of 18.2 μmol mol^{-1} (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_o–δ_e, see below) of 0.709‰, the values of g_m 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_m.

Equation 10 is often further simplified by assuming that the draw-down of CO_2 between the substomatal cavities and the chloroplast stroma is negligible and that fractionation associated with respiration and photorespiration is also negligible; then

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

This equation is the most used model of discrimination and predicts a linear relationship between Δ_i and C_i/C_a. Hence, one can estimate the value of Δ from the value of C_i/C_a measured by gas exchange on the basis of Equation 11 with its underlying assumptions. Comparisons between the expected Δ values and those actually measured (Δ_{obs}) provide an insight into the magnitude of mesophyll conductance and of the draw-down of CO_2 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_i and Δ_{obs} compared with the former, mainly attributable to lower g_m.

The actual estimate of g_m can be performed using different methods, consisting of the determination of Δ_{obs} usually by isotope ratio mass spectrometry and the calculation of the expected Δ_i from C_i/C_a 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.

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_m in a measured leaf of *Arabidopsis thaliana* displaying an A_n rate of 12.1 μmol CO_2 m^{-2} s^{-1} with a stomatal conductance (g_s) of 0.280 mol H_2O m^{-2} s^{-1}

As measured in a small gas exchange cuvette (2 cm^2) at an external CO_2 concentration (C_e) of 400 μmol mol^{-1}, the leaf created a CO_2 draw-down in the cuvette of 18.2 μmol mol^{-1} (i.e. ξ=2.0). The measured δ_o–δ_e was 0.709‰.

b (‰)	Δ_i (‰)	g_m (mol CO_2 m^{-2} s^{-1})	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%

Instrument precision

There are several methods to measure the C isotopic composition in CO_2, 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 $\delta^{13}C$ 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 $^{13}CO_2$ is translated into precision errors in the measurement of g_m.

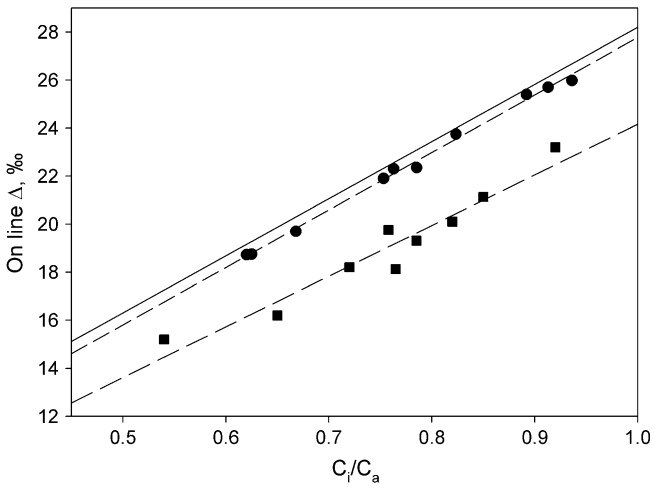

Fig. 3. Relationships between online isotopic discrimination (Δ) and C_i/C_a in bean (*Phaseolus vulgaris* L., circles) and in beech (*Fagus sylvatica* L., squares) leaves. Differences are due to substantially different g_m. The solid line represents the predicted Δ_i from the equation Δ=a+(b–a) C_i/C_a with a=4.4‰ and b=28.2‰. The dashed lines are the regression equations: y=3.83+23.95x, r^2=0.99 for bean; y=3.07+21.1x, r^2=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^2 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^{-2} s^{-1}, with an uncertainty of 0.005 mol m^{-2} s^{-1} 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^{-2} s^{-1} 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_2 draw-down (Fig. 4). In the well-watered *Arabidopsis* leaf already described, with CO_2 draw-down ranging from 100 μmol mol^{-1} to 12 μmol mol^{-1}, 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_2 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_2 draw-down, particularly when photosynthesis rates are low.

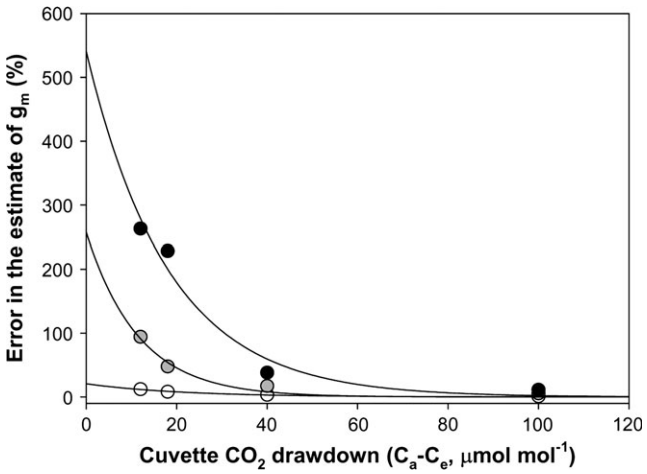

Fig. 4. A sensitivity analysis showing percentage errors in the calculated g_m as a function of total CO_2 draw-downs for precisions of 0.02 (white dots), 0.1 (grey dots), or 0.2‰ (black dots) in a leaf of well-watered *Arabidopsis thaliana* displaying an A_n of 12.1 μmol CO_2 m^{-2} s^{-1}, stomatal conductance (g_s) of 0.28 mol H_2O m^{-2} s^{-1}, and a g_m of 0.15 mol CO_2 m^{-2} s^{-1}.

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_2 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_2 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_2 collected was transferred into a mass spectrometer to determine $\delta^{13}C$. Because of isotopic discrimination during photosynthesis, the air leaving the chamber will be enriched in ^{13}C compared with that entering the chamber. Measuring this difference and measuring the CO_2 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 δ_e and δ_o being the isotopic compositions of the CO_2 (relative to the standard Pee Dee Belemnite) in the air entering and leaving the leaf chamber, respectively. A more complex expression of online Δ, to account for refixation of respired and photorespired CO_2, 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_2 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 Δ (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 Δ value measured using either IRMS or TDLAS should depend on the CO_2 concentration in the chloroplast stroma (C_c), according to Equation 10, while the simplified

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

Subtracting Equation 10 from Equation 11 we obtain

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

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

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

Equation 15 is the basis of the 'slope method' to assess g_m. It shows that the deviation between the observed Δ 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, CO_2 concentrations, or air humidity) to obtain a range of A_n/C_a values.

As mentioned above, a large draw-down of 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 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, CO_2 concentration, or both are varied. This assumes that mesophyll conductance does not vary with changes in irradiance or $[CO_2]$. However, it has been shown (Centritto *et al.*, 2003; Flexas *et al.* 2007*b*; Hassiotou *et al.*, 2009) that g_m can strongly respond to changes in $[CO_2]$, which would impair this assumption. Nevertheless, recent tests using the ^{13}C discrimination method (Tazoe *et al.*, 2009) have shown for wheat leaves that g_m was independent of changes in PFD between 200 μmol m^{-2} s^{-1} and 1500 μmol m^{-2} s^{-1} and independent of C_i between 80 μmol mol^{-1} and 500 μmol mol^{-1}. Tazoe *et al.* (2009) also found that the isotopic composition of the source CO_2 was important because compressed CO_2 cylinders typically differ considerably from atmospheric CO_2. This affects the apparent fractionation factor associated with respiratory fractionation if respiratory 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 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 C_3 species. In addition, measurements of gas exchange and online discrimination both utilize the same CO_2 signal from the entire leaf enclosed in the chamber, whereas fluorescence methods compare a 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 Δ measurement. Hence, gas exchange and online Δ 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 $[CO_2]$, 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.* (2007*b*) has shown similar g_m values obtained under normal air or when <1% 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 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 Δ 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 Δ in leaf soluble sugars is correlated with an assimilation-weighted average of C_i/C_a (and C_c/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 Δ and that of bulk-biomass Δ integrating the entire lifespan of the plant/organ analysed.

This method uses Equation 16 to calculate g_m as described above, except that Δ_{obs} is represented by the isotopic discrimination measured in leaf soluble sugars. The earliest method to analyse Δ 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 $\delta^{13}C$ 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 Δ 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 Δ 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, Δ 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

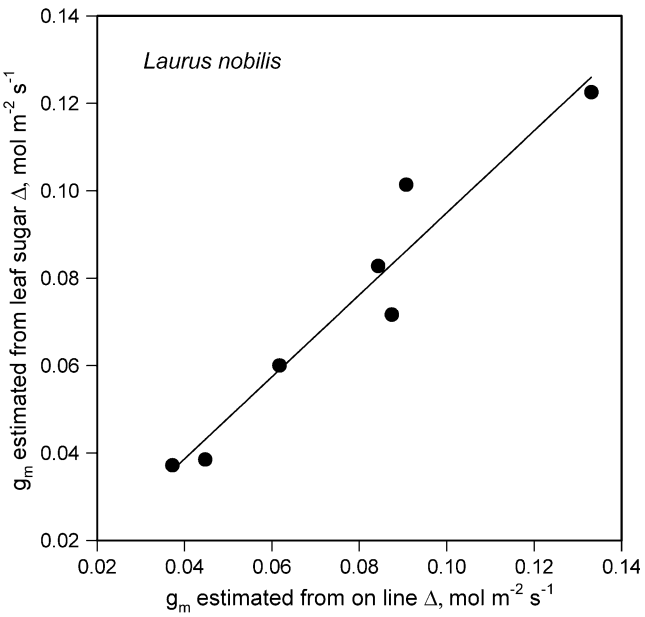

Fig. 5. Relationship between mesophyll conductance (g_m) estimated from isotopic discrimination (Δ) in leaf sugars and that estimated from online Δ, in bay-laurel plants (*Laurus nobilis* L.). Data from E Brugnoli (unpublished results). Plants were subjected to different water availability in order to obtain a wide range of variation in g_m. Gas exchange and online Δ 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

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 Γ^*.
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 Γ^* 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 ξ 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.

when J_A and J_F are not proportional. Other critical parameters to estimate g_m using these methods are Γ* and R_L. These can be estimated using the so-called 'Laisk method'. Alternatively, Γ* 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 Γ*, there is increasing evidence for species-specific variation. As with a value for Γ*, 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_2 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_2 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.

Acknowledgements

Many of the ideas reflected in the present manuscript arose from discussions held at the Workshop on 'Mesophyll conductance to CO_2: mechanisms, modelling and ecological implications', held in Mallorca, Spain, in September 2008. For their contributions to these discussions, we would like to acknowledge: Matthias Barthel, Mauro Centritto, Gabriel Cornic, Miguel Costa, Antonio Díaz-Espejo, Cyril Douthe, Erwin Dreyer, Alex Gallé, Jeroni Galmés, Foteini Hassiotou, Ralf Kaldenhoff, Francesco Loreto, Ülo Niinemets, Alfonso Pérez, Ilja Reiter, Tom Sharkey, Guillaume Tcherkez, Ichiro Terashima, Danny Tholen, Tiina Tosens, Tsonko Tsonev, and Charles Warren. Tom Sharkey is particularly acknowledged for useful comments on the curve-fitting section.

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